1999

Clusterin: a novel chaperone protein with putative cytoprotective properties

David Thomas Humphreys
University of Wollongong

CLUSTERIN: A NOVEL CHAPERONE PROTEIN WITH PUTATIVE CYTOPROTECTIVE PROPERTIES.

A thesis submitted in fulfilment of the

requirements for the award of the degree

Doctor of Philosophy

From

THE UNIVERSITY OF WOLLONGONG

By

David Thomas Humphreys (B.Sc. Hons)

Department of Biological Sciences

1999

Abstract

Clusterin is a highly conserved protein that has been found in every mammalian species examined and in two species of aves. Clusterin is distributed widely throughout the mammalian body and interacts with a diverse array of proteins. These many binding interactions have led to suggestions that clusterin is involved in an equally diverse array of functions. Clusterin is also widely reported to be dramatically upregulated in times of stress and during apoptosis indicating that it may regulate cell death. However, no genuine physiological function for clusterin has yet been established.

One of the two main goals of this project was to determine whether clusterin expression was involved in regulating cell death. This was investigated by transfecting two different expression systems containing human clusterin cDNA into L929 cells and determining if the upregulated expression of clusterin altered the rate of cell death induced by either TNFα, TNFα + actinomycin D, staurosporine, colchicine, or azide. Cells transfected with an expression system containing a cytomegalovirus (CMV) promotor were found to be resistant against TNFα-induced cell death. These transfected cells were not protected from any other cytotoxic agent tested. The other expression system (which resulted in lower levels of clusterin expression) did not provide cells with protection against TNFα or any of the other cytotoxic agents. Further studies suggested that clusterin, normally a secreted product, acted intracellularly to protect L929 cells from TNFα-induced death. However, a control L929 cell line that overexpressed green fluorescent protein was also shown to be protected from TNFα-induced death. Therefore it is possible that the cytoprotection associated with overexpression of clusterin is an artifact resulting from non-specific effects of high levels of protein expression.

It has been deduced from the amino acid sequence of clusterin that it contains three amphipathic helixes, which are required for hydrophobic interactions. It has also been demonstrated that clusterin has a high affinity for hydrophobic surfaces as it can be purified from ram rete testis fluid on the basis of it's strong hydrophobic interaction with Affi-blue gel. Other reports have suggested that clusterin's association with complement, apolipoprotein A-I and cell aggregation are due to hydrophobic interactions. Thus, it appears likely that many of clusterin's binding interactions involve binding to regions of exposed hydrophobicity, a similar property to that of chaperone proteins which bind to regions of exposed hydrophobicity on other proteins. It was also recently demonstrated that a conserved region in the clusterin promotor binds heat shock factor 1, a transcriptional activator of the heat shock protein chaperones. This element can also mediate heat-shock-induced transcription suggesting that expression of clusterin and heat shock proteins may be co-regulated. The second main goal of this project was to test the hypothesis that clusterin has chaperone-like activity.

Clusterin was shown to prevent catalase and GST from heat-induced precipitation as well as preventing BSA and α-lactalbumin from reduction-induced precipitation. It was demonstrated by ELISA that clusterin binds with higher affinity to the denatured form of these proteins. Futhermore, gel filtration analyses of solutions containing any one of these protein together with clusterin, following stress treatment, revealed that high molecular weight complexes had formed between the two proteins. It is proposed that clusterin protects proteins from stress-induced precipitation by binding to hydrophobic regions that become exposed to the solvent to form solubilized high molecular weight complexes. The ability of clusterin to non-specifically protect various proteins from stress-induced precipitation confirms that clusterin has chaperone-like activity and suggests that clusterin has a complementary role to heat shock proteins *in vivo*.

Declaration of authenticity

This thesis is submitted in accordance with the regulations of the university of Wollongong in fulfillment of the requirements for the degree doctor of philosophy. It does not incorporate any material previously published or written by any person except where due reference is made in the text. The experimental work in this thesis is original work and has not been previously submitted for a degree or diploma in any university.

16/9/99

Acknowledgements

This thesis would have not been possible without the help and support of many people. Firstly I would like to thank Dr. Mark Wilson, my superviser over the last 5 years. Your guidance and patience throughout both my honours year and PhD project have been invaluable. I would also like to thank John Carver and Robyn Lindner for their input and their knowledge about chaperone proteins. I would also like to thank everyone who has passed through the "Wilson lab" during my PhD (Jenny, Sally, Marc, KD, Kerry, Melissa, Alison, Katrina, Helen, Karolyn, Rachel, and Spoon). I wish you and all the department the best for the future.

The projects I have completed here in Wollongong would not have been possible without my wonderful family. Thanks for the support and encouragment you all have given throughout my studies at Wollongong University.

Table of contents

List of Figures

List of tables

ABBREVIATIONS

7-AAD	7-amino-actinomycin D
Ab	Antibody
Act D.	Actinomycin D
AD	Alzheimer's disease
ANS	1-anilinonaphthalene-8-sulfonate
AO	Acridine Orange
BSA	Bovine Serum albumin
Bisacrylamide	NN-methylenebisacrylamide
CLE	Clusterin element
CMV	Cytomegalovirus
dH_2O	Distilled water
cDNA	Complementary deoxyribonucleic acid
DMEM:F12	Dulbeccos modified eagles medium: Hams F12
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
FCM	Fibroblast condition medium
FCS	Foetal calf serum
HDC	Heat Denatured casein
HMW	High molecular weight
HRP	Horseradish peroxidase
HSE	Heat shock element
HSF	Heat shock factor

HSP	Heat shock protein
hTNFα	Human TNFα
Kb	Kilo base
MAb	Monoclonal antibody
MES	2-[N-morpholino]ethanesulfonic acid
mTNFα	Murine TNFα
PAGE	Polyacrylamide agarose gel electrophoresis
PBS	Phosphate buffered saline
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RO	Reverse osmosis
SDS	Sodium dodecyl sulphate
sHSP	small heat shock protein
sn	Supernatant
TC	Tissue culture
TGFβ	Transforming growth factor beta
TNFα	Tumour necrosis factor alpha
TEMED	N,N,N'N'-Tetramethyl-Ethylenediamine
Tris	Tris-(Hydroxymethylminomethane)
v/v	volume/volume
w/v	weight/volume

CHAPTER 1

Introduction

Clusterin: a review of the existing literature.

1.1 THE GENERAL FEATURES OF CLUSTERIN

Clusterin is one of many names assigned to a glycoprotein which has been discovered several times in diverse research areas and whose function is still to be elucidated. Each new discovery assigned a different name to this protein (Table 1.1). The name clusterin arose from an early finding of Blaschuk *et al.* (1983) who reported that this protein was capable of clustering Sertoli cells. Clusterin is now the internationally accepted name for the protein (First clusterin workshop, 13^{th}-16^{th} September 1992, Cambridge UK).

Table 1.1:-Alternative names of clusterin originating from different research areas. (Modified from Table 1 in Rosenberg *et al.*, 1993)

Name	Source	Site where clusterin was found	Reference
Clusterin	Ram	Reproduction/ cell aggregating	Blaschuk *et al.*, 1983
Sulphated glycoprotein 2 (SGP-2)	Rat	Sertoli cells/ reproduction	Collard and Griswold, 1987
Glycoprotein-III (GP-III)	Bovine	Adrenal Medulla/ Chromaffin granules	Fischer-Colbrie *et al.*, 1984 Palmer and Christie, 1990
Testosterone repressed prostrate message-2 (TRPM-2)	Rat	Prostate/apoptosis	Leger *et al.*, 1987
Glycoprotein 80 (Gp80)	Canine	Renal Cells/ Vectorial secretion	Hartmann *et al.*, 1991
Serum protein-40,40 (SP-40,40)	Human	Serum (liver)/complement regulation	Kirszbaum *et al.*, 1989
Cytolysis inhibitor (CLI)	Human	Serum (Liver)/ complement regulation	Jenne and Tschopp, 1989
T64	Quail	Neuroretinal cells/ cell transformation	Michel *et al.*, 1989
Apolipoprotein J (Apo J)	Human	Blood/ Lipid transport	James *et al.*, 1991

Clusterin has been detected in a variety of mammals and two non-mammalian species, the quail (Rosenberg *et al.*, 1993) and recently the chicken (Mahon *et al.*, 1999). Complete nucleotide sequencing of clusterin complementary DNA (cDNA) from these different species has shown that human and dog clusterin products are the most similar with 78% identity (Jenne and Tschopp, 1992; Hartmann *et al.*, 1991). Clusterin from the human and rat are also very homologous with 77% identity (May and Finch, 1992). Human and quail clusterin are the least similar with 49% identity (Jenne and Tschopp, 1992). The most conserved region between clusterin molecules from different species is between residues lue 87 and met 150 (homology 98% (Jenne and Tschopp, 1992)) which contains five disulphide bonds. This high level of conservation suggests that this region is important in the function of clusterin.

Clusterin is widely distributed throughout the body and is transcribed from a single gene located on chromosome 8p12-21 in humans (Purrello *et al.*, 1991; Dietzsch *et al.*, 1992; Fink *et al.*, 1993) and chromosome 14 in the mouse (Bikenmeier *et al.*, 1993). Clusterin cDNA is comprised of 16,580 bp which is organised into nine exons and eight introns (Wong *et al.*, 1994 a). There are significant differences in the 5' untranslated sequence between clusterin sequences isolated from different human tissues, suggesting that a similar clusterin multigene family exists (Wong *et al.*, 1994 a). Analysis of the promotor region of clusterin revealed AP1, AP2, SP1, and NF-2 motifs which are thought to be essential for clusterin regulation (Wong *et al.*, 1993). In addition cAMP response elements, heat shock protein binding and growth hormone factor 1 binding motifs have also been identified within the promotor region (Wong *et al.*, 1993).

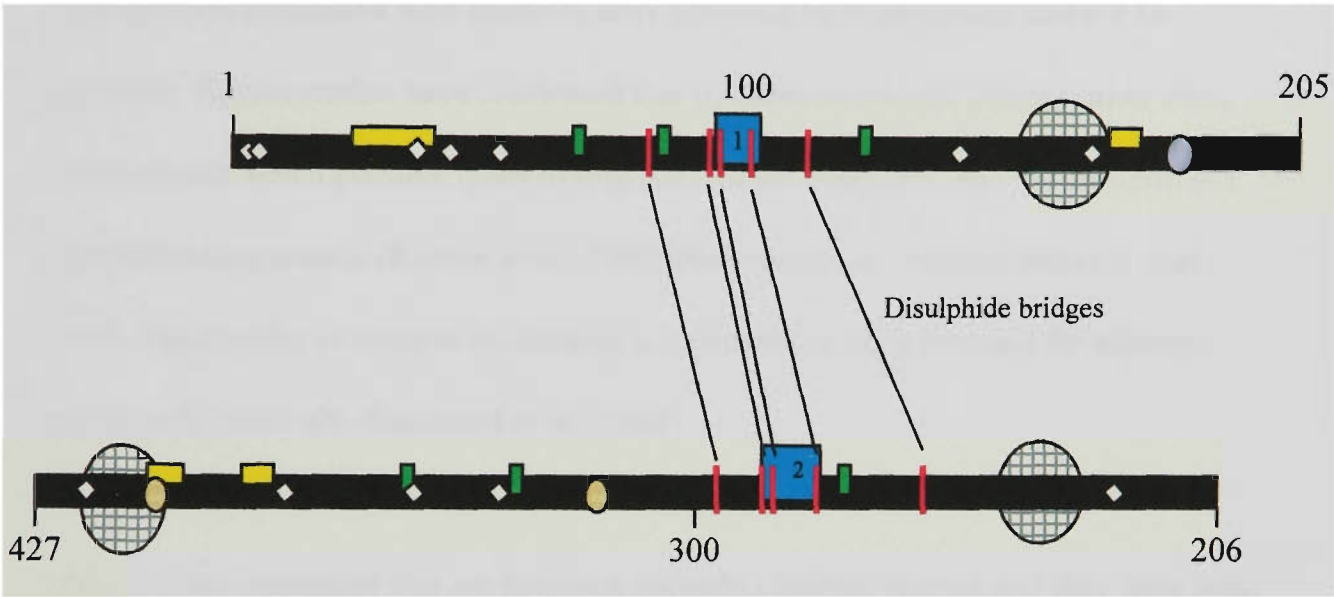
Clusterin has been located in bodily fluids including human plasma (50-100 μg/ml) and blood platelets (Murphy *et al.*, 1988; Jenne and Tschopp, 1989), human breast milk (de Silva *et al.*, 1990a), urine (O'Bryan *et al.*, 1990), spinal fluid (Hochstrasser *et al.*, 1988), on the surface of mature sperm and in seminal plasma (250-500 μg/ml; Jenne and Tschopp, 1989; de Silva *et al.*, 1990a; O'Bryan et al., 1990). In humans, clusterin mRNA has been detected in cells of most organs, although at different levels of expression. The brain, ovary, testis, stomach and liver express clusterin at the highest level while the kidney, heart, spleen, lung, and breast express clusterin mRNA at a much lower level with no expression detected in T-cells (de Silva *et al.*, 1990a). It has generally been found that epithelial cells, especially those that border fluid filled spaces, constitutively express higher levels of clusterin than other cell types (Jordan-Starck *et al.*, 1992).

Early studies showed clusterin to be a heavily glycosylated protein which in its mature secreted form has a molecular mass of 70-80 kDa and has low pI in the range of 4.9-5.4 (Blaschuk *et al.*, 1983; de Silva *et al.*, 1990b and c). The size of unglycosylated clusterin determined by an *in vitro* assay revealed a 50 kDa species (de Silva *et al.*, 1990b), however pulse chase studies in HepG2 cells only identified unprocessed clusterin to have a size of 58 kDa (Burkey *et al.*, 1991). Recently a report analysing purified clusterin with matrix-assisted laser desorption ionization mass spectrometry revealed that two molecular weight species of unglycosylated clusterin exist, and have sizes of 58,505 and 63,507 (Kapron *et al.*, 1997).

Mature secreted clusterin exists as a heterodimer and is comprised of two subunits which are similar in amino acid sequence but which are nevertheless distinct (de Silva *et al.*, 1990c). The subunits are referred to as α and β, with glycosylated masses of 34-36 kDa and 36-39 kDa, respectively (de Silva *et al.*, 1990b). The subunits are linked together by 5 disulphide bonds which are distributed over a span of 29 amino acid residues rich in cysteine (de Silva *et al.*, 1990b, Choi-Miura *et al.*, 1992b). The disulphide bonds are inter-subunit linkages which are conserved between all clusterin homologues indicating a possible importance in conformation (Choi-Miura *et al.*, 1992b; figure 1.1). Both subunits of clusterin are translated from a single 1.9 kb mRNA transcript (de Silva *et al.*, 1990a). Therefore clusterin originally exists as a single chain polypeptide in which the predicted sequence contains 449 amino acids, including a 21-22 N-terminal amino acid secretory sequence (de Silva *et al.*, 1990a, de Silva *et al.*, 1990b; Wong *et al.*, 1994a). Ninety five percent of clusterin reaches the extracellular environment as a heterodimer; sometime following translation and preceding secretion the disulphide bonds form followed by cleavage of clusterin (Burkey *et al.*, 1991). The position of this cleavage is the same for all clusterin homologues and occurs between amino acid residues 205 and 206 by an unknown enzyme (Jordan-Starck *et al.*, 1992; Jenne and Tschopp, 1992). The cleavage creates a 205 amino acid α subunit and a 222 amino acid β subunit.

A lot of information about putative structural features of clusterin has been deduced from the amino acid sequence of the protein. Figure 1.1 displays the approximate position of the four heparin binding domains, six glycosylation sites, numerous phosphorylation sites, and three amphipathic helices that are predicted to exist on the

α SUBUNIT



β SUBUNIT

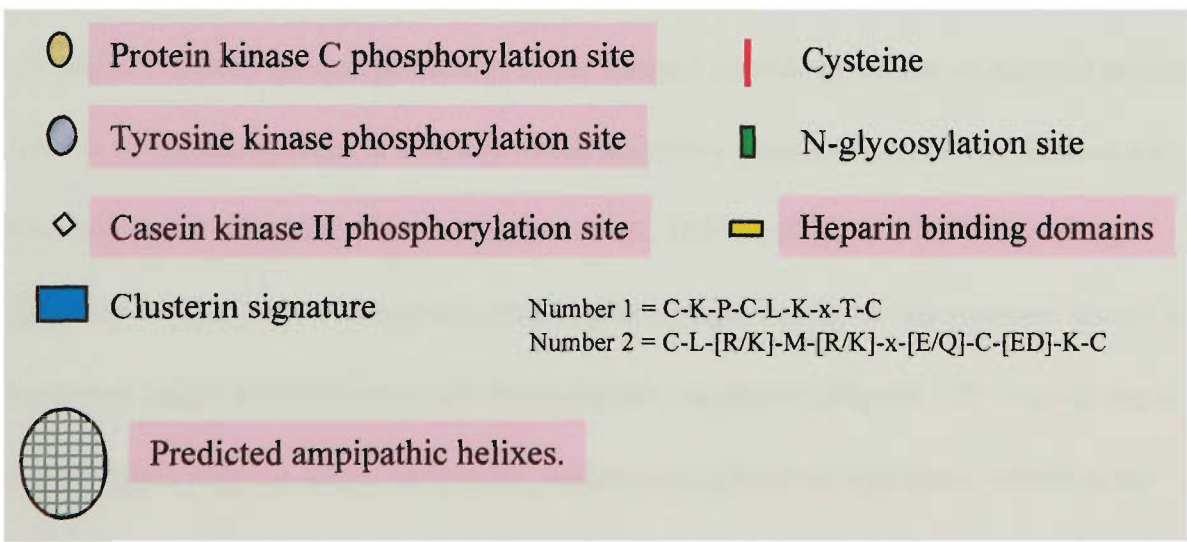


Figure 1.1: Schematic representation of the structure of clusterin showing the location of various known and predicted features (predicted features highlighted in pink in legend). All predicted features (except ampipathic helixes) were determined through software analysis performed at the following web site: http://prowl.rockefeller.edu/cgi-bin/sequence. Ampipathic helixes regions were determined by de Silva *et al.* (1990a). Glycosylation sites have been confirmed by Kapron *et al.*, 1997.

clusterin subunits. The glycosylation sites in clusterin are asparagine linked (or N-linked, i.e. have amino acid motifs of Asn-X-Ser/Thr, where X represents any amino acid except for proline (Alberts, 1989)), as Burkey *et al.* (1991) showed that carbohydrates associated with clusterin were removed by N-glycanase and not O-glycanase. Recent studies have confirmed that (i) clusterin has six glycosylation sites, which contain seven possible types of oligosaccharide structures, and (ii) clusterin is a heparin binding protein (Kapron *et al.*, 1997; Hennessy *et al.*, 1997; Pankhurst *et al.*, 1998). The binding of heparin to clusterin is independent of cations and the affinity increases at acidic pH (Pankhurst *et al.*, 1998).

There are two sequences that are common between clusterin species and they have been defined by the Prowl protein internet database (USA) as clusterin signatures (Figure 1.1). Using these electronic databases to compare protein sequences reveals that clusterin is a truly unique protein as it has limited homology to any sequenced protein. The most similar protein is a newly found secretory protein expressed in human rod photoreceptor cells (Shimizumatsumoto *et al.*, 1997) and has a 17.8% homology (identity) to clusterin. A sequence matrix plot comparing these two proteins shows a scattered linear distribution of the homologous sequences (Figure 1.2). One of these homologous regions aligns within the number one clusterin signature, which is the region most conserved between different species of clusterin, and indicates that these proteins are related.

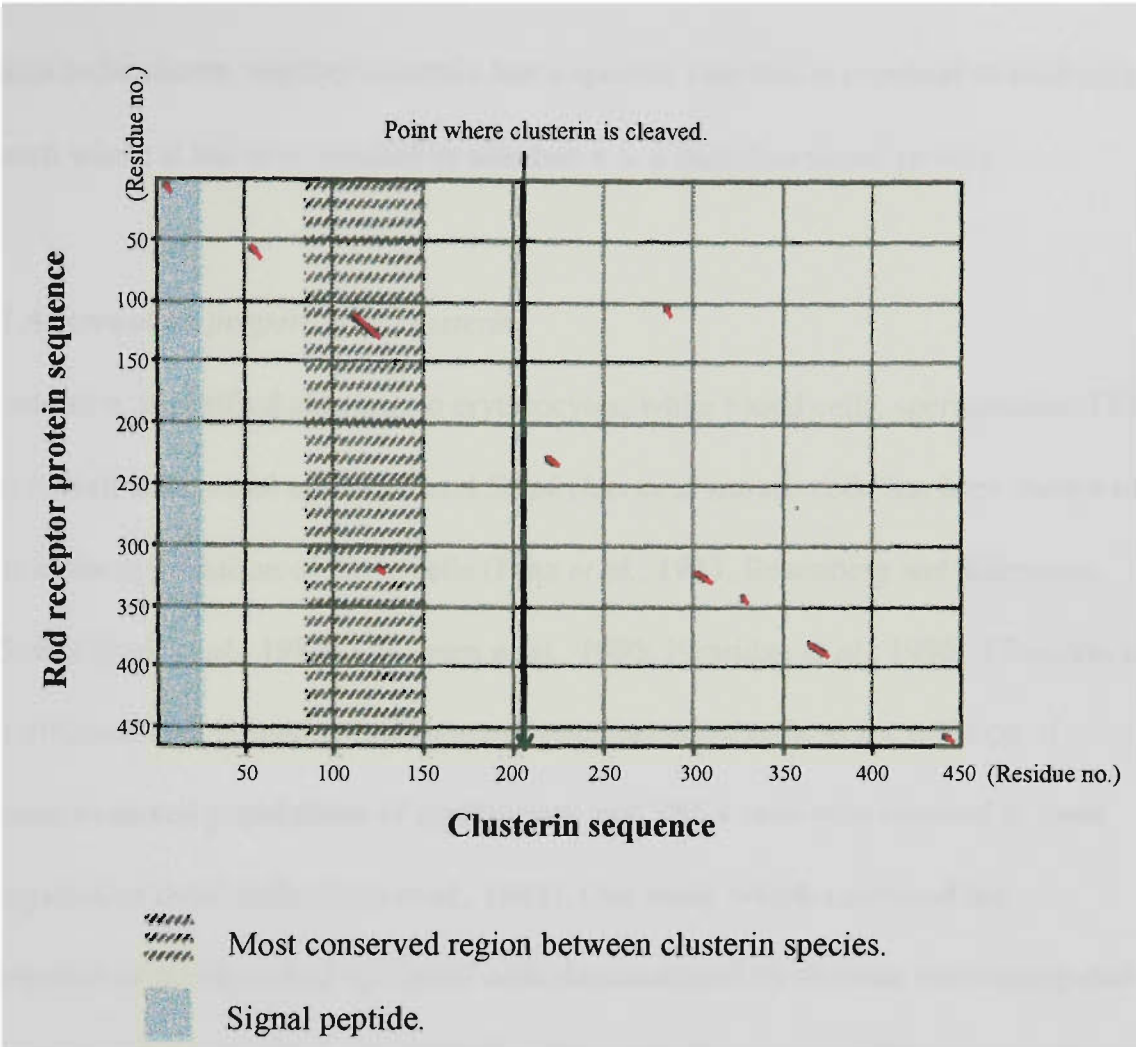


Figure 1.2: Scoring matrix comparing amino acid sequences of clusterin to the rod receptor protein as determined by MacVector 4.1.4 software. The red line corresponds to amino acid sequences that are at least 60 % homologous between proteins. Note a perfect match would be represented as a diagonal line from the top left hand corner to the bottom right hand corner. Software settings were: hash value = 2; and minimum % score = 60. Protein sequences were retrieved from the Entrez database (Rod photoreceptor protein sequence ID 961457; clusterin sequence ID 4502905).

1.2 CLUSTERIN INTERACTIONS

One of the problems associated with identifying clusterin's function is the wide variety of proteins to which it binds. Clusterin has been found associated with many proteins and is expressed in a variety of different cell types. Studies have linked clusterin to cell aggregation, reproduction, complement regulation, lipid transport, neurodegenerative diseases of the brain (eg Alzheimer, scrapie and Pick disease), and cell death. Unfortunately the function of clusterin in each of these areas has not been established. It

remains to be shown whether clusterin has a specific role that is common to each area of research where it has been studied or whether it is a multifunctional protein.

1.2.1 Aggregation properties of clusterin.

The addition of purified clusterin to erythrocytes, white blood cells, spermatozoa, TM-4 cells, Sertoli cells, renal epithelial and *Staphylococcus aureus* cells has been shown to result in the aggregation of those cells (Fritz *et al.*, 1983, Rosenberg and Silkensen, 1995; Blaschuk *et al.*, 1983; Silkensen *et al.*, 1995; Partridge *et al.*, 1996). Clusterin is most efficient in aggregating homogenous populations of cells as the addition of clusterin to mixed populations of erythrocytes and TM-4 cells only resulted in loose aggregation of those cells (Fritz *et al.*, 1983). One study which examined the aggregation of porcine renal epithelial cells demonstrated by electron microscopy that the addition of clusterin enhanced the formation of cell junctions (Silkensen *et al.*, 1995). This finding suggests that clusterin enhances homogenous cell interactions by binding to surface ligand(s) that are present only on particular cell membranes.

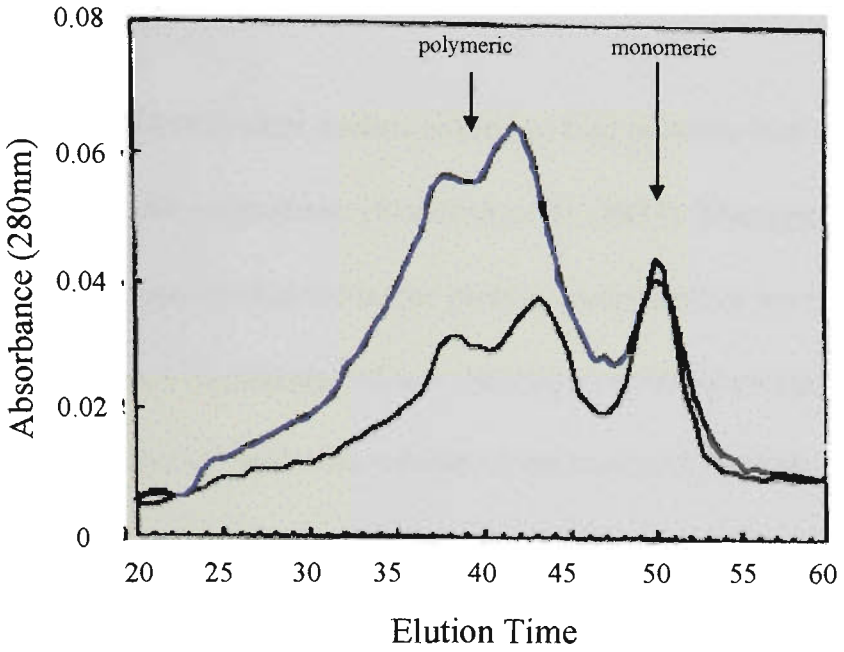
There is also evidence which suggests that clusterin is involved in more complex cell aggregation interactions. For example clusterin is upregulated in smooth muscle cells that form nodules but not in smooth muscle cells that grow in a monolayer (Diemer *et al.*, 1992). Futhermore Schwochau *et al.* (1998) demonstrated that the aggregation of cells due to the presence of clusterin *in vitro* enhanced their viability when exposed to H_2O_2 when compared to cells left in suspension. However it is likely that the increased viability was not a direct effect of clusterin as the aggregation of cells into the shape of a sphere resulted in inner cells becoming shielded from the cytotoxic agents by the outer

cells. It is unlikely that clusterin aggregates cells into the shape of a sphere *in vivo* because this shape is physiologically unfavourable as the inner cells would be starved of nutrients.

Clusterin is the main protein component of ram rete testis fluid and it is likely to be the factor in this fluid responsible for the aggregation of Sertoli cells *in vitro* (Fritz *et al.*, 1983). It has not been reported whether other bodily fluids, which contain clusterin, are also capable of aggregating cells *in vitro*. However, it appears that the aggregative effects of clusterin on various cell types *in vitro* are likely to be non-specific as there has been no evidence for high affinity clusterin binding sites on these cells (Fritz, 1995). Interestingly, the *in vitro* aggregating effect of clusterin has been shown (for one cell type) to be inhibited by the addition of carnitine or chemically related compounds (Fritz and Burdzy, 1989). Carnitine possibly interacts with clusterin or it could generally bind to and mask cell membranes thereby inhibiting the aggregation of cells.

Clusterin is also capable of aggregating weakly with itself to form dimers and tetramers (Blaschuk *et al.*, 1983, Griswold *et al.*, 1986). Gel filtration studies have shown that in purified clusterin preparations the ratio of aggregated clusterin to monomer clusterin increases with concentration (Figure 1.3a). This self-interaction is created by non-ionic forces as aggregated clusterin dissociates into monomers in the presence of 6M urea (Blaschuk *et al.*, 1983). Electrostatic forces may be partly responsible for aggregation as gel filtration studies demonstrate that aggregated clusterin is unstable at low pH (Figure 1.3b).

A



B

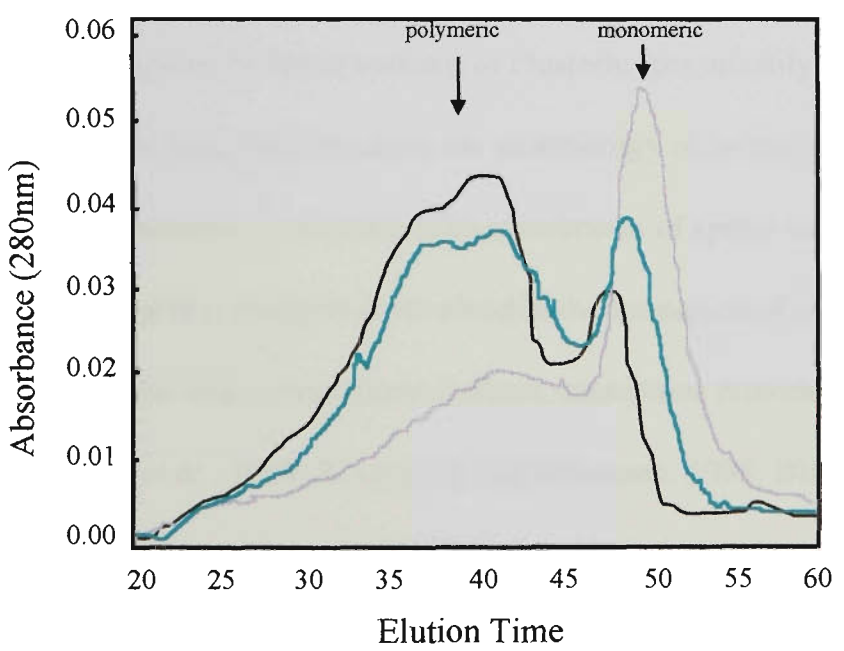


Figure 1.3: (**A**) The elution profile of 100 µl of immunoaffinity purified clusterin at 2 mg/ml (dark blue line), and 1 mg/ml (black line) in PBS (pH 7.4) when injected onto a Superose 6 gel filtration column (0.5 ml/min) (**B**) Effect of pH on the polymeric/monomeric ratio of immunoaffinity purified clusterin. 500 µl of clusterin at 1 mg/ml in MES/PBS (1:1) was adjusted to pH 5.5 (purple line), 6.5 (green line), or 7.5 (black line) and subjected to FPLC using a Superose 6 gel filtration column (0.5 ml/min). Experiments were performed by Tim Hochgrebe (manuscript submitted).

1.2.2 Reproduction

Clusterin was first identified in studies aiming to find proteins that were involved in somatic cell-germ cell interactions (Blaschuk *et al.*, 1983). Through these early findings it was found that clusterin was the major protein component of the testis fluid (18% of total protein content is clusterin) and was the major secretory product of Sertoli cells (epithelial cells of the seminiferous tubules of the testes) (Blaschuk *et al.*, 1983). As the function of Sertoli cells is to support and nourish immature sperm cells, this indicates that clusterin could be an important factor in the generation of mature sperm cells. More recently clusterin has been detected in the acrosome and tail region of almost all mature sperm cells (Sylvester *et al.*, 1984; Sylvester *et al.*, 1991). Different monoclonal antibodies that recognise different variants of clusterin (presumably different glycosylations) have been used to assess the morphology of human sperm (Obryan *et al.*, 1994). The association of clusterin with membranes of sperm cells have led to proposals suggesting that clusterin is involved in the formation of cytoskeletal components of sperm tails, complement defence, membrane remodelling and cell to cell interactions (Jenne *et al.*, 1989; Rosenberg and Silkensen, 1995; Blaschuk *et al.*, 1983).

1.2.3 Complement regulation

Complement is part of the humoral immune system which destroys invading pathogen cell membranes by forming, through a complex cascade of protein interactions, a membrane attack complex (MAC). Complement is tightly regulated and the disruption of any step in the cascade of protein interactions stops the formation of MAC. Clusterin has been shown to associate with complement complexes. In particular, clusterin binds to the complement proteins C7, C8b, and C9 (Tschopp *et al.*, 1993a). It has been

suggested that clusterin may regulate the formation of the MAC as stepwise additions of clusterin followed by complement proteins C5-6, C7, C8 and C9 (each at a concentration of 1 μg/ml) inhibited haemolysis of erythrocytes *in vitro* (Choi-Miura *et al.*, 1989; Murphy *et al.*, 1989). McDonald and Nelsestuen (1997) also reported similar findings. These studies are supported by an *in situ* experiment carried out on rat kidneys perfused with plasma that had or had not been depleted of clusterin. Complement-induced glomerular injury was increased in kidneys perfused with clusterin-depleted plasma (Saunders *et al.*, 1994). Clusterin has also been reported to localise with complement in various affected tissues (French *et al.*, 1994a; Vakeva *et al.*, 1993; Silkensen *et al.*, 1998). These findings led to claims that clusterin was a complement inhibitor *in vivo*, for example, it was proposed that clusterin on the surface of sperm reduced the chance of complement attack in the female genital tract (Kirszbaum *et al.*, 1989).

However, other studies strongly suggest that clusterin does not regulate complement *in vivo*. Complement is activated in infarcted tissue (Weisman *et al.*, 1990) and as clusterin colocalises at these sites (Vakeva *et al.*, 1994), this suggests that clusterin is inefficient at inhibiting the formation of the MAC. Similarly in a "physiological" assay containing a 1:100 dilution of human serum to which clusterin was added to 200 μg/ml (twice its physiological concentration), the rate of haemolysis of rabbit blood cells was not affected (Hochgrebe *et al.*, 1999). Futhermore, the extrapolation of this data set estimated that clusterin concentrations two orders of magnitude greater than is found in serum would be required to protect cells from complement in physiological conditions. In addition, the induction of cell surface human clusterin by transfected L929 cells did

not protect these cells from complement lysis in human serum (Hochgrebe *et al.*, 1999).

1.2.4 Binding to immunoglobulins

The passage of human plasma over an immunoaffinity column bearing covalently attached immunoglobulin G (IgG) suprisingly purified clusterin (Wilson *et al.*, 1991). It was found that clusterin specifically binds to the Fc and Fab regions of Ig by a multivalent mechanism (Wilson *et al.*, 1991; Wilson and Easterbrook-Smith, 1992). Initial experiments demonstrated that purified clusterin could enhance the formation of antibody-antigen complexes (Wilson *et al.*, 1991). However, this was later shown to be the effect of the complement protein C1q which unknowingly copurified with clusterin (Roeth and Easterbrook-Smith, 1996).

1.2.5 Lipid transport

Isolation of clusterin from human plasma by immunoaffinity chromatography led to the finding that a 28 kDa protein co-purified with clusterin (Jenne *et al.*, 1991). This 28 kDa protein was identified as apolipoprotein A-I (apo A-I) and the interaction between it and clusterin was demonstrated to be hydrophobic as they dissociated in the presence of non-ionic detergents (Jenne *et al.*, 1991). However this interaction appears to be specific as serum clusterin binds to immobilised apo A-I (Jenne *et al.*, 1991; Kunutake *et al.*, 1994) and the mole ratio of apo A-I copurifying with clusterin in serum remains constant (Stuart *et al.*, 1992). Apo A-I is the major protein component of high density lipoprotein (HDL) complexes that are involved in the transport of lipids around the body (Stryer, 1988). Interestingly clusterin has also been purified from a subclass of serum HDL particles that contain apo A-I and cholesteryl ester transfer protein activity (de

Silva *et al.*, 1990c; Hennessy *et al.*, 1997; James *et al.*, 1988).

The association of clusterin with HDL particles suggests that clusterin could be involved in reverse cholesterol transport. The reverse cholesterol transport mechanism, proposed by Glomset (1968), predicts that HDL particles are scavangers for cholesterol and once they are loaded transport it via the circulatory system to the liver for excretion or recycling. One study has shown that only 5% of the apo A-I-HDL species were responsible for the transfer of cholesterol from fibroblasts to plasma (de Silva *et al.*, 1990a). Wilson *et al.* (1995) suggests that this small subspecies of apo A-I-HDL could be unique in that it contains clusterin (it is estimated that 2-4% of total apo A-I contains clusterin (Jenne *et al.*, 1991)) which may be the essential ingredient allowing the HDL particle to remove cholesterol. Clusterin's affinity for the various immune components may (i) direct HDL complexes to cells that are to be removed, and (ii) link HDL particles to cell membranes allowing transfer of cholesterol from the cell surface to the HDL particle (Figure 1.4).

Interestingly a distinct HDL subspecies has been reported to exist which not only contains apo A-I and clusterin but a 44 kDa enzyme called paraoxonase (Blatter *et al.*, 1993). Paraoxonase is one member of a poorly understood multigene family (Primo-Parmo *et al.*, 1997) and is classed as an "A"-esterase which catalyses the hydrolysis of organophosphates, aromatic carboxylic acid esters, and carbamates (La Du, 1992). It is unknown whether this HDL subspecies is capable of transporting cholesterol. However a recent study indicates that it may be capable of tranporting cholesterol as this

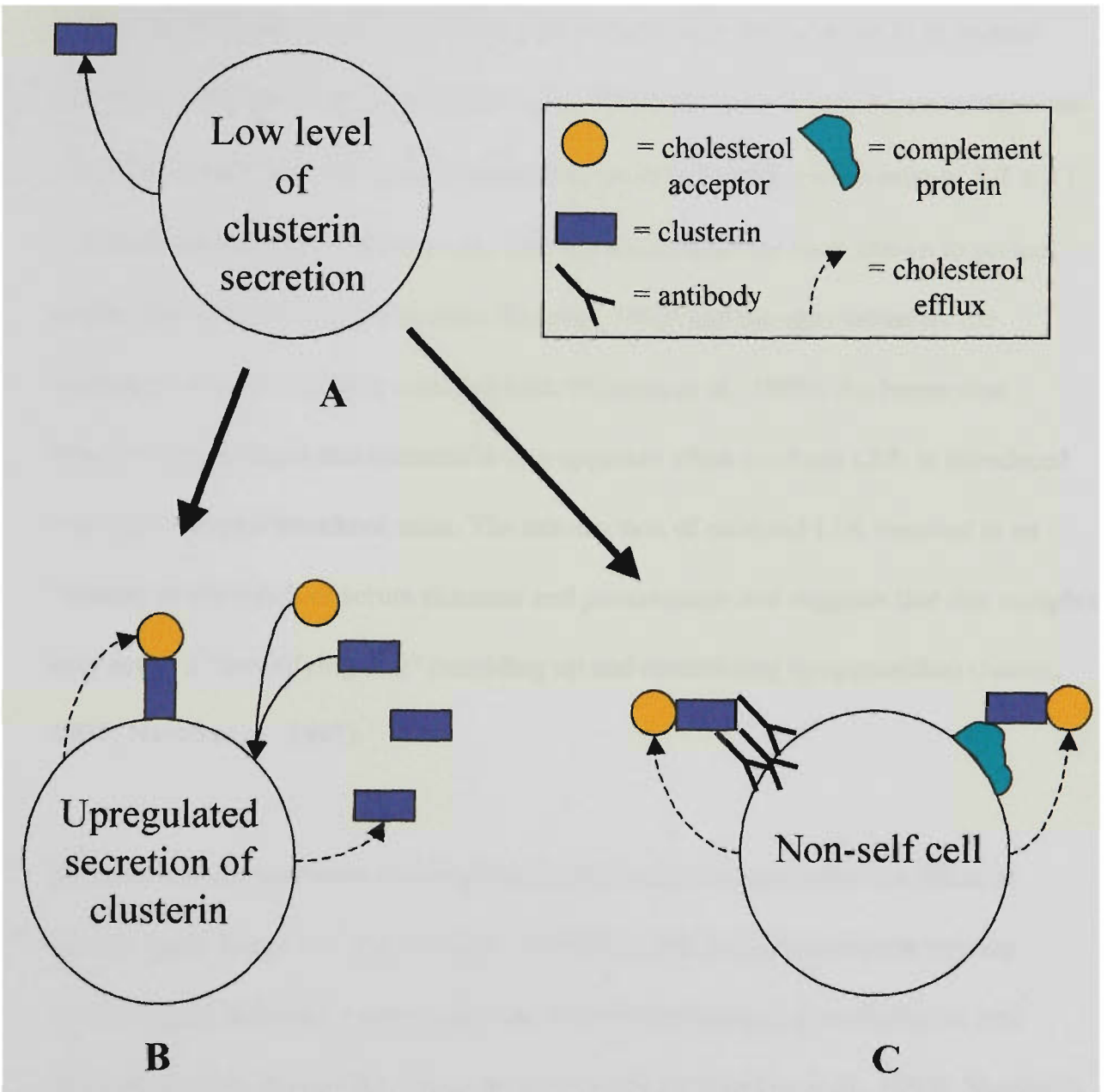


Figure 1.4: The lipid recycling hypothesis. Under non-stressed conditions cells typically express low amounts of clusterin (**A**). Cells which are damaged, or are about to be destroyed tend to upregulate clusterin production (**B** and **C**). It is proposed that clusterin may interact with both Ig (or MAC) and apolipoprotein A-I/HDL. Such an interaction would allow HDL particles to be transported to cells, which are to be broken down, and perhaps anchor the complex onto the membrane surface. There the HDL complex could collect cholesterol from the cell surface before taking it to be recycled. By a similar mechanism clusterin may also escort HDL particles to foreign cells, and aid in their removal by collecting cholesterol from the membrane. Modified from figure 5.5 in Wilson *et al.*, 1995.

subspecies of HDL was detected in artery walls atherosclerotic plaques (Mackness *et al.*, 1997). Paraoxonase has also been reported to co-purify with clusterin from human plasma by immunoaffinity chromatography. The association between paraoxonase and clusterin was shown to be specific and saturable *in vitro* with a mole ratio of 8.2 ± 2.1 (clusterin:paraoxonase) (Kelso *et al.*, 1994). Paraoxonase has been shown to protect against chlorpyrifos toxicity in mice (Li *et al.*, 1995) and can also modulate the biological activity of mildly oxidized LDL (Watson *et al.*, 1995). An interaction between paraoxonase and clusterin is also apparent when oxidized LDL is introduced into LDL receptor knockout mice. The introduction of oxidised LDL resulted in an increase in the levels of serum clusterin and paraoxonase and suggests that this complex may act as a "detoxifying unit" (rounding up and neutralising lipoperoxides) (James, 1995; Navab *et al.*, 1997).

Evidence has accumulated showing that clusterin alone can enhance the efflux of various lipids from cells. For example, in HepG2 cells, secreted clusterin exports various lipids including triglycerides, unesterified cholesterol, phospholipids, and cholesterol esters presumably from the cell membrane (Burkey *et al.*, 1992). Similarly clusterin assembles into lipoprotein particles when secreted from retinal Muller glial cells (Shanmugaratnam *et al.*, 1997). Furthermore clusterin has been shown to enhance the efflux of cholesterol from macrophage foam cells (Gelissen *et al.*, 1998). This lipoprotein like activity of clusterin supports its possible involvement in cholesterol transport.

1.2.6 GP330 - clusterin receptor

Glycoprotein 330 (gp330, previously known as megalin) is a membrane associated glycoprotein which is associated with the autoimmune disorder Heymann's nephritis, which is the animal model of human membranous glomerulonephritis. This disease initiates when circulating antibodies bind to antigens on the surface of epithelial cells of glomeruli. The antigen-antibody complexes then shed and collect in the base membrane causing nephritis (Alousi *et al.*, 1969). gp330 is expressed in epithelial cells of the kidney; it is an antigen which is recognised by antibodies circulating in affected glomeruli (Kerjaschki and Farquhar, 1983).

gp330 is related to the low density lipoprotein (LDL) receptor and the LDL receptor related protein (LRP) which all have the following common features:-

(i) cysteine-rich repeats,

(ii) YWTD repeats,

(iii) a single membrane spanning region and,

(iv) one or more NPXY internalization signals (Brown *et al.*, 1991).

Several ligands have been shown to bind *in vitro* to gp330, some of these include receptor associated protein (RAP), lipoprotein lipase, lactoferrin, Ca^{2+}, albumin, thyroglobulin and tissue/urokinase plasminogen activator : plasminogen activator inhibitor -1 complexes (Willnow *et al.*, 1992; Moestrup *et al.*, 1993; Hjalm *et al.*, 1996; Cui *et al.*, 1996; Zheng *et al.*, 1998). While the interaction between gp330 and any one of these ligands are specific they don't help identify the function of gp330 because (a) the distribution of these ligands throughout the body is different to gp330 and; (b) these

ligands are not specific for gp330 as they also have high affinity for the more widely distributed LPR. Further studies attempting to find a specific ligand for gp330 and not LPR successfully isolated a protein from milk that was identified as clusterin (Kounnas *et al.*, 1995). Following specific binding to gp330, clusterin is internalised and subsequently degraded by F9 cells (Kounnas *et al.*, 1995). Clusterin therefore appears to be a specific gp330 ligand and further research is required to elucidate the purpose of this interaction.

1.2.7 Neurodegenerative diseases and amyloid β peptide

Alzheimer's disease (AD), Pick's disease (PD) and Huntington disease (HD) are all neurodegenerative diseases which affect neurons of the hippocampus. Clusterin has been found to be upregulated in these damaged tissues and its specific role in these diseases is unclear (Duguid *et al.*, 1989). Most of the research to date has focused on clusterin's association with AD.

In AD brain tissue clusterin has been detected interacting specifically with the affected dystrophic neurites and associating in neuropil threads (McGeer *et al.*, 1992). Another classical pathological feature of AD is the formation of amyloid deposits, with which clusterin also associates (McGeer *et al.*, 1992). Little is known about the toxicity of these plaques or how they form. The principle component of these deposits is amyloid β (aβ) peptide which is a product from the cleavage of amyloid precusor protein (Sisodia and Price, 1995). Soluble aβ has been found in plasma, cerebrospinal fluid and cell supernatants and associates with clusterin (Ghiso *et al.*, 1993). The interaction between clusterin and aβ is specific (dissociation constant = 2×10^{-9} M) and is not dissociated in

the presence of other aβ affinity ligands (Matsubara *et al.*, 1995). Presumably aβ is the ligand to which clusterin binds in amyloid deposits.

It is unclear whether clusterin has any effect on the formation of amyloid deposits *in vivo*. Clusterin has a higher affinity for soluble aβ than the aggregated polymer form (Matsubara *et al.*, 1995). The origin of aβ in the brain of AD patients is unknown. Perfusion experiments have been performed to determine whether clusterin is capable of transporting aβ to the brain. In one perfusion experiment, aβ-clusterin complexes were demonstrated to be transported more efficiently across the blood brain barrier into the brain than aβ alone (Zlokovic *et al.*, 1994). Further studies implicated that aβ-clusterin complexes were transferred across the blood brain barrier via the binding of clusterin to gp330 (Zlokovic *et al.*, 1996). However, in another study where radiolabelled clusterin was injected intravenously, no transfer of radiolabelled clusterin to the brain was detected (Shayo *et al.*, 1997). This suggests that clusterin does not cross the blood brain barrier *in vivo* which therefore indicates that it is not capable of transporting aβ into the brain. In addition, clusterin purified from the brain is 5 kDa smaller than clusterin from serum (Oda *et al.*, 1994), which suggests that the clusterin associated with aβ in the brain is locally produced. Clusterin produced in the brain may also regulate the formation of amyloid plaques as clusterin is capable of partially blocking the aggregation of aβ (Oda *et al.*, 1995), however further research in this area is required.

aβ has been shown to be neurotoxic to primary neuron cultures (Yankner *et al.*, 1990; May *et al.*, 1992a), which implies that the presence of amyloid plaques in AD contribute to cognitive decline. However, it is unclear whether clusterin can interfere with the

toxicity of aβ. One study found that clusterin-aβ complexes were more lethal (as determined by an oxidative stress assay) than soluble aβ to PC12 cells (Oda *et al.*, 1995), while another demonstrated clusterin protected rat hippocampal cultures from the toxic effects of aβ (Boggs *et al.*, 1996).

Clusterin is also associated with other neuron diseases. For example clusterin colocalises in apoptotic photoreceptor cells (Agarwal *et al.*, 1996), and is also deposited in senile plaques in brains of Down syndrome patients (Kida *et al.*, 1995). Similarly the expression of clusterin is increased in cells of the rodent brain after transient global ischemia (May *et al.*, 1992b; Kida *et al.*, 1995), neurons of wobbler mice (Popper *et al.*, 1997), in retinitis pigmentosa (Jomary *et al.*, 1993a and b; Jones *et al.*, 1992; Smith *et al.*, 1995; Wong *et al.*, 1994), and in the hippocampus following entorhinal cortex lesioning (Lampert-Etchells *et al.*, 1991). These reports suggest that clusterin plays an important role in diseased or stress affected neurons.

1.2.8 TGFβ

TGFβ has been shown to regulate clusterin gene expression in a cell dependent fashion. In several fibroblast and epithelial cell lines TGFβ has been shown to upregulate clusterin expression (Jin and Howe, 1997) which in one case resulted in a truncated 43 kDa form of clusterin becoming localised to the nucleus (Reddy *et al.*, 1996). Similarly, TGFβ was shown to upregulate clusterin expression in neurons and astrocytes (Laping *et al.*, 1994). The upregulation of clusterin in cells treated with TGFβ involves protein kinase C signalling and requires the AP1 binding site in the clusterin promotor (Jin and Howe, 1997). However TGFβ has also been found to downregulate clusterin expression

(Thomas-Salgar and Millis, 1994). The different regulatory effects of TGFβ indicate that other unidentified factors are responsible for the control of clusterin expression. For example, treatment of astrocyte cultures with TGFβ results in the downregulation of clusterin expression, however, when oligodendrocytes and microglia are present, TGFβ treatment results in the upregulation of clusterin expression in astrocytes (Morgan *et al.*, 1995).

Clusterin has also been shown to interact with the intracellular domains of both type I and type II TGFβ receptors *in vitro* (Reddy *et al.*, 1996). However it seems unlikely that clusterin is directly involved in TGFβ signaling, as clusterin is normally a secreted protein and thus is not confined to the cytosol of the cell.

1.3 CLUSTERIN AND APOPTOSIS

Apoptosis is a form of programmed cell death which is initiated when cells are not needed (e.g. self reactive thymocytes (Coleman, 1992)). Apoptosis is a selective event and therefore must be tightly regulated. Clusterin may also be involved in regulating apoptosis as clusterin mRNA was found to be one of the dominant upregulated mRNA species in the rat ventral prostate following castration (which is associated with a high level of programmed cell death) (Montpetit *et al.*, 1986, Leger *et al.*, 1987). Castration results in the regression of the ventral prostate due to the ablation of androgens normally required to maintain homeostasis. The regression of this tissue appears to require protein synthesis, and as clusterin expression is upregulated specifically in these dying cells (Buttyan *et al.*, 1989), as well as in other apoptotic cells, it has been suggested that it plays a functional role in apoptotic cell death.

Research performed by Akakura *et al.*(1996) suggest that clusterin plays a protective role in the cell death of castrated rat prostate cells. This study created androgen-independent cell lines by repeatedly castrating and replanting tissue into other mice. Cells which became androgen-independent by not dying after castration were found to have more intracellular clusterin compared to androgen-dependent cells which die after castration (Akakura *et al.*, 1996). Clusterin in the androgen-independent cells localized to the nucleus suggesting it may help regulate protein expression. However, it is possible that the expression of clusterin in these cells may only be coincidental to cell survival. Further research in this area is therefore required.

Other studies have also found clusterin upregulated in tissues/cells undergoing apoptosis where it may also have a cytoprotective effect. For example, inducing status epilepticus in rats results in apoptosis of brain nerve cells which upregulate clusterin expression (Dragunow *et al.*, 1995); rat mammary carcinoma cells treated with anti-estrogen toremifene which inhibits growth and causes cell death results in those cells upregulating clusterin (Huovinen *et al.*, 1993); and numerous cell lines induced to undergo apoptosis also upregulate clusterin expression (Kyprianou *et al.*, 1991, Simboli-Campbell *et al.*, 1996). Similarly, it may explain the correlation between high clusterin expression and tumour potency seen in glioma and prostate cells (Danik *et al.*, 1991; Steinberg *et al.*, 1997). The possible cytoprotective capabilities of clusterin may also explain its presence and upregulation in neurodegenerative diseases. However it is unclear how clusterin could act as a cytoprotective agent. Because clusterin is normally a secreted product, it may function locally outside the cell, perhaps on membranes of

stressed cells. Immunofluoresence studies by Simboli-Campbell *et al.* (1996) did detect small amounts of clusterin localising to the cell membrane surface of early apoptotic MCF-7 cells. Interestingly, they also detected a large amount of clusterin in apoptotic bodies and secretory vesicles of late apoptotic MCF-7 cells, which could have been accumulated from outside the cell or specifically produced by those cells.

A study carried out by Bursch *et al.* (1995) on rat liver demonstrated that clusterin can not only be upregulated in cells undergoing apoptosis but also in proliferating cells. The study involved administering high doses of the hepatomitogen cyproterone acetate (CPA) which results in the doubling of liver size within six days. During this period of growth, clusterin was found to be upregulated, before returning to background levels once the maximum growth had been achieved (even though CPA treatment continued). Withdrawal of CPA caused a decrease in liver weight resulting from cells dying by apoptosis, which also correlated with high levels of clusterin mRNA expression (Bursch *et al.*, 1995).

There have also been several cases where clusterin upregulation is not detectable in cells undergoing apoptosis. For example, Jomary *et al.* (1995) found that clusterin is not increased in apoptotic photoreceptor cells, while Garden *et al.* (1991) could not detect clusterin expression in apoptotic cells from the central nervous system or nonneural tissue. Similarly in the hair folicle which undergoes cycles of growth, regression and rest, clusterin was shown only to be expressed during periods of growth (Seiberg and Marthinuss, 1995). Furthermore, in apoptotic thymocytes clusterin was found to be upregulated only in surviving cells and not dying ones (French *et al.*, 1994b). It is

apparent therefore that clusterin expression is not required for cell death to occur. Similarly, clusterin expression does not cause cell death as the viability of transfected cell lines which overexpress clustein was no different to the parental cell lines (Pilarsky *et al.*, 1993; Sensibar *et al.*, 1995; Humphreys *et al.*, 1997).

The different effects clusterin has on cell death may relate to variant clusterin species that have recently been reported. Kimura and colleages (1996 and 1997) identified alternative splicing variants of clusterin mRNA that were produced in various organs of rats injected with cycloheximide or treated with heat. Clusterin variants had deletions in exon 5 resulting from alternative splicing, in which the deleted regions differed not only between species but also between cell types of the same species (Kimura *et al.*, 1997). Similarly, clusterin mRNA variants were also found in prostate, liver and thymus in response to androgen (Izawa, 1998) and two variants were detected in mouse testes (Lee *et al.*, 1993). However, as these independent studies employed rt-PCR which does not correct for complex mRNA conformation structures such as hairpin loops it is possible that these variants are artifacts of the rt-PCR procedure. Further studies will be required to confirm the presence of these alternative transcripts and to determine whether they have different effects on cell death.

1.4 CONCLUSION

Clusterin has been associated with many areas of research, including studies of cell maturation and reproduction, cell death, complement regulation, and lipid recycling. It's expression and interaction with various biological ligands indicates that either clusterin is a multifunctional protein or that it has a single unifying function.

The aims of this thesis were two fold:

i) To investigate the involvement of clusterin expression in cell death by constructing transfected cell lines which inducibly or constitutively express clusterin.

ii) To test the hypothesis that clusterin may have a chaperone-like action (Introduced in chapter 5).

These aims were addressed by:

(i) Identifying agents which induce apoptosis in L929 cells.

(ii) Creating the following stably transfected L929 cell lines:-

(a) A cell line which can inducibly secrete human clusterin.

(b) A cell line which constitutively expresses high levels of human clusterin.

(c) Appropriate control cell lines:-

- a cell line that constitutively expresses high levels of green fluorescent protein (GFP).

- cell lines transfected with the same plasmids as in (a) or (b) but lacking the clusterin cDNA insert.

(iii) Using these transfectant cell lines to analyse whether human clusterin expression provides protection against various forms of stress.

The L929 cell line was chosen for this study because:-

- It is a murine derived cell line and therefore does not express human clusterin. Transfection of L929 cells with human clusterin cDNA allowed specific detection of over-expressed protein using human-specific monoclonal antibodies.
- It is a hardy cell line that is easily transfected by electroporation.

(iv) Sequence analysis predicts that clusterin has three putative amphipathic α-helical regions known to be important for interactions with hydrophobic molecules. It has also been demonstrated that clusterin has a high affinity for hydrophobic surfaces as it can be purified from ram rete testis fluid on the basis of it's strong hydrophobic interaction with Affi-blue gel (Blaschuk *et al.*, 1983). Other reports have also suggested that clusterin's association with complement protein C9, apolipoprotein A-I and cell aggregation are due to hydrophobic interactions (Tschopp *et al.*, 1993; Jenne *et al.*, 1991; Fritz, 1995). During these studies, Michel *et al.* (1997) reported that the clusterin promotor contains an element that specifically recognises the transcriptional activator heat shock factor 1 (HSF 1). It was also demonstrated that this element can mediate heat-shock-induced transcription (Michel *et al.*, 1997) suggesting that expression of clusterin and heat shock proteins may be co-regulated. Against this background it was hypothesized that clusterin may have some small heat shock protein-like chaperone activity. Therefore, several *in vitro* heat and reduction-mediated denaturing assays, commonly used to identify heat shock proteins (Jakob *et al.*, 1993; Horwitz, 1992), were used to determine whether clusterin has any chaperone activity.

CHAPTER 2

General Materials and Methods

2.1 CELL CULTURE

2.1.1 Tissue Culture Reagents

DMEM:F-12 tissue culture medium was purchased from Gibco BRL (Melbourne, Australia). Fetal bovine serum (FBS), trypsin/EDTA (1:250), and all other tissue culture reagents were obtained from Trace Biosciences Pty Ltd (Sydney, Australia).

Table 2.1 General recipes of TC products. Note: all items were filtered sterilised or autoclaved and only opened in a laminar flow hood.

Item	Recipe	pH
PBS	68.5 mM NaCl, 1.3 mM KCl, 0.74 mM KH_2PO_4, 4 mM Na_2HPO_4	7.4
EDTA/PBS	5 mM EDTA in half strength PBS (diluted in RO water)	7.4
DMEM:F12	As manufacturers instructions + 2 g/l $NaHCO_3$ + 2.383 g/l HEPES	7.2
Freeze mix	50% (v/v) FCS, 10% (v/v) DMSO, 40% DMEM:F12	

Table 2.2 Preparations of various stock solutions of fluorescent stains used in tissue culture. Note: as these items were used for short incubation times they were not sterilised.

Stain	Stock concentration	Dissolved in
Propidium iodide	1 mg/ml	RO water
7-amino-actinomycin D	5 mg/ml	DMSO
Acridine orange	1 mg/ml	DMSO

Table 2.3 Preparations of various cytotoxic agents used in tissue culture.

Cytotoxic agent	Stock concentration	Dissolved in
TGFβ	2 μg/ml	PBS
TNFα	0.5 mg/ml	PBS
Actinomycin D	1-5 mg/ml	DMSO
Colchicine	2 mM	DMSO
Staurosporine	1 mM	DMSO
Azide	10% (w/v)	RO water

2.1.2 General methods

2.1.2.1 Maintenance

All cell lines were grown in DMEM:F12 growth media supplemented with 10% (v/v) foetal calf serum (FCS). Cells were incubated in a Forma Scientific humidified incubator at 37°C, 5% CO_2 unless otherwise mentioned.

2.1.2.2 Freezing cells

Cells to be frozen were pelleted at 2000 rpm (Beckman TJ-6) for 5 minutes. The pellet was resuspended in cold freeze mix (defined in section 2.1) and aliquoted (1 ml) into cryovial tubes (Interpath, Sydney). Cryovials were incubated at -80°C overnight in a cryogenic freeze container which lowered the temperature at a rate of approximately 1°C per minute. Thereafter cryovials were permanently stored in liquid nitrogen until needed.

2.1.2.3 Thawing cells

Cryovials were quickly thawed in a 37°C water bath. The cellular suspension was washed and pelleted in PBS at 2000 rpm (Beckman) for 5 minutes. The pellet was resuspended in growth media and maintained in tissue culture flasks. After thawing, cells were not used in experiments for approximately one week to allow them time to recover from being frozen.

2.1.2.4 Passaging

Suspension cells: 95% of the growth media was poured off and collected if needed. Warm fresh media was returned into the tissue culture flask.

Adherent cells: Growth media was poured off and collected if needed. Enough EDTA/PBS was put on to cover cells. Cells were incubated for 5 minutes at 37°C. Approximately 80% of the cells were then removed, using a pipette, by vigorous passage of EDTA/PBS over the inner surface of the culture vessel. The EDTA and cells were removed from the flask and replaced with fresh growth medium.

2.1.3 Cell Lines used in this study

L929: Fibrosarcoma cell line; Murine.

HL60: Myeloid leukemia cells; Human.

HeLa: Cervix epithelial carcinoma cells; Human.

MCF-7: Breast cancer cells; Human.

G7: Murine hybridoma cell line received as a gift from Dr B Murphy (St Vincents Hospital, Melbourne, Australia). Secretes monoclonal $IgG_1\kappa$ antibodies which specifically recognise human clusterin.

78E: Murine hybridoma cell line which secretes monoclonal $IgG_1\kappa$ antibodies which specifically recognise human clusterin (Wilson & Easterbrook-Smith, 1993).

41D: Murine hybridoma cell line which secretes monoclonal $IgG_1\kappa$ antibodies which specifically recognise human clusterin. 41D was made by conventional methods using immunoaffinity purified human serum clusterin as antigen (Wilson M.R, unpublished).

DNP-9: Murine hybridoma cell line. Secretes monoclonal $IgG_1\kappa$ antibodies which recognise dinitrophenol groups (Wilson & Easterbrook-Smith, 1993).

2.2 ELECTROPHORESIS

2.2.1 SDS/PAGE Buffers

* Acrylamide/bisacrylamide stock solution 30% : 0.08 % (w/v) in milli Q water.

Table 2.4: Recipe for either 0.75mm or 1mm thick SDS-PAGE gels. All values are in ml unless otherwise specified.

Gel thickness	0.75 mm			1.0 mm		
[Acrylamide] %	10%	12.5%	15%	10%	12.5%	15%
Acrylamide/bisacrylamide	3.3	4.13	4.95	4.95	6.2	7.43
0.75 M tris cl, pH 8.8	5.0	5.0	2.5	7.5	7.5	3.75
10% SDS	0.1	0.1	0.1	0.15	0.15	0.15
TEMED	0.007	0.007	0.007	0.01	0.01	0.01
1 % ammonium persulfate	0.5	0.5	0.5	0.75	0.75	0.75
H_2O	1.1	0.26	1.95	1.65	0.39	2.93

* Stacking gel:

Acrylamide/bisacrylamide	0.33 ml
1.0 M tris CL, pH 6.8	0.42 ml
10% SDS	0.033 ml
H_2O	2.4 ml
TEMED	0.007 ml
1% ammonium persulphate	0.17 ml

* 2x Sample Buffer: 0.5 M tris Cl (pH 6.8)

20% glycerol (v/v),

5% SDS (w/v) ,

0.005% bromophenol blue (w/v).

* Running Buffer: 0.025 M tris

0.192 M glycine

0.1% SDS

pH 8.3

* Coomassie blue stain: 0.25% (w/v) coomassie blue (R250)

500 ml Methanol

500 ml RO H_2O

100 ml Glacial AcCOOH

* Destain solution: 500 ml Methanol

500 ml RO H_2O

100 ml Glacial AcCOOH

2.2.2 SDS/PAGE Methods

2.2.2.1 Loading the acrylamide solution

The acrylamide solutions were set in either a "tall mighty small" (Pharmacia, Melbourne, Australia) or Novex (Sydney, Australia) gel casting apparatus sealed at the bottom by a 1% agar mix. While the separating gel was setting 1-2 ml of ethanol was placed on top to remove any air bubbles and to create a horizontal interface for the stacking gel. When the separating gel had set the ethanol was washed off with dH_2O, any remaining water was removed with blotting paper.

The stacking gel was loaded with the appropriate toothed comb and after it had polymerised the comb was removed and wells were washed and outlined on the

glass with a marker pen to help position the samples. Gels that were not to be used immediately were kept at 4°C in running buffer.

2.2.2.2 Sample preparation and electrophoresis of SDS PAGE gels

Samples were generally prepared in PBS + 0.02% azide to which a final equal volume of sample buffer was added. Reduced samples had the addition of 50 mM β-mercaptoethanol. Samples were boiled for approximately 5 minutes before being loaded into the wells.

After the samples were loaded, gels were electrophoresed at 130 V for approximately 2 hours. The gel was quickly rinsed with RO water and then gently removed from the ceramic backing plate.

2.2.2.3 Coomassie blue staining of SDS PAGE gels

SDS PAGE gels were placed in a plastic container followed by enough Coomassie blue R250 stain to cover the gel. A typical 10% SDS PAGE gel was stained for approximately 1 hour on a shaker at room temperature, while higher percentage gels were stained for longer time periods.

Stained gels were then destained by adding the destain solution and left on a shaker until the background was negligible. The destain solution was replaced when a considerable amount of Coomassie blue had leached out of the gel. For overnight destaining an equal volume of the destain solution and water was used. Destained gels were dried using the Novex Gel drying kit as described by

the manufacturer's instructions.

2.2.3 Western blotting

2.2.3.1 Semi-dry Transfer

SDS/PAGE gels were electrophoresed as described (section 2.2.2.2) without the staining procedure. After electrophoresis gels were equilibrated in transfer buffer (0.037% (w/v) SDS; 48 mM tris base; 29 mM glycine; 20% (v/v) methanol) for at least 30 minutes at room temperature. Ten pieces of blotting paper of identical size to the gel were also soaked in transfer buffer. Similarly, one piece of nitrocellulose (Sartorius, Melbourne, Australia) or PVDF (Millipore, Sydney, Australia) was cut to the exact size of the gel and soaked in transfer buffer.

Five layers of blotting paper were placed onto the semi dry transfer apparatus (Biorad, Hercules, CA, USA) followed by the nitrocellulose and then the gel

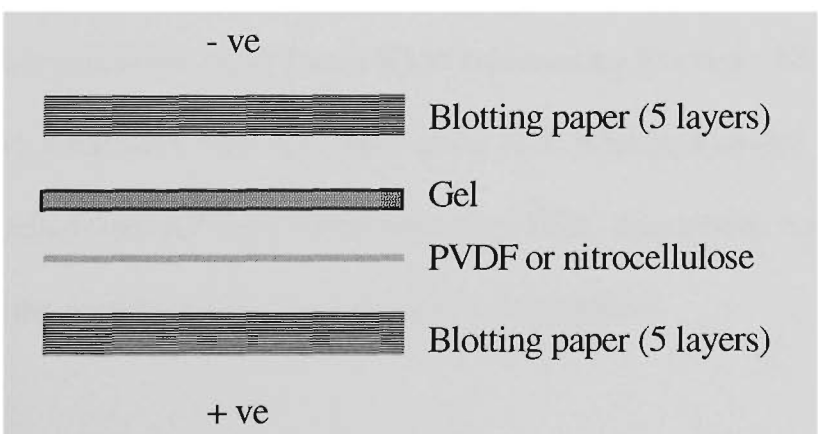


Figure 2.1: Arrangement for a semi-dry western transfer of proteins from a gel onto a PVDF or nitrocellulose membrane. Five pieces of blotting paper are layered followed by a sheet of PVDF or nitrocellulose membrane and then the gel. Another five layers of blotting paper are then layered on top. All components are cut to an equivalent size and are layered one piece at a time to avoid trapping air bubbles.

(Figure 2.1). Another five layers of blotting paper were then placed on top of the gel. The transfer was run at 15 volts constant for approximately 30 minutes (10% SDS PAGE gels) or 1 hour (15% SDS PAGE gels).

2.2.3.2 Immunolabelling western blots.

After proteins from SDS PAGE gels had been transfered to nitrocellulose or PVDF membrane they were blocked with HDC for 1 hour at room temperature. Membranes were incubated with the appropriate antibody, which was diluted in HDC or in many cases consisted of TC sn from hybridoma cultures. In each case, membranes were sealed in plastic bags, and then left on a shaker for 1 hour at room temperature.

The nitrocellulose strip(s) were then thoroughly washed in RO H_2O before being incubated in plastic bags with either sheep-anti-mouse Ig-HRP or sheep-anti-mouse Ig-AP conjugated antibody at a dilution of 1:1000 in HDC for 1 hour at room temperature. The nitrocellulose strips were again thoroughly washed in 0.5% (v/v) Triton X100 followed by RO H_2O. Membranes labelled with HRP were then developed using ECL (Pierce, Sydney), while membranes labelled with AP were developed using ECF (Amersham, Sydney) as described by the corresponding manufacturer's descriptions.

2.2.4 Agarose gel electrophoresis reagents

* 6x Sample buffer: 0.25% (w/v) bromophenol blue

40% (w/v) sucrose

0.25% (w/v) xylene cyanol FF

Made in milliQ water, stored at 4°C

* Running buffer (50x): 242g/l Tris base;

57.1 ml/l galacial acetic acid;

100 ml/l 0.5 M EDTA (pH 8.0).

2.2.5 Agarose gel electrophoresis

Agarose was dissolved in hot running buffer at the desired concentration. The hot agarose solution was poured into the casting tray (BioRad, USA) and then the comb was aligned and inserted. Once the agarose had set the comb was removed and the electrophoresis chamber was filled with running buffer. Gels were electrophoresed at 70 volts until the dye front had migrated the appropriate distance down the gel.

2.3 Bacterial culture

2.3.1 General recipes

* 2YT media: 5 g/l NaCl

16 g/l Tryptone (Oxoid, Melbourne, Australia)

10 g/l Yeast extract (Oxoid, Melbourne, Australia)

pH 7.0 using 5 N NaOH

Sterilized by autoclaving

* 2YT agar media: Same as 2YT media + 15 g/l of agar (Oxoid, Melbourne, Australia). Sterilized by autoclaving

* Wash Buffer: 10% glycerol (v/v in RO water) Sterilized by filtering or autoclaving

2.3.2 Freezing cells

Cells were pelleted and washed once in 10% glycerol. Cells were resuspended in 10% glycerol before being stored indefinitely at -80°C.

2.3.3 Preparing electrocompetent DH5α cells

A fresh colony of DH5α was innoculated in 5 ml of 2YT media and left to incubate overnight at 37°C with shaking. The 5 ml culture was expanded to 250 ml by incubating for 2-3 hours at 37°C until an OD_{550} of 0.8 was reached.

Cells were harvested at 4000 rpm for 10 minutes at 4°C and then resuspended in wash buffer. Cells were pelleted once again and resuspended in 2 mL of wash buffer. 40 µl aliquots were then aliquoted into sterilised Eppendorf tubes before being stored at -80°C.

2.3.4 Transformation of electrocompetent DH5α cells

25 ng of plasmid DNA was mixed with a 40 µl aliquot of DH5α electrocompetent cells. Cells were electroporated using a Biorad Gene Pulser with the following settings: 200 ohms, 25 µF, 2.5 kV.

Cells were left to recover for one hour in 2YT media at 37°C with no selection agent. Cells were plated on petri dishes prepared with 2YT-agar +100 µg/ml ampicillin in dilutions of 1/1, 1/10 and 1/100. Plates were incubated overnight at 37°C. Plate(s) containing distinct colonies were stored at 4°C until needed.

2.3.5 Purification and analysis of plasmids from transformed bacteria

Transformed bacteria were streaked onto 2YT agarose plates containing the appropriate selective agent and left to grow overnight at 37°C. The following morning plates were analysed and typically 2 - 4 isolated colonies were selected and grown up in 5 ml liquid 2YT cultures. Plasmids were purified from transformed cultures using Qiagen mini-prep columns following the manufacturer's instructions. Purified plasmid DNA was then analysed by agarose gel electrophoresis to confirm its identity. Transformed bacteria were frozen for later use.

CHAPTER 3

Producing stably transfected cell lines that inducibly or constitutively express human clusterin.

3.1 Introduction

Clusterin is found in most bodily fluids where it presumably collects from the numerous tissues/organs in which it is expressed. The expression of clusterin in healthy organs appears to be restricted mainly to epithelial cells and in some cases highly specialized cell types (Jordan-Starck *et al.*, 1992). However in times of stress many cells express clusterin and the amount produced is significantly higher than is the case for non-stressed cells. For example, a seventeen-fold increase in clusterin mRNA occurs in castrated rat prostate tissue (Bettuzzi *et al.*, 1989). Similarly, clusterin expression is increased four fold in tumours treated with toremifene (which induces apoptosis) compared to control tumours (Huovinen *et al.*, 1993), while in scrapie-infected brains of hamsters clusterin expression is increased ten fold compared to non-infected brains (Duguid *et al.*, 1989). The timing of clusterin upregulation during cell death has led some to believe that it is a cell death marker and predict that it may have a role in either cell protection or cell death (Buttyan *et al.*, 1989; Alitzky *et al.*, 1992). However, there are numerous reports that show cell death in the absence of clusterin expression. This indicates that clusterin expression is not essential for cell death to occur (refer section 1.3; Jomary *et al.*, 1995; Garden *et al.*, 1991; Seiberg and Marthinuss, 1995).

In order to create transfected cell lines secreting clusterin, use was made of two different commercial expression systems. The Lac Switch expression system from Stratagene (CA, USA) is comprised of two plasmids and allows controllable expression of any desired protein for which cDNA is available (Figure 3.1). Protein expression is controlled via the E.*coli* lactose operon in pOPRSVI. Repressor molecules transcribed from p3'SS have a high affinity for the lactose operon which when bound inhibit the

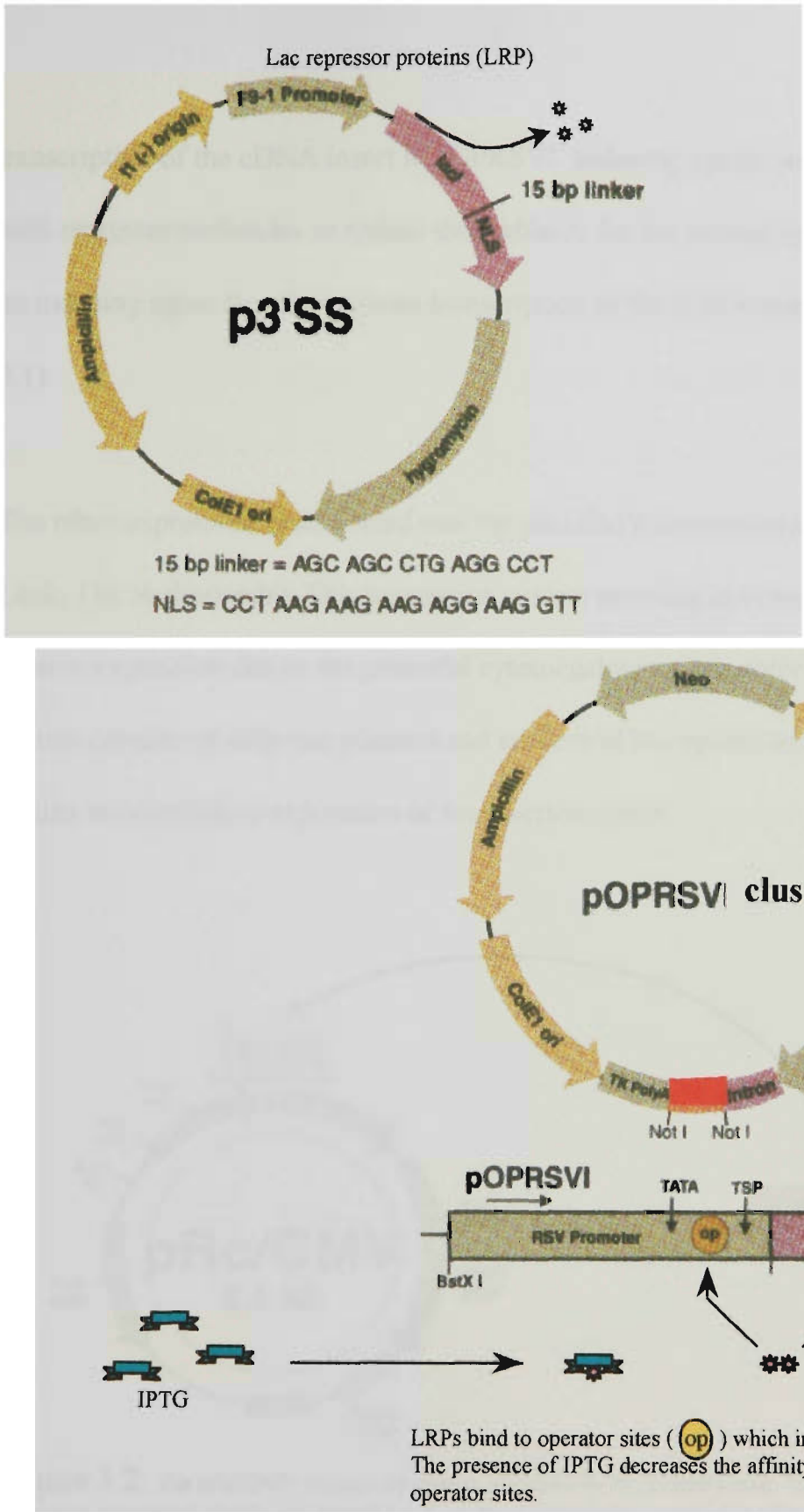


Figure 3.1: The Lac Switch expression system produced by Stratagene (CA, USA). Transfection with one plasmid, p3'SS, results in the endogenous production of lac repressor protein which inhibits the lac promotor on pOPRSVI. The second plasmid, pOPRSVI, is introduced containing a sequence coding for the desired protein, the expression of which is under the control of lac operator sites. Once stably integrated into the genome of the transfected cells, the addition of IPTG to the culture medium results in the inhibition of endogenous lac repressor molecules and thus expression of the desired protein.

transcription of the cDNA insert in pOPRSVI. Inducing agents such as IPTG interact with repressor molecules to reduce their affinity for the lactose operon. The addition of an inducing agent therefore allows transcription of the cDNA insert to proceed (Figure 3.1).

The other expression system used was the pRc/CMV expression system (Invitrogen, Leek, The Netherlands). This expression system provides extremely high levels of protein expression due to the powerful cytomegalovirus promotor (Figure 3.2). This system consists of only one plasmid and successful incorporation into the genome results in constitutive expression of the inserted cDNA.

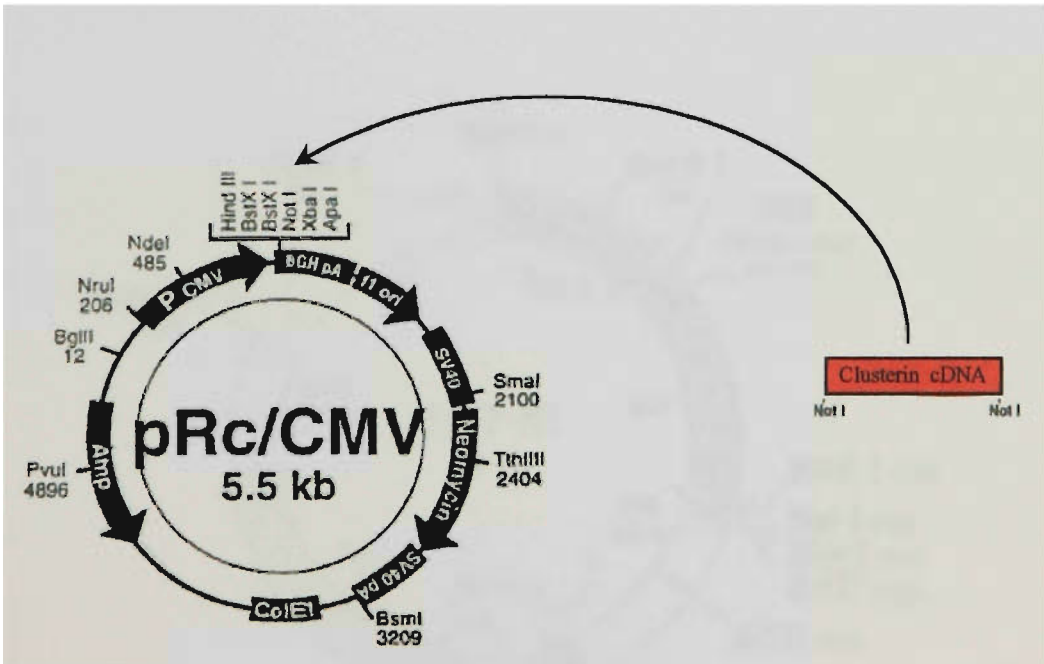


Figure 3.2: The pRc/CMV expression system produced by Invitrogen (Leek, The Netherlands). This system is comprised of only one plasmid and results in constitutive expression of the product coded by the inserted cDNA.

To create a negative control transfected cell line that expressed a non-relevant protein the pEGFP-N1 expression system (Clonetech, CA, USA) was used. The pEGFP-N1 expression system allows constitutive expression of the enhanced green fluorescent protein (EGFP) in mammalian cells. The EGFP is derived from green fluorescent protein (naturally produced by the bioluminescent jellyfish *Aequorea victoria*) and has higher fluorescence intensity than the native protein. The CMV promotor that is present in the CMV/pRc expression system (Figure 3.3) is also present in the EGFP expression system, providing high protein expression. This system was therefore used to act as a negative control for the other expression systems encoding clusterin.

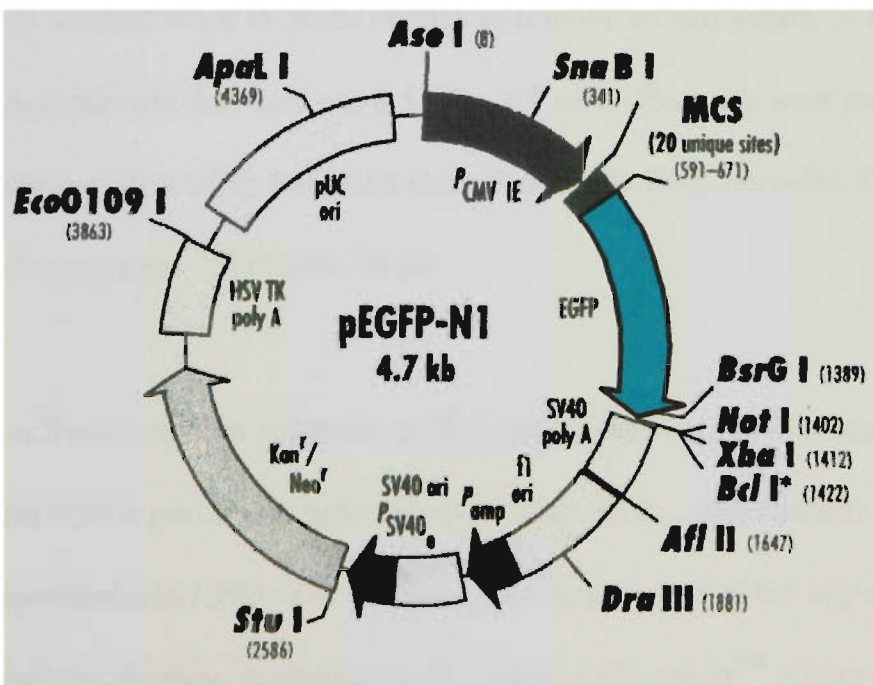


Figure 3.3: The pEGFP-N1 expression system produced by Clonetech (CA, USA). This system is comprised of only one plasmid and results in constitutive expression of EGFP. Note this protein does not contain a secretion signal and therefore expressed protein remains intracellular.

3.2 Methods

3.2.1 Plasmid amplification and purification - The Lac Switch system plasmids (p'3SS and pOPRSVI) were purchased from Stratagene (CA, USA). pRC-CMV was purchased from Invitrogen (Leek, The Netherlands), pEGFP-N1 was purchased from Clontech (CA, USA).

Plasmids were electroporated into DH5α cultures under conditions described in section 2.3.4. Large cultures of successfully transformed bacteria were created and plasmids were purified as described in section 2.3.5.

3.2.2 Electroporation of DNA into eukaryotic cells - Cells were grown to confluency and then washed twice in 50 ml of PBS to remove all calf serum. 5-10 μg of sterile linearised plasmid was used per 0.5 - 1 x 10^6 cells. Plasmids were transfected into cells by electroporation using a BioRad Gene Pulser (BioRad, Hercules, CA, USA) using the following settings- 250 Volts; 96 μF.

The LacSwitch system plasmids, p3'SS and pOPRSVI (which contained full-length clusterin cDNA previously prepared by Wilson, M.R., unpublished), were electroporated into L929 cells and grown in the presence of 0.4 mg/ml hygromycin (Calbiochem, Sydney, Australia) and 0.4 mg/ml Geneticin™ (Gibco Life Technology, Melbourne). Cells were then cloned and screened as described below. As a control, L929 cells were transfected with p3'SS, and pOPRSVI vector which contained the full length clusterin cDNA in reverse orientation.

Full-length human clusterin cDNA had previously been ligated into the pRc/CMV plasmid (Wilson M.R, unpublished). This plasmid was also electroporated into L929, HeLa, and K562 cells under the same conditions as descibed above. Cells were cloned (see below) and grown in media containing 0.4 mg/ml Geneticin^{TM}. As a control, cell lines were also transfected with the pRc/CMV plasmid lacking an insert. Negative control transfected cells that were resistant to Geneticin were selected at random.

The pEGFP-N1 plasmid was also electroporated into L929 cells under the same conditions as described above. Cells were cloned and screened as described below and were grown in media containing 0.4 mg/ml Geneticin.

3.2.3 Cloning of transfected cell lines - After transfection, cells were plated in 96 well trays in DMEM:F12 containing 20% FCS and 20% FCM. Selective agents were added two days after electroporation to kill cells that did not contain plasmid(s). When the individual cultures had grown confluent they were screened by immuno-dot blotting TC (refer section 3.2.4 below). Cells transfected with the pEGFP plasmid were screened for expression of EGFP by fluorescence microscopy.

Once positive colonies of cells had been identified they were cloned twice by limiting dilution as described in (Davis *et al.*, 1994). Briefly, cells were plated in 96 well trays at concentrations of either 1 cell per well or 0.5 cell per well. When colonies of cells had grown confluent they were screened before being cloned again or expanded for further experimentation. Cells were not used for experimentation unless they had been cloned twice.

3.2.4 Screening of clusterin-expressing transfected cell lines - Immuno-dot blots; Cells were grown to confluency in either 96 or 24 well trays and if required were induced to express clusterin for 24 hours. 2 µl of TC sn from transfectant cells was adsorbed onto nitrocellulose and left to dry. Nitrocellulose strips were then blocked with 1% HDC followed by either G7 or DNP-9 TC sn. The secondary antibody was sheep-anti-mouse-HRP antibody (Silenus laboratories, Melbourne, Australia) prepared at a dilution of 1:1000 in 1% HDC. All antibody and blocking steps were carried out for 1 hour on a shaker at room temperature. Membranes were developed with ECL (Pierce, Sydney, Australia) following the manufacturer's instructions.

Flow cytometry, fluorescence microscopy; L929 cells which were transfected with pEGFP-N1 were trypsinized from culture vessels. 10,000 cells were analysed with a flow cytometer (FACSort, Becton Dickinson, Sydney, Australia) and were compared to wild type L929 cells to confirm EGFP expression. Transfected cells were also examined through a Leitz DMIL inverted fluorescence microscope (Leica, Sydney Australia). Photographs were taken through a Wild Photomat MPS45 camera system (Leica, Sydney Australia).

3.2.5 Quantifying clusterin secretion by transfected cell lines- Immuno-dot blots were used to quantify clusterin secretion for different transfected cell lines. Cells were innoculated into 24 well trays and left to adhere to the culture surface overnight. 300 µl of fresh culture media (+ 5 mM IPTG for L929 LS.clus) was then added to the cultures and then left for 48 hours. TC sn were then collected and analysed by immuno-dot blot

(as described above) and cells were trypsinized and counted using a haemocytometer. Immuno-dot blots were analysed by densitometry (Biorad, Hercules, CA,. USA).

3.3 Results

3.3.1 Production of L929 cell lines stably transfected with the Lac switch system

L929 cells transfected with the Lac Switch system containing human clusterin cDNA has previously been described (Humphreys D., Honours thesis, 1994). In summary, cells were transfected by electroporation and cloned twice by limiting dilution to ensure a true clone was obtained. Immuno-dot blot analysis of TC sn probed with the G7 anti-human clusterin mAb identified clusterin secreting clones (Figure 3.4 A). Further analysis of TC sn by western immunoblotting confirmed that secreted clusterin was indistinguishable in size from clusterin purified from human serum (Figure 3.4 B). These inducible clusterin-secreting cells were named L929-LS.clus(F), and the corresponding controls which contain the clusterin cDNA in reverse orientation were named L929-LS.clus(B). Examination by light microscopy did not reveal any morphological differences between L929-LS.clus(F) cells induced to express clusterin and either non-induced L929-LS.clus(F) cells or L929-LS.clus(B) cells.

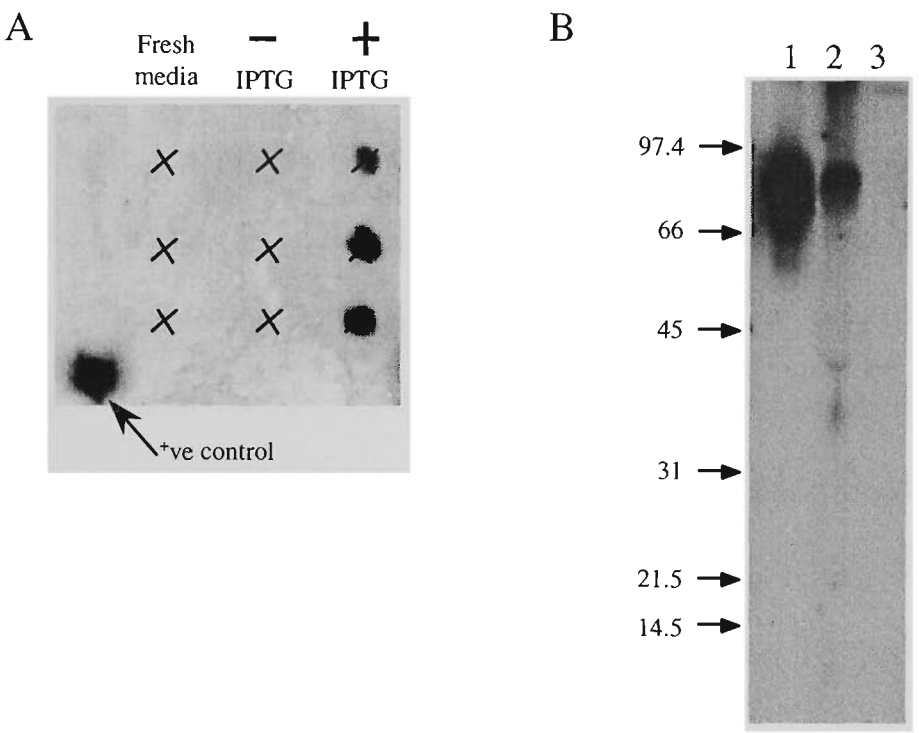


Figure 3.4: (**A**) Dot blot analysis of TC sn from L929 LS.clus(F) clone showing that cells express human clusterin when induced with IPTG. "x" indicates the position where media was dotted onto the membrane. (**B**) Western immunoblot confirming that human clusterin is correctly processed in L929-LS.clus(F) cells. Lane 1, 100 ng of pre purified clusterin purified from human serum; lane 2 and 3, TC sn of L929-LS.clus(F) which had been induced for 24 hours. Lanes 1 and 2 were incubated with G7 antibody that only recognises human clusterin. Lane 3 was incubated with DNP-9, an isotype negative control antibody. Arrows indicate the position of molecular weight markers. (Taken from D Humphreys Honours Thesis, 1994).

3.3.2 Production of L929 cell lines stably transfected with pRc/CMV.

The pRc.clusterin and pRc plasmids were linearised with Bgl II restriction enzyme before being electroporated into L929 cells. The selective agent Geneticin (400 μg/ml) was added to the growth medium two days after transfecting and plating L929 cells. Transfected cells grew quickly and within 10 days there were enough cells to begin screening.

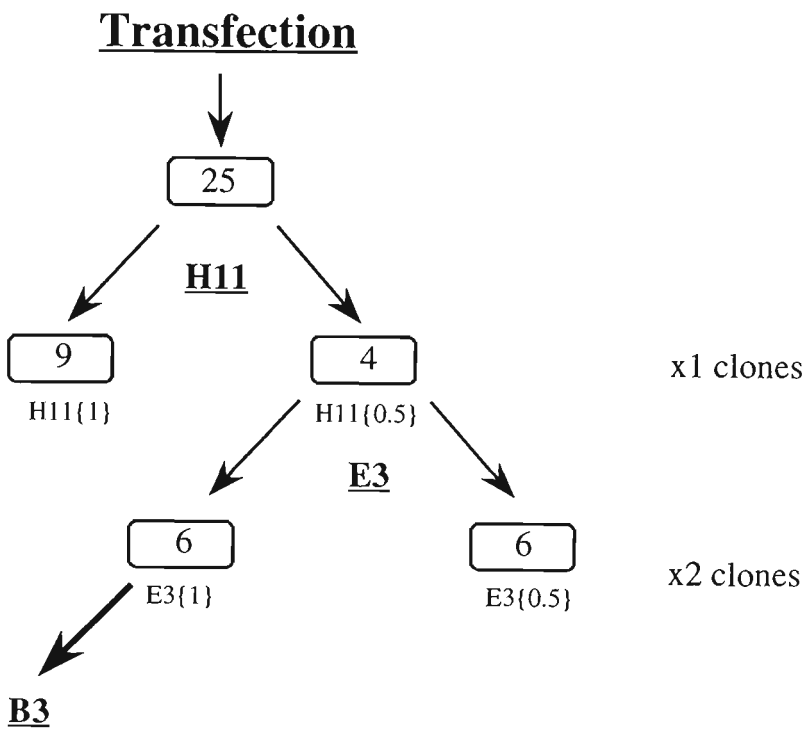
TC sn from individual wells containing transfected pRc.clus cells were screened by immuno-dot blots probed with the G7 anti-human clusterin mAb. Initial screening was

performed on wells that contained viable cells. Clusterin-secreting cells were then cloned (Figure 3.5 A). A total of thirteen colonies grew from the first round of cloning. Analysis of the TC sn from each of these colonies revealed that they secreted different amounts of clusterin. The strongest clusterin secreting colony (E3) was cloned again. The second round of cloning resulted in 12 colonies which each secreted a similar amount of clusterin. Colony B3 was expanded and named L929-pRc.clus (Figure 3.5 A). Clusterin secreted from L929-pRc.clus was indistinguishable from purfied human serum clusterin on western immunoblots and had a molecular mass of about 80 kDa under non-reducing conditions and about 40 kDa under reducing conditions (Figure 3.5 B).

Similarly, pRc transfected L929 cells that did not secrete human clusterin were cloned twice by limiting dilution and one of these colonies of cells was expanded and named L929-pRc. These cells were used as the control cell line for L929 pRc.clus cells. Examination by light microscopy did not reveal any morphological differences between L929-pRc.clus and L929-pRc cells.

The Lac Switch expression system uses the RSV promotor, which is weaker than the cytomegalovirus promotor used by the pRc/CMV system. TC sn from L929-LS.clus(F) and L929-pRc.clus cells were analysed to estimate the amount of clusterin produced by each expression system. Initially, cells were grown in serum-free media in order to maximise the amount of clusterin in the TC sn binding to the dot blot membrane. However, when transfected cell lines were grown in serum-free media for 24 hours no clusterin could be detected. Therefore immuno-dot blot comparisons was performed on

A



B

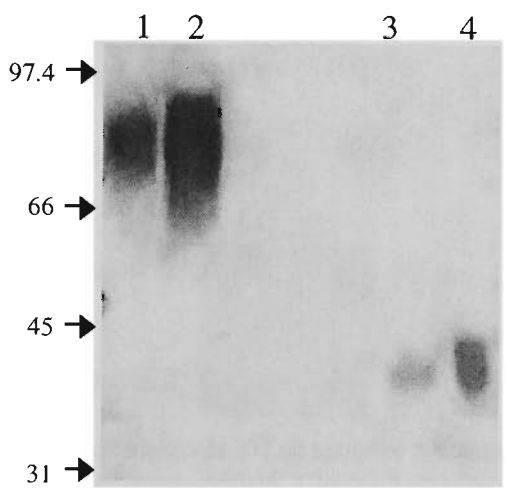


Figure 3.5: (A) The clone history of the L929-pRc.clus transfectants. Each rectangle represents a plating of cells and the number enclosed represents the number of colonies that grew in that plating. Bold underlined text represents the plate position of a colony which was screened and selected for subsequent cloning. Arrows indicate cloning, generally two rounds of cloning were performed on the selected colony. The text underneath each rectangle represents the plate position of the parental colony and the concentration of the plating. ie {1} = 1 cell/well; {0.5} = 0.5 cells/well (refer 3.2.3). (B) Analysis of L929-pRc.clus TC sn by western immunoblotting demonstrates that cells secrete correctly processed human clusterin. Lane 1 and 3, 100 ng purified serum clusterin; Lane 2 and 4, L929 pRc.clus TC sn. Lanes 3 and 4 were electrophoresed under reducing conditions. Note that the intensity of the reduced clusterin bands are considerably less than non-reduced bands due to poor recognition by the G7 anti-clusterin antibody. Arrows indicate the position where molecular mass markers migrated to.

transfected cells TC sn samples which contained 10% FCS (Figure 3.6). To compensate for the amount of serum binding to the membrane, clusterin standards were also made up in growth media containing 10% FCS. Densitometer analysis of the dot blots estimated that L929-pRc.clus cells secreted about 0.2 pg of clusterin per cell over 48 hours while L929-LS.clus(F) cells secreted about 4 fg of clusterin per cell over 48 hours. Therefore, L929-pRc.clus cells secrete human clusterin at a rate about 50x that of IPTG induced L929-LS.clus(F) cells.

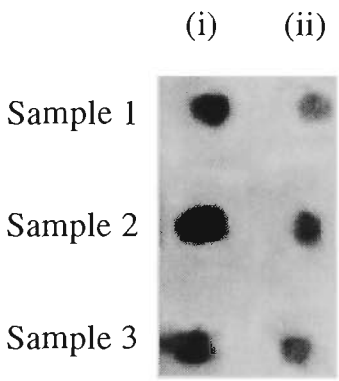


Figure 3.6: Immuno-dot blots of triplicate TC sn samples obtained from (i) L929-pRc.clus cells or (ii) induced L929-LS.clus cells after 48 hours growth. Clusterin standards were also dotted onto the same membrane before probing with the anti-clusterin G7 antibody (not shown). Densitometer analysis of these dots indicated that L929-pRc.clus cells secreted approximately 50 x more human clusterin than L929-LS.clus cells.

3.3.3 Production of HeLa cell lines stably transfected with pRc/CMV.

The pRc/CMV plasmids were transfected into HeLa cells by electroporation. The selective agent Geneticin (400 µg/ml) was added to the growth medium two days after transfecting and plating of the HeLa cells. Transfected cells grew slowly because cells did not grow in a monolayer, but rather they grew in clumps forming nodules. Cells were therefore regularly trypsinized to maintain a monolayer of cells which enhanced growth.

TC sn from individual wells containing transfected HeLa-pRc.clus cells were screened by immuno-dot blots probing with the G7 mAb. Initial screening of the first round clones indicated that 4 of the 96 wells contained cells which constitutively secreted clusterin (Figure 3.7 A). Cells from one of these wells (H11) was then cloned. After the first round of cloning 6 of the 14 wells in one tray contained cells which secreted clusterin. Each clone secreted different amounts of clusterin. The strongest secreting clone (G7) was cloned for the second time. The second round of cloning isolated clones which secreted a similar amount of clusterin (Figure 3.7 B). Cells growing in well E4 were expanded and named HeLa-pRc.clus (Figure 3.7 A). HeLa-pRc transfected cells containing no clusterin cDNA were also created by electroporation and cloned twice by limiting dilution.

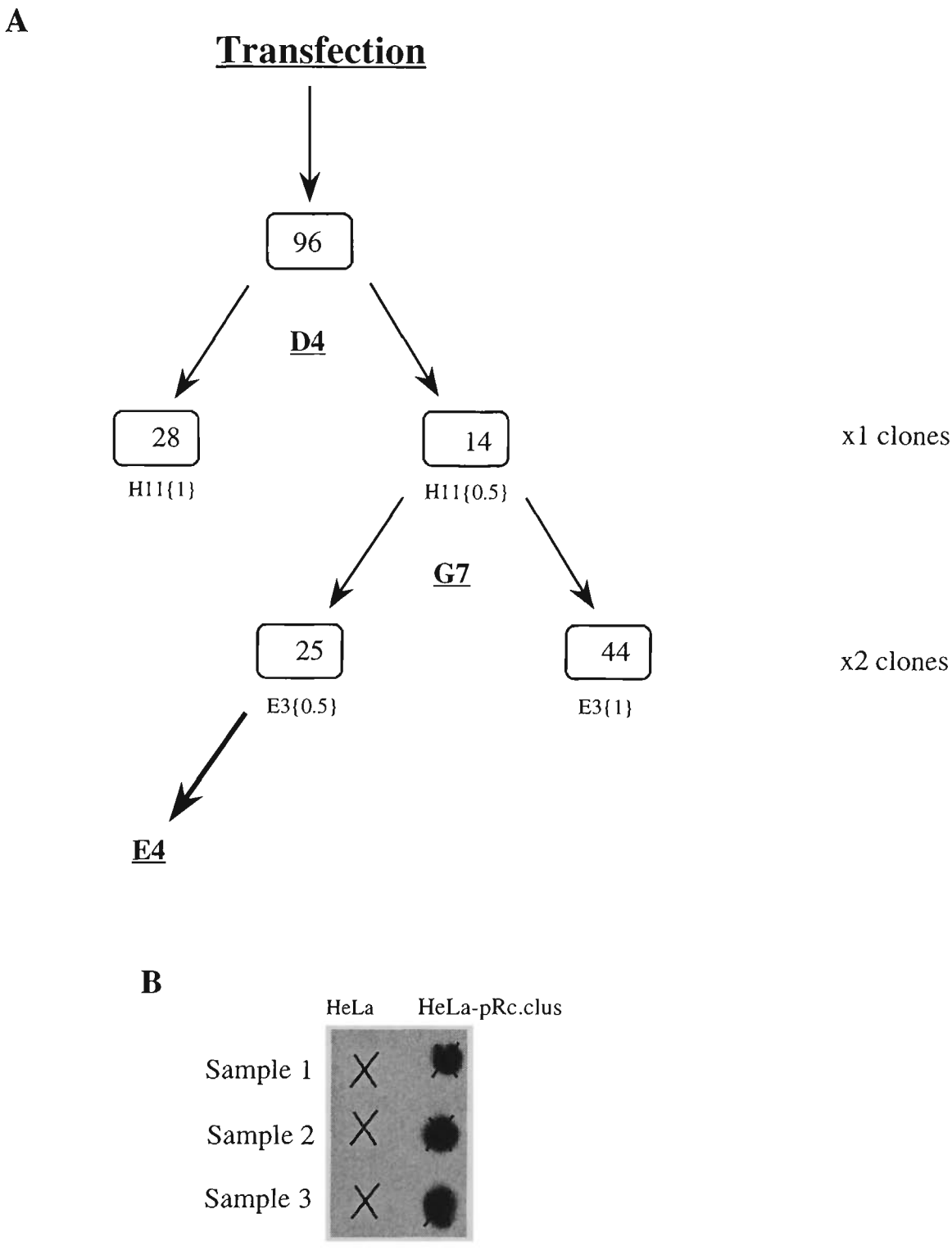


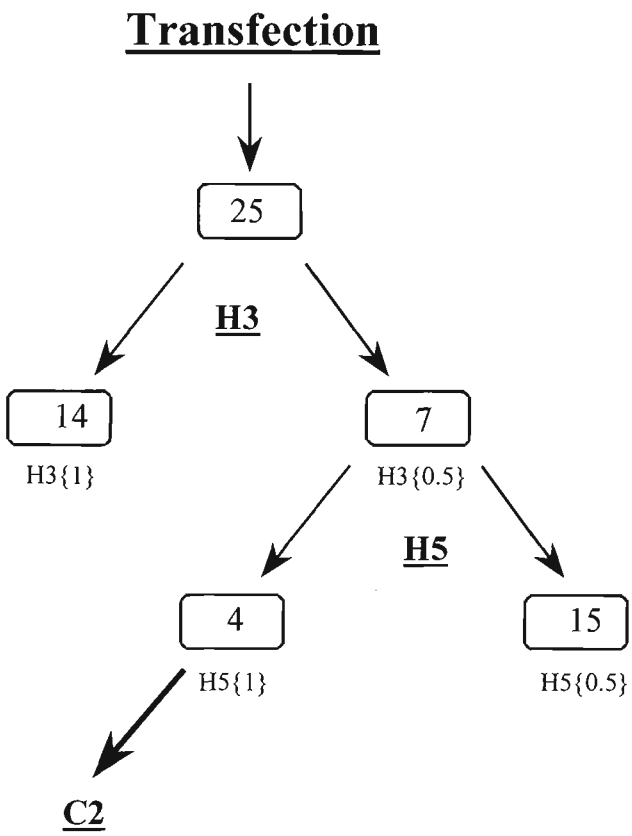
Figure 3.7: (A) The clone history of the HeLa-pRc.clus transfectants. Each rectangle represents a plating of cells and the number enclosed represents the number of colonies that grew in that plating. Bold underlined text represents the plate position of a colony which was screened and selected for subsequent cloning. Arrows indicate cloning, generally two rounds of cloning were performed on the selected colony. The text underneath each rectangle represents the plate position of the parental colony and the concentration of the plating. ie {1} = 1 cell/well; {0.5} = 0.5 cells/well (refer 3.2.3). **(B)** Analysis of HeLa-pRc.clus TC sn by immuno-dot blot demonstrating that transfected cells secrete more human clusterin compared to HeLa WT cells. "x" represents the positions where TC sn were dotted. Clusterin was detected with the G7 mAb.

3.3.4 Production of L929 cell lines stably transfected with pEGFP-N1.

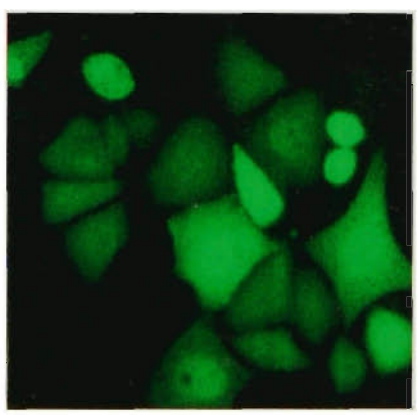
pEGFP-N1 plasmid was purified from transformed DH5α bacteria and then linearised with Dra III restriction enzyme. The pEGFP-N1 plasmid was transfected into L929 cells by electroporation. The selective agent Geneticin (400 μg/ml) was added to the growth medium two days after transfection and plating of the L929 cells. Electroporated cells grew quickly and wells containing cells expressing EGFP were identified by fluorescence microscopy within four days.

Transfected cells were cloned twice by limiting dilution (Figure 3.8 A), and the final clone from well C2 was expanded and called L929-EGFP. These cells express high levels of GFP protein which was easily visualised by fluoresence microscopy (Figure 3.8 B). Interestingly, there were variations in the level of EGFP production within cultures of cloned cells. Flow cytometric analysis of the C2 clone shows a broad green fluorescent peak which ranges from negligible fluorescence (ie same fluorescence as L929 wild type cells) to approximately 10-100 fold brighter green fluorescence than L929 wild type cells (Figure 3.8 C). The C2 cell line was maintained for long periods of time (months), and throughout this time the cells did not lose their ability to produce EGFP. The reason(s) for this wide cariation in the levl of expression of EGFP in individual transfected cells is uncertain. Examination by light microscopy did not reveal any morphological differences between L929-EGFP and the parental L929 cell lines.

A



B



C

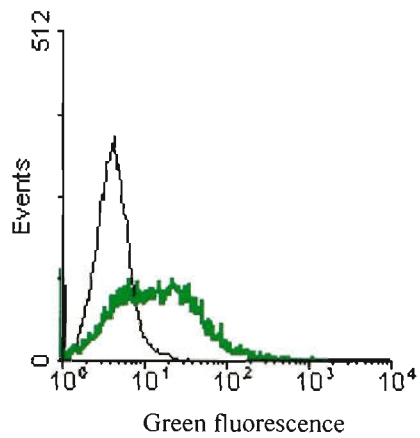


Figure 3.8: **(A)** The clone history of the L929-EGFP transfectants. Each rectangle represents a plating of cells and the number enclosed represents the number of colonies that grew in that plating. Bold underlined text represents the plate position of a colony which was screened and selected for subsequent cloning. Arrows indicate cloning, generally two rounds of cloning were performed on the selected colony. The text underneath each rectangle represents the plate position of the parental colony and the concentration of the plating. ie {1} = 1 cell/well; {0.5} = 0.5 cells/well (refer section 3.2.3). **(B)** Fluoromicrograph of L929-EGFP cells (x400). **(C)** Flow cytometry analysis showing the fluorescence of L929-EGFP cells (thick, green line) versus the fluorescence of wild type L929 cells (thin, black line). Most L929-EGFP cells have high green fluorescence compared to L929 wild type cells due to the expression of EGFP.

3.3.5 Production of K562 cell lines stably transfected with pRc/CMV.

The pRc.clus and pRc plasmids were transfected into K562 cells by electroporation. The selective agent Geneticin (400 μg/ml) was added to the growth medium two days after transfecting and plating the K562 cells. Electroporation resulted in the death of a large number of cells. Surviving cells grew very slowly. Dot blot analyses did not detect any secreted clusterin in TC sn of electroporated K562 cells. This procedure was performed many times without success. The reason(s) for the inability to produce clusterin-secreting transfected K562 cells are uncertain.

3.4 DISCUSSION:

This chapter described the transfection of L929 and HeLa cell lines with expression systems containing human clusterin cDNA. The Lac Switch expression system and the pRc/CMV expression systems were both trialled in L929 cells while the latter was only used in HeLa cells. The Lac Switch expression system is comprised of two plasmids, p3'SS and pOPRSVI, and allows controllable expression of inserted cDNA. Human clusterin cDNA had previously been inserted into pOPRSVI in the two possible orientations, foward (sense information allowing expression) and reverse (non-sense information, no expression).

L929 cells were transfected by electroporation with either of the expression systems before being cloned twice by limiting dilution. The final secondary clones secreted human clusterin which had a molecular mass of 75 - 80 kDa under non-reducing conditions and a molecular weight of 40 kDa under reducing conditions (ie

indistinguishable from purified clusterin from human serum). This indicates that the murine L929 cell line can secrete human clusterin that is glycosylated and proteolytically processed.

Negative control cell lines were also made. In the case of the Lac Switch expression system, the negative control consisted of transfected cells containing pOPRSVI in which clusterin was present in the reverse orientation (called L929-LS.clus(B)). In the case of the pRc/CMV expression system, the negative control consisted of transfected cells containing pRc plasmid with no clusterin insert (called L929-pRc). These cells were transfected by electroporation and as these cell lines could not be screened for protein expression they were selected solely on the basis of their ability to grow in selective agents. The morphology of the transfected L929 cell lines were identical to the parental L929 cells.

L929-pRc.clus cells were expected to express more clusterin per cell than L929-LS.clus(F) cells due to the powerful CMV promotor. Immuno-dot blot comparisons of TC sn confirmed this. L929-pRc.clus cells secreted about 0.2 pg of clusterin per cell over 48 hours while IPTG-induced L929-LS.clus(F) cells secreted about 4 fg of clusterin per cell over 48 hours.

There are two previous reports of other transfected cells lines which over-express clusterin (Pilarsky *et al.*, 1993; Sensibar *et al.*, 1995). Pilarsky *et al.* transfected baby hamster kidney (BHK-21) cells with the vector pBEH in which canine clusterin cDNA was inserted. The parental cell line was reported not to naturally express clusterin under

normal culturing conditions. However, the question of whether these cells secreted endogenous clusterin when stressed was not addressed. The transfected cell line constitutively expressed high levels of canine clusterin (Pilarsky *et al.*, 1993). Sensibar *et al.* (1995) transfected LNCaP cells (a human prostatic cancerous cell line) with the vector pBK/CMV in which mouse (SGP-2) clusterin cDNA was inserted. There was no indication as to whether the parental LNCaP cells secreted endogenous clusterin under normal culturing conditions or when cells were stressed. However the transfected cell lines constitutively expressed high levels of mouse clusterin. The rate of clusterin secretion by each of these transfectant cells was not determined. The expression of clusterin by these transfected cell lines was found not to affect cell morphology or viability indicating that the expression of clusterin did not cause cell death (Pilarsky *et al.*, 1993; Sensibar *et al.*, 1995).

The level of naturally produced clusterin mRNA in L929 cells is low under normal culturing conditions (Kyprianou *et al.*, 1991). However, the high level of human clusterin expression did not appear to affect morphology or viability, as judged by light microscopy. As found in other transfected cell types the high levels of clusterin expression in L929 cells under normal culture conditions did not result in death. The effect of clusterin expression on stress-induced cell death was also of interest and the findings of this study are described in chapter 4.

The L929-EGFP transfectants were created to act as another negative control for the clusterin-expressing cells. This cell line was created by electroporating the pEGFP-N1 construct into L929 cells. This construct, like the pRc/CMV expression system,

contained a CMV promotor. However the pEGFP-N1 expression system differed from those used to express clusterin in that the cDNA for EGFP contained no secretion signal and thus when expressed the protein remained intracellular, which enabled quick screening by flow cytometry and fluorescence microscopy.

The pRc/CMV expression system was also transfected into HeLa and K562 cell lines. Clusterin was secreted at high levels by HeLa cell transfectants and these cells were called HeLa-pRc.clus. The control cell line that contained the pRc/CMV expression system without the cDNA insert was called HeLa-pRc. Unfortunately no K562 transfectants were produced. In each attempt cultures grew very slowly in the presence of selective agents, and no clusterin was ever detected in TC sn. It is possible that high levels of clusterin expression cells are toxic in K562 cells.

In conclusion, various L929 cell lines were created that secreted different levels of human clusterin. L929-LS.clus(F) cells expressed human clusterin only upon the addition of IPTG while L929-pRc.clus cells constitutively expressed high levels of clusterin. L929-pRc.clus cells secreted approximately 50x more clusterin than L929-LS.clus(F) cells into the TC sn. The morphology and viability of each of these cell lines was not affected by human clusterin expression. These cells lines are useful tools for the analysis of clusterin's effects on cell death (Chapter 4).

CHAPTER 4

Mode of L929 cell death in response to various cytotoxic agents and effects of clusterin overexpression.

4.1 INTRODUCTION

4.1.1 The morphology of cell death

The inevitable death of cells within the body is a frequent event that can occur either when cells come in contact with damaging external stimuli or as a planned event whereby the body directly instructs cells to die. Necrosis is a term used to describe uncontrolled cell death that occurs when cells are exposed to external noxious stimuli. Apoptosis describes cell death that results from a specific signal(s) directed to a cell that in turn activates a complex cascade of intracellular reactions. Apoptosis and necrosis are distinguished by morphological and biochemical criteria.

The distinguishing morphological characteristics of apoptosis include condensed, fragmented cell nuclei, reduction of cell volume and cell membrane blebbing (Figure 4.1A). The blebbing of the cell membrane leads to the formation of apoptotic bodies. Apoptotic bodies hold fragments of the cells such as organelles, DNA, and cytoplasm and allow for quick phagocytosis by other cells such as macrophages where they are broken down and/or recycled (refer Savill *et al.*, 1993). The formation of apoptotic bodies partly explains how the cell decreases in size. The reduction of cell volume may also results from a loss of water and ions (Cohen, 1993).

In contrast, the distinguishing morphological characteristics of necrosis include pronounced swelling of the nucleus, mitochondria and cell membrane (Figure 4.1). These characteristics result because the cell cannot maintain homeostasis. Eventually the swelling of the cell results in the rupture of the outer cell membrane releasing cellular contents into the surrounding tissue. Also released are noxious compounds that provoke inflammation of the local tissue.

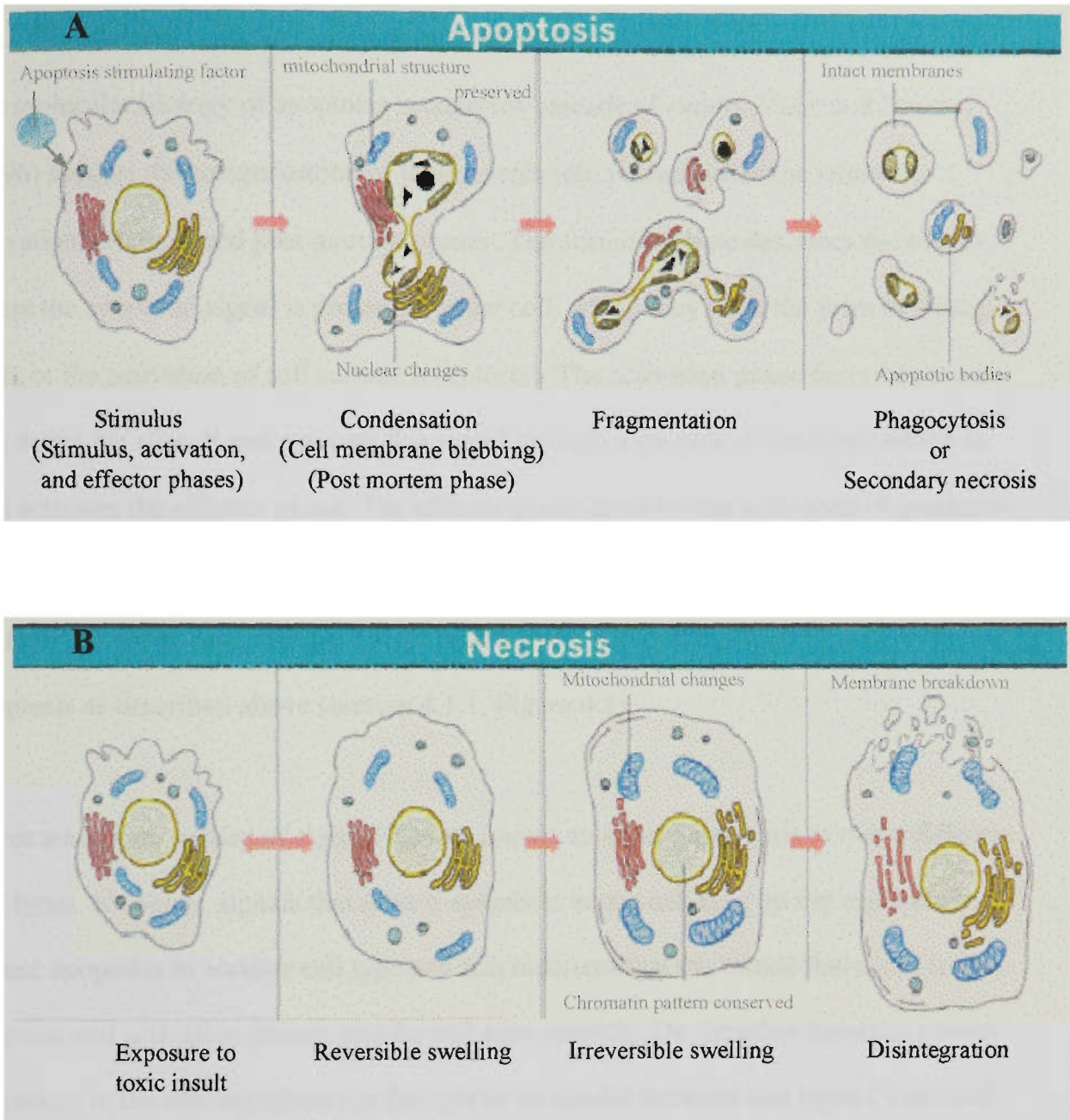


Figure 4.1: The typical morphological cascade of events that occur during apoptosis and necrosis. (**A**) Apoptosis results in condensation of the cell volume through the loss of water and ions. The nucleus also condenses and fragments. The outer cell membrane begins to bleb, fragmenting the cell into apoptotic bodies. Apoptotic bodies are thought to be recognised and phagocytosed by macrophages or other cells. Throughout apoptosis the cell membrane integrity is retained. (**B**) Necrosis is initiated from the exposure of a toxic insult that initially causes reversible swelling of the cell. Continued presence of the insult results in irreversible swelling of the cell and pronounced swelling of the mitochondria and nucleus. This finally results in cell rupture and total disintegration of the cell. Figure modified from Boehringer internet catalogue (http://biochem.roche.com/techserv/apoptosis/slide1.html).

4.1.2 The molecular biology of apoptotic cell death

The molecular biology of apoptosis is complex cascade of events. Vaux and Strasser (1996) suggest the categorization of these events into phases called the stimulus, activation, effector, and post mortem phases. The stimulus phase describes the events where the apoptotic signal is presented to the cell, which may be in the form of a drug, toxin or the activation of cell surface receptor(s). The activation phase describes events that detect the stimuli and transmit this signal through a cascade of reactions which in turn activates the effector phase. The effector phase involves the activation of proteases and other destructive events which initiate the physical death of the cell, while the postmortem phase describes the events which cause the morphological features of apoptosis as described above (section 4.1.1, Figure 4.1).

There are a wide variety of signals that are known to induce apoptosis in many different cell types. However, signals that induce apoptosis in one cell type do not necessarily induce apoptosis in another cell type and this indicates that the events that occur in the stimulus and activation phases may be cell-type specific. On the other hand the events that occur in the effector phase are thought to be similar between cell types (Vaux and Strasser, 1996). The effector phase includes the activation of numerous cysteine proteases that are mammalian homologues to ced-3 found in the nematode *Caenorhabditis elegans* (Nicholson and Thornberry, 1997). These proteases are called caspases and require specific cleavage of their tetrameric polypeptide chain that creates two active heterodimeric enzymes, each consisting of a small and large chain (Salvesen and Dixit, 1997). Of the fourteen caspases that have been identified three (caspases 3, 2, 10) appear to be essential for the effector phase in apoptosis (Nicholson and Thornberry, 1997). Caspases 6, 7, and 8 also appear to be involved in apoptosis and are thought to promote various protein cascades, while the other caspases are involved in the

activation of proinflammatory cytokines (Salvesen and Dixit, 1997).

Mitochondria have also recently been identified as an important regulatory element of apoptosis. Mitochondria of apoptotic cells have been shown to undergo a large reduction in the mitochondrial membrane potential (Kroemer et al., 1997), which is thought to result from a phenomenon called permeability transition (an increase in the permeability of the inner mitochondrial membrane) (Zamzami *et al.*, 1995). Mitochondrial proteins have also been shown to be released from mitochondria into the cytosol during apoptosis. Cytochrome c and apoptosis inducing factor (AIF) are two such proteins and are important for the apoptotic pathway to proceed. AIF is capable of inducing apoptosis in many cell types (Zamzami *et al.*, 1996) and is thought to play a key role in apoptosis as it can induce nuclear condensation and fragmentation in the absence of other cytosolic components (Susan *et al.*, 1996). Cytochrome c has been shown to trigger the activation of caspase-3, which then triggered events required to induce DNA fragmentation (Kluck *et al.*, 1997a,b; Liu *et al.*, 1996). It is unclear at present whether the release of such proteins from the mitochondria is directly linked to the reduction of mitochondrial membrane potential or permeability transition. However, the events that lead to the disruption of mitochondrial membrane potential appear to be linked to the effector phase, especially as this only occurs in cells that are committed to apoptotic cell death (Kroemer *et al.*, 1997).

In contrast necrotic death does not involve any protein activation or regulatory events. This is because cells are exposed to situations that are intolerable and therefore undergo rapid death caused by the inactivation of basic cell functions.

4.1.3 Biochemical properties of apoptotic cells

The changes that occur in the late stages of apoptosis (postmortem phase) are unique and can be exploited to specifically identify apoptotic from healthy or necrotic cells. Biochemical characteristics of apoptotic cells that are not found in necrotic or healthy cells include an increase in cell membrane permeability, exposure of phosphatidylserine (PS) on the outer surface of the cell membrane, mitochondrial depolarisation, and internucleosomal fragmentation of chromosomal DNA (Gorman *et al.*, 1994). Each of these characteristics along with the changes in cell morphology are routinely used to identify apoptotic cells. Unfortunately most studies only analyse one or possibly two characteristics to determine apoptosis.

The membranes of apoptotic cells have been shown to be more permeable to small molecules than healthy viable cells, but yet less permeable than membranes of dead cells. This was demonstrated with the use of small fluorescent dyes which are impermeable to healthy cells but freely pass into dead cells and stain apoptotic cells to an intermediate level (Schmid *et al.*, 1994; Dive *et al.*, 1992; Zamai *et al.*, 1996; Idziorek *et al.*, 1995; Lyons *et al.*, 1992). However, the reason for the increase in cell membrane permeability during apoptosis are unclear. Structural changes to the outer membrane such as the redistribution of PS to the outer leaflet of the membrane may explain in part the increased permeability.

PS is a negatively charged phospholipid that is an important membrane component of mammalian cells (Alberts, 1989). In healthy cells PS is confined to the inner leaflet of the plasma membrane; however, during apoptosis, PS redistributes to the outer leaflet of the plasma membrane (Martin *et al.*, 1995). It has been proposed that PS acts as a ligand that is recognized by macrophages and instucts them to remove the dying cell (Fadok *et*

al., 1992a,b).

The nucleus is another organelle that undergoes dramatic changes in the final stages of apoptosis. Chromosomal DNA of the nucleus is normally tightly wound about proteins called histones (approximately 180 bp per histone) to form nucleosomes (Alberts, 1989). In apoptotic cells, exposed DNA that links nucleosomes together is cut by activated endonucleases which results in mono and oligonucleosome fragments (Wyllie, 1980: Nature). DNA is initially cleaved into >700 and 200-250 kbp size fragments by a Mg^{2+} dependent enzyme (Sun and Cohen, 1994; Oberhammer *et al.*, 1993), before being further degraded into multiples of 200 bp fragments. Analysis of DNA from apoptotic cells was the initial technique used to identify apoptotic cells as electrophoresis of the fragmented DNA resolves a characteristic "DNA ladder" (Wyllie, 1980). The reason(s) for the fragmentation of DNA is not clear but it may be a method by which the cell makes sure no "readable" DNA escapes and transfects other cells. This would be undesirable especially if the dying cell contained viral information, or if the DNA induced an autoimmune response (Peitsch *et al.*, 1994). The thorough fragmentation of DNA is obviously irreversible and therefore the completion of this step guarantees cell death. DNA laddering is now considered to be one of the hallmarks of apoptosis.

4.1.4 TNFα induced death in L929 cells

The high level of interest in the field of apoptosis has lead to identification of many agents that cause apoptosis. TNFα, a cytokine secreted by many cell types, is capable of inducing apoptosis in several cell lines (Sarraf, 1994). It is also capable of causing necrosis in tumours (hence its name) while not affecting "normal" cells in surrounding tissues (Sarraf, 1994). Many studies use the L929 mouse fibrosarcoma cell line to test the cytotoxic effects of TNFα (Flick *et al.*, 1984; Levesque *et al.*, 1995; Mishra *et al.*,

1995; Trost *et al.*, 1994). Independent studies analysing the mode of cell death in L929 cells have reported TNFα to induce either necrosis (Schulze Osthoff *et al.*, 1992; Grooten *et al.*, 1993) or apoptosis (Fady *et al.*, 1995; Kyprianou *et al.*, 1991).

TNFα interacts with cells through two cell surface receptors known as TNF-R1 and TNF-R2. The high affinity type I (TNF-R1) receptor is 55 kD while the low affinity type II receptor (TNF-R2) is 75 kD (Sarraf, 1994). The extracellular domains of these receptors have three or four subdomains with a strong conserved pattern of cysteine residues (Loetscher *et al.*, 1990; Schall *et al.*, 1990). Sequence analysis reveals that the extracellular domains of the TNF receptors are most similar to the extracellular domains of NGF-R and FAS, while their cytoplasmic domains show no homology to any known protein (Schall *et al.*, 1990; Loetscher *et al.*, 1990).

As TNFα can bind up to three receptors it was proposed that receptor aggregation is required to activate an intracellular signal (Song *et al.*, 1994). This theory has been supported by studies using transfected L929 cells that contain constructs for chloramphenicol acetyltransferase (CAT), a well known cytosolic trimeric protein, linked to TNF-R1. The inducible expression of CAT-TNF-R1 led to forcible trimerisation of the TNF receptors which resulted in cell death, just as if TNFα had been used (Fiers *et al.*, 1996). Intracellular signalling resulting from the activation of TNF-R1 is initiated by the death domain protein called TRADD, which recruits other recently identified proteins called RIP, TRAF2 and FADD/MORT1 (Kronke *et al.*, 1996 and references therein). FADD/MORT1 recruits caspase-8 or caspase-10 to this assembly where either is proteolytically activated (Boldin *et al.*, 1996; Muzio *et al.*, 1996; Vincenz and Dixit, 1997).

One of the earliest events detected following the treatment of cells with TNFα is an increase in protein phosphorylation (Van-Lint *et al.*, 1992). The most prominant protein that is phosphorylated is HSP25/27 (Mehlen *et al.*, 1995). Van-Lint and colleagues (1992) demonstrated that TNFα treatment of L929 cells results in the stimulation of numerous serine and threonine protein kinases, which included two MAP kinases. The stimulation of MAP kinases is thought to be important because they have not only been shown to phosphorylate proteins in the cytosol but are responsible for the activation of various nuclear transcription factors (Fiers *et al.*, 1996). For example, p38 MAP kinase is activated in TNFα treated L929 cells and is responsible for the activation of an unknown nuclear transcription factor required for interleukin-6 expression (Beyaert R *et al.*, 1996). While the activation of various MAP kinases has been shown to be essential for the activation of cell death, their activation alone does not necessarily result in cell death. One such example is Jun kinase, a member of the mitogen-activated protein kinase (MAPK) family. This is activated in L929 cells following TNFα treatment (Gardner and Johnson, 1996) and is essential for cell death to occur (Kronke *et al.*, 1996). Co-treating cells with fibroblast growth factor-2 diminishes the cytotoxic effects of TNFα but does not inhibit the activation of Jun kinase (Gardner and Johnson, 1996).

The TNF signalling cascade also involves lipids, as ceramide production is increased in TNFα-treated L929 cells (Hayakawa *et al.*, 1996; Jayadev *et al.*, 1997). Ceramide is produced from the sphingomyelin pathway and is a product of the hydrolysis of sphingomeylin (Kronke *et al.*, 1996). Activation of the sphingomeylin pathway involves other unknown reactions which result in an increase of arachidonic acid (AA) production and may require cytosolic phospholipase A_2 (Jayadev *et al.*, 1994; Jayadev

et al., 1997). Research comparing the effects of TNFα treatment on wild type L929 cells to the TNFα resistant L929 cell line, C12, revealed that the production of AA, and hence ceramide, are crucial to activate cell death (Jayadev *et al.*, 1997).

Mitochondria have been proposed to be the first targets of TNFα damage (Hennet *et al.*, 1993; Schulze-Ostoff *et al.*, 1993). In L929 cells, TNFα treatment has been shown to generate reactive oxygen species (ROS) in the mitochondria (Schulze-Osthoff *et al.*, 1992) which is dependent on the presence of glutamine (Goossens *et al.*, 1996). It has been suggested that ROI generated in TNFα-treated L929 cells are the cytotoxic agents as the addition of antioxidants and iron chelators counteract the effects of TNFα (Schulze-Osthoff *et al.*, 1992; Goossens *et al.*, 1995). ROI have also been implicated as causing DNA damage in TNFα-treated L929 cells (Shoji *et al.*, 1995).

The mode of death in L929 cells exposed to TNFα has previously been reported to be either apoptotic (Fady *et al.*, 1995; Kyprianou *et al.*, 1991; Tomasovic *et al.*, 1994) or necrotic (Schulze Osthoff *et al.*, 1992; Grooten *et al.*, 1993; Schulze Osthoff *et al.*, 1993; Goossens *et al.*, 1996). These claims have been based on studies of DNA fragmentation and general cell morphology. This chapter describes research which analysed a variety of L929 cell characteristics to help distinguish the mode of TNFα-induced death. The parameters studied included changes in membrane permeability (7-AAD), redistribution of PS to the cell surface, DNA fragmentation, and general cell morphology. In addition, transfected L929 cells which secrete high levels of clusterin were also examined to determine whether clusterin expression had any effects on TNFα-induced cell death.

4.2 MATERIALS AND METHODS

4.2.1 Materials - Actinomycin D (Act D), 7-aminoactinomycin D (7-AAD), acridine orange (AO), propidium iodide (PI), staurosporine, colchicine and azide were all obtained from Sigma (St. Louis, MO, USA). Annexin V-FLUOR (FITC conjugate) was obtained from Boehringer Mannheim (Sydney, Australia). Recombinant human TNFα was a gift from Dr D Rathgen (Peptide Technology Ltd, Sydney, Australia). TGFβ was purchased from Sigma. All other general chemicals were purchased from Sigma or Ajax Chemicals (Sydney, Australia).

4.2.2 Tissue culture - Cells were seeded into 24 well plates and left to aclimate for at least 16 hours before being treated. To induce death, cells were incubated with one of the following; 100 ng/ml of recombinant human TNFα, 100 ng/ml TNFα + 4 μg/ml Act D, 2 μM colchicine, 1 μM staurosporine, or 0.6% (w/v) sodium azide. After various durations, media containing non-adherent (floating) cells were removed while the remaining adherent cells were incubated with trypsin/EDTA for 5 minutes at 37°C to remove them from the plastic. Both populations of cells were pooled before staining.

When testing for (i) the effects on cell survival of secreted proteins adsorbed to the culture vessel surface, or (ii) the expression of clusterin at the cell surface, adherent cells were removed from the plastic surface by removing the culture medium and overlaying the cells with 0.5x PBS/EDTA (refer 2.1.1). After a 5-10 min incubation, the cells were removed by vigorous washing with 0.5x PBS/EDTA.

4.2.3 Flow cytometry - Flow cytometry data was collected using a Becton Dickinson FACSort and analysed using CELLQuest software (Becton Dickinson, Sydney,

Australia). For each flow cytometric analysis, data were acquired for 10,000 events.

4.2.3.1 Determining the rate of death in L929 cells caused by each cytotoxic agent- Cells were seeded into 24 well tissue culture plates for 16 hours before treatment. Cells were treated with each cytotoxic agent and at various time points, remaining adherent cells were removed with trypsin and pooled with non-adherent cells before being stained with 1 μg/ml PI in PBS for 10 minutes and analysed by flow cytometry. Viable cells were identified by their lack of staining with PI.

4.2.3.2 Membrane permeability of cells- The red fluorescing nuclear stain 7-AAD was used to discriminate between dead, apoptotic and viable cells, as described in (Schmid *et al.*, 1994). To summarise, cells were washed in PBS and then resuspended in a small volume (50 μl) of PBS containing 20 μg/ml of 7-AAD and incubated in the dark on ice for 20 minutes. Cells were then resuspended in 100 - 500 μl of PBS before being analysed by flow cytometry. In some cases experiments only required discrimination of viable and dead cells. In these cases cells were stained with 1 μg/ml of PI for 5 minutes and then immediately analysed by flow cytometry.

4.2.3.3 Phosphatidylserine exposure- The fluorescent FITC-labelled annexin V protein (Boehringer, Sydney, Australia) was used to identify the presence of PS on the outer cell membrane. Cells were washed in annexin V buffer (10 mM Hepes, 140 mM NaCl, 5 mM $CaCl_2$) and then resuspended in a small volume of annexin V buffer containing annexin V (according to the

manufacturer's description) and 7-AAD (1 μg/ml). Dead cells (cells which were most permeable to 7-AAD) were electronically gated out from the analysis.

4.2.3.4 Nuclear DNA content- Nuclear DNA content was assayed with propidium iodide (PI) as described in (Nicoletti *et al.*, 1991). Briefly cells were washed and pelleted in PBS before being permeabilized with 0.3% Triton X-100 (in PBS) overnight at 4°C. The following morning permeabilised cells were stained with 1 μg/ml of PI before being analysed by flow cytometry.

4.2.3.5 Determining the level of intracellular clusterin- In one type of experiment it was necessary to establish the level of intracellular clusterin for live versus dead cells in the same culture. This was done in the following way. For each test, 2.5 x 10^6 cells were pelleted by centrifugation, resuspended in 50 μl of 20 μg/ml 7-AAD in PBS, and incubated on ice for 20 min. The cells were then washed 3x with cold PBS and permeabilized by resuspension in 1 ml of ice cold 50% (v/v) ethanol in PBS, followed by vortexing for 1 min and incubation on ice for a further 4 min. The cells were then washed 3x with cold PBS before incubating them with G7 MAb or a control MAb, followed by a sheep-anti-mouse Ig-FITC (SaMIg-FITC) conjugate (Silenus Laboratories, Melbourne, Australia). Dead cells were discriminated by their high red fluorescence, while cells that in culture still had an intact cell membrane were discriminated by their relatively low red fluorescence.

4.2.3.6 Determining the amount of L929-pRc.clus cell surface clusterin- Cells were removed from TC flasks by constantly withdrawing and dispelling large volumes of 0.5x PBS/EDTA over the culture vessel surface. Cells were washed 3x with cold PBS. Cells were then incubated with G7 Mab or a control Mab, followed by a SaMIg-FITC conjugate before being analysed by flow cytometry.

4.2.4 Analysis of DNA fragmentation- To isolate DNA, 2-3 x10^6 cells were washed in PBS before being lysed in 0.5 ml of 10 mM Tris, 6 mM EDTA, 0.5% SDS (w/v), pH 8.0, containing 300 µg/ml of proteinase K (Bresatec, Adelaide). Lysates were incubated for 16 hours at 55°C before being subjected to two phenol:chloroform extractions followed by one chloroform extraction (as described in Kyprianou *et al.*, 1991). DNA was precipitated from the extracts by adding two volumes of cold 100% ethanol, 1/10 volume of 3 M sodium acetate and 1/100 volume of 1 M magnesium chloride followed by overnight incubation at -20°C. DNA was pelleted at 13,000 rpm in a bench top microfuge before being washed in cold 80% (v/v) ethanol, air dried and then redissolved in 20-50 µl of 10 mM Tris, 1 mM EDTA, pH 8.0, and quantified by A_{280}. Contaminating RNA was removed by adding 2 µl of RNAse cocktail (Bresatec, Adelaide, Australia) to each DNA sample and incubating for 1 h at 37°C. Purified DNA was subjected to electrophoresis on 1.6% agarose gels and, after staining with ethidium bromide (0.5 µg/ml), visualized with a Novaline gel documentation system (Novex, Sydney).

4.2.5 Microscopy- L929 cell morphology in response to various treatments was studied using a Leitz Diavert white light inverted microscope (Leica, Germany). Cells were observed in cultures without the aid of stains. For the examination of nuclear morphology, cells were removed from flasks with trypsin before being stained with 5 µg/ml of acridine orange and then being washed twice with PBS. Micrographs and fluorographs were taken on a Wild MPS45 photoautomat system through a Leitz DMIL (Leica, Germany) inverted fluorescence microscope following the manufacturer's instructions.

4.2.6 Preparation of cell lysates, fluoro-imaging and dot blot assays- To prepare cytoplasmic fractions (i.e. cytosol plus ER/Golgi), cells were lysed in TTX buffer (20 mM Tris HCl, pH 8.0, 100 mM NaCl, 0.2% Triton X-100) on ice for 15 min (Reddy *et al.*, 1996). The lysates were centrifuged at 10,000 rpm in a microfuge for 5 min and the supernatants mixed 1:1 with sample buffer (SB; 0.5 M Tris.Cl, 20% (v/v) glycerol, 5% (w/v) sodium dodecyl sulfate, 0.005% bromophenol blue, pH 6.8) and electrophoresed on 10% SDS polyacrylamide gels. Nuclear extracts were made as described in (Reddy *et al.*, 1996); briefly, cell were lysed as above and the total lysate overlayed on a cushion of 10% sucrose in TTX buffer before centrifuging at 800 g for 10 min. The pelleted nuclei were resuspended in SB and boiled for 5 min before loading on to 10% SDS polyacrylamide gels and electrophoresis. Total cell extracts were prepared by washing cells once in PBS, and then lysing them directly in SB and boiling them for 10 min before loading on to SDS polyacrylamide gels. Samples separated by SDS/PAGE (refer 2.2.2) were electrophoretically transferred to nitrocellulose membrane as described in section 2.2.3. Fluoro-imaging was performed using a Storm 840 fluoroimager (Molecular Dynamics, Sunyvale, CA, USA) and all data analyses carried out using the ImageQuant^{TM} software (Molecular Dynamics, Sunyvale, CA, USA).

4.2.7 Electroporation of proteins into L929 cells- L929 cells were prepared in electroporation buffer (1.26 mM $CaCl_2$, 5.37 mM KCl, 0.52 mM KH_2PO_4, 0.64 $MgCl_2$, 0.63 $MgSO_4$, 85.5 mM NaCl, 5.8 mM $NaHCO_3$, 0.50 mM NaH_2PO_4, 12.5 mM Hepes) as described by Glogaver and McCulloch (1992). Cells were electroporated in electroporation buffer that contained 0.5 mg/ml of either BSA or clusterin, which had or had not been labelled with FITC. Electroporation was performed using a BioRad Gene pulser (BioRad, Hercules, CA, USA) with the following settings: 200 or 400 volts, 96 μF. Thereafter, cells were trypsinized (with trypsin/EDTA mix from TRACE, Melbourne, Australia) for various times (maximum 15 minutes) to establish how much protein became incorporated into the cell rather than on or in the outer membrane. The viability (refer section 4.2.3.2) and the amount of protein incorporated into cells was then analysed by flow cytometry.

4.3 RESULTS:

4.3.1 Rate of L929 cell killing effected by different cytotoxic agents

There have been numerous reports describing agents capable of inducing apoptosis. These agents do not necessarily cause apoptosis in all cell types as they often involve specific ligand interactions. Therefore, several reported apoptotic inducers were tested on L929 wild type cells and transfected cells to determine whether they induced apoptosis. Table 4.1 lists the cytotoxic agents tested and the concentrations used.

Table 4.1 Cytotoxic agents at the concentrations used to test the mode of death in L929 cells.

Cytotoxic Agent	Known Functions	Concentration
TNFα	Cytokine	100 ng/ml
TNFα + actinomycin D	Protein synthesis inhibitor	100 ng/ml 4 μg/ml
Staurosporine	Protein kinase inhibitor	1 μM
Colchicine	Disrupts microtubules	2 μM
Azide	Respiratory poison	0.6 % (w/v)

Of the agents tested, TNFα/Act D killed L929 cells most rapidly, killing >90% of cells within 12 hours (Figure 4.2). The next most rapidly acting agent was staurosporine, which killed >90% of cells within 18 hours (Figure 4.2). TNFα, colchicine and azide were slower acting in that each agent required more than 48 hours to kill >90% of cells (Figure 4.2).

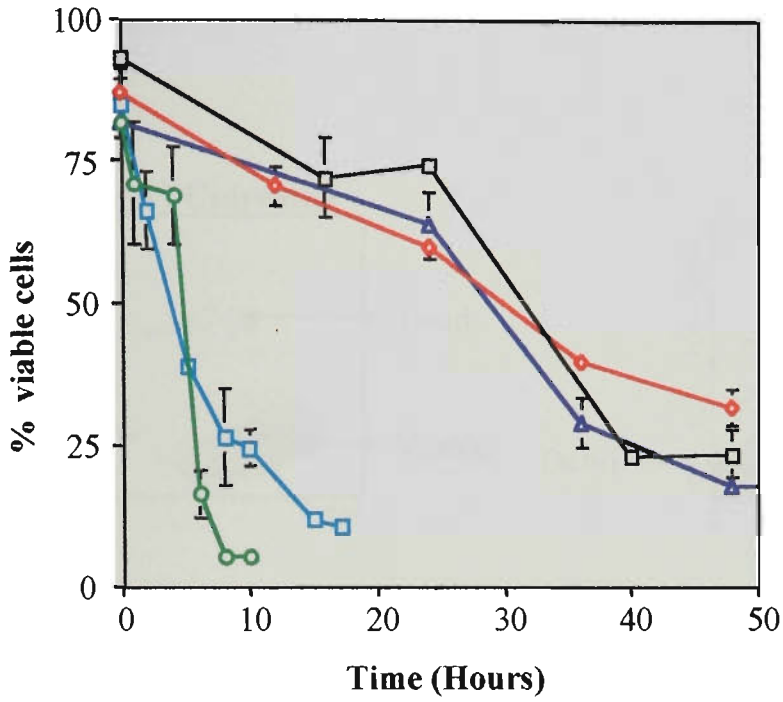


Figure 4.2: Comparing the viability of L929 cells over time when treated with various cytotoxic agents. Viability determined on the basis of the ability of cells to exclude PI. Treatments are as follows:- □ 2 μM colchicine; ◆ 100 ng/mL TNF; ○ 100 ng/mL TNF + 4 μg/mL Act D; □ 1 μM staurosporine; ▲ 0.6% Azide. All time points shown represent the mean for three independent cultres of cells and error bars are the standard deviation of the mean. In some cases the error was too small to be visable.

4.3.2 Effects of cytotoxic agents on cell size and cell membrane permeability

Particles differing in size can be identified by flow cytometry as populations distinguished on the basis of the forward scatter signal. Classically, cells undergoing necrosis initially increase in size before losing membrane integrity. In contrast, cells undergoing apoptosis shrink and maintain a smaller size than healthy cells. These size differences can be detected by flow cytometry (Darzynkiewicz *et al.*, 1992).

The DNA stain 7-AAD can be used to discriminate viable, apoptotic and dead cell populations (Schmid *et al.*, 1994). Flow cytometry was used to measure changes in cell size (forward scatter) and the extent of staining with 7-AAD (red fluorescence) in L929 cells treated with each of the cytotoxic agents. Analysis of TNFα-treated cells stained with 20 μg/ml 7-AAD resolved three populations (Figure 4.3B). There was consistently

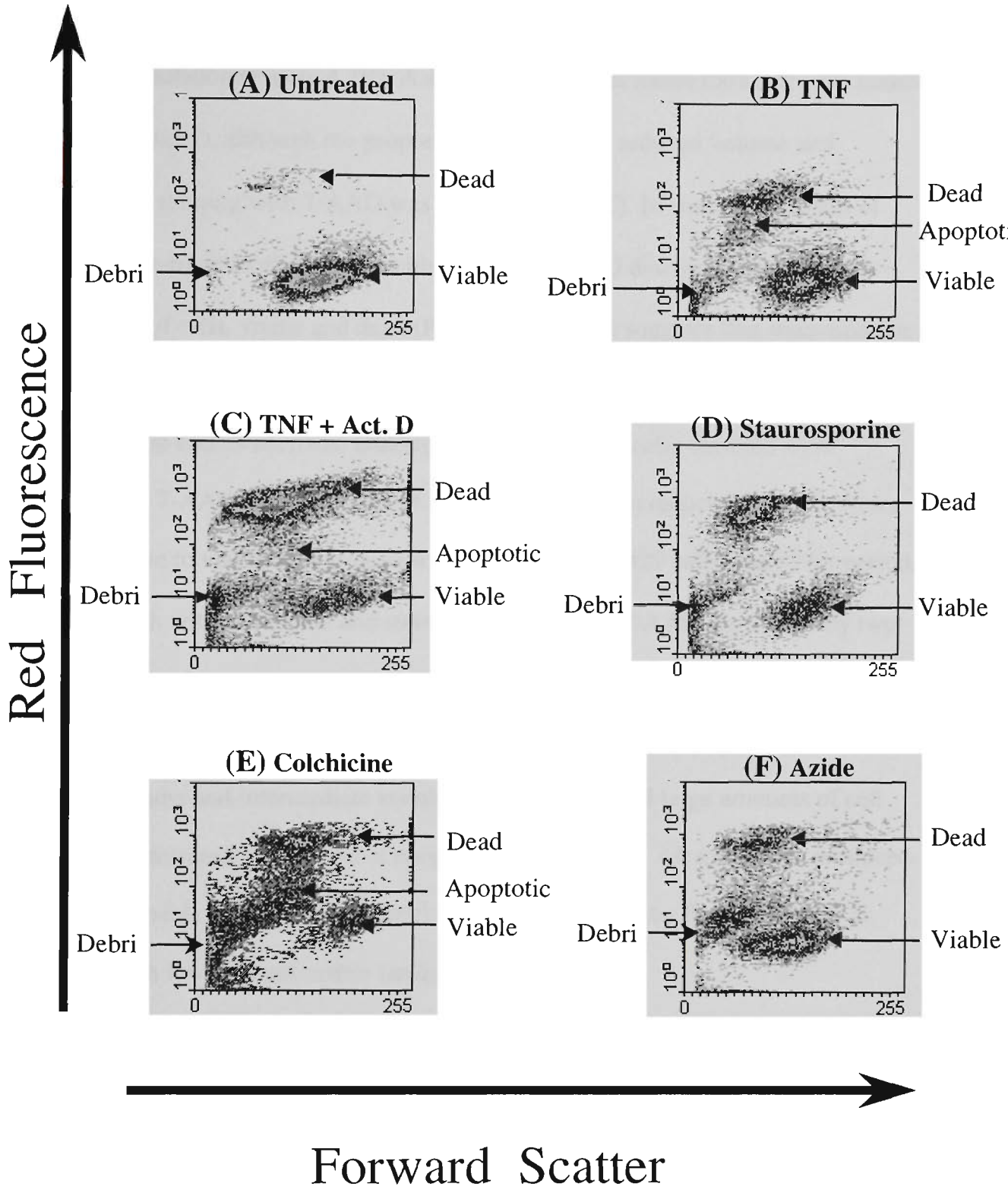


Figure 4.3: Flow cytometry density plots of 7-AAD uptake versus forward scatter in L929 cells exposed to the treatments indicated. The treatments were: **A)** Untreated L929 cells; **B)** 100 ng/ml TNFα for 24 hours; **C)** 100 ng/ml TNFα + 4 μg/ml actinomycin D for 6 hours; **D)** 1μM staurosporine for 10 hours; **E)** 2 μM colchicine for 36 hours; **F)** 0.6% (w/v) sodium azide for 24 hours. The identity of different cell populations is identified with labelled arrows. The results shown are representative of many independent experiments.

a small population of cells with a reduced size and an intermediate to high level of 7-AAD staining, suggestive of apoptosis. This population was not fully resolved from the dead cell population (Figure 4.3B). A similar pattern was found for L929 cells treated with TNFα/Act D, although the proportion of cells with reduced volume and intermediate staining with 7-AAD was less (Figure 4.3C). In contrast, analysis of staurosporine-treated cells stained with 20 μg/ml 7-AAD discriminated only two populations of cells, viable and dead (Figure 4.3D). This suggests that staurosporine causes necrosis in L929 cells. A similar pattern was found for azide-treated cells which suggests it too causes necrosis; although there were some cells detected with intermediate 7-AAD staining, most of these did not show a reduced forward scatter signal relative to viable cells (Figure 4.3F). Analysis of L929 cells treated for periods less than 36 h with colchicine and stained with 20 μg/ml 7-AAD resolved only two populations, viable and dead. However, when the same analysis was performed with cells exposed to colchicine for periods longer than 36 h, a large population of cells with reduced volume and intermediate staining with 7-AAD (and large amounts of cell debris) was detected, suggestive of apoptosis (Figure 4.3E). All treatments produced cell debri (visible by light microscopy) which was detected by flow cytometry as particles with low forward scatter (indicated in Figure 4.3).

4.3.3 Effects of cyotoxic agents on exposure of PS at the cell surface

Annexin V is a protein that belongs to the family of annexins that bind calcium and phospholipids. Annexin V has a high affinity for PS and has been used by many groups to identify PS exposed on the outer surface of apoptotic cells (Martin *et al.*, 1995; Fadok *et al.*, 1992a and b; Vermes *et al.*, 1995; Koopman *et al.*, 1994). Treated and untreated L929 cells were dual-stained with annexin V-FITC and 1 μg/ml 7-AAD and analysed

by flow cytometry. Dead cells identified by 7-AAD staining were electronically excluded from the analysis. With the exception of azide, all treatments resulted in significant binding of annexin V to intact L929 cells, indicating exposure of PS at the cell surface (Figure 4.4).

4.3.4 Flow cytometric analysis of DNA fragmentation induced by cytotoxic agents

DNA fragmentation that occurs in the nucleus of cells can be identified by flow cytometric analysis of permeabilized cells stained with the DNA intercalating fluor PI (Nicoletti *et al.*, 1991). Permeabilisation of cells with detergent-containing isotonic buffer allows any small fragments of DNA to diffuse out from the cell. In preliminary experiments, this technique was used to test what treatments elicited DNA fragmentation in L929 cells.

Flow cytometric analysis of healthy untreated L929 cells identified two main populations of cell nuclei which had similar but distinct levels of PI staining (Figure 4.5 A). The nuclei which stained most intensely with PI were from cells in the G_2/M stage of mitosis (as these cells are about to undergo cell division and contain twice the amount of DNA), while the other main population of nuclei belonged to cells in the G_1 stage of mitosis. Nuclei from cells in the S phase were detected in the region between these two populations (Nicoletti *et al.*, 1991).

Treatment of L929 cells with each cytotoxic agent resulted in a population of nuclei which had low PI stained nuclei (Figure 4.5 B-F). This indicates that all treatments resulted in some form of DNA degradation. TNFα + Act D and staurosporine treatments were by far the most potent inducers of DNA fragmentation, as fragmentation was first detected at 6 hours. TNFα, azide and colchicine treatments

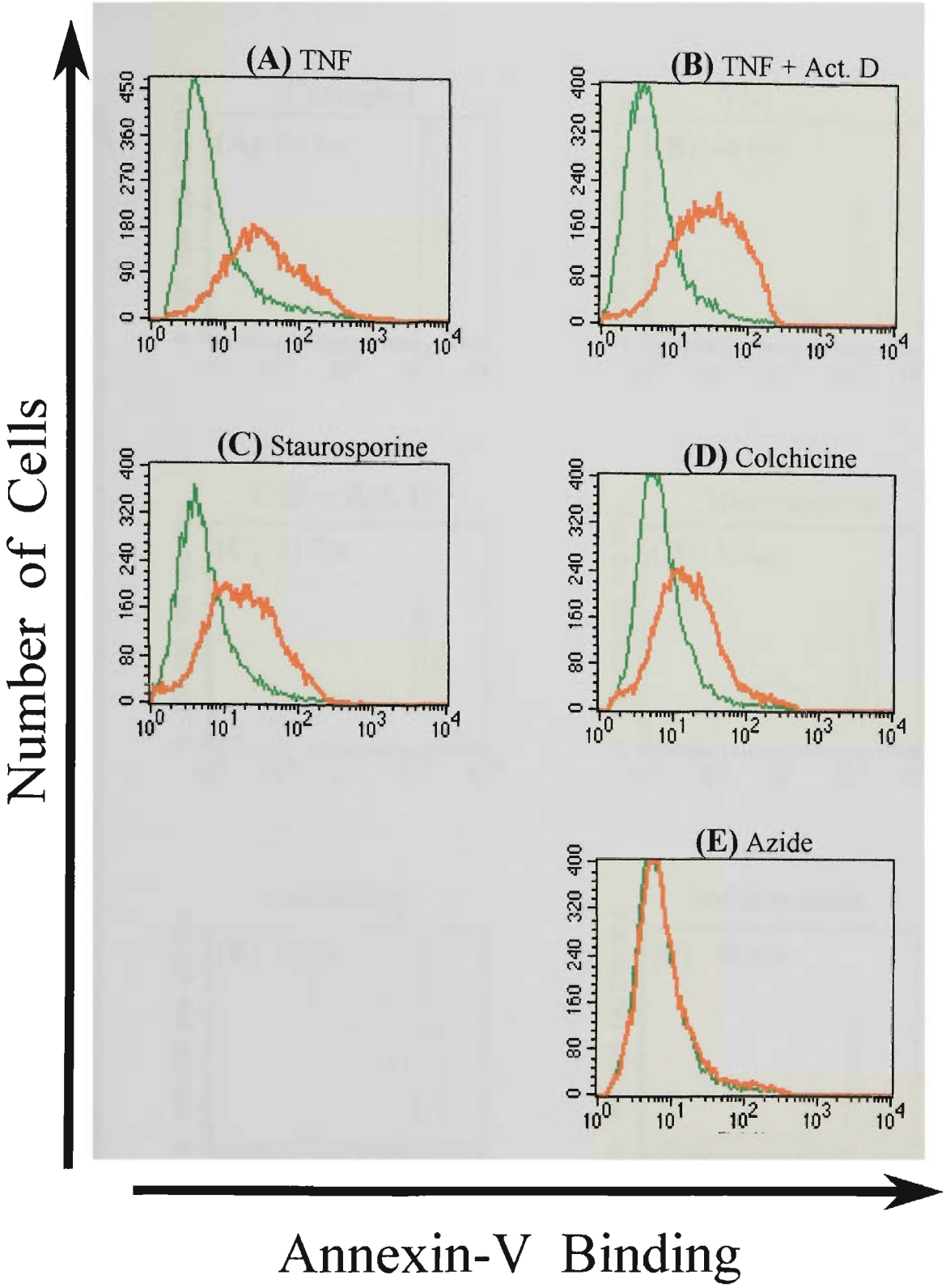


Figure 4.4: Flow cytometry histogram overlays showing binding of annexin-V-FITC to untreated L929 cells (green line) versus L929 cells treated (orange line) with the agent indicated. Dead cells (identified by their permeability to PI) were electronically excluded from histograms. The treatments were **A)** 100 ng/ml TNFα for 48 hours; **B)** 100 ng/ml TNFα + 4 μg/ml actinomycin D for 4 hours; **C)** 1 μM staurosporine for 7 hours; **D)** 2 μM colchicine for 24 hours; **E)** 0.6% (w/v) sodium azide for 24 hours. The results shown are representative of many independent experiments.

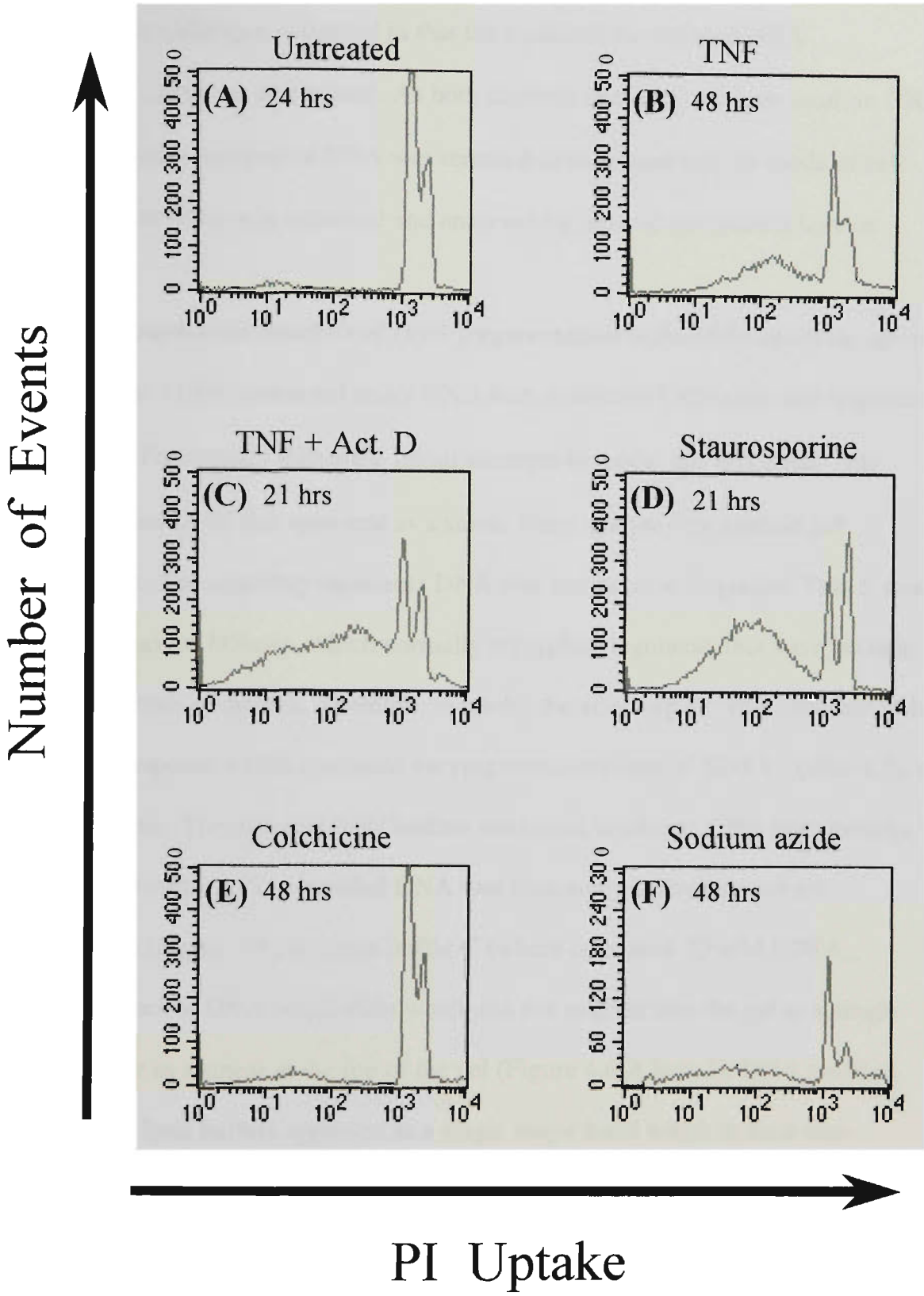


Figure 4.5: PI fluorescence of permeabilised L929 cells which had previously been treated with various cytotoxic agents for time periods shown. **A)** untreated; **B)** 100 ng/ml TNFα; **C)** 100 ng/ml TNFα + 4 μg/ml act D; **D)** 1 μM Staurosporine; **E)** 2 μM Colchicine; **F)** 0.6% Sodium azide.

were slower inducers of DNA fragmentation, which was first detected at 36 hours. This flow cytometric technique is limited in that the nature of the induced DNA fragmentation cannot be determined. As both necrosis and apoptosis can result in DNA degradation further analysis of DNA was required to help establish the mode of cell death. Therefore DNA was extracted and analysed by agarose gel electrophoresis.

4.3.5 Gel electrophoretic analysis of DNA fragmentation induced by cytotoxic agents

Kyprianou *et al.* (1991) extracted intact DNA from untreated L929 cells and fragmented DNA from TNFα treated L929 cells. Initial attempts to repeat this procedure only yielded degraded DNA that appeared as a smear when analysed by agarose gel electrophoresis. The smearing represents DNA that had become degraded. This is most likely due to various DNases, which normally are tightly regulated, that have become released upon lysis of the cell. Therefore to inhibit the affecting DNases different lysis buffers were prepared which contained varying concentrations of EDTA (Table 4.2), a protease inhibitor. The different lysis buffers were used to extract DNA from healthy untreated L929 cultures. The purified DNA was then analysed by agarose gel electrophoresis (Figure 4.6 A). Lysis buffer C (which contained 25 mM EDTA_ resulted in a viscous DNA preparation which did not migrate into the gel as a single band, but rather as a smear at the top of the gel (Figure 4.6 A lane 3). DNA purified using the other lysis buffers appeared as a single major band which in each case migrated a small distance into the gel (Figure 4.6 A lanes 1 and 2). Lysis Buffer B (which contained 12 mM EDTA) resulted in the least amount of DNA smearing indicating less DNase damage. This buffer was used for all further extractions of genomic DNA from L929 cells.

Table 4.2 Composition of different lysis buffers used to extract genomic DNA from L929 cells.

Lysis Buffer	Concentration of EDTA	Components common to all buffers
A	5 mM	25 mM Tris
B	12 mM	0.25% SDS
C	25 mM	pH 8.0

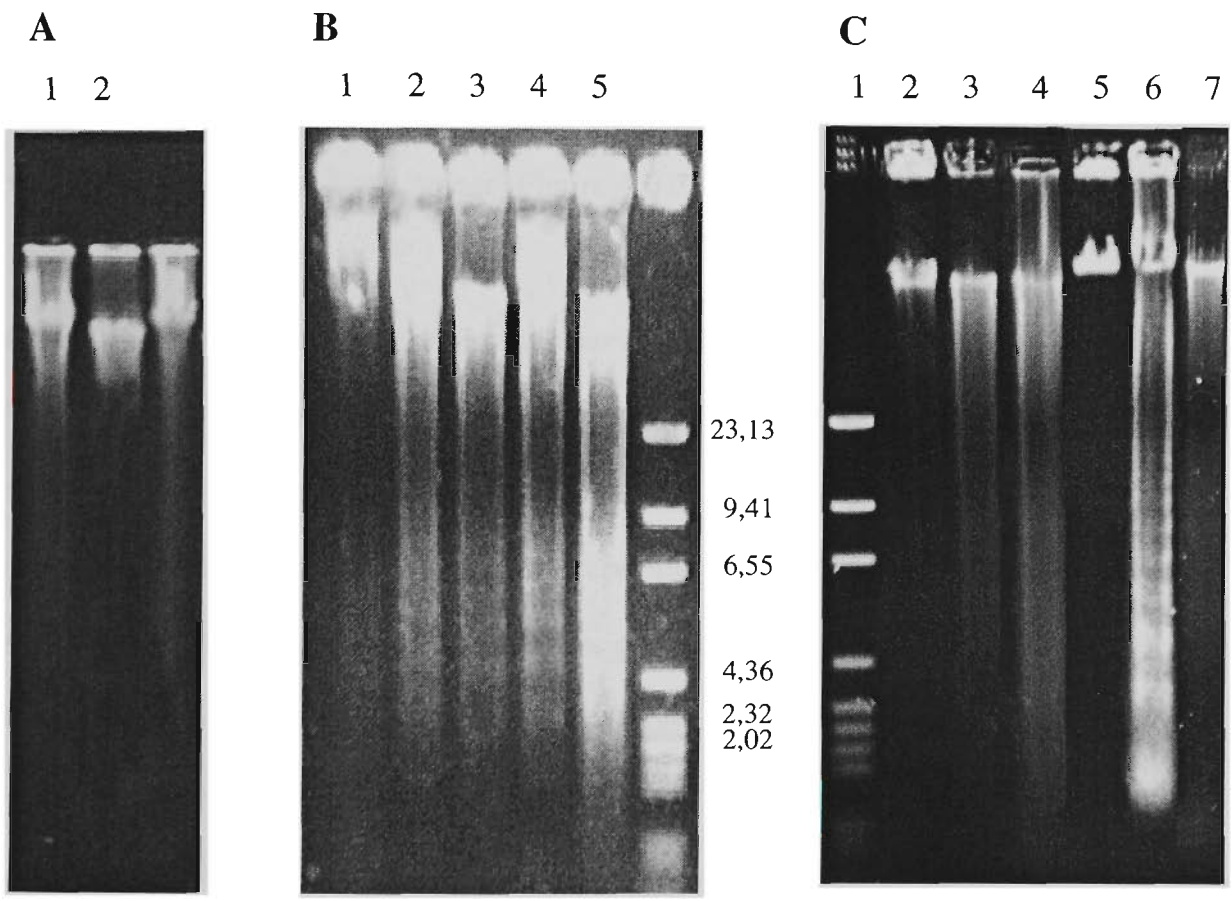


Figure 4.6: 1.6% agarose gel electrophoresis of DNA extracted from L929 cells treated as follows:- **A)** DNA extracted from untreated L929 cells using buffer A (lane 1); buffer B (lane 2), buffer C (lane 3); refer table 4.2. **B)** Lane 1: untreated L929 cells; Lane 2: 1 μM staurosporine for 7 hours; Lane 3: 1 μM staurosporine for 24 hours; Lane 4: 100 ng/ml TNF + 4 μg/ml actinomycin D for 7 hours; Lane 5: 100 ng/ml TNF + 4 μg/ml actinomycin D for 24 hours; Lane 6: DNA markers; **C)** Lane 1 DNA markers; Lane 2: untreated cells; Lane 3: 100 ng/ml TNF for 48 hours; Lane 4: 2 μM colchicine for 48 hours; Lane 5: untreated HL60 cells; Lane 6: etoposide treated HL60 cells (ie positive control for DNA laddering); Lane 7: 0.6% azide.

Analysis of DNA extracted from L929 cells treated with staurosporine or TNFα/Act D revealed DNA "ladders", a typical feature of apoptosis (Figure 4.6 B, lanes 2/3 & 4, respectively). DNA laddering in staurospine treated cells was most apparent in cultures that had been treated for between 7 and 24 hours. Treating cells with TNFα + act D for periods longer than 16 hours resulted in extensive DNA degradation which on gel electrophoresis appeared as a smear (Figure 4.6 B, lane 5). DNA laddering was not detected in L929 cells treated with either TNFα, colchicine or azide at any time point up to 48 hours. However, L929 cells treated with these cytotoxic agents for 24 hours or longer resulted in non-specific cleavage of DNA which when analysed by gel electrophoresis appeared as a smear (Figure 4.6 C, lanes 3, 4 and 7, respectively).

4.3.6 Effects of cytotoxic agents on nuclear and cell morphology

AO is a fluor that stains single and double stranded DNA as well as RNA. It is cell-permeable and is a useful stain to visualise nuclear morphology. Nuclei of healthy cells are large, intact and stain uniformly while apoptotic nuclei are generally small, compact and fragmented. Nuclei of necrotic cells are generally larger than healthy cell nuclei and do not show condensation or fragmentation (Gorman *et al.*, 1994). Following the various treatments, cell morphology was examined by light microscopy. Cells were trypsinized and pooled before staining with AO; nuclear morphology was analysed by fluorescence microscopy.

Nuclei of untreated cells were large intact structures which stained heavily but uniformly with AO (Figure 4.7 A). In untreated L929 cultures more than 95% of cells remained attached to the culture vessel surface. Nuclei from TNFα-treated cells were either (i) small and weakly stained, or (ii) normal size and brightly stained (Figure 4.7

B). Rounded-up, detached cells were common under these conditions and a very small proportion of the adherent population showed extensive membrane blebbing (estimated ~1%) (Figure 4.7 B; no blebbing visible in the field shown). The appearance of nuclei in TNFα/Act D-treated cells was similar to those of cells treated with TNFα alone, although fewer large brightly stained nuclei were visible at corresponding time points and some fragmented nuclei were detected (Figure 4.7 C). The addition of Act D greatly enhanced the sensitivity of cells to TNFα and, between 5 and 7 hours after the initial exposure, induced violent blebbing of the cell membrane (Figure 4.7 C). When removed from culture vessel surfaces by treatment with trypsin, a fraction of cells treated with TNFα or TNFα/Act D were observed to be smaller in size than untreated cells.

The AO-stained nuclei of staurosporine-treated L929 cells showed a variety of morphologies, consisting of the following types: (i) brightly stained, condensed and fragmented, (ii) condensed but dimly stained and (iii) brightly stained and intact (Figure 4.7 D). Staurosporine induced membrane blebbing within 1 hour of treatment (Figure 4.7 D) and after trypsinization many staurosporine-treated cells were noticably smaller than untreated cells. Similarly, the AO-stained nuclei of colchicine-treated cells showed a variety of morphologies, consisting of the following types: (i) condensed, lightly stained nuclei, (ii) brightly stained, heavily fragmented nuclei, or (iii) brightly stained intact nuclei (Figure 4.7 E). Following trypsinization, cells treated with colchicine for periods of 48 hours or longer were noted to be smaller in size than untreated cells.

AO-staining of nuclei within azide-treated cells revealed two distinct morphologies: (i) nuclei which only stained adjacent to the nuclear membrane and within one or more small intra-nuclear patches and (ii) brightly, uniformly stained nuclei (Figure 4.7 F). These nuclei were of equivalent size to non-treated cell nuclei. Following

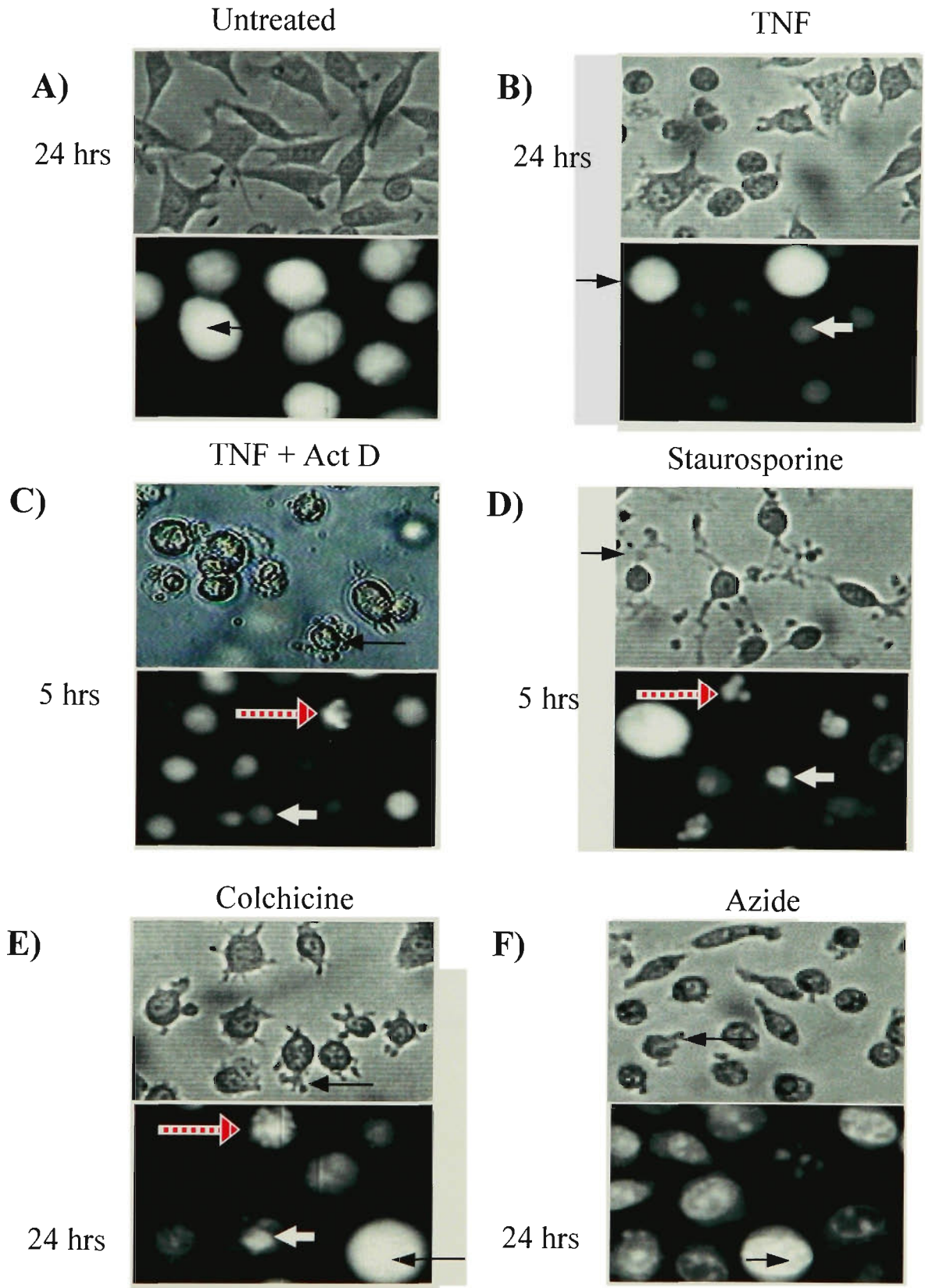


Figure 4.7: Light micrographs (top; magnification 100x) and the corresponding fluorographs (bottom; magnification 400x) of AO-stained L929 cells treated as indicated (note fields of view are not coincident). (A) Untreated cells; (B) 100 ng/ml TNF; (C) 100 ng/ml + 4 μg/ml Act D; (D) 1 μM staurosporine; (E) 2 μM colchicine; (F) 0.6% azide. On fluorographs, thin black arrows point to intact brightly stained nuclei, thick white arrows indicate condensed faintly stained nuclei, thick red striped arrows indicate fragmented nuclei. On light micrographs, the arrows indicate membrane blebbing.

trypsinization, L929 cells treated with azide were of similar size to untreated cells. Interestingly, azide did induce some membrane blebbing (Figure 4.7 E).

4.3.7 Human clusterin expression does not affect TNFα induced cell death in L929-LS transfectants.

There were were no differences in morphology detectable by light microscopy between L929-LS.clus(F), L929-LS.clus(B) and L929 wild type cells. Similarly, when L929-LS.clus cells were induced to secrete clusterin, no changes in cell viability or morphology were apparent. The effects of TNFα, TNFα/Act D and colchicine treatments on L929-LS transfectants were then analysed. The mode of cell death in transfected cells in response to the cytotoxic agents was similar to L929 wild type when analysed by 7-AAD staining (Figure 4.8 vs 4.3) or light microscopy. The induction of human clusterin expression in L929-LS.clus(F) cells did not provide any protection to any of the cytotoxic agents when compared with non-induced L929-LS.clus(F) or L929-LS.clus(B) cells (Figure 4.8 B & C). Previous kinetic studies on L929-LS transfectant cells revealed that clusterin is first detected in the TC sn 6 hours after induction with 1 mM IPTG (Humphreys, 1994). Even inducing clusterin expression in cells 12 hours prior to treatment did not provide any protection against the cytotoxic agents.

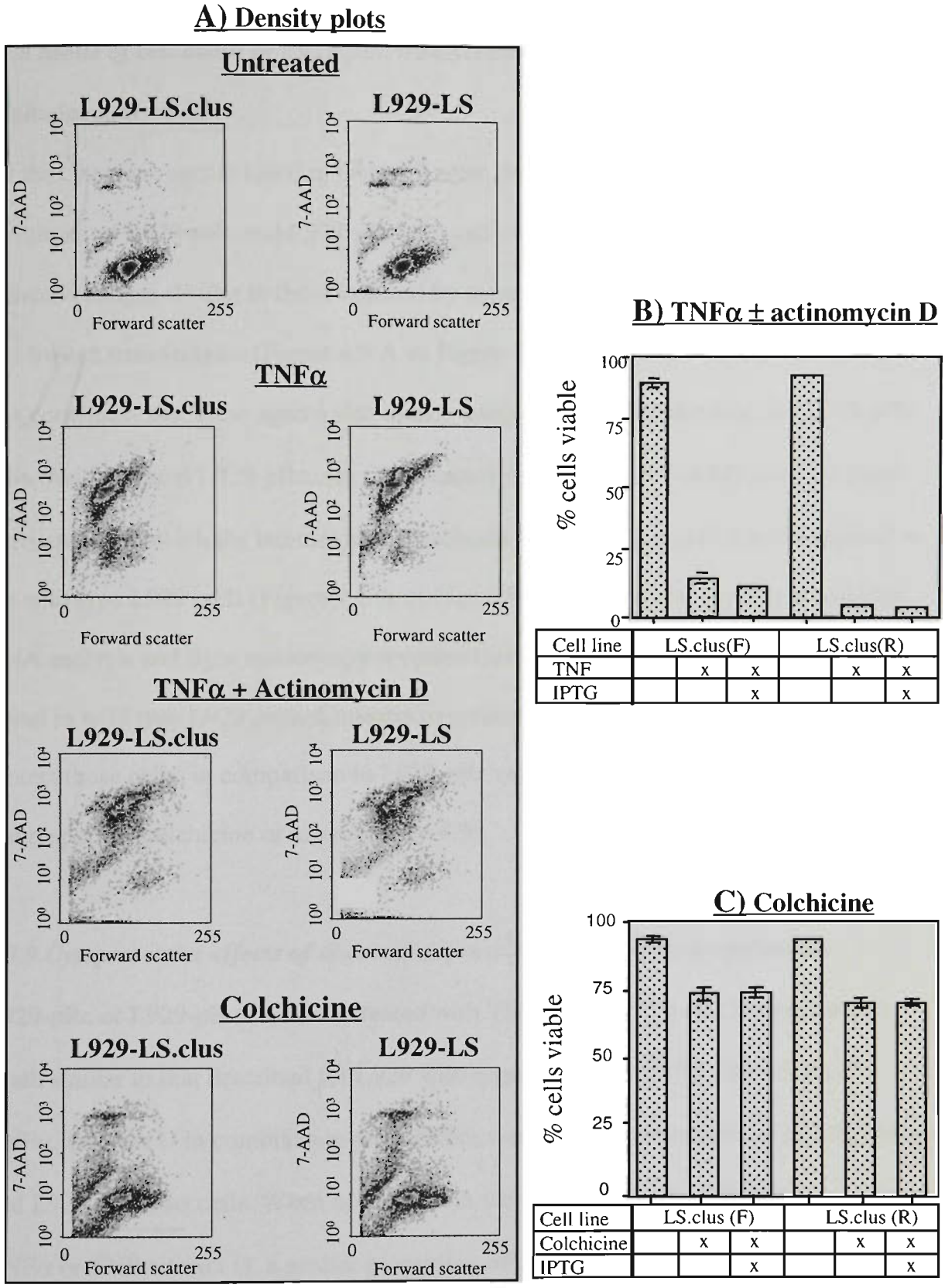


Figure 4.8: A) Flow cytometry density plots of 7-AAD uptake versus forward scatter in L929-LS.clus(F) and L929-LS.clus(B) transfectants exposed to the various cytotoxic agents indicated. The treatments were 100 ng/ml TNFα for 48 hours; 100 ng/ml TNFα + 4 μg/ml Act D; 2 μM colchicine. **B)** Histograms showing average percent of viable cells (as determined in figure 4.3) in cultures exposed to either 100 ng/ml TNFα or 100 ng/ml TNFα + Act D. The treatments are indicated by a corresponding "x" in the table below the histogram. **C)** Histograms showing average percent of viable cells (as determined in figure 4.3) in cultures exposed to 2 μM colchicine. The treatments are indicated by a corresponding "x" in the table below the histogram. For each histogram error bars represent standard error of the mean and in some cases are too small to be visible.

4.3.8 Mode of cell death in L929-pRc transfectants in response to staurosporine, colchicine and azide.

All the cytotoxic agents listed in table 4.2 were tested on the L929-pRc transfectants. Treatment of L929-pRc and L929-pRc.clus cells with staurosporine or colchicine induced changes similar to those induced by these agents in wild type L929 and L929 Lac Switch transfectants (Figure 4.9 A vs Figure 4.3; refer section 4.3.2). Therefore it was concluded that these agents also induce apoptosis in L929-pRc.clus and L929-pRc cells. Azide-treated L929-pRc.clus cells stained with 20 μg/ml 7-AAD resolved three populations in which the intermediate population was more apparent when compared to the wild type L929 cells (Figure 4.9 A vs Figure 4.3 F). However, annexin V staining, DNA analysis and light microscopy revealed that azide resulted in necrotic death as found in wild type L929 cells. Clusterin expression in L929-pRc.clus cells did not protect those cells, in comparison to L929-pRc cells, from the cytotoxic effects of staurosporine, colchicine or azide (Figure 4.9).

4.3.9 Cytoprotective effects of clusterin expression in L929-pRc transfectants

L929-pRc or L929-pRc.clus cells treated with TNFα or TNFα + Act D resulted cell death similar to that described for L929 wild type (section 4.3.2-6). The effects of TNFα, and Act D in combination with TNFα, were tested on the survival of L929-pRc and L929-pRc.clus cells. When non-clusterin secreting L929-pRc cells were exposed to TNFα or TNFα + Act D, a greater proportion of cells were non-viable (as determined by 20 μg/ml 7-AAD staining) relative to L929-pRc.clus cells treated with the same agents (Fig 4.10 A and B). Interestingly, TNFα treatment of L929-pRc.clus cells resulted in characteristics of cell shrinkage and intermediate staining with 7-AAD being more apparent in L929-pRc.clus cells than in control L929-pRc cells (e.g. Figure 4.10 A

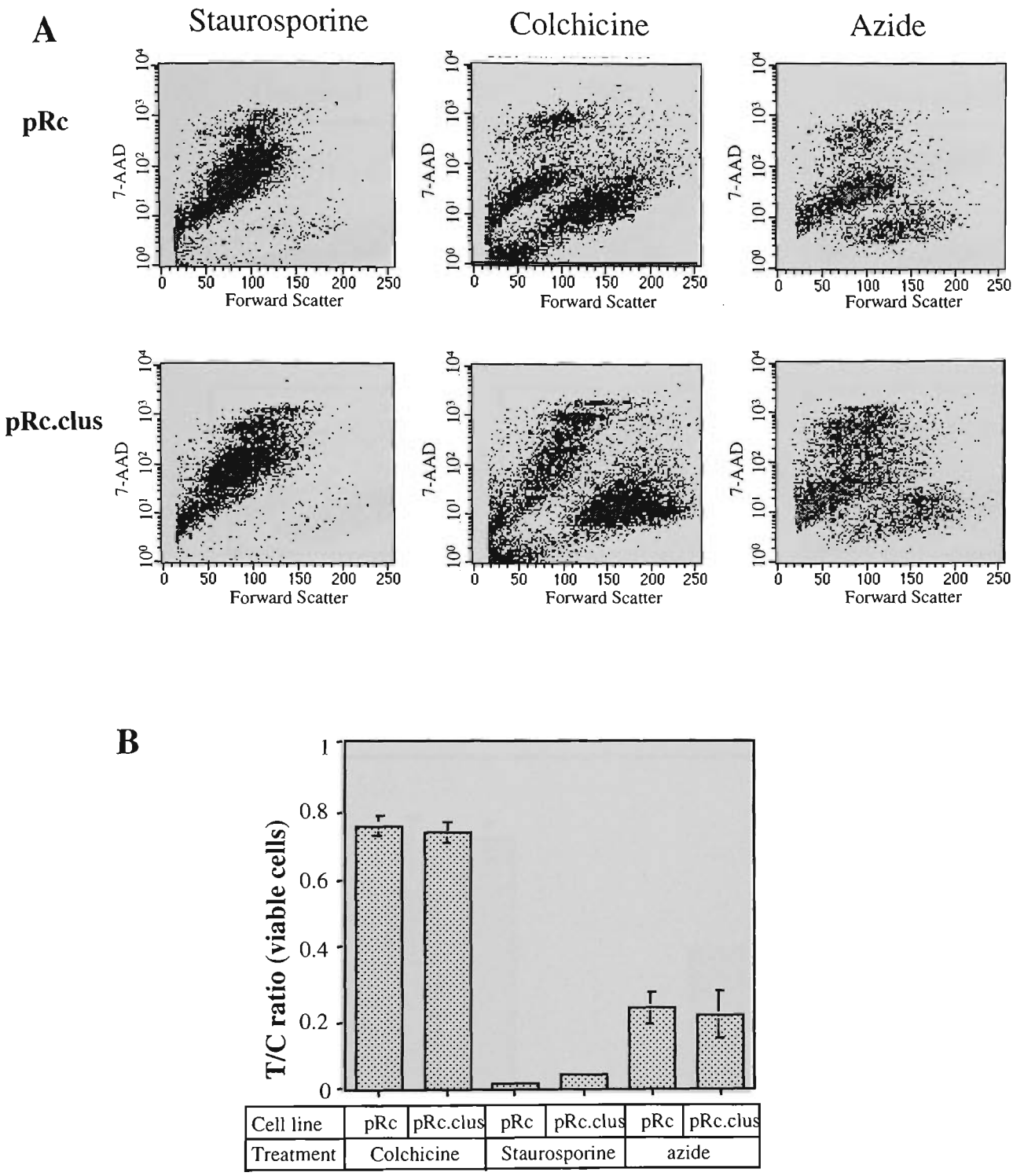


Figure 4.9: A) Flow cytometry density plots of 7-AAD uptake versus forward scatter in L929 pRc.clus and pRc cells treated with various cytotoxic agents indicated; **B**) Histogram showing the treatment:control ratio (T/C ratio) for the percentage of viable L929-pRc (pRc) and L929-pRc.clus (pRc.clus) cells in cultures exposed to the treatments indicated compared with control untreated cultures (Calculated by dividing the % viable cells in treated cultures by the % viable in untreated cultures). In each case, the table below the x-axis indicates the cell line and the conditions applied. The treatments were 2 μM colchicine for 48 h, 1 μM staurosporine for 16 h and 0.6% (w/v) sodium azide for 24 h. Viable cells were determined by 7-AAD staining and flow cytometric analysis (see figure 4.3). Each histogram represents the mean of three replicate measurements and the error bars shown represent SD of the mean. In a number of cases the SD is too small to be visible. None of the differences between L929-pRc and L929-pRc.clus cells are statistically significant ($p>0.05$, Student's t-test).

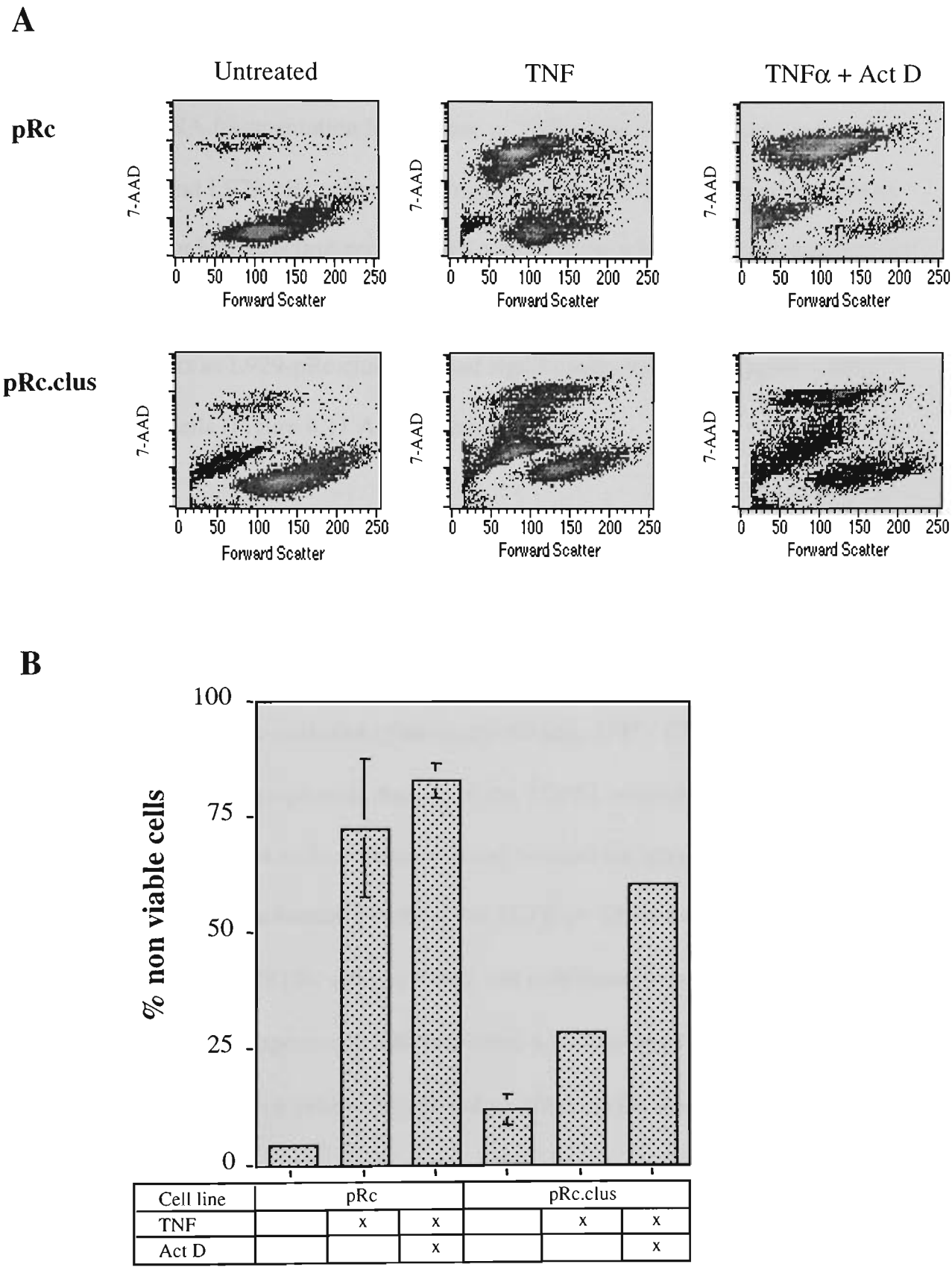


Figure 4.10: **A)** Flow cytometry density plots of 7-AAD uptake versus forward scatter in L929-pRc.clus and L929-pRc cells treated with 100 ng/ml TNFα or 100 ng/ml TNFα + 4 µg/ml Act. D. **B)** Histogram showing average percent of viable cells after treatment as determined by flow cytometry analysis of 7-AAD stained cultures (refer figure 4.3). The treatments are listed in the table below the histogram and are indicated by an "x". Error bars are the standard deviation of the mean and in some cases are too small to be visible.

vs Figure 4.3).

The extent of DNA fragmentation in response to TNFα was also compared between L929-pRc.clus and L929-pRc cells. DNA fragmentation was both analysed in cells by flow cytometry or by extracting genomic DNA from cells, which was then analysed by agarose gel electrophoresis. Both techniques confirmed that L929-pRc cells were more sensitive to TNFα as L929-pRc.clus cells had significantly less DNA fragmentation than L929-pRc cells (Figure 4.11 A and B respectively).

4.3.10 Overexpression of clusterin affects the sensitivity of L929 cells to TGFβ and TNFα

It was previously reported that exposure of L929 cells to TGFβ provides them with protection against TNFα-mediated cytotoxicity (Chang, 1995). Clusterin has also been reported to bind to the cytoplasmic domain of the TGFβ1 receptor (Reddy *et al.*, 1996). Therefore, L929-pRc.clus cells were used to test whether the upregulated expression of human clusterin would influence the effects of TGFβ on TNFα-mediated cell death. The pre-exposure of L929.pRc cells to TGFβ was confirmed to protect cells from death induced by subsequent exposure to TNFα (Figure 4.12 7 versus 3). In contrast, pre-exposure of L929-pRc.clus cells to TGFβ had no effect on the sensitivity of the cells to TNFα (Figure 4.12 8 versus 4). Furthermore, L929-pRc.clus cells show significant cell death in response to TGFβ, while L929-pRc cells did not die under the same conditions (Figure 4.12 6 versus 5).

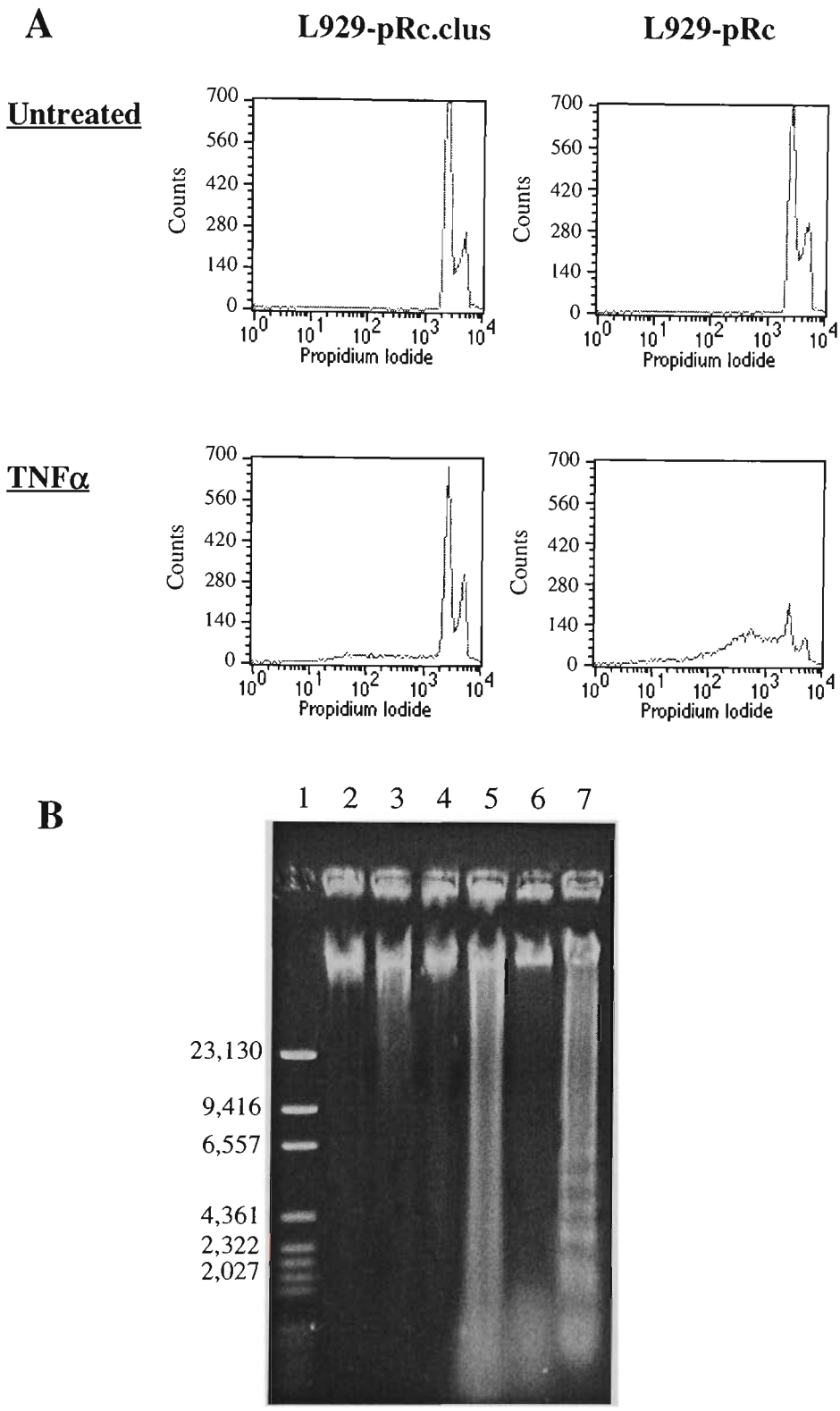
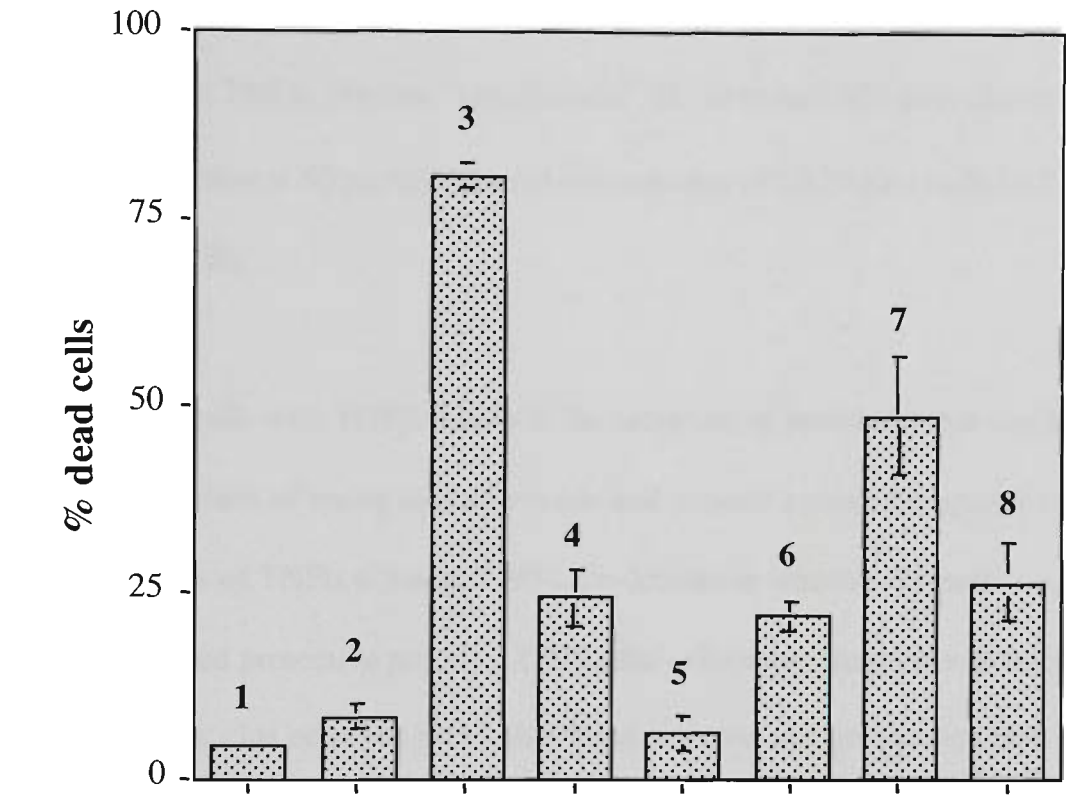


Figure 4.11: A) PI fluorescence of permeabilised L929-pRc.clus and L929-pRc cells which had been left untreated or treated with 100 ng/ml TNFα for 48 hours. **B)** 1.6% agarose gel electrophoresis of DNA extracted from L929 cells treated as follows:- Lane 1: markers; Lane 2: L929-pRc.clus untreated cells; Lane 3: L929-pRc.clus TNFα treated (100 ng/ml) cells; Lane 4: L929-pRc untreated cells; Lane 5: L929-pRc TNFα treated (100 ng/ml) cells; Lane 6: HL60 untreated cells; Lane 7 HL60 cells treated with etoposide (20 μg/ml). DNA from HL60 cells confirmed that the DNA extraction method was capable of extracting internucleosomally fragmented DNA. Thus TNFα treatment does not result in internucleosomal DNA fragmentation but rather random DNA fragmentation in L929-pRc cells.



Cell line	pRc	pRc.clus	pRc	pRc.clus	pRc	pRc.clus	pRc	pRc.clus
TNF			x	x			x	x
TGF					x	x	x	x

Figure 4.12: Percentages of 7-AAD stained cells defined by flow cytometric analysis as dead (refer figure 4.3) from cultures of L929-pRc (pRc) or L929-pRc.clus (pRc.clus) cells exposed to 2 ng/ml of TGFβ for 48 h and/or 100 ng/ml TNFα for 24 h. Cultures treated with both TGFβ and TNFα first had a 24 h incubation with TGFβ followed by a 24 h incubation with both TGFβ and TNFα. In each case, the table below the x-axis indicates the cell line and the conditions applied (indicated by "x"). Each data set represents the mean for triplicate cultures and the error bars shown represent SD of the mean in each case. The result shown is representative of three independent experiments. There was no significant difference in the proportion of dead cells in untreated pRc versus pRc.clus cultures (**1** vs. **2**; Student's t-test, $p > 0.5$). Whether exposed to TNFα alone, or to TGFβ followed by TNFα, there was significantly less cell death in pRc.clus versus pRc cells (**4** vs. **3**, $p<0.001$, and **8** vs. **7**, $p<0.05$, respectively; Student's t-tests). There were significantly more dead cells in cultures of pRc.clus cells exposed to TGFβ relative to pRc cultures treated the same way (**6** vs. **5**; $p<0.005$, Student's t-test). Following exposure to TNFα, there were significantly more dead cells in control pRc cultures relative to those that had been pre-exposed to TGFβ (**3** vs. **7**; $p<0.001$, Student's t-test). Under the same conditions, there was no significant difference between the level of dead cells in TNFα-treated pRc.clus cultures versus those that had been pre-exposed to TGFβ prior to treatment with TNFα (**4** vs. **8**; $p>0.2$, Student's t-test).

4.3.11 Secreted or exogenous clusterin does not protect cells from TNFα-mediated cytotoxicity

The data in figure 4.10 (A, B) show that L929-pRc.clus cells are protected against TNFα-mediated death. In order to establish the cellular site at which clusterin provides this protective effect, L929-pRc cells were cultured in "conditioned" L929-pRc.clus TC media containing secreted clusterin or media containing added exogenous clusterin and then treated with TNFα. Neither "conditioned" TC sn from L929-pRc.clus cells nor exogenous clusterin at 60 μg/ml affected the response of L929-pRc cells to TNFα (Figure 4.13 A, B).

Treating L929 cells with TGFβ results in the secretion of protein(s) that can become bound to the surface of tissue culture vessels and provide protection against the cytotoxic effects of TNFα (Chang, 1995). To determine whether clusterin-secreting cells also secreted protective proteins, L929-pRc cells were grown in culture vessels where L929-pRc.clus cells has previously been cultured and gently removed. There was no significant difference in viability following TNFα treatment of L929-pRc cells grown on either new culture wells or culture wells in which L929-pRc or L929-pRc.clus had previously been grown (Figure 4.13 C).

The cell surface was the only other possible extracellular site where clusterin may be responsible for TNFα resistance. However, using flow cytometry, negligible clusterin was found to be associated with the surface of L929-pRc.clus cells (Figure 4.13 D), suggesting that cell surface-bound clusterin was not producing the effect. One interpretation of these results is that they suggest that protection against TNFα-mediated cell death is afforded by an intracellular action of clusterin protein.

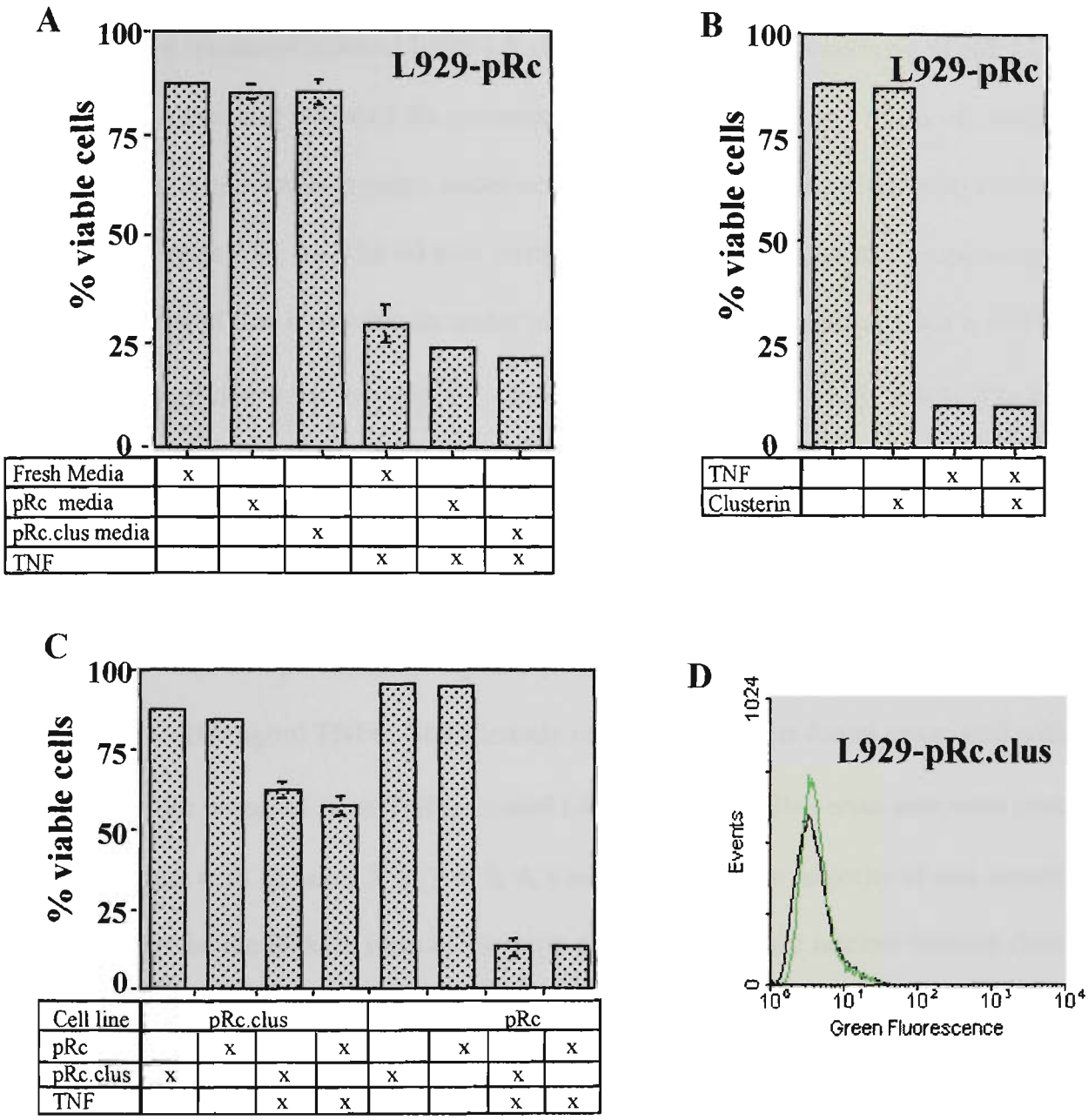


Figure 4.13: Influence of secreted molecules and added exogenous clusterin on the cytotoxic effects of TNFα on L929 cells. Histograms showing the average percentages calculated from triplicate cultures of viable L929-pRc cells, defined by 7-AAD staining and flow cytometric analysis (refer figure 4.3), from cell cultures exposed to a range of conditions. **A)** Where TNFα was applied, pRc cells were exposed for 24 h to 100 ng/ml TNFα added to fresh culture medium or to culture supernatant removed from other cell cultures (which had previously been incubated for 24 h). In each case, the table below the x-axis indicates the conditions applied (indicated by "x"). There were no significant differences in the percentages of viable cells in cultures exposed to the different culture media, with or without TNFα (single factor ANOVA, $0.05<p<0.025$). **B.** pRc cells were incubated with TNFα with or without the addition of 60 μg/ml of purified exogenous human serum clusterin. In each case, the table below the x-axis indicates the conditions applied (indicated by "x"). The addition of exogenous clusterin had no effect on the viability of the cells under any of the conditions tested ($p>0.2$, Student's t-test). In a number of cases the SD is too small to be visible. **C)** Viability of cultures treated with TNFα, where indicated, after growing on surfaces which previously contained the indicated cell type (designated by "x") or fresh cultures. **D)** Flow cytometry histogram showing that no surface bound human clusterin is detectable on L929-pRc.clus cells. Green line = L929-pRc.clus cells stained with G7, 78E, 41D Ab cocktail; black line = L929-pRc.clus cells stained with an isotype control Ab.

4.3.12 Analysis of intracellular forms of clusterin in transfected L929 cells

Analysis of lysates of induced L929-LS.clus and L929-pRc.clus cells by SDS/PAGE and immunoblotting revealed the presence in both cell types of two forms of clusterin with apparent molecular masses, under non-reducing conditions, of about 80 kDa and 64 kDa (Figure 4.14 A). The 80 kDa form was also found in cell culture supernatants and dissociated into two subunits under reducing conditions, indicating that it had been appropriately cleaved at the Asp^{205} - Ser^{206} bond (Kirszbaum *et al.*, 1989). The 64 kDa form was not found in cell culture supernatants and did not dissociate into subunits on reduction.

Analyses were also performed on lysates prepared from L929 pRc.clus cells exposed for 48 hours to 100 ng/ml TNFα. Significantly more clusterin was found associated with all cell fractions prepared from TNFα-treated L929-pRc.clus cells versus untreated control cells (Figure 4.14 B; lanes 3, 5, 7 vs 2, 4, 6 respectively). The majority of this increase appeared to be the 80 kDa form of clusterin associated with the nuclear fraction (lane 7 vs 6), however, there was also a noticable increase in both the 80 and 64 kDa clusterin species present in the cytoplasmic fractions (lane 5 vs 4). The nuclear-associated clusterin (Figure 4.14 B; lane 7) was physically indistinguishable from purified human serum clusterin (Figure 4.14 B; lane 1), which is itself indistinguishable from cell-secreted clusterin (Figures 4.14 A; lane 3).

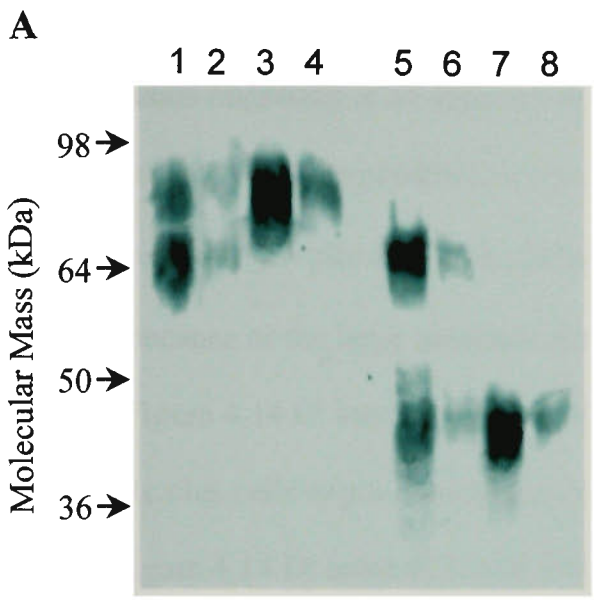
In addition, the cytoplasmic and total cell fractions prepared from TNFα-treated L929-pRc.clus cells contained a form of clusterin which was detected on immunoblots that was difficult to detect in fractions prepared from untreated L929-pRc.clus cells. This novel form of clusterin is most clearly resolved under reducing conditions, where it can

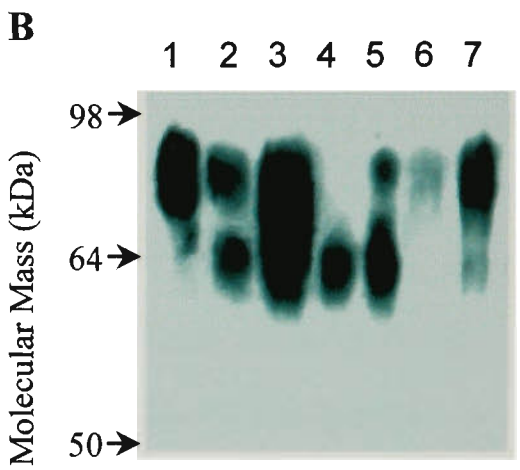
Figure 4.14: A) Immunoblot of whole cell lysates and tissue culture supernatants from L929-LS.clus cells and L929-pRc.clus which were grown in 5 ml of culture medium in 25 cm^2 flasks for 24 h. L929-LS.clus cells were grown in the presence of 5 mM IPTG to induce clusterin expression. About 3 x 10^6 cells were recovered from each flask after the 24 h period. Cells were removed from the flasks with trypsin/EDTA, washed, and then lysed in a volume of 1 ml as described in section 4.2.6. Aliquots of cell lysates (40 µl) and culture supernatants (20 µl) were electrophoresed on a 10% SDS-polyacrylamide gel and then electrophoretically transferred to nitrocellulose membrane. Membranes were probed with a cocktail of G7, 78E and 41D MAbs (which only recognise human clusterin). Lane 1, whole cell lysate from L929-pRc.clus; lane 2, whole cell lysate from induced L929-LS.clus; Lane 3, culture supernatant from L929-pRc.clus; Lane 4, culture supernatant from induced L929-LS.clus. Lanes 1-4 were run under non-reducing conditions. Lanes 5-8 contain identical samples to those in lanes 1-4 but were run under reducing conditions.

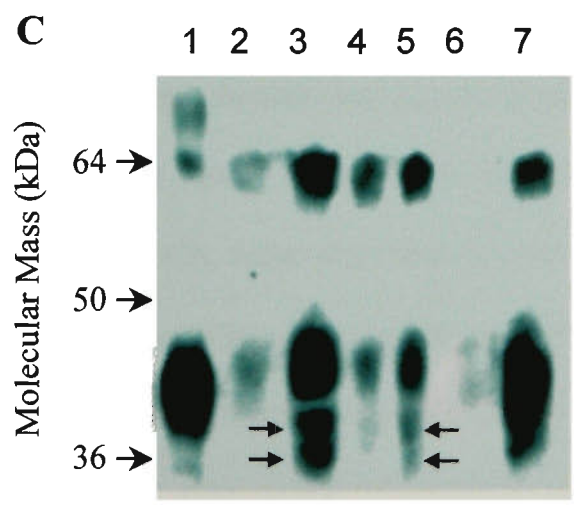
B) Immunoblot of various cell fractions prepared from L929-pRc.clus cells that had been exposed to 100 ng/ml TNFα for 48 h, or untreated controls. Cells were removed from the flasks with trypsin/EDTA, washed, and then lysed as described in section 4.2.6. Fractions from about 1.5 x 10^6 cells were loaded onto each track. Samples were electrophoresed under non-reducing conditions on a 10% SDS-polyacrylamide gel and then electrophoretically transferred to nitrocellulose membrane Lane 1, 320 ng of purified human serum clusterin; Lane 2, total cell fraction, untreated controls; Lane 3, total cell fraction, TNFα-treated; Lane 4, cytoplasmic fraction, untreated controls; Lane 5, cytoplasmic fraction, TNFα-treated; Lane 6, nuclear fraction, untreated controls; Lane 7, nuclear fraction, TNFα-treated.

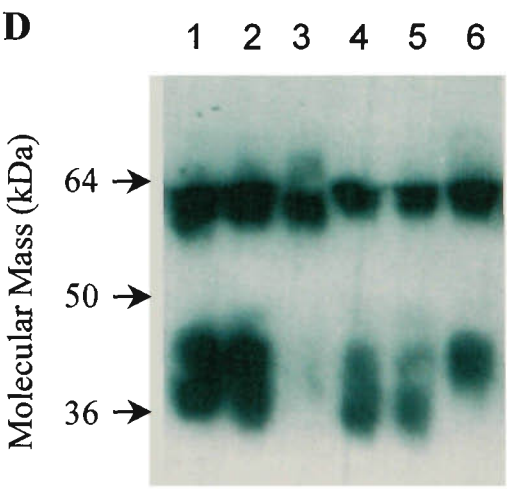
C) Same as B except samples were reduced with β-mercaptoethanol. Arrows indicate the position of two clusterin bands at about 36 and 38.5 kDa in the total cell and cytoplasmic fractions prepared from TNFα treated cells.

D) Immunoblot of whole cell lysates from L929-pRc.clus treated with various cytotoxic agents. Cells were removed from the flasks with trypsin EDTA, washed and lysed as described in section 4.2.6. Fractions from about 1.5 x 10^6 cells were loaded onto each track. Samples were reduced with β-mercaptoethanol before being loaded onto SDS PAGE gels. After electrophoresis, proteins were transferred onto nitrocellulose membrane and probed with the G7 anti-human clusterin antibody. Lane 1 TNFα treated (48 hrs); Lane 2 colchicine treated (48 hrs); Lane 3 azide treated (48 hrs); Lane 4 staurosporine treated (16 hrs); Lane 5 TNFα + Act D treated(16 hrs); Lane 6 untreated (48 hrs).


A
1 2 3 4 5 6 7 8
98
64
50
36
Molecular Mass (kDa)


B
1 2 3 4 5 6 7
98
64
50
Molecular Mass (kDa)


C
1 2 3 4 5 6 7
64
50
36
Molecular Mass (kDa)


D
1 2 3 4 5 6
64
50
36
Molecular Mass (kDa)

be seen as a closely spaced pair of bands migrating at apparent sizes of about 36 and 38.5 kDa (Figure 4.14 C; lane 3 and 5, bands indicated with arrows). In the same preparations, the normal processed form of clusterin is detected as a poorly resolved pair of bands migrating at an apparent size of about 43-45 kDa. The 36/38.5 kDa form of clusterin may also be present to a lesser extent in nuclear fractions prepared from TNFα-treated L929-pRc.clus cells, although, by immunoblot analysis, this is difficult to discern because of the large amounts of normal processed clusterin bound to dead cell nuclei (Figure 4.14 C; lane 7). The 36/38.5 kDa clusterin is also detectable in lysates of L929-pRc.clus cells exposed to staurosporine, colchicine, and to a much lesser extent azide (Figure 4.14 D; lanes 4, 2, and 3 respectively). Like the constitutive form of clusterin, the 36/38.5 kDa form has clearly been internally proteolytically cleaved.

Wilson *et al.* (1995) has reported that when purified exogenous clusterin is added to nutrient-deprived cultures of various cell types it readily binds to the nuclei of dead cells. Therefore it was suspected that in TNFα-treated cultures of L929-pRc.clus cells, cell-secreted clusterin was simply binding to the nuclei of dead cells once their cell membrane was no longer intact. This hypothesis was tested by first staining L929-pRc.clus cells, either untreated or treated with TNFα, briefly with 1 μg/ml 7-AAD, and then permeabilizing the membranes of the cells before detecting intracellular clusterin with G7 MAb followed by SaMIg-FITC (refer section 4.2.3.5). Two colour flow cytometric analysis then allowed the determination of the relative amounts of clusterin associated with dead cells (discriminated as "strongly" stained with 7-AAD) versus cells still possessing an intact cell membrane (discriminated as "weakly" stained with 7-AAD) (Figure 4.15 A). This analysis confirmed that in TNFα-treated L929-pRc.clus cultures, relative to cells with an intact cell membrane, dead cells had greater amounts

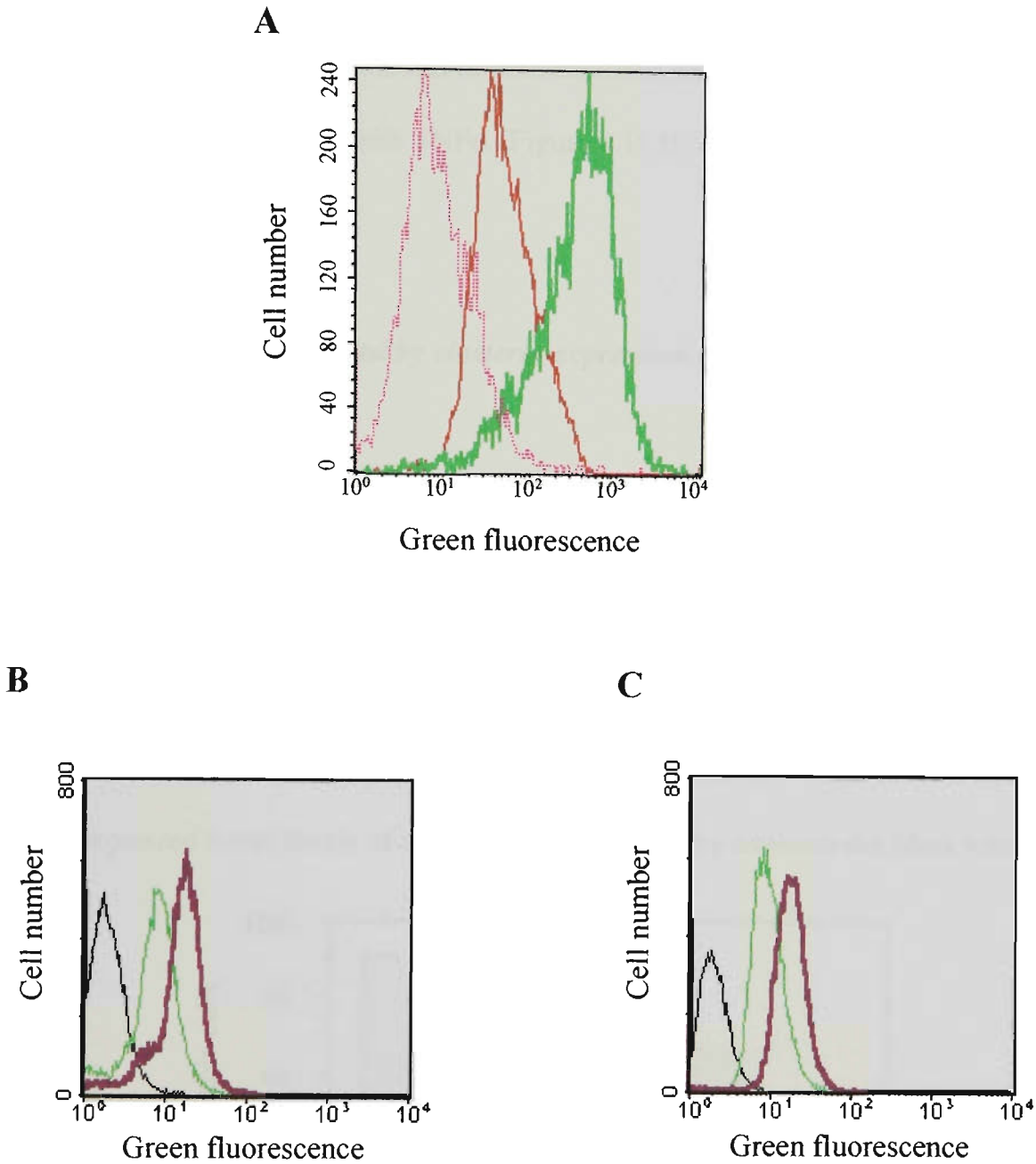


Figure 4.15: **A)** Flow cytometry overlay histograms showing the level of cell-associated clusterin for ethanol-permeabilized L929-pRc.clus cells that had been exposed to TNFα for 48 hours. Dead cells (that in culture lacked an intact cell membrane) were discriminated on the basis of high red fluorescence (staining with 7-AAD; refer section 4.2.3.2). Cell-associated clusterin was detected as described in section 4.2.3.5 and is represented by green fluorescence (dead cells - green line; cells that in culture had an intact cell membrane - red line). The dotted line represents dead cells that were stained with DNP-9 (an isotype-matched control antibody) then SaMIg-FITC. **B)** Flow cytometry overlay histograms showing the level of clusterin-FITC (purple line) or BSA-FITC (green line) that bound to permeabilized L929 WT cells treated with TNFα. Black line represents unlabelled cells. **C)** same as **B** except cells were not treated with TNFα. Results shown are representative of three independent experiments

of cell-associated clusterin. Inspection of the stained cells by fluorescence microscopy indicated that clusterin was predominantly localized on the nuclei and cell membranes of dead cells. Flow cytometry was also used to demonstrate that both clusterin-FITC and BSA-FITC bound at high levels to permeabilised wild type L929 cells which had previously had been treated with TNFα (Figure 4.15 B) or left untreated (Figure 4.15 C).

4.3.13 The protection provided by clusterin expression is dependent upon the amount of clusterin expressed.

The first round of cloning L929-pRc.clus cells produced several clones that expressed different levels of clusterin. The highest expressing clone (E3) was chosen for a second round of cloning and used for experimentation (refer Figure 3.5). To confirm that resistance to TNFα seen in L929-pRc.clus cells was directly related to clusterin expression, the cytotoxic effects of TNFα was tested on the first round clones. Clones which expressed lower levels of clusterin as determined by immuno-dot blots were

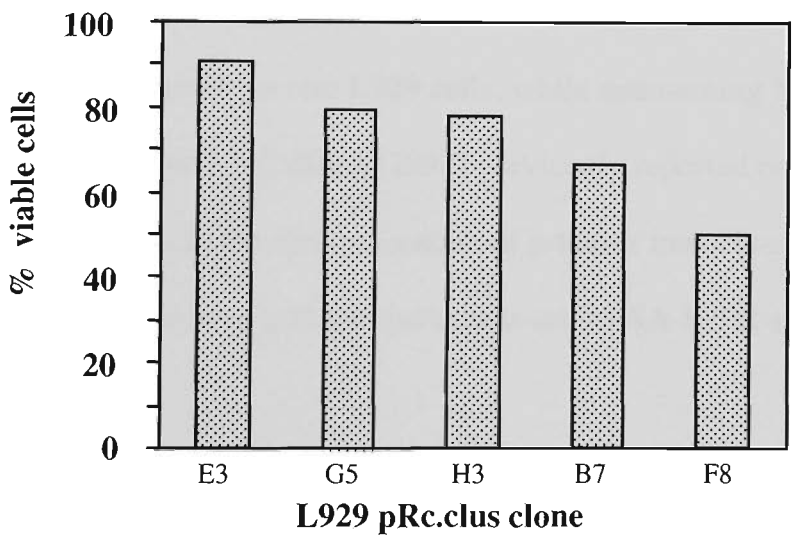


Figure 4.16: Viability of x1clones of L929-pRc.clus cells treated with 100 ng/ml of TNFα for 24 hours. Viability was determined using 7-AAD staining (refer figure 4.3). Each clone secreted different levels of clusterin. Clones are placed in order of the amount of clusterin secreted as determined by immunodot-blot, with E3 being the highest secreting clone and F8 being the weakest secreting clone. Results shown are single determinations and are representative of two independent experiments

more sensitive to TNFα compared to clones which expressed higher levels of clusterin (Figure 4.16).

4.3.14 The expression of TNF-receptors are not modified in L929-pRc.clus cells

The high expression of clusterin in L929-pRc.clus cells may indirectly affect the expression of other proteins crucial to the TNF pathway thereby providing protection against TNF. Two commercial antibodies specific for TNFR1 and TNFR2 were purchased and the expression of these proteins on the surface of L929 cell lines was analysed by flow cytometry. Both receptors were detected, expressed at a low level, on the surface of each L929 cell line (Figure 4.17). This suggests that clusterin expression did not affect the expression of TNF receptors.

4.3.15 Introducing clusterin protein into the cytosol of intact L929 cells

The presence of clusterin in TC sn was shown not provide protection to TNFα (Figure 4.13 C). Therefore purified clusterin was electroporated into L929 cells to see whether this provided protection against the cytotoxic effects of TNFα treatment. First, conditions to electroporate proteins into L929 cells, while maintaining high viability, were examined. Glogaver and McCulloch (1992) previously reported conditions they found to be most favourable in the electroporation of proteins into fibroblasts. To avoid using the finite stocks of clusterin initial experiments used BSA-FITC as a "tracer".

L929 cells prepared in electroporation buffer containing BSA-FITC were electroporated at either 200 or 400 volts. Electroporation using these conditions resulted in high mortality and many of the dead cells clumped together to form a large hydrophobic mass. Approximately 30% of the cells that were returned to the culture were determined to be dead. These cells were easily identified by their high level of staining with PI

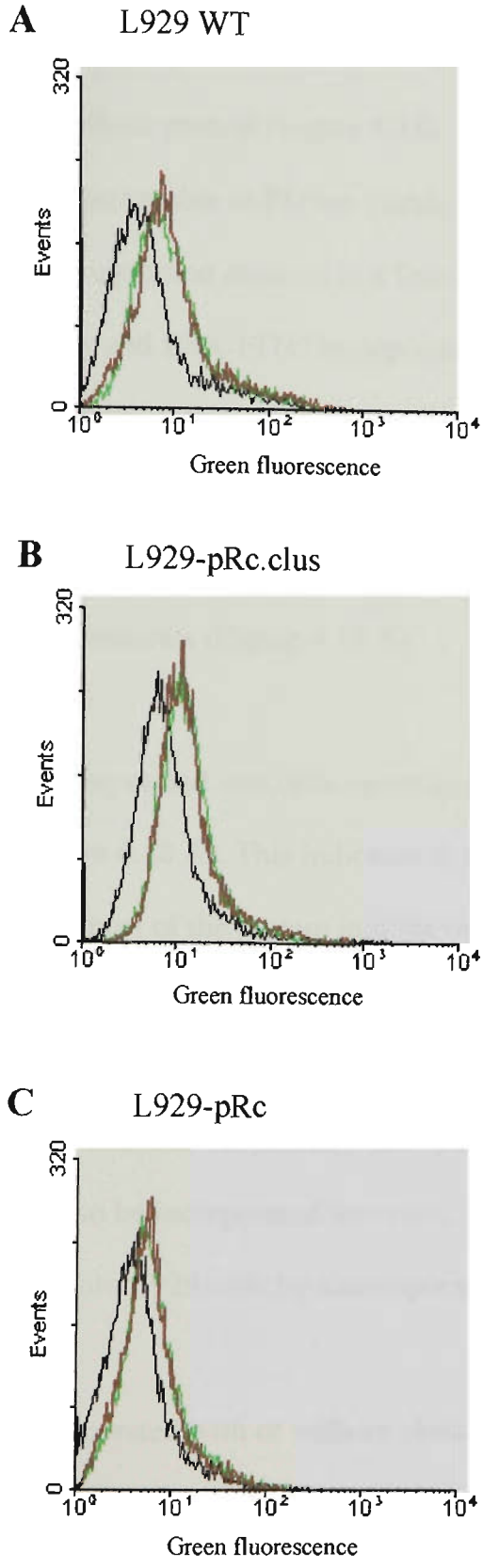
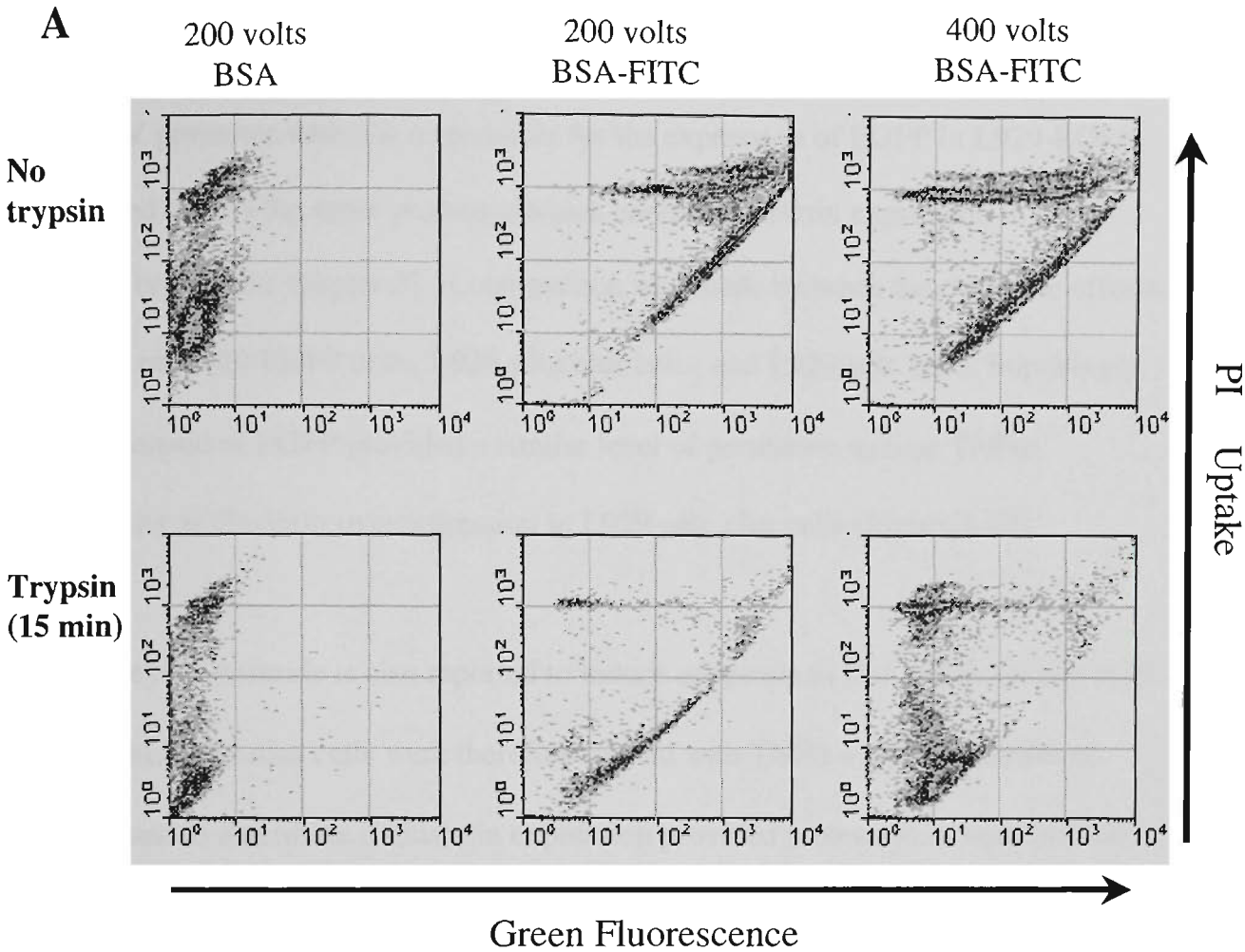


Figure 4.17: Flow cytometry histograms showing analyses of untreated **A)** L929 WT, **B)** L929-pRc.clus and **C)** L929-pRc cell lines. Cells were labelled with antibodies that recognised either TNF-R1 (green histogram), TNF-R2 (brown histogram) or an isotype control antibody (black line) followed by sheep-anti-mouse-Ig-FITC secondary antibody.

(Figure 4.18 A). A similar amount of BSA-FITC was incorporated into cells, as determined by flow cytometry, from each electroporation setting relative to cells which were electroporated with no protein (Figure 4.18). Immediately after electroporation many cells were more permeable to PI than viable healthy cells (Figure 4.18). Interestingly, the electroporation resulted in a linear distribution of cells that varied in membrane permeability and BSA-FITC incorporation (Figure 4.18 A). Cells with high membrane permeability (high PI fluorescence, except dead cells as indicated in figure 4.18 A) tended to have incorporated more BSA-FITC (high green fluorescence) and vice versa. The membrane integrity of these cells returned to levels similar to that of untreated cells after 15 minutes (Figure 4.18 A).

Most of the protein incorporated into cells electroporated at 400 volts was destroyed by trypsin treatment (Figure 4.18 A). This indicated that electroporation of L929 cells at 400 volts incorporates most of the protein into the outer membrane. Most of the protein incorporated into cells electroporated at 200 volts was not destroyed by trypsin treatment (Figure 4.18 A), indicating that under these conditions proteins were internalised. As BSA-FITC was successfuly incorporated into cells this suggests that clusterin protein can also be incorporated into cells. It was found that clusterin-FITC was also incorporated into L929 cells by electroporation at 200 volts (data not shown).

L929 cells were electroporated with or without clusterin before comparing the cytotoxic effects of TNFα. The electroporation of clusterin into cells did not alter cell viability relative to cells electroporated with BSA or no protein (Figure 4.18 B). Surprisingly clusterin incorporated into L929 cells did not provide any significant protection against TNFα when compared with cells electroporated with no protein or BSA (Figure 4.18 B).



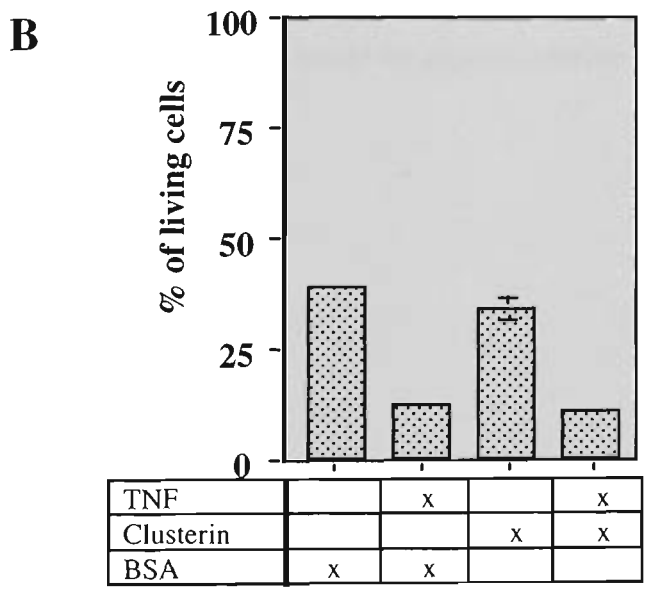


Figure 4.18: A) Density plots of L929 cells which had been electroporated at the indicated voltage with BSA (labelled with FITC or unlabelled as indicated) and then either treated (15 minutes) or not treated with trypsin. Density plots show green fluorescence (BSA-FITC uptake) versus PI uptake. Cell that had a greater PI uptake than the blue line are dead. **B**) Average viability of L929 cells which had been electroporated with clusterin or BSA at 200 volts and then treated with 100 ng/ml of TNFα for 24 hours. (Viability was determined by flow cytometric analysis of cells stained with PI; refer section 4.2.3.2). Table below the histogram indicates (with "x") the protein electroporated into cells (clusterin or BSA) and whether cells were treated with TNFα. Error bars represent standard deviation of the mean in each case. In some cases the standard deviation is too small to be seen. Results shown are representative of three independent experiments.

4.3.16 Effects of TNFα on other transfected cell lines

The CMV promotor which is responsible for the expression of EGFP in L929-EGFP transfected cells is the same promotor which controls clusterin expression in L929-pRc.clus cells (refer chapter 3). A comparison was made between the cytotoxic effects of TNFα on L929-EGFP cells, L929-pRc.clus cells, and L929-pRc cells. Suprisingly, overexpression of EGFP provided a similar level of protection against TNFα cytotoxicity as clusterin overexpression in L929-pRc.clus cells (Figure 4.19).

TNFα + cycloheximide is also reported to induce apoptosis in HeLa cells (White *et al.*, 1992). HeLa-pRc.clus cells were therefore treated with TNFα + cycloheximide or TNFα alone to determine if clusterin expression provided protection as seen in L929-pRc.clus cells. HeLa transfected cells were not sensitive to TNFα alone, however the addition of cycloheximide resulted in cell death. Surprisingly, HeLa-pRc.clus cells were *more* sensitive to TNFα + cycloheximide treatment relative to HeLa-pRc cells (Figure 4.19).

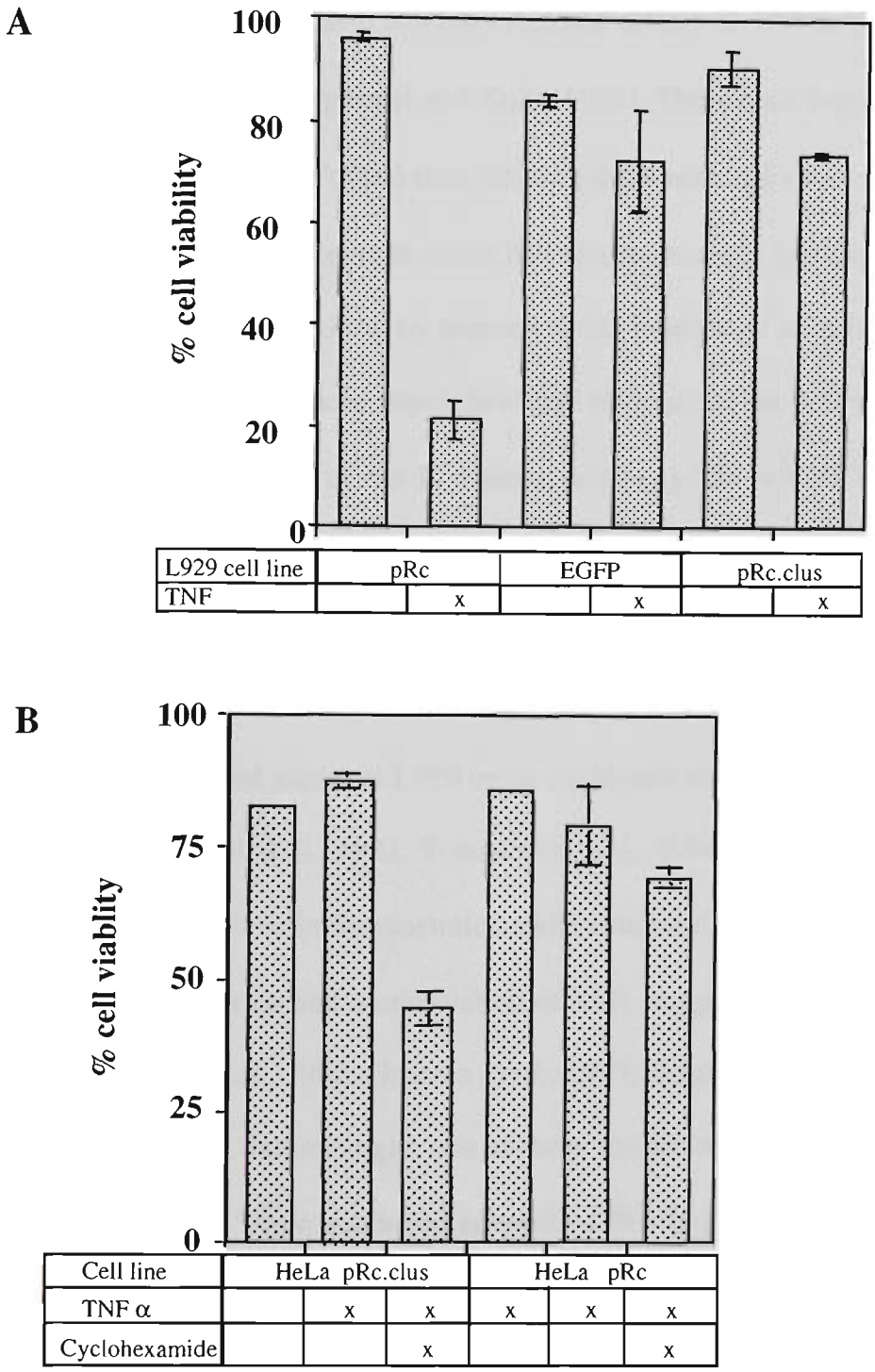


Figure 4.19: **A)** Histogram showing average cell viability of L929-EGFP, L929-pRc.clus and L929-pRc cells following treatment with 100 ng/ml TNFα for 48 hours. **B)** Average cell viability of HeLa-pRc.clus and HeLa-pRc.clus transfectants treated with either 100 ng/ml TNFα alone or in combination with 10 μg/ml cycloheximide. The tables below the histograms indicate (with an "x") treatments. In each case viability was determined by flow cytometric analysis of cells stained with 7-AAD (refer figure 4.3). Means were calculated from values determined for three independent cultures and errors bars are standard deviation of the mean. In some cases the standard deviation is too small to be visible.

4.4 DISCUSSION

The L929 cell line is commonly used to test the cytotoxic effects of TNFα, a procedure that was first described in 1985 (Aggarwal and Kohr, 1985). These tests typically involve exposing L929 cells to TNFα and then staining them with coloured or fluorescent compounds unable to penetrate intact cell membranes (eg Levesque *et al.*, 1995). Loss of cell viability is quantified by measuring the fraction of all cells that have stained with these compounds (ie those which have lost integrity of the cell membrane). The tests often involve the addition of Act D which sensitizes L929 cells to TNFα, reducing the incubation times required (Ostrove and Gifford, 1979).

Initial reports demonstrated that TNFα induced oligonucleosomal DNA fragmentation and condensed and fragmented nuclei in L929 cells, consistent with apoptotic cell death (Fady *et al.*, 1995; Kyprianou *et al.*, 1991; Tomasovic *et al.*, 1994). However, other studies of TNFα-treated L929 cells demonstrated swift structural degeneration of mitochondria and the production and accumulation of ROS, suggesting a necrotic mode of death (Schulze Osthoff *et al.*, 1992; Grooten *et al.*, 1993; Schulze Osthoff e*t al.*, 1993; Goossens *et al.*, 1996). Interestingly, one of these studies reported that extended treatment of L929 cells with TNFα produced apoptotic DNA fragmentation (although this data was not shown; (Schulze Osthoff e*t al.*, 1992)). Research described in this chapter examined the mode of death of L929 cells following exposure to various cytotoxic agents including TNFα, TNFα/Act D, staurosporine, colchicine and azide. The mode of cell death was investigated using the DNA stain 7-AAD to identify "leaky" apoptotic membranes, annexin V-FITC to identify exposure of PS on the outer surface of the cell membrane, the DNA/RNA stain acridine orange (AO) to visualise nuclear morphology, agarose gel electrophoresis to analyse DNA fragmentation, and

light microscopy to examine cellular morphology. PI staining of permeabilised cells has been reported to allow detection of DNA fragmentation (Nicoletti *et al.*, 1991). However, this method did not discriminate apoptotic from necrotic cells as both modes of death eventually resulted in the degradation of DNA (Gorman *et al.*, 1994). Therefore this measurement was not used in determining the mode of cell death. A review of the literature reveals that 7-AAD and annexin V have not been used before in analyses of L929 cell death.

4.4.1 Mode of L929 cell death induced by TNFα

Flow cytometric analysis of TNFα-treated L929 cells stained with 20 μg/ml of 7-AAD identified a population with reduced cell volume and cell membrane permeability between that of healthy viable cells and dead cells (Figure 4.3B), characteristic of apoptotic cells. Examination of untreated and TNFα-treated cells by light microscopy indicated that TNFα induced cell shrinkage, supporting the flow cytometry results. The redistribution of PS from the inner to the outer leaflet of the plasma membrane in apoptotic cells is thought to constitute a phagocyte recruitment signal and facilitate disposal of the cell corpse (Fadok *et al.*, 1992 a and b). Annexin V bound to the surface of TNFα-treated L929 cells (Figure 4.4A) indicating that these cells had externalised PS, consistent with them undergoing apoptosis. However, extensive membrane blebbing, common in apoptotic cells, was only detected in a small fraction (~1%) of TNFα-treated L929 cells.

One of the hallmarks of apoptosis is the cleavage of DNA into fragments that are multiples of 180-200 bp in length. DNA from TNFα-treated L929 cells was non-specifically fragmented (detected as a broad smear by agarose gel electrophoresis;

Figure 4.6C). Similar DNA degradation occurred in L929 cells treated with sodium azide (a known inhibitor of the respiratory chain which results in necrosis in many cell types; Fady *et al.*, 1995; Lizard *et al.*, 1995; Vaux *et al.*, 1996) and during heat-induced necrosis of L929 cells (Tomasovic *et al.*, 1994). The nuclei of TNFα-treated L929 cells were not fragmented (Figure 4.7B). Examination of TNFα-treated L929 cells stained with AO identified two populations discriminated on the basis of differences in nuclear morphology. One population consisted of cells with normal sized brightly stained nuclei, while the second population, which appeared after 24 hours, had small weakly stained nuclei (Figure 4.7B). The intensity of staining with AO gives an indication of the amount of DNA present in the nucleus. Therefore, the small weakly stained nuclei probably belong to cells that had undergone extensive degradation and lost DNA. This pattern of DNA degradation without nuclear fragmentation is consistent with a necrotic mode of cell death.

4.4.2 Relationship to other studies of the effects of TNFα on L929 cells

As previously mentioned, other studies have identified the mode of L929 cell death induced by TNFα as either apoptotic or necrotic. There are a number of possible explanations for the discrepancies between these earlier reports and between this report and those. Firstly, a number of these reports relied exclusively or largely on the demonstration of DNA "laddering" to establish apoptosis. Furthermore, some of these studies used recombinant murine TNFα to induce death while others used recombinant human TNFα. It has been shown that although murine TNF-R1 binds with equal affinity to mTNFα and hTNFα, the latter is bound with relatively poor affinity by murine TNF-R2 (Lewis *et al.*, 1991). Thus, differential recognition by murine TNF-R2 of TNFα from different species may in part explain some of the reported discrepancies.

However, this cannot explain all discrepancies because it was reported that L929 cells treated with human TNFα (i) showed clear evidence of classical oligonucleosomal DNA fragmentation after only 12 hrs (Kyprianou *et al.*, 1991), or (ii) showed extensive non-specific fragmentation of DNA with evidence of limited oligonucleosomal fragmentation only after 48 hrs of exposure (Sanchezalcazar *et al.*, 1997). A possible explanation for the differences between these latter published results might be the different concentrations of TNFα used ((Kyprianou *et al.*, 1991)- 100 ng/ml; (Sanchezalcazar *et al.*, 1997)- 25 ng/ml).

It is interesting to note that much earlier studies reported both necrosis and apoptosis occurring simultaneously in L929 cultures treated with TNFα (Mcquilkin and Earle, 1961; Russel *et al.*, 1972). It was suggested that there are two distinct subpopulations of cells within L929 cultures (Laster *et al.*, 1988). It is therefore possible that over time, as a result of different culturing conditions between research groups, one or other of these subpopulations has become dominant, accounting for the contrasting results obtained by different groups. Nevertheless, the research described in this chapter was performed on L929 cells that were obtained from ATCC in 1996. When treated with TNFα these cells exhibited features that could be regarded as typical of both necrosis and apoptosis. This is not a unique finding as Simm *et al.* (1997) also found features of both necrosis and apoptosis in serum-depleted fibroblasts.

4.4.3 Mode of death induced by TNFα/Act D

It was confirmed that Act D sensitized L929 cells to TNFα as the rate of death was increased approximately five fold relative to treatment with TNFα alone (Figure 4.2). Flow cytometric analysis of 7-AAD-stained TNFα/Act D-treated L929 cells

discriminated two major populations of cells, viable and dead. A minor population of cells was also identified that had a reduced volume and stained to an intermediate level with 7-AAD (Figure 4.3C). As death induced by TNFα/Act D was very rapid it is possible that the stage of intermediate cell membrane permeability is short lived in this system. The mode of death in L929 cells exposed to this combination of agents was apoptotic as cell shrinkage (Figure 4.3C and light microscope observations), membrane blebbing (Figure 4.7C), exposure of PS at the cell surface (Figure 4.4B), nuclear condensation/fragmentation (Figure 4.7C) and DNA laddering (Figure 4.6B) were all detected.

4.4.4 Modes of death induced by staurosporine, colchicine and azide

Staurosporine and colchicine are cytotoxic agents which are commonly used to induce apoptosis in a variety of cell types. The inhibitory effects of staurosporine on protein kinases has recently been reported to sensitise L929 cells to the effects of TNFα (Oconnell *et al.*, 1997; Beyaert *et al.*, 1993). Here it is shown that exposure of L929 cells to staurosporine induced cell shrinkage, extensive membrane blebbing (Figure 4.7D), exposure of PS at the cell surface (Figure 4.4 C), condensation and fragmention of the nuclei (Figure 4.7D) and cleavage of DNA into oligonucleosomal fragments (Figure 4.6B). Flow cytometric analysis of staurosporine-treated cells discriminated only two populations, viable and dead (Figure 4.3C). Staurosporine-induced cell death was very rapid (Figure 4.2). Thus, it is possible that in this system the stage of intermediate cell membrane permeability is very short lived (or absent). Taken together, these results indicate that staurosporine induces rapid apoptosis of L929 cells.

Colchicine is an agent that potently disrupts microtubule structure. Previous studies have examined the effects of colchicine on L929 cells but these studies focussed on

changes in microtubules and their effects on the structure of the cell (Moskalewski and Thyberg, 1988; Kessel and Katow, 1984). Colchicine-treated L929 cells were found to undergo a stage of intermediate staining with 7-AAD (Figure 4.3E), cell shrinkage (Figure 4.3E), exposure of PS at the cell surface (Figure 4.4E), condensation of the nucleus together with a unique pattern of nuclear fragmentation (Figure 4.7F; earlier described in (Kessel and Katow, 1984)) and membrane blebbing (Figure 4.7F). Surprisingly, colchicine treatment of L929 cells induced non-specific cleavage of L929 nuclear DNA (Figure 4.6A). However, it is known that not all cases of apoptosis involve oligonucleosomal fragmentation of DNA (Schulze Osthoff *et al.*, 1994; Zamai *et al.*, 1996; Nakamura *et al.*, 1995; Fournal *et al.*, 1995; Xu *et al.*, 1996). Therefore, it is concluded that colchicine induces a form of apoptosis in L929 cells which lacks specific cleavage of nuclear DNA at internucleosomal sites.

Cell death induced by azide was relatively slow, taking more than 48 hrs for all cells to die, compared with approximately 12 hrs for cells treated with TNFα/Act D (Figure 4.2). Examination of untreated and azide-treated cells by light microscopy indicated that azide did not induce cell shrinkage. Although a small fraction of azide-treated cells stained to an intermediate level with 7-AAD, most of these were not reduced in size compared to viable cells (Figure 4.3D). Azide did not induce exposure of PS at the cell surface (Figure4.4D), nor did it induce condensation or fragmentation of the nucleus (Figure 4.7E). Azide induced non-specific fragmentation of DNA in L929 cells (Figure 4.6A). These results are consistent with azide inducing necrosis of L929 cells, consistent with its effects in many other cell types (Fady *et al.*, 1995; Lizard *et al.*, 1995; Vaux *et al.*, 1996).

4.4.5 Death of clusterin-secreting L929 transfectants in response to various cytotoxic agents

The mode of cell death in clusterin-secreting L929 transfectants in response to the various cytotoxic agents was similar to the parental cell line. The rate of cell death in L929-LS.clus(F) and L929-LS.clus(B) transfectants treated with the various cytotoxic agents was not altered when cells expressed clusterin. With the exception of TNFα a similar pattern was observed in L929-pRc.clus cells when treated with the various cytotoxic agents.

The overexpression of clusterin in L929-pRc.clus cells was found to provide them with resistance to TNFα-mediated cytotoxicity, relative to control L929-pRc cells (Figure 4.10 and 4.11). Importantly, this protection was specific to TNFα, since L929-pRc and L929-pRc.clus cells were equally susceptible to death mediated by colchicine, staurosporine or azide (Figure 4.9). The level of clusterin expression appeared to correlate with the level of protection against TNFα. L929-LS.clus cells, which express approximately 50 times less clusterin than L929-pRc.clus cells (refer Figure 3.R.4), were not protected from the cytotoxic effects of TNFα. Furthermore, comparing L929-pRc.clus x1 clones, the levels of resistance to TNFα appeared to relate to the amount of clusterin expressed (Figure 4.16). Clearly, further research is required to analyse the relationship between clusterin expression and TNFα toxicity in L929 cells. The Tet off/on expression system (Clonetech, CA, USA), which only became available late in this study, would be an ideal expression system for such a study as it allows "graded" expression of a desired protein.

As a further control to determine whether protein expression was responsible for the

protection against TNFα another transfectant cell line was created when the studies on L929-pRc.clus were well advanced. The pEGFP-N1 expression system appeared ideal as (i) it contained the same CMV promotor found in the CMV/pRc expression system, and (ii) EGFP is not known to have any effects on mammalian cell metabolism. The only non-ideal characteristic of the pEGFP-N1 expression system (for this application) is that the expressed protein remains intracellular. Surprisingly, L929-EGFP cells had a similar level of protection against TNFα cytotoxicity as L929-pRc.clus cells. As GFP is a natural protein produced by *Aequorea victoria* (a bioluminescent species of jellyfish), it was not expected to specifically interact with the TNF pathway. It is possible, however, that the protective effect provided by EGFP expression may be non-specific. For example, the high EGFP intracellular content may have "absorbed" the cytotoxic effects of ROI that are responsible for TNFα cytotoxicity in L929 cells (Schulze Osthoff *et al.*, 1992; Goossens *et al.*, 1995; refer section 4.1.4). Alternatively, as high protein expression is common to both cell lines, it is possible that the expression of either protein limits cell resources which in turn interferes with intracellular signalling. Future studies will be required to discriminate between these alternative possibilities.

However, clusterin overexpression may have a "real" effect on the TNFα and TGFβ signalling pathways by virtue of some non-specific chemical or physical property of clusterin protein. It would be valuable to quantify the level of expression of endogenous clusterin protein during exposure of L929 cells to TNFα. However, as murine-specific anti-clusterin antibodies were not available, this was not possible. In support of the contention that expression of clusterin can protect cells from TNFα-mediated cytotoxicity, Sensibar *et al.* have shown that anti-sense clusterin mRNA enhances sensitivity of LNCaP cells to TNFα, and that similar protection is provided by

overexpression of clusterin in the same cell type (Sensibar *et al.*, 1995). Interestingly, they showed that a transfected clone expressing low levels of clusterin was not protected from TNFα, while a clone that expressed higher levels was given complete protection, which agree with the results of this study. Unfortunately, the effects of other cytotoxic agents were not studied in the LNCaP system (Sensibar *et al.*, 1995).

4.4.6 Cytoprotective effect of clusterin and its relationship to the TGFβ pathway in L929 cells

Using the two-hybrid system and other techniques, clusterin has recently been identified as binding with the intracellular domains of TGFβ receptors type I and II (Reddy *et al.*, 1996). It has also been independently demonstrated that in L929 cells TGFβ1 induces resistance to TNFα-mediated cytotoxicity (Chang, 1995). It was reported that the exposure of L929 cells to TGFβ induces phosphorylation of a variety of cellular proteins and induces the secretion of proteins that bind to the extracellular matrix, which can protect other L929 cells against TNFα cytotoxicity. Both of these induced effects were implicated in protecting L929 cells from TNFα-mediated cytotoxicity (Chang, 1995). These results were partly confirmed in that the exposure of L929 cells to TGFβ protected the cells from cytotoxicity associated with subsequent exposure to TNFα (Figure 4.12). It was thought that the protective effects of clusterin expression against TNFα cytotoxicity may be linked with the TGFβ pathway. Therefore, L929-pRc.clus cells were tested to determine whether the cytoprotective mechanism against TNFα cytotoxicity was similar to that of L929 cells pre-treated with TGFβ. It was found that L929-pRc.clus transfectants did not secrete protein(s) capable of protecting L929-pRc or L929 wild type cells against TNFα cytotoxicity (Figure 4.13 C).

The high level of clusterin expression in L929-pRc.clus cells treated with TGFβ alone potentiated low levels of cell death. Pre-exposure of L929-pRc.clus cells to TGFβ did not alter the already high resistance to TNFα-mediated cytotoxicity (Figure 4.12). These results indicate that the protective effects of clusterin overexpression and TGFβ against TNFα-mediated cell death are not additive and suggests that both clusterin and TGFβ may antagonise the TNFα-mediated cell death pathway via a common mechanism. While the suggestion that high-level clusterin expression may potentiate the cytotoxicity of TGFβ on the one hand, yet on the other hand provide protection from TNFα-mediated cytotoxicity might at first appear paradoxical, the ability of a single type of molecule to be involved in signalling for the opposing outcomes of cell death versus survival is not unprecedented. Both Fas and TNFα induce cell death in tumour cells and lymphocytes but can also enhance cell proliferation in these same systems (Ware *et al.*, 1996). Furthermore it is known that TNFα induces resistance to its own cytotoxic effect (Ware *et al.*, 1996).

High protein expression is a common factor between L929-pEGFP and L929-pRc.clus transfectant cells and may explain the resistance each has against the cytotoxic effects of TNFα. For example during TNFα treatment the high level of protein expression in these transfected cells may have resulted in the use of cell resources that are normally required for full activation of the TNF pathway. However, it is possible that the human clusterin protein expressed in L929-pRc.clus cells may specifically interact with components of the TNF pathway. If clusterin is capable of specifically protecting L929 cells against TNFα cytotoxicity then the results described here suggest that it is most likely to act intracellularly, since: (i) L929-pRc.clus cells did not secrete molecules

capable of protecting other L929 cells against TNFα cytotoxicity (Figure 4.13 A); (ii) protection was not provided by the addition of exogenous clusterin (Figure 4.13 B); and finally (iii) protection could not be ascribed to clusterin bound to the cell surface, since no surface clusterin was detected by flow cytometry (Figure 4.13 D).

4.4.7 Analysis of secreted and intracellular forms of clusterin from L929-pRc.clus transfectants

A truncated, non-glycosylated 43 kDa form of clusterin lacking the first 33 N-terminal amino acids, and hence the hydrophobic signal peptide, has been reported in TGFβ-treated HepG2 and CCL64 cells (Reddy *et al.*, 1996). This shorter form of clusterin is clearly translated on free ribosomes and is not translocated into the lumen of the endoplasmic reticulum (Reddy *et al.*, 1996). The intracellular form of clusterin could therefore interact with the cytoplasmic domains of TGFβ (or other) receptors. Analysis of the forms of clusterin present in lysates of transfected L929 cells was performed as a first step in examining the possibility that a cytosolic form of clusterin might interact with cytoplasmic domains of receptors and hence influence the cellular response to TNFα. When analysed under non-reducing conditions, cell lysates of L929-pRc.clus and IPTG-induced L929-LS.clus cells contain two major species of clusterin, one is similar to the secreted form with a molecular mass of about 80 kDa, and the other is an uncleaved form of apparent molecular mass about 64 kDa (Figure 4.14).

Similar forms of clusterin, at 58 kDa and 70 kDa, were also present in lysates of HepG2 cells (Burkey *et al.*, 1991). The 70 kDa form was shown to represent mature glycosylated clusterin, processed via the endoplasmic reticulum and Golgi systems, and internally cleaved to produce the two subunits. It has been suggested that this form is

found within the cell inside Golgi and post-Golgi vesicle compartments. The 58 kDa form has been shown to represent a non-glycosylated, uncleaved precursor form to the 70 kDa species (Burkey *et al.*, 1991). Two forms of intracellular clusterin have also been identified in MDCK cells: an uncleaved, incompletely glycosylated 65 kDa form and a cleaved and fully glycosylated 80 kDa form (Urban *et al.*, 1987). The two forms of clusterin present in L929 cell lysates almost certainly correspond to the intracellular forms described in untreated HepG2 and MDCK cells (Burkey *et al.*, 1991; Urban *et al.*, 1987). The recent demonstration of a novel 43 kDa truncated form of clusterin in TGFβ-treated HepG2 and CCL64 cells and the association of this form with the cell nucleus (Reddy *et al.*, 1996a), led to the examination of L929 cells to establish whether exposure to TGFβ or TNFα induced any changes in the level of synthesis of different molecular forms of clusterin or their association with the nucleus.

In response to TNFα, L929-pRc.clus cells demonstrate an increased association of fully processed clusterin, physically indistinguishable from cell-secreted clusterin, with the nucleus (Figure 4.14). Flow cytometric analysis demonstrated that there is an increased association of clusterin specifically with dead cells lacking an intact cell membrane (Figure 4.15). This most probably results from secreted clusterin binding to dead cell nuclei. The demonstration that BSA also binds to dead cell nuclei under these same conditions and that clusterin and BSA bound to the nuclei of permeabilised untreated cells indicates that the interaction of clusterin with the nucleus is in this case unlikely to have physiological relevance (Figure 4.15).

An intracellular form of clusterin was also detected on immunoblots (following SDS/PAGE under reducing conditions) as a pair of bands at about 36 and 38.5 kDa

which was found in increased amounts in L929-pRc.clus cells treated with TNFα, TNFα + Act D, TGFβ, azide, colchicine or staurosporine. These bands were unique as the constitutively produced clusterin in either untreated or TNFα-treated L929-pRc.clus cells appeared on the same immunoblots as a pair of poorly resolved bands at about 43-45 kDa (Figure 4.14). The clusterin variant is clearly not the truncated 43 kDa unprocessed form reported associated with nuclei in TGFβ-treated HepG2 and CCL64 cells (Reddy *et al.*, 1996). The TNFα-induced form of clusterin in L929-pRc.clus cells is internally proteolytically cleaved and is not specifically associated with the nucleus. An exon-skip mechanism has been hypothesized to account for a truncated clusterin mRNA in rat tissues treated with heat-shock or inhibitors of protein synthesis (Kimura & Yamamoto, 1996). Therefore, the apparently smaller size of the clusterin subunits induced by toxic agents could result from a variety of mechanisms, including modifications to transcription (Kimura & Yamamoto, 1996) or translation (Reddy *et al.*, 1996), additional proteolytic processing, or changes in glycosylation. The proportion of clusterin detected as the 36/38.5 kDa form was increased in response to all of the toxic agents tested. This indicates that this novel form may not be responsible for providing protection from TNFα. However, it still remains a possibility.

4.4.8 Electroporation of clusterin into L929 cells does not provide protection from TNFα

To confirm that clusterin was acting intracellularly in protecting L929 cells from TNFα mediated death a method was established to introduce purified clusterin into intact L929 cells. Electroporation has previously been shown to succesfully incorporate exogenous proteins into fibroblast cells (Glogauer and McCulloch, 1992). L929 cells were also shown to incorporate exogenous proteins following this method. Surprisingly, the

incorporation of purified clusterin into L929 cells did not provide any protection against TNFα (Figure 4.18).

Electroporation is an invasive technique and may have inhibited clusterin from protecting L929 wild type cells against TNFα. For example, clusterin or other proteins may have leaked out from electroporated cells after a short period of time. An alternative interpretation of this result is that it suggests that the protection gained in clusterin-expressing cell lines is an artifact and is not specifically due to the expression of clusterin.

4.4.9 The effect of TNF on other transfected cell lines.

Another transfected cell line created late in this study was the HeLa-pRc.clus. TNFα induces apoptosis in HeLa cells, an effect that can be augmented by the addition of cycloheximide (White *et al.*, 1992). The overexpression of clusterin in HeLa-pRc.clus did not provide protection against TNFα cytotoxicity as seen in the L929 transfectants. Instead, the cytotoxic effect of TNFα was enhanced by the overexpression of clusterin. The differences seen in HeLa and L929 transfectants in response to TNFα may be explained by different pathways being activated by TNFα. As HeLa and L929 originate from different species this may mean that TNFα activates different pathways, hence resulting in different outcomes.

4.4.10 Conclusion

The results presented in this chapter describe examinations of a variety of morphological and biochemical parameters commonly used to discriminate between apoptotic and necrotic cells. These studies determined the mode of death occurring in L929 cells in response to treatment with TNFα and other cytotoxic agents. The parameters examined included changes in membrane permeability (measured by staining with 7-AAD) and exposure of PS at the cell surface (detected by binding of annexin V-FITC). The literature reveals that these techniques have not been used before in studies of L929 cell death. Features of both necrosis and apoptosis were detected in L929 cells exposed to TNFα alone. The augmentation of the cytotoxicity of TNFα with Act D induced classical apoptosis in L929 cells. This study also demonstrated for the first time that staurosporine and colchicine both induce apoptosis of L929 cells.

There has been a clear association between increased clusterin expression and a wide variety of instances of cellular "stress". It has been suggested that clusterin expression may be a general cellular response to a variety of noxious insults. The effect of clusterin expression in L929 cells treated with the various cytotoxic agents was also studied. The overexpression of clusterin was shown to protect transfected L929 cells from death mediated by TNFα but not from death induced by colchicine, staurosporine or azide. The results also indicate that high level expression of clusterin potentiates the induction of cell death in L929 cells by TGFβ. The results are compatible with the suggestion that clusterin and TGFβ may act through a common mechanism to protect L929 cells from TNFα cytotoxicity. The intracellular action of clusterin protein may be responsible for the protection against TNFα cytotoxicity . However, this cannot be accounted for by

synthesis of a 43 kDa truncated form of clusterin reported in other systems, or a cytokine-induced specific association of clusterin with the nucleus. Unexpectedly, high expression of EGFP also protects L929 cells against TNFα which raises the possibility that in each case the effects measured are non-specific and only due to high levels of protein expression. However, it remains possible that each protein specifically protects cells from TNFα.

A subset of the results presented in this chapter have been published in the following two journal papers:

Humphreys D., Hochgrebe T. T., Easterbrooksmith S. B., Tenniswood M. P. and Wilson M. R. (1997) Effects Of Clusterin Overexpression On TNF-Alpha- and TGF-Beta-Mediated Death Of L929 Cells. *Biochemistry* **36,** 15233-15243

Humphreys D.T., andWilson M.R. (1999) Modes of L929 cell death induced by TNFα and other cytotoxic agents. *Cytokine*. In press.

These papers are attached in the appendix.

CHAPTER 5

Chaperone-like activity of clusterin.

5.1 INTRODUCTION

Clusterin has been shown to bind a wide array of biological molecules which include immunoglobulins (Wilson and Easerbrook-Smith, 1992), lipids (Gelissen *et al.*, 1998), heparin (Pankhurst *et al.*, 1998), glutathione-S-transferase (GST) (S B Easterbrook-Smith, unpublished results), the surfaces of pathogenic isolates of *Staphylococcus aureus* (Partridge *et al.*, 1996), terminal complement components C7, C8 and C9 (Tschopp *et al.*, 1993), apolipoprotein A-I (Jenne *et al.*, 1991), paraoxonase (Kelso *et al.*, 1994), amyloid β peptide (Oda *et al.*, 1995), gp330 (Kounnas *et al.*, 1995) and a protein secreted by *Streptococcus pyogenes* (Akesson *et al.*, 1996). Each description of a new clusterin binding interaction has proposed that clusterin is involved with the specific biological function of the binding partner. Consequently, functions proposed for clusterin are as diverse as its reported binding partners (refer chapter 1). None of the previously proposed functions has been established as genuine.

Sequence analysis predicts that clusterin has three putative amphipathic α-helical regions, a type of secondary structure thought to be important in mediating interactions with hydrophobic molecules (de Silva *et al.*, 1990a; Tsuruta *et al.*, 1990; Figure 1.1). Many of the reported biological ligands of clusterin are largely or partly hydrophobic in character. Therefore, the interactions of these molecules with clusterin may reflect a general propensity of clusterin to bind to hydrophobic regions of molecules, regardless of their biological function. In this context, the recent demonstration that a highly conserved 14-bp element (clusterin element or CLE), shared by all vertebrate clusterin proximal promoters, specifically recognizes the heat shock factor 1 (HSF1) transcription factor is of significant

interest. This 14-bp element was shown to be capable of mediating heat-shock-induced transcription (Michel *et al.*, 1997). These authors suggested that this heat-responsive element provides an explanation for the high sensitivity of clusterin expression to environmental changes and proposed that clusterin may function as an extracellular heat-shock protein (Michel *et al.*, 1997).

Heat shock proteins (HSP) are cytoprotective proteins that were first identified by their upregulated expression in response to cellular stresses such as heat, hypoxia, and metabolic stress. HSP are categorised into five classes based on size, which include: HSP100, HSP90, HSP70, HSP60 and the small HSP (sHSP). Each class of HSP contains proteins that have high sequence homology to one another and all facilitate the folding of other proteins. The upregulation of these proteins in times of stress are essential for cell survival as they prevent cell death by inhibiting permanent denaturation of proteins. The ability of HSP to interact with the folding and unfolding of proteins led to the term molecular chaperone.

The transcriptional activation of HSP upon heat shock results from the trimerisation of proteins called heat shock factors (HSF) which bind to specific regions called heat shock elements (HSE) located in the HSP promotor (Westwood *et al.*, 1991). All HSP typically have between three and six HSE in the upstream promotor sequence, which are inversely repeated, and have a length of five base pairs (5' -nGAAn- 3') (Amin *et al.*, 1988). The CLE differs by only one base to three HSE inverted repeats (CLE: nTTCnnGAAnn**C**TCn; triple HSF repeats: nTTCnnGAAnnTTCn). Mutation experiments that altered the central

GAA motif in CLE reduced the heat shock induced transcriptional activity of the clusterin promotor sequence (Michel *et al.*, 1997). This indicates that the one copy of CLE in the clusterin promotor is essential for heat induced transcription activity. If HSF is responsible for regulating clusterin expression via CLE *in vivo* then HSP70 proteins, one of the most important classes of HSP that regulate the stress response in eukaryotic cells by binding and inactivating HSFs, may also regulate clusterin expression.

The members of the sHSP are less conserved than other HSP, but all have a conserved domain refered to as the "α-crystallin domain" (Ehrnsperger *et al.*, 1997; Leroux *et al.*, 1997). The upregulated expression of sHSP provides thermotolerance to some cells. The pattern of activation of sHSP is different to that of other HSP. For example, they are upregulated at lower temperatures than other HSP and are the most highly expressed proteins relative to other HSPs (Arrigo and Landry, 1994). During stress, sHSP localise to the nucleus (Collier and Schlesinger, 1986; Arrigo *et al.*, 1988; Collier *et al.*, 1988; Nover *et al.*, 1989; Rossi and Lindquist, 1989; Lavoie *et al.*, 1993a). However sHSP are not always upregulated in times of stress, for example, they are also upregulated in response to various hormones (eg hsp27 estrogen (Fuqua *et al.*, 1989)). The upregulated expression of sHSP in response to stress is of the same order of magnitude as clusterin. Recently it was shown that the upregulation of sHSP protected L929 cells from the cytotoxicity of TNFα (Mehlen *et al.*, 1995). The level of protection was similar to that measured in L929-pRc.clus cells expressing clusterin (chapter 4). There is no substantial sequence homology between clusterin and any known sHSP. However, there are three small regions of sequence similarity between human clusterin and bovine α-crystallin (an archetypal sHSP;

Figure 5.15). These regions correspond to human clusterin residues 203-210, 227-233 and 243-249 (numbered from the first translated residue; Figure 5.1). Interestingly, the last of these regions (K70 to V76 in αA-crystallin) corresponds to a part of the "α-crystallin domain" which is the most conserved region in all sHSP.

bovine αA-crystallin

^{33}E Y D L L P F L ^{40}..... ^{54}R T V L D S G ^{60}..... ^{70}K F V I F L D ^{76}

human clusterin

^{203}T Y H Y L P F S ^{210}..... ^{227}R S L M P F S ^{233}.....^{243}M F Q P F L E ^{249}

Figure 5.1: Regions of sequence similarity between human clusterin and bovine αA-crystallin (analysed using MacVector v4.14). Clusterin residues are numbered from the N-terminal amino acid of the pre-pro-protein (ie prior to removal of the 22 amino acid signal peptide).

The function of clusterin expressed during cellular stress, like the sHSP, may be to act in a chaperone-like manner and bind to hydrophobic regions of partly unfolded, stressed proteins, thereby "solubilizing" them and protecting cells from the cytotoxic consequences of protein precipitation. The aim of work described in this chapter was to test one part of this hypothesis by examining the ability of clusterin to protect a variety of proteins from stress-induced precipitation *in vitro*.

5.2 MATERIALS AND METHODS:

5.2.1 Reagents- Bovine serum albumin (BSA), catalase, 1-chloro-2, 4-dinitrobenzene, glutathione (GSH), H_2O_2, iodoacetamide (IAA), α-lactalbumin and 1-anilino-8-naphthalene sulfonate (ANS) were all obtained from Sigma (St. Louis, MO, USA). Dithiothreitol (DTT) was obtained from Boehringer Mannheim (Sydney, Australia). All buffer salts were obtained from Ajax (Sydney, Australia). Glutathione-S-transferase (GST) from *Schistosoma japonicum* was prepared by thrombin cleavage of recombinant Jun leucine zipper-GST fusion protein and purified by GSH-agarose affinity chromatography as described by (Heuer *et al.*, 1996).

5.2.2 Immunoaffinity purification of clusterin-

5.2.2.1 Preparation of serum- Human serum packs were stored at -80°C. Serum packs were quickly thawed in a 37°C water bath. 1 mM of phenylmethanesulfonl fluoride and 5 mM of EDTA (protease inhibitors) was added to the serum. The serum was filtered through a 7 cm glass fibre filter paper (GFC, Whatman, Crown Scientific, Sydney).

5.2.2.2 Isolation of clusterin- PBS + 0.02% azide was passed through G7 anti-clusterin monoclonal antibody column and a UV monitor until a baseline had formed on the connected chart recorder (Figure 5.2). The UV monitor, which had a 280 nm flow cell, was set at 0.5 absorbance units, the chart recorder 100 mV and the flow rate was set at 0.5 ml/ minute. Serum was then passed through the G7 column followed by washing with PBS + 0.02% azide until the base line was

reached. 0.5% Triton X100 in PBS + 0.02% azide was then passed through the system to remove any bound apolipoprotein (Apolipoprotein A-I co-purifies with clusterin purified from human serum; Jenne *et al.*, 1991). PBS + 0.02% azide was once again flushed through the column until the base line was reached. Clusterin was then eluted from the column with 2 M guanidine hydrochloride (prepared in PBS, pH 7.4).

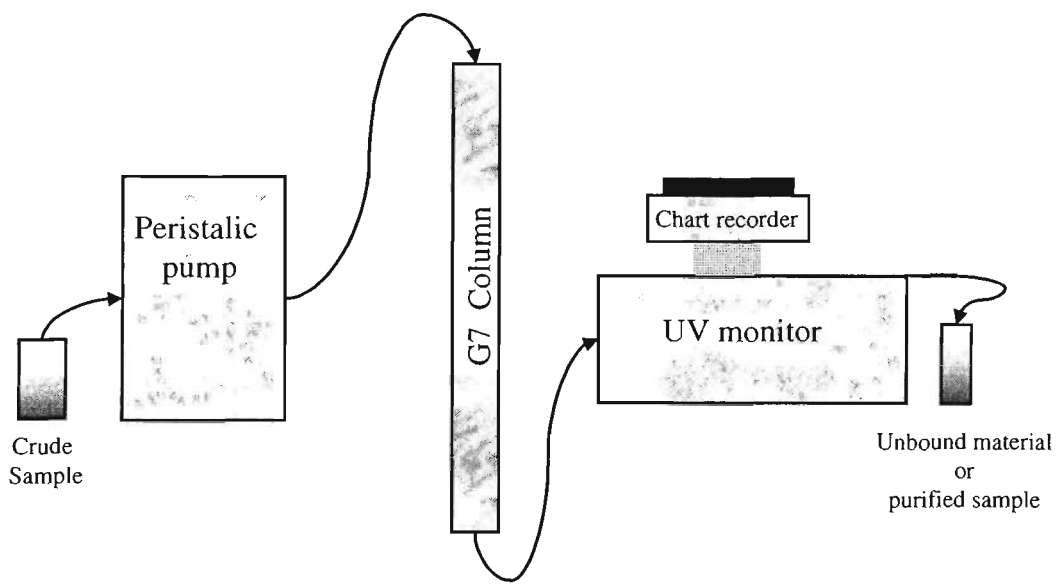


Figure 5.2: Immunoaffinity chromatography setup. Crude sample is pumped through the system at a rate controlled by a peristalic pump. Clusterin is retained in the column while the remaining molecules pass out via the UV monitor. After all crude material has been washed from the system the molecule of interest can be eluted from the column and collected.

5.2.2.3 Secondary purification of Clusterin- Guanidine hydrochloride solutions containing clusterin were dialysed against two 3 L volumes of PBS + 0.02 % azide. Thereafter clusterin was passed through a Protein G column (Sigma, Sydney) to remove contaminating IgG. Chart recorder settings and the flow rate were the same as described for the G7 immunoaffinity column. The fraction which passed through the Protein G column contained clusterin which was free of

IgG. 2 M guanadine hydrochloride (pH 7.4) was used to elute off any Ig-clusterin complexes. Thereafter PBS + 0.02 % azide was passed through the column until the base line was reached.

5.2.2.4 Concentrating dilute stocks of clusterin- Each clusterin sample was then concentrated in dialysis tubing where water + salts were absorbed from samples by covering the dialysis tubing with dextran (7 million Daltons in size, ICN; Sydney, Australia). The amount of clusterin present in a sample was calculated by an absorbance reading at 280 nm. Every batch of purified clusterin was analysed by SDS-PAGE electrophoresis (Refer 2.2.1)

5.2.3 Protein precipitation assays - Individual solutions of clusterin (10-200 μg/ml), catalase (200 μg/ml) or GST (200 μg/ml), or mixtures of clusterin with catalase or GST at the same final concentrations, were prepared in 0.7 ml of 50 mM sodium phosphate containing 0.1M NaCl and heated at 60°C. The light scattering of the solution at 360 nm was measured every 30 s for a total of 25 min in an automated seven chambered diode array spectrophotometer (Hewlett Packard GMBH, Germany). Individual solutions of clusterin (10-200 μg/ml), α-lactalbumin (2.5 mg/ml) or BSA (2.5 mg/ml), or mixtures of clusterin with α-lactalbumin or BSA at the same final concentrations, were prepared in 0.3 ml of 50 mM sodium phosphate containing 0.1 M NaCl (or $NaSO_4$ or NaSCN where indicated) and incubated at 37°C with or without 20 mM DTT. During this period absorbance readings at 360 nm were acquired every 5 min for a total of 5 h in a Spectramax 250 plate reader (Molecular Devices, Sunnyvale CA).

5.2.4 Enzyme assays - Catalase was prepared at a concentration of 200 μg/ml in 0.1 M sodium phosphate (pH 7.0) with or without clusterin (100 μg/ml). Mixtures were heated at 37°C or 55°C for 30 min. 1.0 μl of the catalase/clusterin mixture was incubated with 1.0 ml of H_2O_2 substrate solution (0.12% (v/v) H_2O_2 in 50 mM Na_2HPO_4, pH 7) at 37°C for 5 min before the reaction was stopped with 150 μl of 4 M NaOH. The absorbance of H_2O_2 was measured at 250 nm on a Spectramax 250 plate reader. Enzyme activity was measured as a decrease in absorbance. GST was prepared at a concentration of 200 μg/ml in 50 mM Na_2HPO_4 (pH 7.0) with or without clusterin (100 μg/ml). Mixtures were heated at 37°C or 50°C for 30 min. GST was then diluted into substrate solution (1 mM GSH, 2 mM 1-chloro-2,4-dinitrobenzene in 0.1 M phosphate solution pH 7.4) to a final concentration of 4.5 μg/ml and incubated at 37°C for 5 min before measuring the absorbance at 350 nm. Enzyme activity was measured as an increase in absorbance, corresponding to the appearance of 1-S-glutathionyl-2,4-dinitrobenzene.

5.2.5 ELISA - GST and catalase, at 20 μg/ml in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4) were adsorbed onto ELISA trays (Disposable Products, Adelaide, Australia) for 1 h at 37°C. In some cases, after the coating step, the trays were heated to 60°C for 30 min to partially denature the adsorbed proteins. BSA and α-lactalbumin were applied to ELISA trays in PBS at concentrations between 1 and 5 mg/ml, with or without 20 mM DTT, and incubated overnight at 37°C. Plates were then blocked with 1% (w/v) heat-denatured casein (HDC) prepared in PBS, pH 7.4 (ie 1% HDC). Plate-bound, DTT-treated proteins were then incubated with 5 mM IAA for 1 h at

37°C, in order to exclude the possibility of subsequent formation of disulfide bonds between clusterin and the other proteins. Clusterin, initially at 10 μg/ml, was then serially diluted in binary steps across the ELISA tray, using 1% HDC as diluent, and incubated at 37°C for 1 h. To minimize non-specific binding of clusterin to the ELISA plates, three washes were then performed with 0.1% (v/v) Triton X-100 in PBS. A cocktail of G7, 78E and 41D anti-clusterin monoclonal antibodies was used to detect bound clusterin, and DNP-9 was used as a isotype control. All antibodies were used in the form of unpurified hybridoma culture supernatants. Bound primary antibodies were detected with sheep-anti-mouse-Ig-HRP (Silenus, Sydney, Australia) using *o*-phenylenediamine dihydrochloride (OPD; 2.5 mg/ml in 0.05 M citric acid, 0.1 M Na_2HPO_4, pH 5.0, containing 0.03% (v/v) H_2O_2) as substrate.

5.2.6 Size exclusion chromatography and native gel electrophoresis - Individual solutions of clusterin (1 mg/ml), or GST, catalase, BSA or α-lactalbumin (each at 2 mg/ml), or mixtures of clusterin with one of the other proteins (at the same respective final concentrations) were left untreated or treated with heat (60°C for 30 min) or DTT (20 mM at 37°C for 5 h) and then centrifuged (1 min at 10,000 rpm in a benchtop microfuge) to remove precipitated protein. Each solution was then analysed by loading 50 μl onto a 25 x 1 cm column of Sephacryl 300 (Pharmacia Biotech, Melbourne, Australia) equilibrated in PBS containing 0.02% (w/v) azide. Separations were performed at a flow rate of 0.25 ml/min using a low pressure liquid chromatography system equipped with a 280 nm flow cell (Econosystem; BioRad, Sydney, Australia). Aliquots (10-15 μg total protein) of solutions prepared as above were also electrophoresed on 1% agarose gels. After

electrophoresis gels were often stained with Coomassie blue for 30 minutes at room temperature before being destained in destain solution overnight (refer section 2.2.1 and 2.2.2.3). In some cases, proteins were excised from gels to be further analysed by SDS PAGE electrophoresis. Coomassie blue stained gels could not be used for this purpose as the staining procedure precipitates proteins. Native proteins were removed from gels in which duplicate samples had been loaded. After electrophoresis half the gel was stained with Coomassie blue while the other half was stored at -20°C to stop proteins diffusing out of the gel. After the stained gel was destained (using destain, refer sections 2.2.1 and 2.2.2.3) the protein bands were identified and the corresponding section of the frozen gel was excised. Excised gel pieces were boiled in Eppendorf tubes with the addition of SDS and bromophenol blue (final concentrations of 0.01% (w/v) and 0.05% (w/v) respectively) prior to loading onto polyacrylamide gels.

5.2.7 ANS fluorescence studies - Individual solutions of clusterin (100 μg/ml), or GST or catalase (each at 200 μg/ml), or mixtures of clusterin with one of the two other proteins (at the same respective final concentrations) were left untreated or heated at 60°C for 30 min. Individual solutions of clusterin (1.0 mg/ml) or BSA or α-lactalbumin (each at 2.5 mg/ml), or mixtures of clusterin with one of the two other proteins (at the same respective final concentrations) were left untreated or were incubated with DTT (20 mM at 37°C for 5 h). A total of 10-20 μg of protein was then loaded into wells of a black 96 well tray (Nunc, Denmark). At room temperature, ANS was progressively added to each well to give a final concentration of up to 150 μM. After each addition of ANS, the fluorescence (390 nm excitation/480 nm emission) of the wells was measured with a Biolumin 960 fluorescence

plate reader (Molecular Dynamics, Melbourne, Australia). The fluorescence values obtained were corrected by subtracting the fluorescence of equivalent amounts of ANS in blank wells containing buffer alone. In some cases, possibly as a result of quenching of the fluorescence of ANS in solution by protein, this correction produced small negative fluorescence values.

5.2.8 Clusterin binding to DNA + DNA degradation assay- Genomic DNA was purified from untreated L929 cells as described in section 4.2.4. RNA was purified from L929 cells using the RNA isolation reagent (Integrated sciences, Sydney). 200 ng of DNA was dotted onto nitrocellulose membrane and baked at 70°C for 1 hour. Membranes were then blocked with 1% HDC for 1 hour. Membranes were then incubated in solutions of 1% HDC containing 20 μg/ml of clusterin, before being washed with PBS with or without 0.1% Triton X-100. Bound clusterin was detected with TC sn from G7 hybridoma cultures followed by sheep-anti-mouse-HRP secondary antibody. Blots were developed with ECL (Pierce, Sydney, Australia) following the manufacturer's instructions.

For the DNA degradation assay, tubes were set up containing DNA (3-10 μg), DNase (0-15 μg), and either clusterin, BSA, gelatin or casein (100 μg/ml) in a total volume of 15 μl. Tubes were incubated for 30 minutes at 37°C before samples were immediately electrophoresed in 1% agarose gels.

5.2.9 Studies of the effects of heat and H_2O_2 on cell viability- L929-pRc and L929-pRc.clus cells were cultured in 24 well TC trays. For analysing the effect of heat on cell viability, cultures were prepared in 24 well TC trays and then incubated in fresh media at either 37°C, 41°C or 50°C for set periods of time. For analysing the effect of H_2O_2 on cell viability, cultures were prepared in 24 well TC trays and then incubated in fresh media containing 0.4 mM H_2O_2 for 24 hours. After either treatment cells were removed from TC trays with trypsin and stained with PI before being analysed by flow cytometry (as described in section 4.2.3.2).

5.3 RESULTS

5.3.1 Purification of clusterin by immunoaffinity chromatography.

The G7 immunoaffinity column was equilibrated with PBS (Figure 5.3A pre-peak A) before 50 ml of serum was passed through. Serum passage was detected by the UV monitor as a large absorbance (Figure 5.3 A peak A). PBS followed serum through the column until the baseline was reached. Apolipoprotein A-I and lipids were dissociated from bound clusterin with 0.1% Triton X-100 (Figure 5.3 A peak B) before clusterin was finally eluted from the column with 2 M guanidine hydrochloride (Figure 5.3 A peak C).

Analysis of purified clusterin on SDS PAGE gels revealed a major protein species with a size of 80 kDa, which ran at 40 kDa under reducing conditions (Figure 5.3 B). Western immunoblots using the human clusterin specific monoclonal antibody confirmed that this protein was clusterin (Figure 5.3 C). SDS PAGE analysis of purified clusterin also revealed a minor protein species with a size of 200 kDa. This was thought to be contaminating IgG, which clusterin binds to (Wilson and Easterbrook-Smith, 1992). Clusterin stocks were therefore passed over a Protein G column to remove contaminating IgG. Clusterin which was free of IgG (as seen in figure 5.3 B) was used for further experimentation.

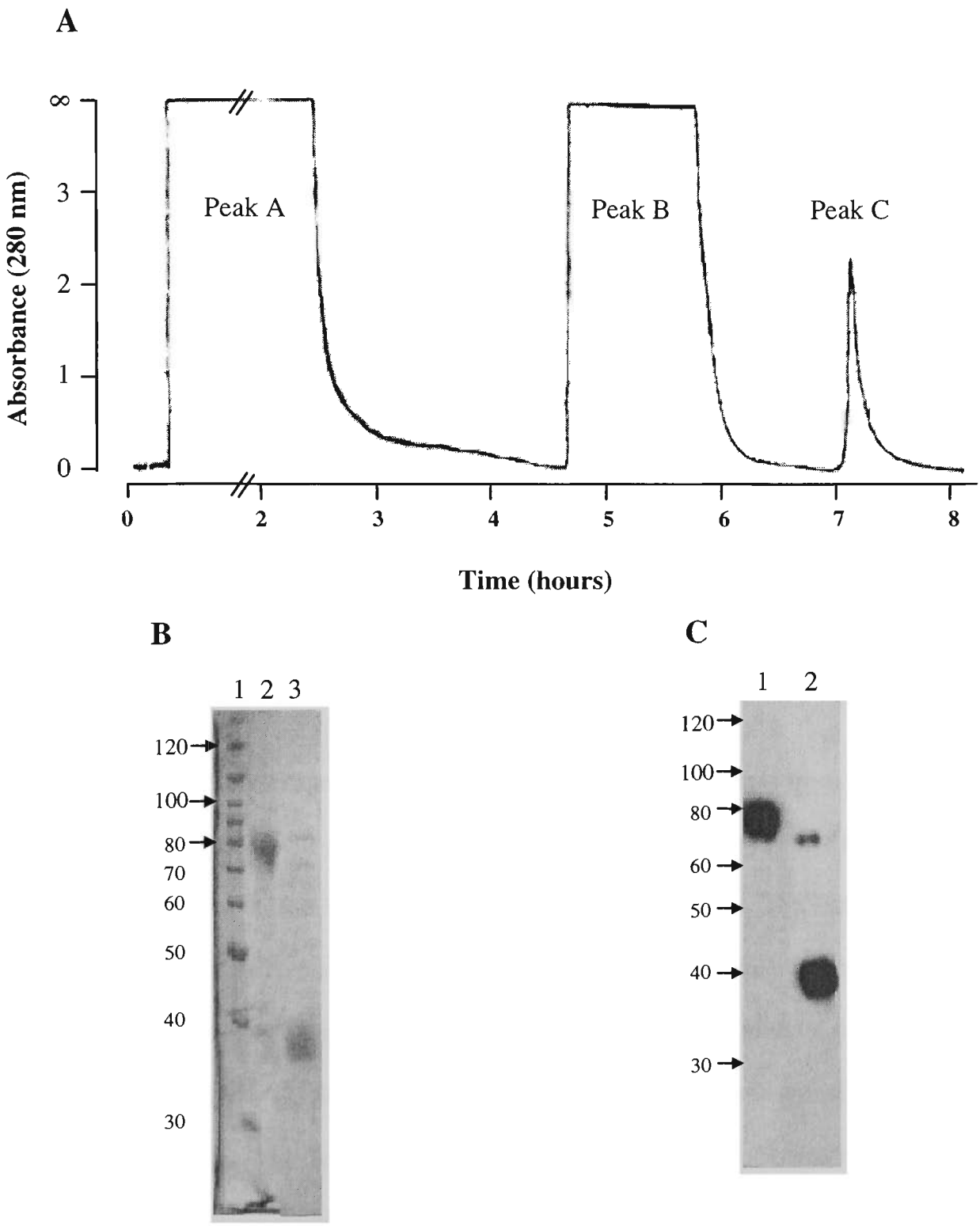


Figure 5.3: A) A trace of a chart recording which measured the absorbance of solutions passed over a G7 anti-clusterin immunoaffinity column. The UV monitor was blanked on PBS. Peak A corresponds to serum being passed through the column followed by PBS. Peak B corresponds to Triton X-100 being passed through the column followed by PBS. Peak C corresponds to 2 M guanidine hydrocholride (pH 7.4) being passed through the column followed by PBS. **B)** 10 % SDS-PAGE electrophoresis of clusterin purified by immunoaffinity chromatography. Lane: 1 Molecular weight markers; lane 2: 10 μg of clusterin; lane 3: 10 μg of clusterin reduced with β-mercaptoethanol. **C)** Immunoblot of an SDS PAGE gel which electrophoresed purified clusterin samples. Lane 1: 1 μg clusterin; lane 2: 1 μg clusterin reduced with β -mercaptoethanol. Clusterin was detected with a cocktail of G7, 41D and 78E antibodies.

5.3.2 Clusterin protects proteins from stress-induced precipitation but does not protect enzymes from heat-induced loss of function

Heating solutions containing GST or catalase at 60°C produced extensive protein precipitation within 30 min (Figure 5.4 A, B). Likewise, reduction of solutions containing BSA or α-lactalbumin with DTT resulted in extensive protein precipitation within 4 h (Figure 5.4 C, D). In contrast, clusterin did not precipitate when heated at 60°C for 30 min or when treated with DTT for 5 h (Figure 5.4 E). Similarly other proteins that were stable at high temperatures did not prevent other proteins from precipitating (Figure 5.4 F). When co-incubated with any of the proteins subjected to heat or DTT-mediated reduction, clusterin potently inhibited protein precipitation (Figure 5.4 A-D).

To investigate whether clusterin is also capable of protecting enzymes from stress-induced loss of function, the enzyme activity of GST and catalase was tested before and after exposure to 60°C for 30 min in the presence or absence of clusterin. The enzyme activity of GST was determined by an increase in absorbance at 350 nm which corresponded to the conversion of 1-chloro-2, 4-dinitrobenzene and glutathione to 1-S-glutathionyl-2,4-dinitrobenzene. Catalase is an enzyme found in peroxisomes and is responsible for catalysing $2H_2O_2$ into H_2O and O_2 (Alberts, 1989). A spectral scan showed that H_2O_2 absorbed light at wavelengths between 200 and 260 nm (Figure 5.5 A). Therefore the enzyme activity of catalase was measured at 215 nm, where a decrease in absorbance represented consumption of H_2O_2. The presence of clusterin (at sufficient concentration to provide protection against precipitation; Figure 5.5 A,B) had no effect on the loss of enzyme activity in either case (Figure 5.5 B,C). Overall, these results

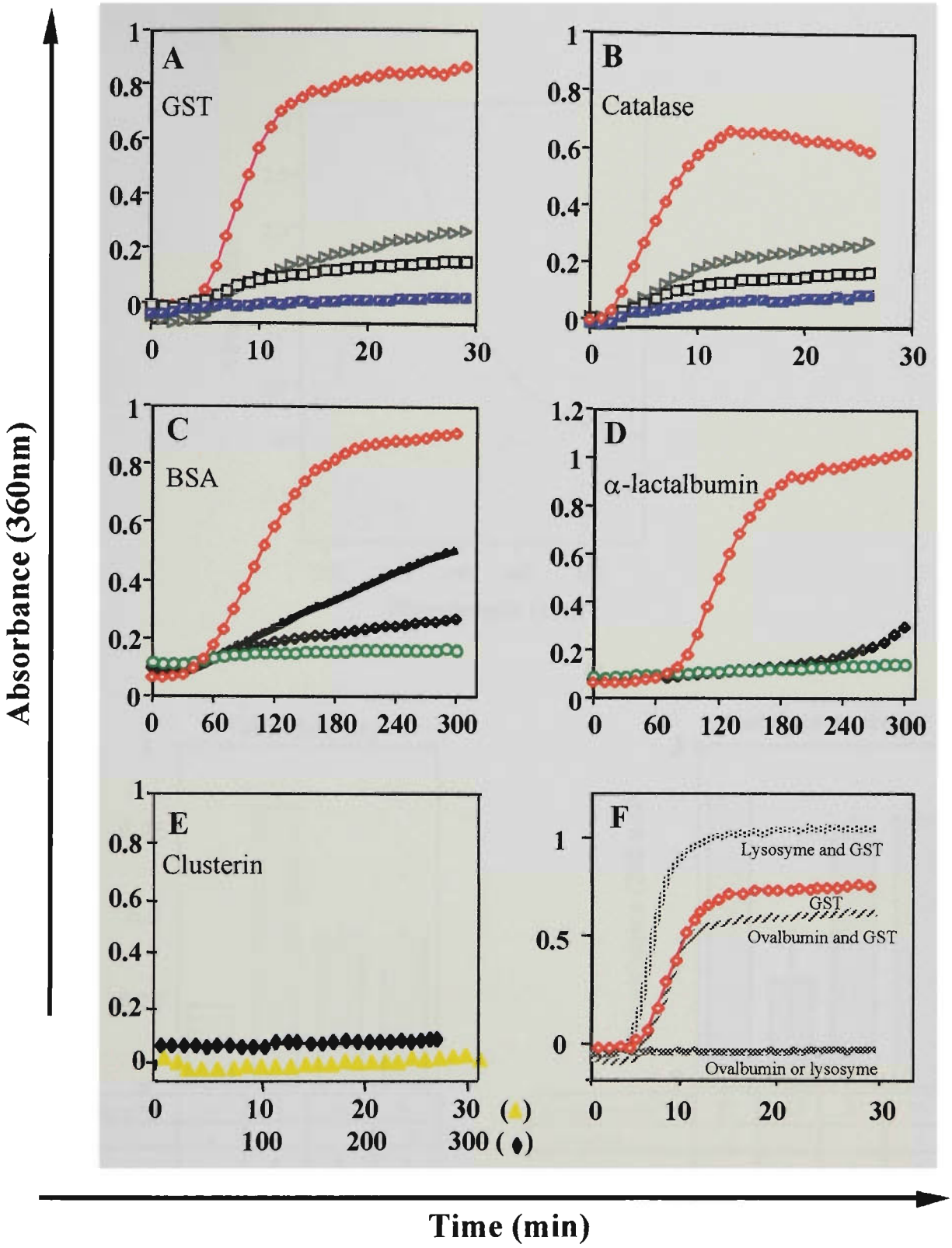


Figure 5.4: Absorbance at 360 nm, as a function of time for (**A**) GST (200 μg/ml) or mixtures of GST (200 μg/ml) and clusterin (at the concentrations indicated) heated at 60°C; (**B**) catalase (200 μg/ml) or mixtures of catalase (200 μg/ml) and clusterin (at the concentrations indicated) heated at 60°C; (**C**) BSA (750 μg/ml) or mixtures of BSA (750 μg/ml) and clusterin (at the concentrations indicated) treated with 20 mM DTT at 37°C; (**D**) α-lactalbumin (750 μg/ml) or mixtures of α-lactalbumin (750 μg/ml) and clusterin (at the concentrations indicated) treated with 20 mM DTT at 37°C; (**E**) clusterin (100 μg/ml)) untreated, treated with 20 mM DTT (◆) or treated at 60°C ( ); (**F**) GST or mixtures of GST (200 μg/ml) and lysosyme or ovalbumin (100 μg/ml; indicated by label below each graph) at 60°C. Concentrations of clusterin in mixtures; (●) 0 μg/ml, (▷) 25 μg/ml, (□) 50 μg/ml, (■) 100 μg/ml, (▬) 250 μg/ml, (◆) 330 μg/ml, (○) 667 μg/ml.

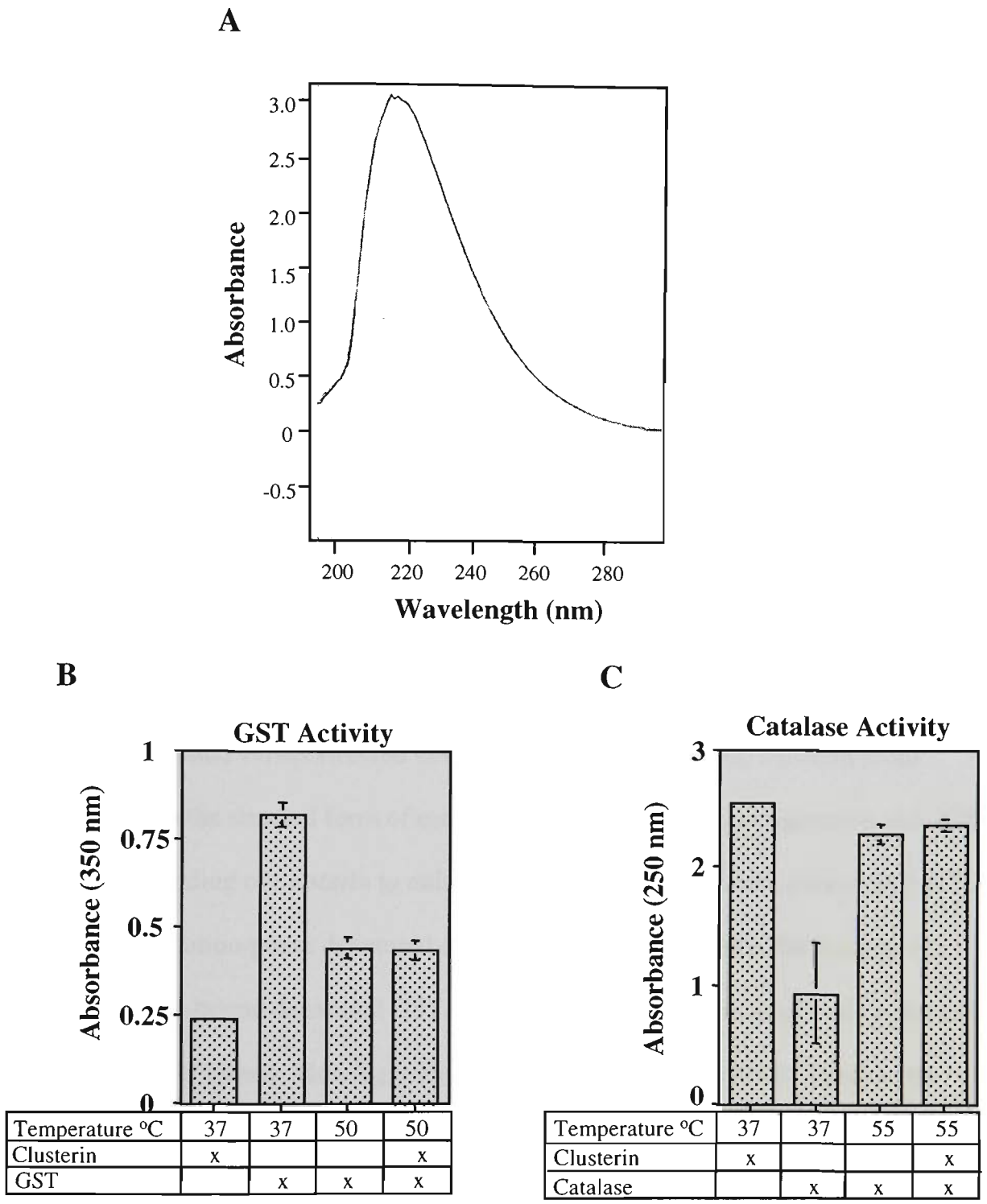


Figure 5.5: Enzyme activity of (**A**) GST and (**C**) catalase following incubation at either 37°C or 50°/ 55°C. Solutions containing the specified enzyme ± clusterin were heated for 30 minutes at the temperature indicated before measuring enzyme activity. In each case, the table below the x-axis indicates the conditions applied (indicated by an "x"). GST activity was measured as an increase in absorbance at 350 nm, representing the appearance of 1-S-glutathionyl-2,4-dinitrobenzene. Catalase activity was measured as a decrease in absorbance at 250 nm, representing the disappearance of H_2O_2 which absorbs light at this wavelength. **A)** spectral absorbance scan of H_2O_2 between 190 and 300 nm. Further description of these activity assays is provided in Experimental Procedures. Each histogram represents the mean of three replicate measurements and the error bars shown correspond to standard errors (SE) of the mean. In some cases, the SE are too small to be visible.

indicate that clusterin has potent heat- and reduction-stable chaperone-like properties that are capable of protecting stressed proteins from precipitation but which cannot protect GST or catalase from heat-induced loss of enzyme activity.

5.3.3 Clusterin binds preferentially to stressed proteins

Clusterin did not bind significantly to any of the non-stressed proteins tested, with the exception of GST (Figure 5.6). Binding of clusterin to unstressed GST had been previously noted (S. Easterbrook-Smith, unpublished results). Clusterin showed significantly increased binding to heat-stressed GST (Figure 5.6 A) and significant binding to DTT-treated BSA and α-lactalbumin (Figure 5.6 C, D). Thus, the ELISA results indicate that, comparing untreated versus stressed GST, BSA and α-lactalbumin, clusterin binds preferentially to the stressed form of each protein. In the ELISA configuration tested, there was minimal binding of clusterin to either untreated or heat-stressed catalase (Figure 5.6 B). However solution-phase denatured catalase was shown to inhibit the binding of clusterin to plate-bound denatured GST, while solution-phase non denatured catalase did not (Figure 5.6 B insert). This suggests that clusterin also preferentially binds to the stressed form of catalase.

5.3.4 Clusterin interacts with stressed proteins to form a HMW complex

Chaperones such as the sHSP are known to form HMW complexes with partly unfolded proteins under stress conditions (Rao *et al.*, 1993; Carver *et al.*, 1994). Experiments were carried out to determine whether clusterin interacted with stressed proteins to form HMW complexes. Size exclusion chromatography was used to analyse the following proteins or

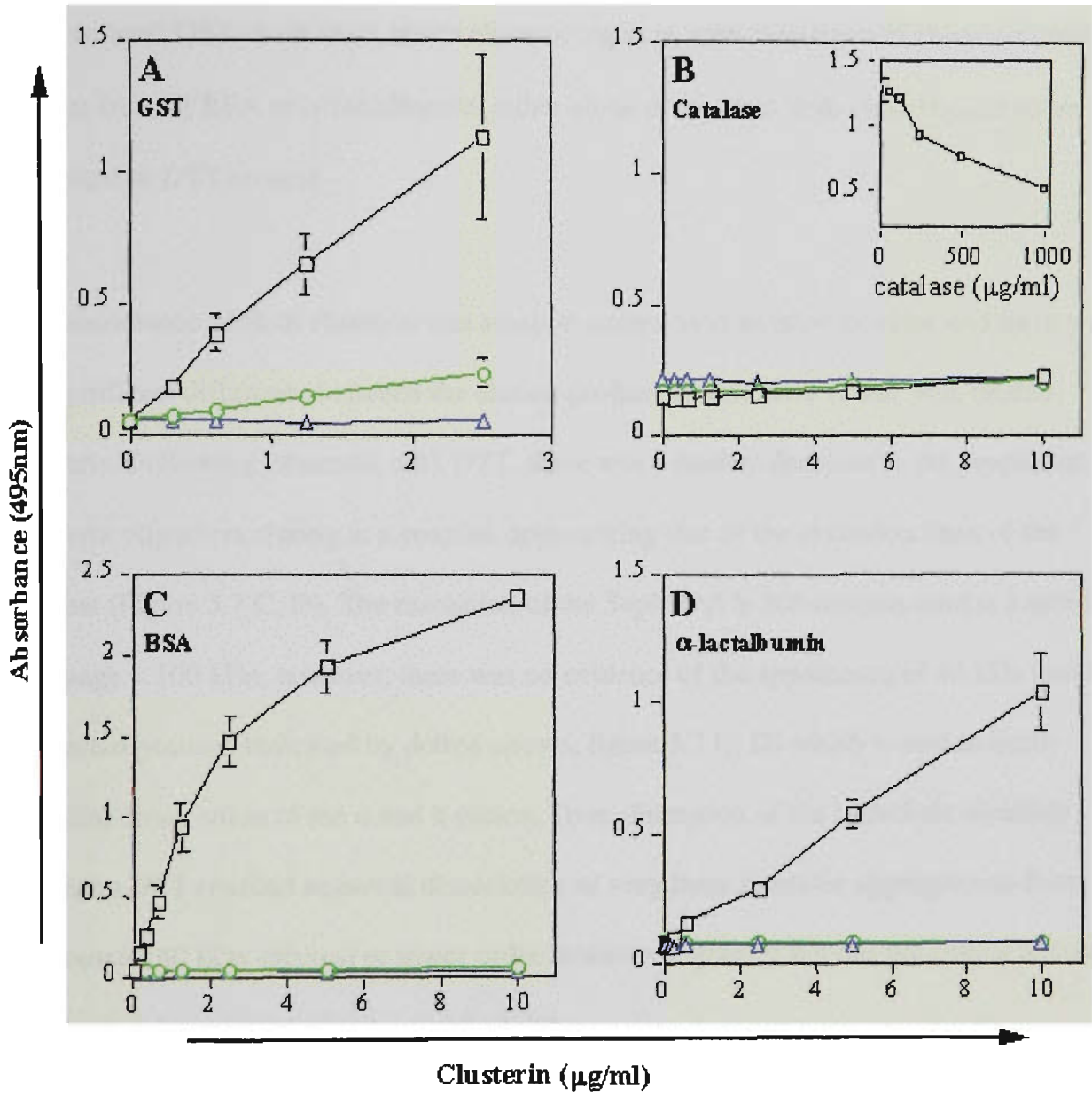


Figure 5.6: Results of ELISA measuring the binding of solution-phase clusterin to native (O) or denatured (□ and Δ) adsorbed proteins: **(A)** GST, heated at 60°C for 40 min to denature; **(B)** catalase, heated at 60°C for 40 min to denature. Insert shows solution phase denatured catalase inhibiting the binding of clusterin to denatured GST which was adsorbed to the plate; **(C)** BSA, incubated with 20 mM DTT at 37°C for 5 h to denature; **(D)** α-lactalbumin, incubated with 20 mM DTT at 37°C for 5 h to denature. Bound clusterin was detected with the G7 (anti-clusterin) monoclonal antibody (□ , O) and DNP-9 (Δ) was used as an isotype control; refer Experimental Procedures. The results shown are representative of three independent experiments. Each data point represents the mean of three replicate measurements and the error bars shown are SE of the mean in each case. In some cases, the SE are too small to be visible.

mixtures of proteins initially dissolved in PBS: clusterin alone, either untreated or heat- or DTT-treated; GST or catalase, either alone or together with clusterin and either untreated or heat-treated; BSA or α-lactalbumin, either alone or together with clusterin and either untreated or DTT-treated.

The absorbance peak of clusterin was small in comparison to other proteins and there was no significant difference between the elution profiles of untreated versus heat-treated clusterin. Following treatment with DTT, there was a modest decrease in the proportion of clusterin oligomers eluting at a position approaching that of the exclusion limit of the column (Figure 5.7 C, D). The resolution of the Sephacryl S-300 column used is limited in the range < 100 kDa, however, there was no evidence of the appearance of 40 kDa species (expected position indicated by dotted arrows, figure 5.7 C, D) which would indicate physical dissociation of the α and β chains. Thus, disruption of the interchain disulfide bonds by DTT resulted in partial dissociation of very large clusterin aggregates to form monomers (80 kDa species) or lower order clusterin oligomers but did not appear to cause substantial dissociation of the α and β chains.

In all cases, when clusterin was added to proteins undergoing heat- or DTT-mediated stress, analysis of the mixtures by size exclusion chromatography indicated pronounced formation of HMW complexes (Figure 5.7 A-D). The HMW complexes were not detected in analyses of clusterin alone or any of the individual proteins, whether these molecules were stressed or not. Nor were they detected in mixtures of clusterin and any of the other proteins in the absence of experimental stress (Figure 5.7 A-D). Next SDS PAGE

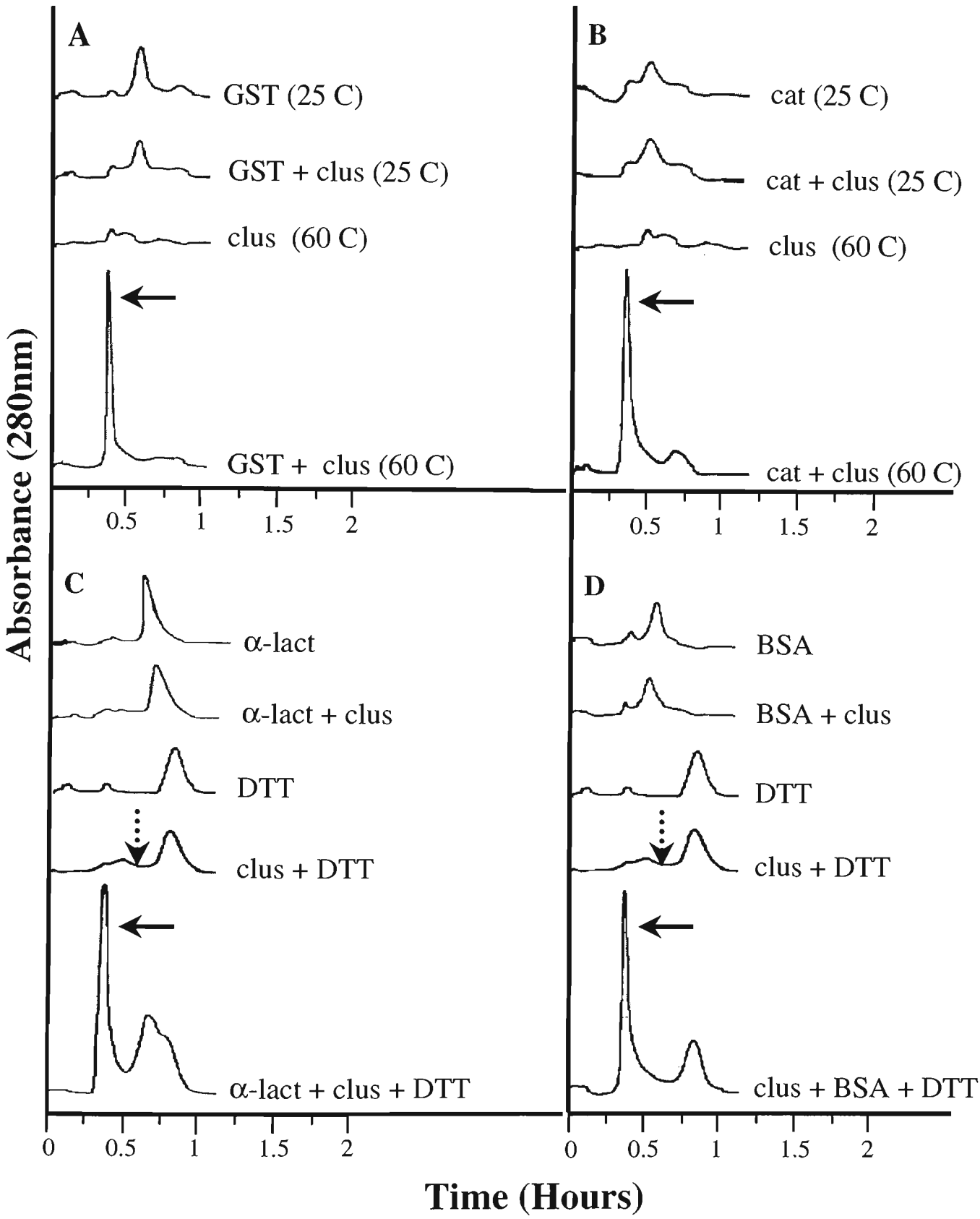


Figure 5.7: Size exclusion chromatographic analyses of individual solutions of clusterin, GST, catalase, BSA or α-lactalbumin, or mixtures of clusterin with one of the other proteins, either untreated or treated with heat (60°C for 30 min) or DTT (20 mM at 37°C for 5 h). Specific treatments are indicated adjacent to the individual plots. The results shown are representative of at least two independent experiments. Dark arrows indicate the HMW species, dotted arrows indicate the approximate elution position of a 40 kDa protein. Each analysis used 50 μg of each protein.

electrophoresis was performed on HMW fractions collected from size exclusion chromatography. In each case it was shown that these fractions contained both clusterin and the stressed protein (Figure 5.8). The equivalent size exclusion chromatography samples from untreated mixtures contain minimal amounts of protein (determined by UV absorbance traces Figure 5.7). This suggests that clusterin was binding to stressed proteins and forming HMW complexes.

To confirm that clusterin was binding to stressed proteins in solution and forming HMW complexes, samples were prepared as for size exclusion chromatography and analysed by native agarose gel electrophoresis. The position of proteins was determined by staining gels with Coomassie blue. The results were consistent with those obtained from size exclusion chromatographic analyses. In all cases, when clusterin was present together with proteins undergoing heat- or DTT-mediated stress, a broad protein band was detected with electrophoretic mobility distinct from that obtained by electrophoretic analysis of the individual proteins in the mixture (Figure 5.9). Furthermore, the band of unique electrophoretic mobility was only detected when the mixtures had been exposed to experimental stress (Figure 5.9). It seems probable that these stress-induced complexes correspond to the stress-induced HMW complexes detected by size exclusion chromatography. Next areas of agarose gels where proteins migrated to were physically cut out of the gel (e.g. areas defined in Figure 5.9) and analysed by SDS PAGE electrophoresis. Agarose gel cutout sections, which corresponded to the distinct migrating bands from protein mixtures which had been exposed to experimental stress, resolved into two distinct bands by SDS PAGE electrophoresis (Figure 5.10). In each case these bands

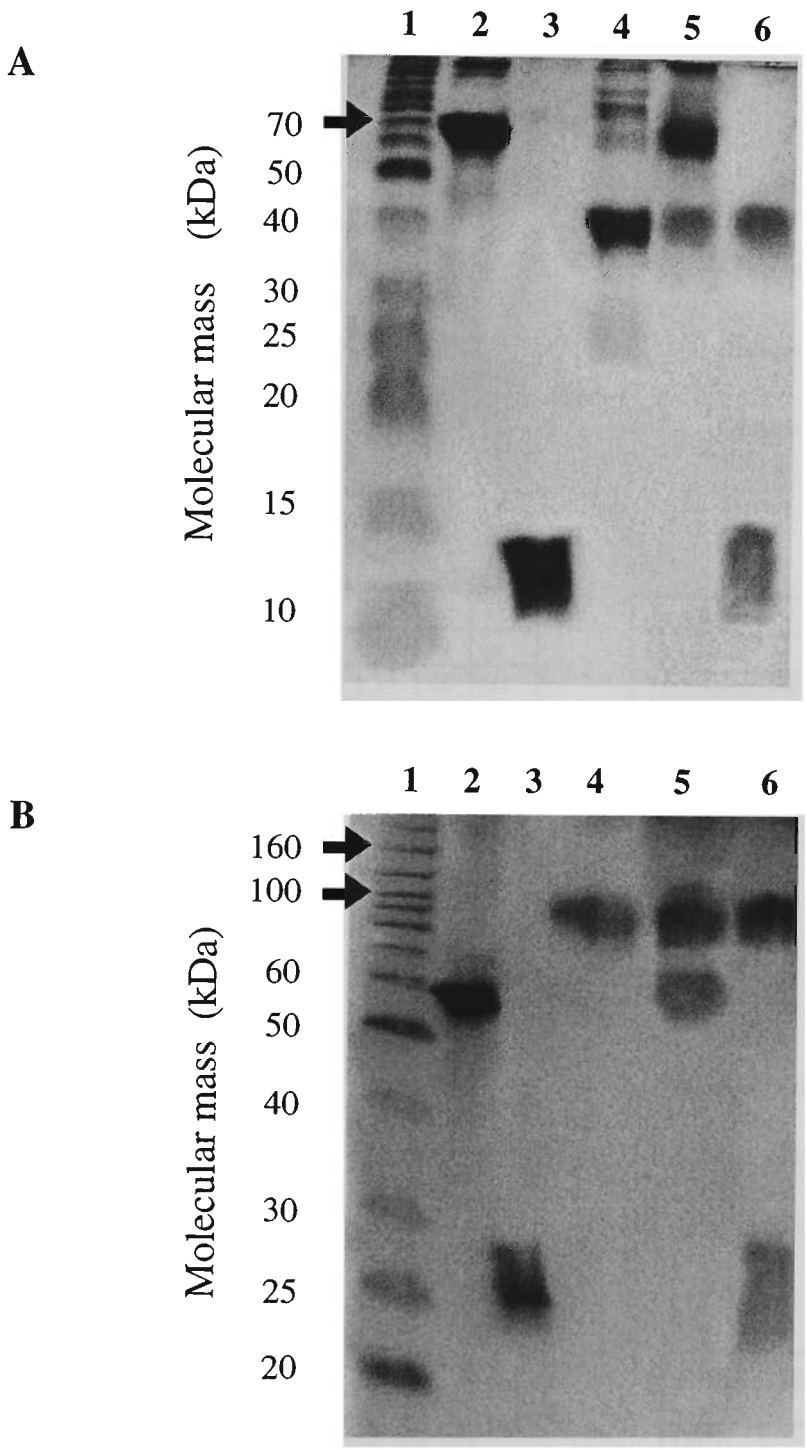
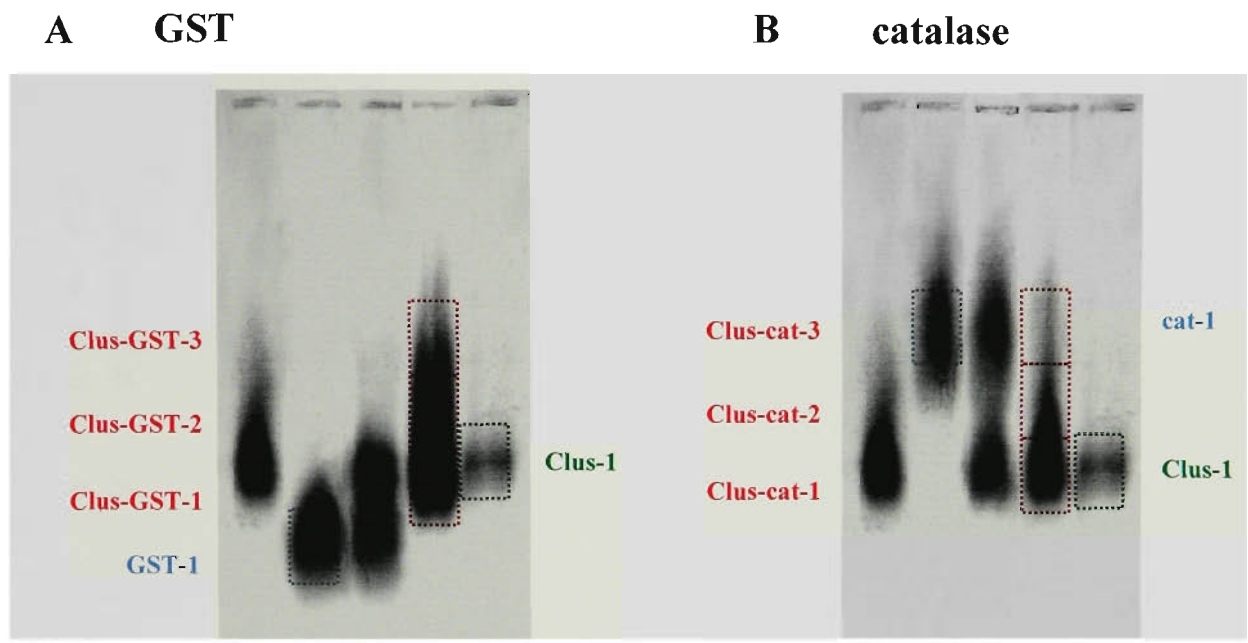
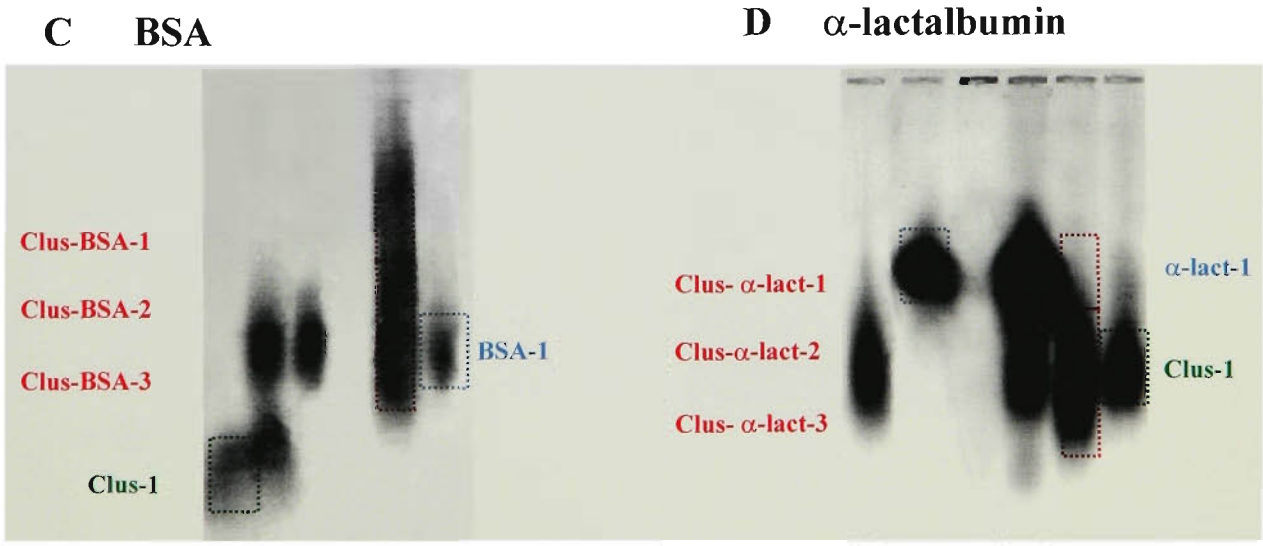


Figure 5.8: SDS-PAGE gel analyses of HMW complexes which were collected by size exclusion chromatography (refer fig 5.R.6 where samples collected from peaks indicated with solid arrows). **A**) 15% SDS-PAGE gel. Lane 1: Molecular weight markers; Lane 2: 10 µg catalase; Lane 3: 10 µg GST; Lane 4: 10 µg clusterin; Lane 5: HMW complex formed from clusterin and catalase when heated at 60°C for 30 minutes; Lane 6: HMW complex formed from clusterin and GST when heated at 60°C for 30 minutes. **B**) 12.5 % SDS PAGE gel. Lane 1: Molecular weight markers; Lane 2: 10 µg BSA; Lane 3: 10 µg α-lactalbumin; Lane 4: 10 µg clusterin; Lane 5: HMW complex formed from clusterin and BSA when treated with DTT; Lane 6: HMW complex formed from clusterin and α-lactalbumin when treated with DTT.



GST		x	x	x	
Clusterin	x		x	x	x
Temperature(°C)	37	37	37	60	60

Catalase		x	x	x	
Clusterin	x		x	x	x
Temperature(°C)	37	37	37	60	60



BSA	x	x			x	
Clusterin		x	x	x	x	x
DTT				x	x	x

α-lactalbumin		x	x	x	
Clusterin	x		x	x	x
DTT				x	x

Figure 5.9: Native agarose gel electrophoresis of clusterin, GST, catalase, BSA or α-lactalbumin, or mixtures of clusterin with one of the other proteins, either untreated or treated with heat (60°C for 30 min) or DTT (20 mM at 37°C for 5 h). Individual proteins or protein mixtures were treated prior to electrophoresis as indicated in the table below each image (indicated with an "x"). Dotted rectangles represent regions which were excised and analysed by SDS-PAGE, further described in figure 5.10.

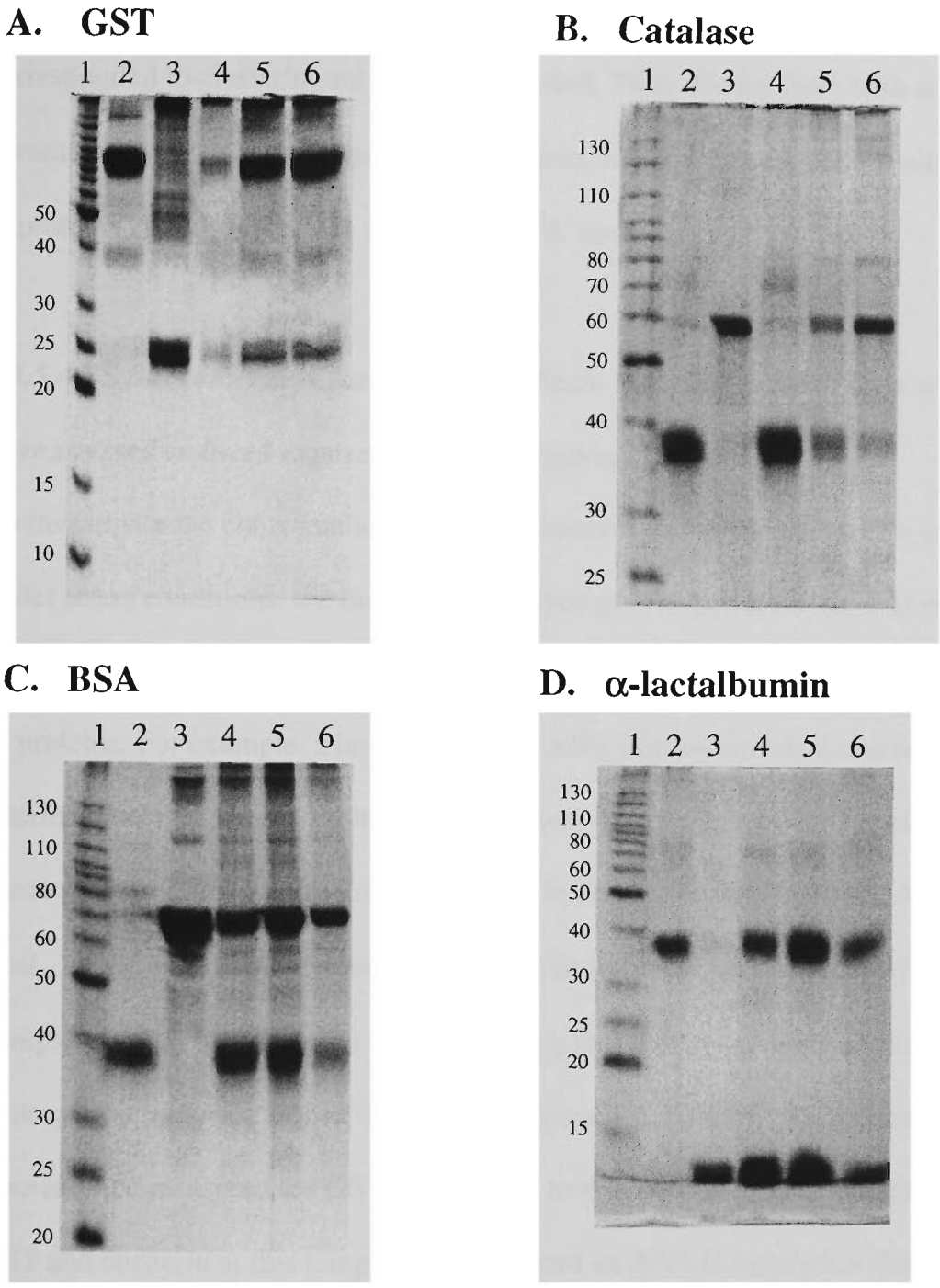
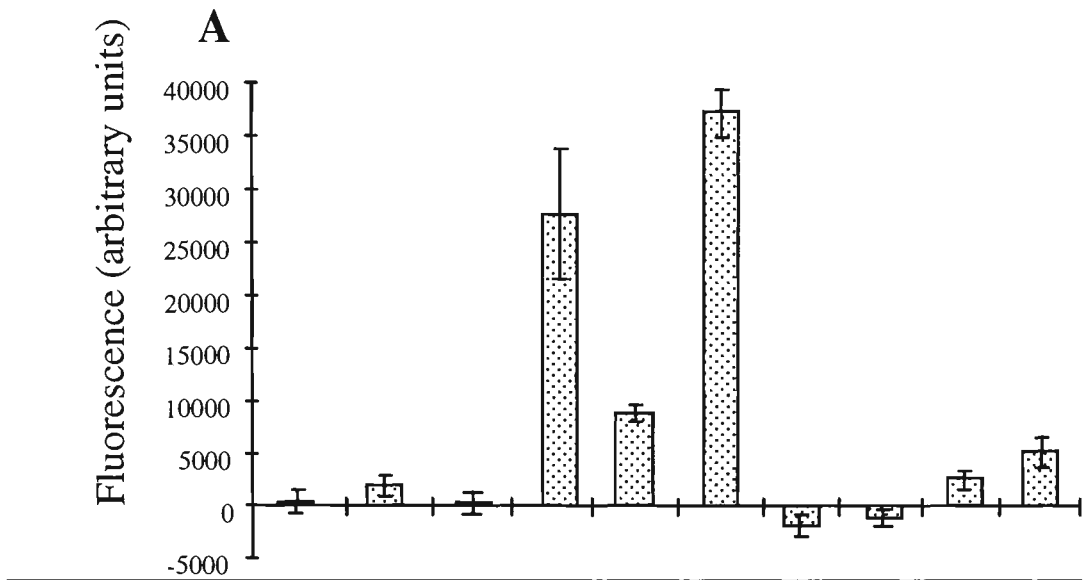


Figure 5.10: SDS PAGE electrophoresis of agarose gel sections containing clusterin, GST, catalase, BSA, α-lactalbumin, or fractions of the broad migrating band occuring from mixtures of clusterin with one of the other proteins when treated with heat (60°C for 30 min) or DTT (20 mM at 37°C for 5 h). The lanes contain protein bands excised from agarose gels following native gel electrophoresis (see figure 5.9). A) Lane 1: Makers; Lane 2: clusterin (clus-1); Lane 3: GST (GST-1); Lane 4-6 clusterin and GST heated at 60 C for 30 mins (clus-GST-1, clus-GST-2, clus-GST-3 respectively). B) as for A except Lane 3: catalase (cat-1); Lane 4-6 clusterin and catalase heated at 60 C for 30 mins (clus-cat-1, clus-cat-2, clus-cat-3, respectively). C) as for A except Lane 3: BSA (BSA-1); Lane 4-6 clusterin and BSA treated with 20mM DTT for 5 hours (clus-BSA-1, clus-BSA-2, clus-BSA-3 respectively). D) as for A except Lane 3: α-lactalbumin (α-lact-1); Lane 4-6 clusterin and α-lactalbumin treated with 20mM DTT for 5 hours (clus-α-lact -1, clus- α-lact -2, clus- α-lact -3 respectively). The results shown are representative of two independent experiments.

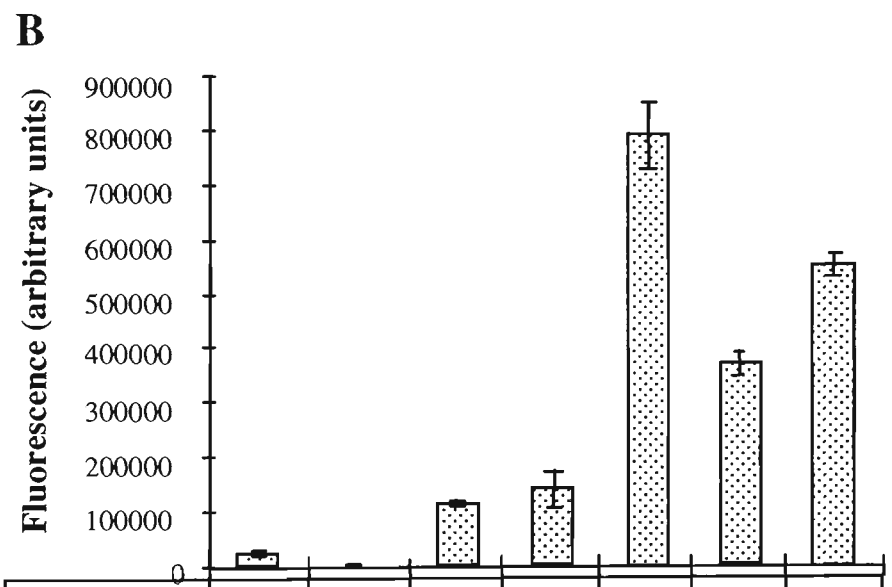
corresponded to clusterin and the protein tested. Thus, results from both size exclusion chromatography and native gel electrophoresis indicate that clusterin binds preferentially to proteins undergoing stress to form a HMW complex.

5.3.5 ANS fluorescence measurements indicate that clusterin interacts with proteins that have stressed-induced exposed regions of hydrophobicity

To investigate the conformational state of clusterin and the proteins with which it interacts under stress conditions, the binding of the hydrophobic probe, ANS, was monitored via fluorescence spectroscopy. ANS binds to clustered, solvent-exposed hydrophobic regions of proteins. For example, a large amount of ANS fluorescence is a characteristic of intermediately structured molten globule states of proteins which have elements of secondary structure present and an exposed hydrophobic core to which ANS binds (Ptitsyn *et al.*, 1990). Clusterin exhibited an increase in fluorescence of bound ANS at 60°C compared to 25°C (Figure 5.11 A) suggesting that it exposes some additional regions of hydrophobicity to solution at the higher temperature. At 25°C, the amount of ANS fluorescence measured for GST was similar to that measured for clusterin. A mixture of GST and clusterin at this temperature produced an ANS fluorescence that was significantly greater than the sum of the two individual values ($p < 0.05$). This result suggests that there is a conformational change in one or both of the proteins upon mixing at 25°C that exposes more hydrophobic surface. The implication from this result is that there is an interaction between the two proteins under physiological conditions which is consistent with the ELISA data (Figure 5.6 A).



Clusterin	x	x			x	x			x	x
GST			x	x	x	x				
Catalase							x	x	x	x
Temperature °C	25	60	25	60	25	60	25	60	25	60



Clusterin	x			x			x
BSA					x	x	x
α-lactalbumin		x	x	x			
DTT	x		x	x		x	x

Figure 5.11: Maximum ANS fluorescence measured for clusterin and (A) GST or catalase or (B) BSA or α-lactalbumin (see Experimental Procedures). Individual proteins or protein mixtures were treated as indicated in the table below each plot (indicated with an "x"). The results shown are representative of three independent experiments. Each histogram represents the mean of three replicate measurements and the error bars shown correspond to standard errors (SE) of the mean. In some cases, the SE are too small to be visible. The fluorescence values of blank wells containing an equivalent concentration of ANS in buffer alone have been subtracted from the values shown, resulting in some cases in small negative values (see Experimental Procedures).

A large increase in ANS fluorescence is observed for GST upon heating to 60°C (Figure 5.11 A). This suggests that heating to 60°C induces a significant conformational change in GST which generates an increased solvent-exposed hydrophobic surface on the protein. The ANS fluorescence of a HMW complex between clusterin and GST after heating at 60°C was approximately the sum of the values for the individual proteins at 60°C (Figure 5.11 A). Clearly, interactions between GST and clusterin did not reduce the total ANS fluorescence. It has recently been shown that the subunits of α-crystallin, a sHSP, undergo inter-molecular exchange even in the presence of bound substrate protein (Bova *et al.*, 1997). It is possible that this type of dynamic interchange may also occur in the interactions between clusterin and stressed proteins. It is therefore feasible that, as a result of the dynamic nature of these interactions, ANS may have essentially unrestricted access to solvent-exposed regions of stressed proteins, despite binding interactions with clusterin. As clusterin undergoes only a relatively small change in its ANS fluorescence upon heating (Figure 5.11 A), it is unlikely that the large amount of ANS fluorescence of the HMW clusterin:GST complex arises from an alteration in exposed hydrophobicity of clusterin at 60°C. More likely, this fluorescence is due to the increased exposed hydrophobic surface on the bound GST molecules in the complex.

The pattern of change of ANS fluorescence for clusterin and α-lactalbumin, and mixtures of these two proteins, with and without DTT-mediated stress was found to be similar to that observed for corresponding treatments of clusterin and GST with heat-stress (Figure 5.11 B). The ANS fluorescence values of reduced clusterin and native α-lactalbumin were small. α-Lactalbumin is known to adopt a molten globule conformation upon reduction

(Kuwajima *et al.*, 1990). Consistent with this, a large increase in ANS fluorescence was observed following reduction of α-lactalbumin. The ANS fluorescence of the HMW complex formed between clusterin and α-lactalbumin after reduction with DTT was approximately the sum of the values for the reduced individual proteins (Figure 5.11 B). This pattern is consistent with the two proteins associating such that the majority of the ANS fluorescence arose from the partly unfolded, molten globule state of α-lactalbumin (Figure 5.11 B).

Upon heating catalase to 60°C, there was only a slight increase in ANS fluorescence (Figure 5.11 A). Accordingly, the addition of ANS to a solution of catalase and clusterin that had been heated at 60°C only led to a small increase in ANS fluorescence relative to an unheated control solution of the two proteins (Figure 5.11 A). The relative magnitude of this increase was much less than that observed for the heated solution of clusterin and GST (Figure 5.11 A) suggesting that catalase does not display a large increase in exposed hydrophobic surface upon partial unfolding and formation of the HMW complex with clusterin.

Clusterin, BSA and mixtures of these two proteins, with or without reduction with DTT, were also analysed for ANS binding (Figure 5.11 B). Native BSA exhibited a large amount of ANS fluorescence. Upon reduction of BSA, ANS fluorescence decreased to 48% of its value in the native form (Figure 5.11 B). Reduced BSA is known to adopt a molten globule conformation (Shin and Hirose, 1995). However, the data presented here indicate that unlike most other proteins, the molten globule state of BSA has decreased, although still

significant, exposed hydrophobicity compared to the native protein. The ANS fluorescence of the HMW complex formed under reducing conditions between clusterin and BSA was significantly greater than the sum of the fluorescences of the individual reduced proteins (Figure 5.11 B). This implies that there is also some alteration in the exposed hydrophobicity of clusterin and/or the molten globule-state of BSA upon incorporation into the HMW complex.

5.3.6 Stress precipitation of proteins in solutions containing different salts confirm that proteins interact via hydrophobic interactions.

In solution salts effect the interaction between molecules and water. The Hofmeister series predicts the strength of these interaction caused by different salts (Creighton, 1993). Each of the stress sensitive proteins tested were prepared in solutions containing different salts from this series to confirm that proteins were precipitating as a result of hydrophobic interactions. The Hofmeister series predicts the following order of salts that enhance hydrophobic interactions in solution (listed from highest to lowest): SO_4, Cl, PO_4, followed by SCN. In each case a greater rate of precipitation resulted when proteins were prepared in SO_4 (Figure 5.12). In extended stress treatments the precipitation of some proteins prepared in this salt formed very large aggregations which decreased the amount of light absorbed at 360nm (Figure 5.12, e.g. catalase after 20 minutes). Solutions containing SCN resulted in limited precipitation of any protein when stressed with either heat or DTT. Solutions containing Cl and PO_4 ions also resulted in the precipititaion of proteins when stressed. With the exception of catalase the stress treatment of all proteins prepared in Cl resulted in faster precipitation compared to PO_4 (Figure 5.12). Catalase prepared in either

Cl or PO_4 resulted in a similar rate of precipitation when heated (Figure 5.12 B). The rate of precipitation of proteins prepared in Cl or PO_4 in each instance was less than proteins prepared in SO_4 and greater than proteins prepared in SCN.

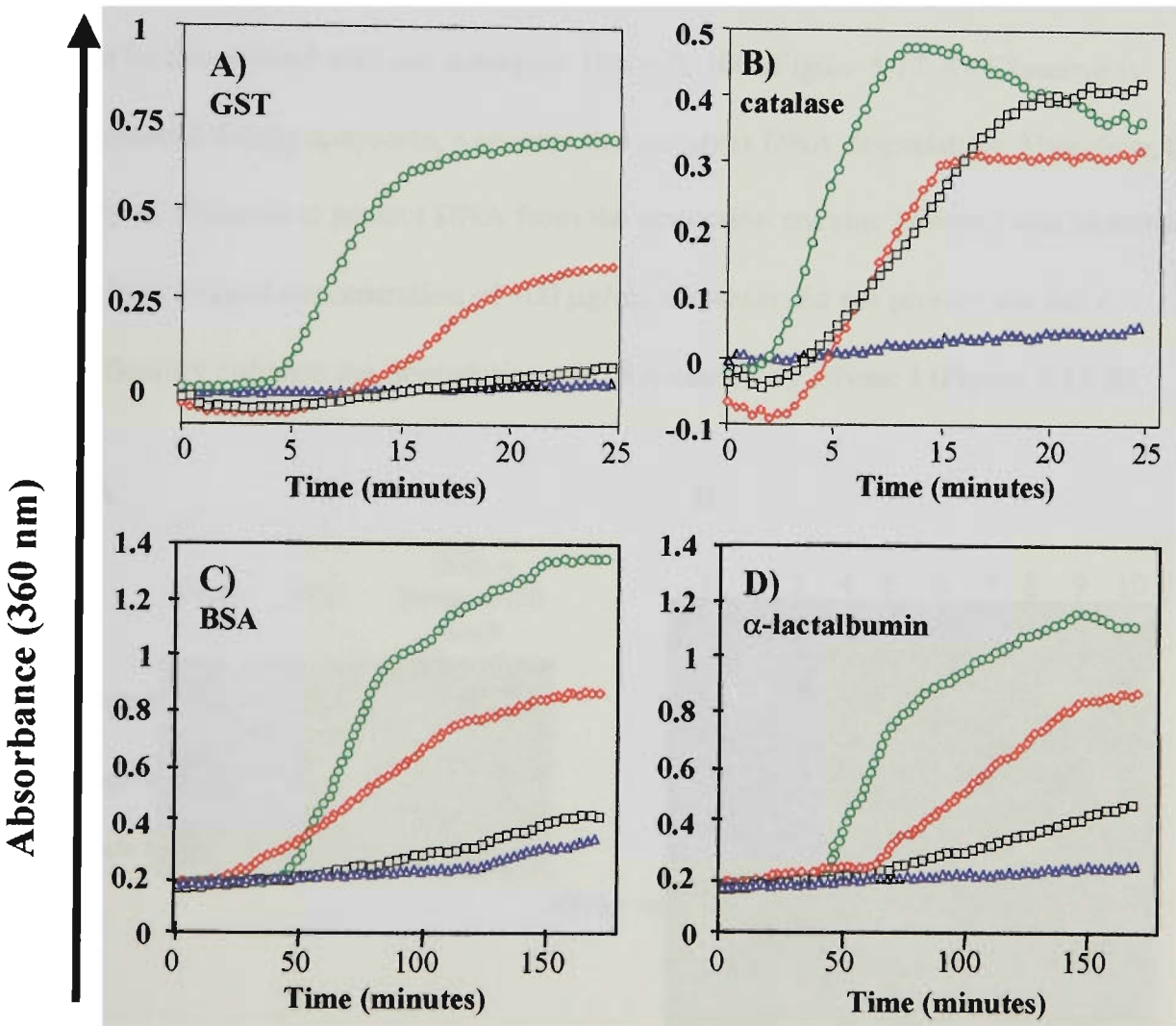


Figure 5.12: Protein precipitation as a function of salt. Absorbance at 360 nm, as a function of time for (**A**) GST (200 μg/ml) heated at 60°C; (**B**) catalase (200 μg/ml) heated at 60°C; (**C**) BSA (750 μg/ml) treated with 20 mM DTT at 37°C; (**D**) α-lactalbumin (750 μg/ml) treated with 20 mM DTT at 37°C. In each case proteins were prepared in either 0.1 M NaCl (◇), 0.1 M $NaSO_4$ (○), 0.1 M NaSCN (△), or 0.1 M $NaPO_4$ (□).

5.3.7 Binding of clusterin to DNA

Clusterin was shown to accumulate in the nuclei of TNFα treated L929 cells (refer section 4.3.12), and reportedly binds to nuclei of nutrient-deprived Hym2 or U937 cells (Wilson *et al.*, 1995). Clusterin was therefore tested to see if it bound to DNA or RNA purified from wild type L929 cells. Clusterin bound to immobilised DNA but not RNA (Figure 5.13 A). The binding interaction between clusterin and DNA is probably hydrophobic as clusterin could be dissociated with the detergent Triton X-100 (Figure 5.13 A). Clusterin is upregulated during apoptosis, a process that involves DNA degradation. Therefore, the ability of clusterin to protect DNA from the restriction enzyme DNase I was examined. At the physiological concentration of 100 μg/ml, clusterin did not protect nor did it significantly enhance the degradation of DNA caused by DNase I (Figure 5.13 B).

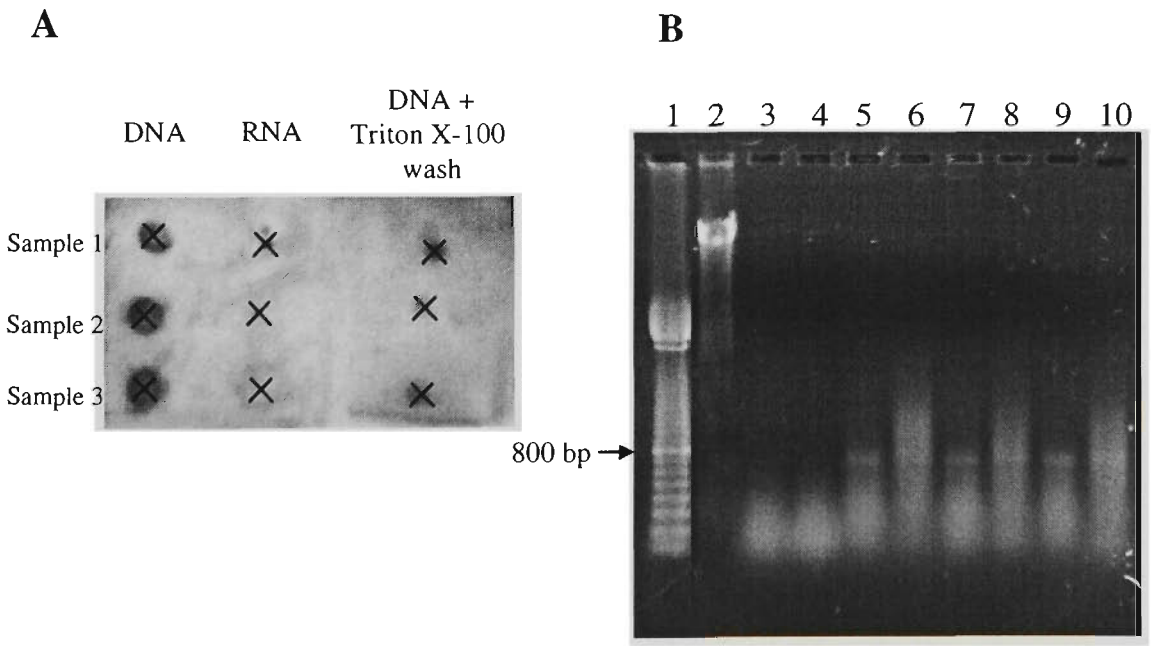


Figure 5.13: **A)** Dot blot in which either DNA or RNA which was dotted onto nitrocellulose and then incubated with clusterin. Clusterin was detected with G7 monoclonal antibody. One membrane was washed with 0.1% triton X-100 in PBS. X mark the positions where DNA or RNA was doted onto membranes. **B)** L929 genomic DNA which were electrophoresed on a 1% agarose gel. Lane 1: molecular weight markers; lane 2: 3 μg untreated DNA. All other lanes were 3 μg of DNA treated with 50 μg/ml DNase I + either 100 μg/ml clusterin (lane 3 and 4), BSA (lanes 5 and 6), gelatin (lane 7 and 8), or casein (lanes 9 and 10) for 30 minutes at 37°C.

5.3.8 Clusterin does not protect L929-pRc.clus cells against heat or H_2O_2.

The clusterin-expressing L929 transfected cell lines were tested to see whether the expression of clusterin provided protection to those cells from heat or H_2O_2. Several cultures of L929-pRc.clus and L929-pRc cells were compared following incubation at either 37°C or 47°C. The rate of cell death, determined by PI staining (refer 4.2.3.2), was not significantly different between the cell lines when cultured at high temperatures (Figure 5.14). However, on occasions, L929-pRc.clus cells were found to be significantly resistant to heat relative to L929-pRc cells. Unfortunately, this result could not be reliably reproduced.

The transfectants were also tested for their resistance to exposure to H_2O_2. Surprisingly, the rate of death due to H_2O_2 exposure was significantly greater ($p < 0.05$; students t-test) for L929-pRc.clus cells than for L929-pRc cells (Figure 5.14).

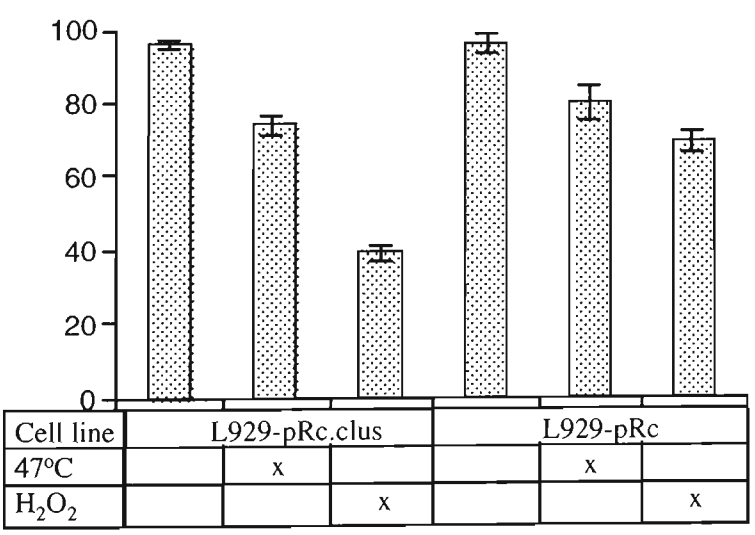


Figure 5.14: Average L929-pRc.clus and L929-pRc cell viability after treatment with either 0.4 mM H_2O_2 or 47°C for 16 hours as indicated. All other cells were incubated at 37°C. Viability was determined by staining cells with PI (refer section 4.2.3.2) and then analysing cells by flow cytometry. Results shown is representative of three independent exeriments. Error bars are standard deviation of the mean.

5.4 DISCUSSION

5.4.1 Clusterin's ability to prevent protein precipitation by forming HMW complexes.

It was previously shown that clusterin reduced the aggregation of a synthetic amyloid β-peptide in aqueous solution (Oda *et al.*, 1995). However the effects of clusterin on the aggregation of stressed proteins has not been reported before. The results presented in this chapter demonstrate for the first time that clusterin has chaperone-like activity. Clusterin potently protected GST and catalase from heat-induced precipitation and α-lactalbumin and BSA from precipitation induced by reduction (Figure 5.4). Clusterin does not have an affinity for these proteins, with the exception of GST, which suggests that it may also be capable of protecting many other proteins from stress-induced precipitation. This property of clusterin is therefore similar to that of sHSP which also are capable of preventing protein precipitation caused by heat or reduction (Jakob *et al.*, 1993; Horwitz 1992). In some cases it has been demonstrated that some sHSP are capable of protecting enzymes from heat-induced loss of catalytic activity. For example a recent report has demonstrated that α-crystallin not only protects catalase from heat-induced precipitation but also retains catalase enzyme activity (Hook *et al.*, 1997). However, in the case of two enzymes tested, GST and catalase, clusterin was unable to protect against heat-induced loss of activity (Figure 5.5).

sHSP have been shown to protect proteins from various stresses by forming HMW complexes (Carver *et al.*, 1994). Clusterin also formed HMW complexes with each of the four proteins tested upon heat or reducing treatment as determined by size exclusion chromatography (Figure 5.7). The peak representing the HMW complex was at the

position of the exclusion volume of the column indicating a mass greater than $1.5x10^6$ Da. As this peak was absent in the analyses of solutions containing (i) unstressed protein mixtures or (ii) individual proteins or clusterin, regardless of whether these proteins had been stressed or not, this indicates that clusterin only formed HMW complexes with stressed proteins.

SDS-PAGE analyses demonstrated the presence, in each case, of two proteins (clusterin and the stressed protein) in the HMW fractions collected from size exclusion chromatography (Figure 5.8). The formation of a complex between clusterin and stressed proteins was also verified in each case by native gel electrophoresis and then subsequent analysis by SDS PAGE (Figure 5.9 & 10). Results from size exclusion chromatography and native gel electrophoresis clearly indicate that, in each case tested, like sHSP, clusterin binds to the denatured protein to form HMW complexes.

5.4.2 Clusterin's attraction to hydrophobic surfaces.

No crystal structure is available for clusterin and very limited experimental data is available concerning the secondary or tertiary structure of the protein. However, sequence analysis indicates that clusterin has three putative amphipathic α-helical regions, secondary structures thought to be important in interactions with hydrophobic molecules (de Silva *et al.*, 1990a; refer Figure 1.1). In addition, sequence analysis predicts a number of short regions of hydrophobicity outside those defined by the putative amphipathic α-helical regions (Figure 5.15). Since clusterin is known to bind to a variety of native proteins with

hydrophobic domains, it is likely that clusterin exerts its chaperone-like activity by binding to exposed hydrophobic regions of stressed proteins.

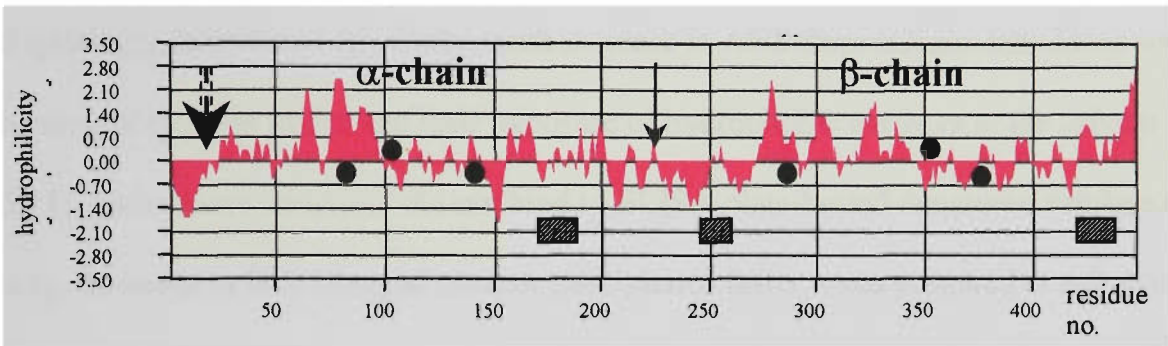


Figure 5.15: Predictions based on analysis of the sequence of human clusterin cDNA. The hydrophobicity index (MacVector v4.14, Hopp-Woods scale) is plotted as a histogram display. Predicted regions of amphipathic α-helices are represented by striped rectangles. Proteolytic cleavage sites for the removal of the signal peptide and internal cleavage to produce the α- and β-chains are indicated by striped and solid arrows, respectively. Asn residues that serve as attachment points for glycolsylation are marked as solid circles.

The stress treatment of proteins prepared in salts which enhanced hydrophobic interactions led to an increased rate of precipitation while the stress treatment of proteins prepared in salts that diminishes hydrophobic interactions resulted in minimal protein precipitation. In addition measurements of ANS fluorescence indicated that stress treatment of GST and α-lactalbumin led to exposure of hydrophobic regions that were accessible to solvent and therefore also clusterin (Figure 5.11). These results indicate that precipitation occurred due to denatured proteins agglomerating together through interactions of hydrophobic surfaces that became exposed to the solvent. It is therefore likely that clusterin prevents their precipitation by non-specifically interacting with these newly exposed hydrophobic regions caused by stress and thereby prevents the uncontrolled aggregation of proteins. There was no significant difference of the ANS flourescence between stressed GST or α-lactalbumin with clusterin and the sum of clusterin and the corresponding stressed protein. The

similarity of the flourescence therefore suggests that clusterin does not "umbrella" the hydrophobic surface of the stressed protein as ANS still bound to the hydrophobic surface.

Suprisingly there was a relatively small increase in ANS fluorescence associated with heating of catalase indicating little exposure of hydrophobic surfaces to the solvent (Figure 5.11). Futhermore, clusterin did not bind to ELISA plate-bound denatured catalase (Figure 5.6). However as heat stressed catalase precipitates faster when prepared in salts that enhanced hydrophobic interactions (Figure 5.12), this suggests that hydrophobic interactions are responsible for the precipitation of this protein. Similarly, solution-phase denatured catalase inhibited the binding of clusterin to plate-bound denatured GST (Figure 5.6 insert), which suggests that clusterin bound to denatured catalase and formed HMW complexes through hydrophobic interactions. In fact the ELISA format which showed that clusterin had negligible affinity to bound denatured catalase may have been due to steric constaints imposed by solid phase adsorption. Further work is therefore required to better define the nature of this interaction.

Native BSA had a high level of ANS flourescence that presumably is a consequence of substantial exposed hydrophobic surface. This may serve the *in vivo* function of BSA in transporting hydrophobic molecules in plasma. The 48% decrease in its ANS fluorescence induced by reduction indicates that a major conformation change resulted (Figure 5.11). This conformational change occurred due to the unfolding of the protein when cystein bonds were broken from DTT treatment. The unfolding of BSA and α-lactalbumin was shown to be very unstable as they both precipitated out of solution when treated with DTT

(Figure 5.4). Precipitation of BSA (like catalase, GST and α-lactalbumin) was due to hydrophobic interactions. This is supported by the results of experiments testing the effects of different salts on protein precipitation (Figure 5.12). Therefore, it is likely that clusterin interacts with hydrophobic surfaces to prevent BSA from reduction-induced precipitation.

With the exception of GST, clusterin was shown not to bind to the native form of each of the four proteins tested in this study by ELISA (Figure 5.6). Clusterin bound more strongly to heat-treated than to untreated GST and also bound significantly to reduced α-lactalbumin and BSA (Figure 5.6). Clusterin also bound strongly to reduced-denatured BSA and α-lactalbumin but not heat-denatured catalase (Figure 5.6). However solution-phase heat-denatured catalase but not native solution-phase catalase prevented clusterin from binding to plate-bound heat-denatured GST (Figure 5.6). This indicates that clusterin has a higher affinity for denatured catalase than native catalase. Alltogether these ELISA results suggest that clusterin is capable of non-selectively binding to denatured proteins. It is proposed that the binding of clusterin to denatured proteins results in the formation of HMW complexes that in turn prevents the denatured protein from precipitating.

5.4.3 The efficiency of clusterin binding.

Since many chaperones (and also clusterin) exist in solution as aggregates of an ill-defined number of monomers, a convention that has been adopted when dealing with the interactions between chaperones and other proteins is to define stoichiometry in relation to the individual subunits of the chaperone and the protein with which it interacts. For these calculations it was assumed that the molecular mass for intact clusterin was 80 kDa and the

subunit mass 40 kDa. An approximate subunit molar ratio (SMR) of clusterin:GST of 1.0:3.2 was the minimum required to virtually abolish reduction-induced precipitation (Figure 5.4 A). Corresponding SMRs for the stabilizing effects of clusterin on the other proteins tested were 1.0:1.3 (catalase), 1.0:2.3 (BSA), and 1.0:11 (α-lactalbumin) (Figure 5.4 B, C & D, respectively). In contrast, even at corresponding SMRs as high as 1.4:1.0, a control protein (ovalbumin) had only a small effect on the stress-induced precipitation of any of the proteins tested (Figure 5.4 F). These SMR values indicate that clusterin is a very efficient chaperone. In comparison, the sHSPs are less efficient in their interactions with stressed proteins; the available data suggest that sHSPs bind stressed proteins at a SMR of one or more subunits of sHSP to one partially folded protein (Farahbakhsh *et al.*, 1995; Lindner *et al.*, 1998).

The SMRs of interaction of heat-stressed GST and catalase with clusterin are proportional to the mass of the stressed protein (Table 5.1). A similar relationship was found for BSA and α-lactalbumin, stressed by DTT-mediated reduction (Table 5.1). However, there is not a simple uniform relationship (for all the proteins tested, regardless of the type of stress) between mass and the SMR effecting inhibition of precipitation. Although the number of data points is small (two per treatment) the close relationship between mass and the SMR effecting inhibition of precipitation for GST and catalase and for BSA and α-lactalbumin (Table 5.1) suggests that there may be a fundamental difference in the mechanism by which clusterin exerts its chaperone-like action under conditions of stress induced by heat versus reduction. Despite this, regardless of the nature of the stress, the data indicate that steric constraints influence the interaction of stressed proteins with clusterin. When

considering proteins stressed by heat, or those stressed by reduction, in each case clusterin was able to interact with and stabilize a greater number of smaller versus larger stressed protein molecules. This is consistent with the suggestion from the ANS studies that stressed proteins bind to the external surface of clusterin. Interestingly, when a similar comparison is made for the interaction of three reduced proteins (α-lactalbumin, BSA and ovotransferrin) with the sHSP, α-crystallin, a close correlation is also observed between the ratios of the molecular masses and SMR (Lindner *et al.*, 1998).

Table 5.1: Relationship between molecular mass of proteins stressed by either heat or DTT-mediated reduction versus the SMR of interaction between the stressed proteins and clusterin effecting inhibition of protein precipitation.

Comparison Between Proteins	**Ratio of Molecular Mass**	**Ratio of SMR**
Catalase vs. GST	2.1	2.3
BSA vs. α-lactalbumin	4.7	4.7

5.4.4 The effects of clusterin expression on cell stress

The upregulation of HSP25 and α-crystallin in L929 cells has been shown to provide thermotolerance to those cells (Aoyama *et al.*, 1993; Chretien and Landry, 1988). Presumably this is due to an intracellular action whereby they prevent important regulatory enzymes and other proteins from becoming inactivated. However, the clusterin-expressing L929 transfectants, L929-pRc.clus were not protected against either heat or H_2O_2 (Figure 5.13). Clusterin cDNA in the L929-pRc.clus cells contain the endogenous signal peptide (chapter 3) which theoretically indicates that all the clusterin produced should be secreted. Therefore clusterin produced in these cells could not protect enzymes and other intracellular proteins from precipitating due to heat and H_2O_2 treatments. This also suggests that the secreted clusterin, despite being present in low concentrations, was not capable of

protecting L929 cells extracellularly from heat and H_2O_2 treatments. Interestingly preliminary work has shown that physiological concentration of clusterin have provided limited protection to other cell lines in response to heat (S. Poon, Unpublished). It would be of interest to determine whether higher concentrations were also capable of protecting L929 cells from the cytotoxic effects of heat and H_2O_2.

An intracellular form of clusterin has been reported (Reddy *et al.*, 1996). Analysis of the clusterin gene that codes for clusterin reveals contains two in frame ATG sites. Translation of clusterin from the first ATG site would include the signal peptide while translation from the second site (located 33 bases downstream) would exclude the signal peptide, and would result in a truncated intracellular form (Reddy *et al.*, 1996). While no truncated form of clusterin was detected by immunoblotting in the L929-pRc.clus cells under normal culturing conditions (refer figure 4.14) it is still possible that small amounts of maybe produced. It would be interesting to determine whether this intracellular clusterin form is capable of protecting cells from various stresses likely to occur in physiological conditions. Creating transfectant cells lines that contain clusterin cDNA starting at the second ATG site would help determine this. If such a study showed that clusterin did protect cells intracellularly from stress would further support its role as a HSP. Additionally other studies would also be required to determine if this intracellular form of clusterin really exists.

5.4.5 Conclusion

The results described in this chapter indicate that clusterin has a chaperone-like function similar to that of the sHSP. Like clusterin, sHSP are known to bind to exposed hydrophobic regions on stressed proteins to form HMW complexes (Carver *et al.*, 1995). The formation of these complexes "solubilizes" stressed proteins, preventing their precipitation. It is also known that, generally, when acting alone, sHSP are unable to protect enzymes from heat-induced loss of function (Carver *et al.*, 1994; Horwitz, 1992). All of these characteristics appear to be shared with clusterin. There are a variety of additional similarities between the sHSP and clusterin which suggest that they may have a similar functional role *in vivo:*

(1) Heat shock factor 1 (HSF1) is a transcriptional activator of both the clusterin gene and sHSP genes (Michel *et al.*, 1997).

(2) Both sHSPs and clusterin are involved in, and/or their expression is upregulated in, a variety of instances of cell stress and disease states, for example, sHSPs in cataract, neurodegenerative disorders such as Alzheimer's, Alexander and Creutzfeldt-Jakob diseases and various cancers (Ehrnsperger *et al.*, 1997) and clusterin in cataract (P. Wong, pers. commun.), retinitis pigmentosa, Alzheimer's and Pick's diseases, gliomas, epileptic foci and many experimental models of stress (Silkensen *et al.*, 1994; Jomary *et al.*, 1993; Jones *et al.*, 1992; Clark and Groswold, 1997; Chapter 1).

(3) sHSPs and clusterin are highly resistant to stress-induced precipitation ((Horwitz, 1992), and results shown here).

(4) Both sHSPs and clusterin can protect cells from stresses ((Ehrnsperger *et al.*, 1997); refer chapter 4).

(5) Both sHSPs and clusterin oligomerise in aqueous solution (oligomeric sHSPs perform the chaperone function (Ehrnsperger *et al.*, 1997; Leroux *et al.*, 1997), refer chapter 1).
(6) The sHSP, HSP25, is often found as a disulfide-linked dimer, which aggregates to form large oligomeric complexes (Zavialov *et al.*, 1998). The oxidized and reduced forms of HSP25 both have very similar chaperone ability (Zavialov *et al.*, 1998). Likewise, clusterin forms disulfide-bonded dimers that behave very effectively as a chaperone under both reducing and non-reducing conditions.
(7) The limited sequence homology between the α-crystallin domain (common to all sHSP) and clusterin residues 243-249.

While much remains to be done to establish the mechanism by which clusterin protects proteins from stress-induced precipitation, predicted structural features of the molecule provide some clues. A yet to be defined sub-set of known hydrophobic regions of clusterin sequence, possibly including one or more of the predicted amphipathic α-helices (Figure 5.15), are likely to be involved in binding to hydrophobic regions exposed on partly unfolded, stressed proteins. The results clearly show that hydrophobic interactions are important in the chaperone-like activity of clusterin. The structure of clusterin is amphipathic, since it also possesses a number of strongly hydrophilic regions, including a region at the extreme C-terminus of the molecule (Figure 5.15). It is interesting to note that sHSPs also have a hydrophilic C-terminal region, which plays an important role in their chaperone action (Carver *et al.*, 1992; Carver *et al.*, 1995; Smulders *et al.*, 1996). Substantial additional hydrophilicity is provided to secreted clusterin by its extensive glycosylation, which is complex and is attached to clusterin via six Asn residues (Kapron

et al., 1997; Figure 7). Like the sHSPs, the amphipathicity of clusterin is very probably critical in effecting solubilization of stressed proteins incorporated into HMW complexes. In this context, an obvious corollary is that at least some of the reported binding interactions between clusterin and other (non-stressed) molecules with regions of hydrophobicity may simply result from the amphipathic character of clusterin and represent artifacts unrelated to any genuine biological function.

A subset of the results presented in this chapter have been published in the following journal paper (a reprint is attached in the appendix):

Humphreys D.T., Carver J.A., Easterbrook-Smith S.B., Wilson M.R. (1999). Clusterin has chaperone like activity similar to that of small heat shock proteins. *J Biol Chem.* **274,** 6875-6881.

CHAPTER 6

Summary and conclusion.

This thesis has described research examining (i) the effect of clusterin expression on cell death and (ii) the ability of clusterin to act as a chaperone. The aims of this project were to:

(i) Generate stable transfected L929 cell lines that inducibly or constitutively secrete human clusterin.

(ii) Use the transfected cell lines to test what effects clusterin expression has on apoptosis or necrosis.

(iii) Determine whether clusterin can act as a molecular chaperone.

6.1 Clusterin's propensity to bind to hydrophobic surfaces

Clusterin has been linked to numerous roles in the body due to its interaction with a diverse array of ligands (refer section 1.2). These interactions indicate that clusterin either has a multifunctional role in the body or that it has a common underlying function. Here it has been shown that clusterin has chaperone-like activities similar to that of small heat shock proteins. These results suggest that clusterin binds to hydrophobic residues on denatured proteins and thereby inhibits protein precipitation (chapter 5). It is also been shown that the upregulation of clusterin can protect L929-pRc.clus cells from TNFα-mediated death (chapter 4). The propensity of clusterin to bind to hydrophobic surfaces may explain how clusterin protects L929 cells from TNFα mediated death as well as explain the wide array of proteins to which clusterin binds.

Clusterin's affinity for hydrophobic surfaces was first reported in 1983 by Blaschuk and colleagues who demonstrated that (i) clusterin aggregated with itself to form dimers and tetramers, (ii) it had a high affinity for the hydrophobic Affi-Gel blue matrix, and (iii) it

aggregated Sertoli cells *in vitro*. From these early findings clusterin was thought to influence cell interactions in the testis or epididymis. On the basis of amino acid sequence, computer analysis predicts three amphipathic α-helical regions in clusterin, which also suggests that clusterin is likely to participate in hydrophobic interactions (de Silva *et al.*, 1990a; Tsura *et al.*, 1990). Research described in chapter 5 also suggested that clusterin participates in hydrophobic interactions as it bound to heat- and DTT-denatured proteins to a significantly greater extent than to the corresponding native proteins. Results presented here suggest that clusterin has sHSP-like chaperone activity as it inhibits heat or DTT-induced precipitation of proteins. Like the sHSP, it appears likely that clusterin binds to exposed hydrophobic regions of denatured proteins to form a solubilized high molecular weight complex (chapter 5). The concentrations of clusterin that protect stressed proteins from precipitation is within the range normally found in some extracellular fluids in mammalian systems (e.g. 50-370 μg/ml in human serum and 2.1-15.0 mg/ml in human seminal fluid; O'Bryan *et al.*, 1990). This raises the possibility that in these environments clusterin may act as a constitutively expressed chaperone.

6.2 The association of clusterin expression with cell death

The numerous findings that clusterin is expressed in most tissues and is upregulated in times of stress has generated a lot of interest and led to the suggestion that it either provokes or prevents cell death. Three independently made transfected cell lines expressing clusterin have each shown that its expression does not affect viability under normal culturing conditions (Pilarsky *et al.*, 1993; Sensibar *et al.*, 1995; section 4.3.9). Others have shown that clusterin is upregulated in proliferating tissue (Bursch *et al.*, 1995; Seiberg and Marthinuss, 1995). These results indicate that clusterin expression

does not cause cell death. However multiple lines of evidence suggest that clusterin acts to protect cells from environmental stresses. For example, it was earlier reported that inhibition of clusterin synthesis by treatment of LNCaP cells with an anti-sense oligonucleotide enhanced the cytotoxicity of TNFα and that overexpression of clusterin in these cells protected them from TNFα-mediated death (Sensibar *et al.*, 1995). Furthermore, following the exposure of U937 cells to UV-B irradiation, *in situ* hybridization detected clusterin mRNA in surviving cells but not in those that had undergone apoptosis (a control mRNA was detected in both live and dead cells) (French *et al.*, 1994). Similarly, increased clusterin mRNA expression was not detected in murine olfactory neurons induced to undergo apoptosis but was detected in surviving glial cells that surounded the neurons (Michel *et al.*, 1997). In addition, it has been shown here that high clusterin expression in L929 cells protected them from TNFα mediated death (Chapter 4). Therefore, it appears that clusterin expression is associated with cell survival.

The upregulation of HSP27 and HSP25 also provides protection against TNFα mediated death in L929 cells (Melhen *et al.*, 1995; Park *et al.*, 1998). In addition, the upregulated expression of other sHSPs also protect cells from stresses by promoting cell viability (Ehrnsperger *et al.*, 1997). Therefore, by analogy with the HSP, the cytoprotective effects of clusterin expression may be due to clusterin's chaperone-like activity.

TNFα reportedly induces cell death in L929 cells by stimulating the production of reactive oxygen intermediates (ROIs) in the mitochondria (Schulze Osthoff *et al.*, 1992; Goossens *et al.*, 1995). It was demonstrated that the expression of clusterin in L929-pRc.clus cells protected them against TNFα through an intracellular mechanism as the

protection could not be passed onto control L929 cell lines though the addition of exogenous clusterin or media from L929-pRc.clus cells (refer 4.3.11). As ROIs are capable of denaturing and thus destroying proteins (Stadtman, 1992), it is possible that the protective action of clusterin was due to it binding to cytosolic damaged protein(s) and thereby inhibiting their precipitation. However, as clusterin contains a signal peptide, this appears unlikely as it should be processed and then immediately secreted from the cell. Despite this, an intracellular form of clusterin has been reported and was postulated to result from the translation of a second in-frame ATG site located 33 bases downstream of the signal peptide (Reddy *et al.*, 1996). The translation of such a product may also occur in L929-pRc.clus cells, however further research will be required to demonstrate this. Alternatively, clusterin may have interacted with denatured proteins in the secretory pathway. Here high concentrations of clusterin may act as a "lubricant" for damaged proteins by inhibiting their tendency to aggregating into large unmanageable complexes, thereby escorting them along the secretory pathway to the surface of the cell.

The protective effect of clusterin overexpression against TNFα may not be specific as L929 cells expressing high levels of EGFP were also protected from TNFα (refer section 4.3.16). This suggests that high level expression of any protein may provide TNFα resistance to L929 cells. The protective effect of the overexpression of EGFP could be an artifact caused by the accumulation of intracellular EGFP. For example, EGFP may have "soaked up" the damaging effects of ROIs which would otherwise have damaged important intracellular proteins. Therefore, another L929 transfected cell line that *secretes* high levels of an unrelated protein will be required to determine whether overexpression of protein provides protection against TNFα mediated cell

death in L929 cells. Until this cell line is created and tested, the possibilty that clusterin expression protects L929 cells from TNFα cytoxocity cannot be excluded.

Clusterin expression did not protect L929 cells from staurosporine, colchicine, or azide induced death (chapter 4). In these cases it is possible that cell death is not directly caused by the denaturation of proteins, thus not allowing clusterin to interfere with the process of cell death. For example, staurosporine and colchicine treatments resulted in apoptosis (chapter 4) which involves many proteolytic cascades rather than denaturing events. However, clusterin may play some role in apoptosis as it is commonly found to be upregulated before the morphological characteristics become apparent (ie very early) in apoptotic cells. Perhaps clusterin is secreted in such situations as a precautionary measure in case cell lysis occurs. Lysis would result in release of noxious agents that would damage proteins of the extracellular matrix and other neighbouring cells. The release of clusterin expressed by the cell would therefore help protect near-neighbour proteins from irreversible damage.

The L929 clusterin secreting transfected cells are not the ideal system to examine the effects of clusterin expression on cell stress. One flaw in this system is that like nearly all cell types, L929 cells secrete endogenous clusterin during times of stress (Kyrprianou *et al.*, 1991). It is therefore possible that synthesis of endogenous clusterin may mask the effects of overexpression of recombinant clusterin. A similar flaw exists in the study of transfected LNCaP cells expressing clusterin and their sensitivity to TNFα (Sensibar *et al.*, 1995). Such a flaw would also exist if the transfected BHK-21 clusterin expressing cells described by Pilarsky *et al.*, (1993) were used in similar studies, as no measurements were reported for these cells of the levels of endogenous

clusterin production in times of stress (Pilarsky et al., 1993). The ideal cell type would be one in which clusterin expression has been knocked out. Transfecting such cell lines with "graded" expression systems would allow the full effect of clusterin expression on cell death to be established. Recently, clusterin knockout mice have been created which will allow such cell lines to be created.

6.3 The site of clusterin action

The studies on L929-pRc.clus cells suggested that clusterin expression protected cells from TNFα cytotoxicity via an intracellular site of action. However this site of action appears unlikely as clusterin is normally a secreted product. Futhermore, there are only two reports describing intracellular forms of processed clusterin. Reddy *et al.*, (1996b) identified a truncated form of clusterin in LNCaP cells when treated with TGF β, while Mahon *et al.*, (1999) recently reported that chicken clusterin is not a secreted product. It is concluded that the protection against TNFα cytotoxicity provided to L929-pRc.clus cells by clusterin overexpression is most likely an artifact caused by high protein expression. This is supported by the demonstration that L929-EGFP cells were also protected from TNFα cytotoxicity. If clusterin is capable of protecting cells from other forms of stress it is predicted that the site of action will be extracellular.

6.4 The association of clusterin with lipid recycling

The proposal that clusterin is involved in lipid recycling due to its interactions with various lipid complexes may also involve its chaperone-like function. It is likely that clusterin binds to lipid complexes because of its affinity for hydrophobic surfaces. Its role in lipid recycling may be a general one as described by James (1995), whereby it cleanses biological systems of hydrophobic harmful molecules. Here, clusterin has been

shown to be resistant to treatments that are damaging to other proteins (Chapter 5). Clusterin may interact with such hydrophobic harmful molecules and possibly neutralise them. The association of clusterin with paraoxonase is also fitting as paraoxonase is thought to inactivate potent forms of lipids (Mackness *et al.*, 1991; Witztum and Steinberg, 1991).

6.5 The association of clusterin with complement

Clusterin's association with purified complement proteins, in particular with C5b-7, has been deduced to be hydrophobic (Choi-Miura *et al.*, 1993). Clusterin's hydrophobic interactions with denatured proteins have been shown to be non-specific (chapter 5), which may also include its interaction with complement. The proposed function of clusterin regulating the formation of the MAC now appears to be dubious as in near physiological conditions clusterin did not affect MAC formation (refer section 1.2.3). Clusterin's co-localisation to regions of complement activation may now be explained by it's chaperone-like activity. In these situations, clusterin may prevent proteins in the extracellular matrix and on the surface of cells from being permanently damaged by noxious compounds released from complement lysed cells. This in turn would help the affected tissue recover from complement attack. The interaction between clusterin and complement components may just be a means by which clusterin is recruited to affected areas. In this scenario clusterin would most likely have to dissociate from complement proteins before attending to proteins that are damaged on or near membranes of unaffected cells to minimise any chance of complement-induced damage to those cells.

6.6 Clusterin and Alzheimers disease

Clusterin has been shown to specifically interact with amyloid β, one of the major components of amyloid plaques that form in Alzheimer's diseased (AD) brains. It has also been demonstrated that clusterin is capable of inhibiting the aggregation of the amyloid $\beta_{(1-42)}$ peptide *in vitro* (Oda *et al.*, 1995). The ability of clusterin to inhibit amyloid$\beta_{(1-42)}$ peptide from aggregating *in vitro* may involve similar mechanisms in which clusterin prevented stressed proteins from precipitating (chapter 5). For example this may result from a non-specific interaction that involves clusterin associating with hydrophobic surfaces on the amyloid $\beta_{(1-42)}$ peptide. However, the association of clusterin with amyloid β plaques *in vivo* indicates that it does not prevent their formation, but novertheless clusterin may inhibit the plaque growth. The presence of clusterin may be advantageous in affected areas of the brain afflicted with AD, as components of these plaques have been implicated as being neurotoxic (Yankner *et al.*, 1990; May *et al.*, 1992a). However, more research in this area is required as one *in vitro* study found that clusterin increased the neurotoxicity of amyloid $\beta_{(1-42)}$ peptide (Oda *et al.*, 1992).

The increased expression of clusterin in tissues affected with Alzheimer's disease may also relate to the chaperone activity of clusterin. As described above, the production of clusterin in stressed tissue may non-specifically protect cells from death by either binding to and preventing irreversible protein denaturation of intracellular or extracellular proteins.

6.7 Summary

The research described in this thesis has demonstrated that clusterin has chaperone-like activity. The basis of this activity comes from its tendency to bind to hydrophobic surfaces and protect proteins from stress induced precipitation. Clusterin also appears to have characteristics similar to the sHSP. For example the expression of either clusterin (chapter 4) or sHSP in L929 cells both protect cells from the cytotoxic effects of TNFα. The recent finding that clusterin expression can be controlled by HSF1 also indicate that clusterin has similar properties to the HSP. The fact that clusterin is generally a secreted product suggests that it may be an exracellular chaperone. No molecular chaperone has been previously reported to act extracellularly and therefore this will require thorough testing. The chaperone-like activity also suggest that many of clusterin's binding partners throughout the body may actually be a non-specific interaction between clusterin and these ligands. It is proposed that clusterin's chaperone-like activity is utilised through interactions with these binding partners.

The findings of this thesis have now opened up exciting new avenues for clusterin research. Some of the immediate questions that require addressing include determining whether clusterin expression can protect cells from various forms of stress other than TNFα treatment. It will also have to be determined how clusterin can protect cells from stress. Answers to these questions will involve identifying the properties of clusterin involved in mediating its interactions with party unfolded proteins and determining whether clusterin has a specific cellular site of action.

REFERENCES

Agarwal N., Jomary C., Jones S. E., Orourke K., Chaitin M., Wordinger R. J. and Murphy B. F. (1996) Immunocytochemical colocalization of clusterin in apoptotic photoreceptor cells in retinal degeneration slow Rds mutant mouse retinas. *Biochem. Biophys. Res. Com.* **225,** 84-91.

Aggarwal B. B. and Kohr W. J. (1985) Human tumor necrosis factor. *Meth. in Enzymol.* **116,** 448-456.

Akakura K., Buchovsky N., Rennie P.S., Coldman A.J., Goldenberg S.L., Tenniswood M., Fox K. (1996). Effects of intermittent androgen suppression on the stem cell composition and the Expression of the TRPM-2 (clusterin) Gene in the Shionogi Carcinoma. *J. Steroid. Biochem. Mol. Biol.* **59,** 501-511.

Akesson P., Sjoholm A.G., Bjorck L. (1996) Protein SIC, a novel extracellular protein of *Streptococcus pyogenes* interfering with complement function. *J. Biol. Chem.* **271,** 1081-1088.

Alberts B., Bray D., Lewis J., Raff M., Roberts K. and Watson J. D. (1989): "Molecular biology of the cell." Garland Publishing, Inc. New York & London.

Alitzky W. K., Schlegel P. N., Wu D., Yan Cheng C., Chen C.-L. C., Shihua Li P., Goldstein M., Reidenberg M. and Bardin C. W. (1992) Measurement of urinary clusterin as an index of nephrotoxicity. *Proc. Soc. for Exp. Biol. and Med.* **199,** 93-96.

Alousi M.A., Post R.A., Heymann W. (1969) Experimental autoimmune nephrosis in rats. Morphogenesis of the glomerular lesion: immunohistochemical and electron microscopic studies. *Am J. Pathol.* **54,** 47-71.

Amin J., Ananthan J., Voellmy R. (1988) Key features of heat shock regulatory elements. *Mol. Cell. Biol.* **8,** 3761-3769.

Aoyama A., Frohli E, Schafer R., Klemenz R. (1993) αB-crystallin expression in mouse NIH 3T3 fibroblasts: Glucocorticoid responsiveness and involvement in thermal protection. *Mol. Cell. Biol.* **13,** 1824-1835.

Arrig A.-P., Landry J. (1994) Expression and functon of the low-molecular weight heat shock proteins. In The biology of heat shock proteins and molecular chaperones. 1st edition, Cold Spring Harbor Laboratory Press. New York, USA.

Arrigo A.-P., Suhan J., Welch W.J. (1988) Dynamic changes in the structure and locale of the mammalian low molecular weight heat shock protein. *Mol. Cell. Biol.* **8,** 5059-5071.

Arrigo, A. P. (1998) Small stress proteins: chaperones that act as regulators of intracellular redox state and programmed cell death. *Biol. Chem.* **379,** 19-26.

Baler R., Welch W. J. and Voellmy R. (1992) Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. *J. Cell. Biol.* **117,** 1151-1159.

Bettuzzi S., Kiipakka R.A., Gilna P., Liao S.T.(1989) Identification of an androgen-repressed mRNA in rat ventral prostate as coding for sulphated glycoprotein 2 by cDNA cloning and sequence analysis. *Biochem. J.* **257,** 293-296.

Bettuzzi S., Troiano L., Davalli P., Tropea F., Ingletti M. C., Grassilli E., Monti D., Corti A. and Franceschi C. (1991) In vivo accumulation of sulfated glycoprotein 2 mRNA in rat thymocytes upon dexamethasone-induced cell death. *Biochem. Biophys. Res. Comm.* **175,** 810-815.

Beyaert R., Cuenda A., Vanden Derghi W., Plaisance S., Lee J.C., Haegeman G., Cohen P., Fiers W. (1996) The p38/RK mitogen activated protein kinase pathway regulates interleukin-6 synthesis in response to tumour necrosis factor. *EMBO J.* **15,** 1914-1923.

Beyaert R., Vanhaesebroeck B., Heyninck K., Boone E., De Valck D., Schulze Osthoff K., Haegeman G., Van Roy F. and Fiers W. (1993) Sensitization of tumor cells to tumor necrosis factor action by the protein kinase inhibitor staurosporine. *Cancer Res.* **53,** 2623-2630.

Birkenmeier E. H., Letts V. A., Frankel W. N., Magenheimer B. S. and Calvet J. P.

(1993) Sulfated glycoprotein-2 (SGP-2) maps to mouse chromosome 14. *Mam. Gen.* **4,** 131-132.

Blaschuk O., Burdzy K. and Fritz I. B. (1983) Purification and characterisation of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. *J. Biol. Chem.* **258,** 7714-7720.

Blatter M. C., James R. W., Messmer S., Barja F. and Pometta D. (1993) Identification of a distinct human high-density lipoprotein subspecies defined by a lipoprotein-associated protein, K-45. Identity of K-45 with paraoxonase. *Eur. J. Biochem.* **211,** 871-879.

Boggs L. N., Fuson K. S., Baez M., Churgay L., McClure D., Becker G. and May P. C. (1996) Clusterin (apo J) protects against *in vitro* amyloid-beta(1-40) neurotoxicity. *J. Neurochem.* **67,** 1324-1327.

Boldin M.P., Goncharov T.M., Goltsev Y.V., Wallach D. (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor induced cell death. *Cell* **85,** 803-815.

Bova, M. P., Ding, L.-L., Horwitz, J., and Fung, B. K.-K. (1997) Subunit exchange of alphaA-crystallin. *J. Biol. Chem.* **272,** 29511-29517.

Brown M.S., Herz J., Kowal R.C., Goldstein J.L., (1991) The low density lipoprotein receptor-related protein: double agent or decoy? *Curr. Opin. Lipidology* **2,** 65-72 .

Burkey B. F., deSilva H. V. and Harmony J. A. (1991) Intracellular processing of apolipoprotein J precursor to the mature heterodimer. *J. Lipid Res.* **32,** 1039-1048.

Burkey B. F., Stuart W. D. and Harmony J. A. (1992) Hepatic apolipoprotein J is secreted as a lipoprotein. *J. Lipid Res.* **33,** 1517-1526.

Bursch W., Gleeson T., Kleine L., Tenniswood M. (1995) Expression of clusterin (testosterone-repressed prostate message-2) mRNA during growth and regeneration of rat liver. *Arch. Toxicol.* 69:253-258.

Buttyan R., Olsson C. A., Pintar J., Chang C., Bandyk M., Ng P.-Y. and Sawczuk I. S. (1989) Induction of the TRPM-2 gene in cells undergoing programmed death. *Mol. Cell. Biol.* **9,** 3473-3481.

Carver, J. A., Aquilina, J. A., Cooper, P. G., Williams, G. A., and Truscott, R. J. W. (1994) Alpha-crystallin molecular chaperone and protein surfactant. *Biochim. Biophys. Acta* **1204,** 195-206.

Carver, J. A., Aquilina, J. A., Truscott, R. J. W., and Ralston, G. B. (1992) Identification by 1H NMR spectroscopy of flexible C-terminal extensions in bovine lens alpha-crystallin. *FEBS Lett.* **311,** 143-149.

Carver, J. A., Esposito, G., Schwedersky, G., and Gaestel, M. (1995 a) 1H NMR spectroscopy reveals that mouse HSP25 has a flexible C-terminal extension of 18 amino acids. *FEBS Lett.* **369,** 305-310.

Carver, J. A., Guerreiro, N., Nicholls, K. A., and Truscott, R. J. W. (1995 b) On the interaction of alpha-crystallin with unfolded proteins. *Biochim. Biophys. Acta.* **1252,** 251-260.

Chang N. S. (1995) Transforming growth factor-beta 1 induction of novel extracellular matrix proteins that trigger resistance to tumor necrosis factor cytotoxicity in murine L929 fibroblasts. *J. Biol. Chem.* **270,** 7765-7772.

Chretien P., Landry J. (1988) Enhanced constitutive expression of the 27-kDa heat shock proteins in heat resistant variants from Chinese hamster cells. *J. Cell Physiol.* **137,** 157-166.

Choi-Miura N.-H., Mazda T. and Tomita M. (1989) A serum protein SP40,40 modulates the formation of membrane attack complex of complement on erythrocytes. *Mol. Immunol.* **26,** 835-840.

Choi-Miura N.H., Sakamoto T., Tobe T., Nakano Y., Tomita M. (1993) The role of

HDL consisting of SP-40,40, apo A-I, and lipids in the formation of SMAC of complement. *J. Biochem.* **113,** 484-487.

Choi-Miura N. H., Takahashi Y., Nakano Y., Tobe T. and Tomita M. (1992 b) Identification of the disulfide bonds in human plasma protein SP-40,40 (apolipoprotein-J). *J. Biochem..* **112,** 557-561.

Clark A. M. and Griswold M. D. (1997) Expression of clusterin/sulfated glycoprotein-2 under conditions of heat stress in rat Sertoli cells and a mouse Sertoli cell line. *J. Androl.* **18,** 257-263.

Cohen J.J. (1993) Apoptosis. *Immunol. Today.* **14,** 126-130.

Collard M. W. and Griswold M. D. (1987) Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells. *Biochem.* **26,** 3297-3303.

Collier N.C., and Schlesinger M. (1986) The dynamic state of heat shock protein in chicken embro fibroblasts. *J. Cell Biol.* **103,** 1495-1507.

Collier N.C., Heuser J., Aach-Levy M., and Schlesinger M. (1988) Ultrastructural and biochemical analysis of the stress granules in chicken embro firoblasts. *J. Cell Biol.* **106,** 1131-1139.

Coleman R.M., Lombard M.F., Sicard R.E., (1992) Fundamental immunology. 2nd edition. Wm. C. Publishers. Dubuque, IA, USA.

Creighton T.E., (1993) Proteins structures and molecular properties. 2nd Edition W.H Freeman and Company, New York.

Cui S., Verroust P. J., Moestrup S. K. and Christensen E. I. (1996) Megalin/gp330 mediates uptake of albumin in renal proximal tubule. *Am. J. Physiol.* **271,** 900-907.

Danik M., Chabot J-G., Mercier C., **et al**. (1991) Human liomas and epileptic foci express high levels of a mRNA related to rat testisclar sulfated glycoprotein 2, a purported marker of cell death. *Proc. Nat. Acad. Sci. USA* **88,** 8577-8581.

Darzynkiewicz Z., Bruno S., Bino G.D>, Gorczyca W., Hotz M.A., Lassota P., Tranganos F. (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry* ***13,*** *79-808.*

Davis J.M., (1994) Basic Cell culture: A practical approach. 1st edition. IRC Press. Oxford. New York.

de Silva H. V., Harmony J. A. K., Stuart W. D., Gil C. M. and Robbins J. (1990 a) Apolipoprotein J: Structure and tissue distribution. *Biochem.* **29,** 5380-5389.

de Silva H. V., Stuart W. D., Park Y. B., Mao S. J. T., Gil C. M., Wetterau J. R., Busch S. J. and Harmony J. A. K. (1990 b) Purification and characterization of apolipoprotein J. *J. Biol. Chem.* **265,** 14292-14297.

de Silva H. V., Stuart W. D., Duvic C. R., Wetterau J. R., Ray M. J., Ferguson D. G., Albers H. W., Smith W. R. and Harmony J. A. K. (1990 c) A 70-kDa apolipoprotein designated ApoJ is a marker for subclasses of human plasma high density lipoproteins. *J. Biol. Chem..* **265,** 13240-13247.

Diemer V., Hoyle M., Baglioni C., Millis A.J.T. (1992) Expression of porcine complement cytolysis inhibitor mRNA in cultured aortic smooth muscle cells. *J. Biol. Chem.* **267,** 5257-5264.

Dietzsch E., Murphy B. F., Kirszbaum L., Walker I. D. and Garson O. M. (1992) Regional localization of the gene for clusterin (SP-40,40; gene symbol CLI) to human chromosome 8p12-p21. *Cytogen. Cell Gen.* **61,** 178-179.

Dive C., Gregory C. D., Phipps D. J., Evans D. L., Milner A. E. and Wyllie A. H. (1992) Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim. Biophys. Acta* **1133,** 275-285.

Dragunow M., Preston K., Dodd J., Young D., Lawlor P. and Christie D. (1995) Clusterin accumulates in dying neurons following status epilepticus. *Mol. Brain Res.* **32,** 279-290.

Duguid J.R, Bohmont C.W., Liu N.G., Tourtellotte W.W., (1989) Changes in brain gene expression shared by scrapie and Alzhiemer's disease. *Proc. Natl. Acad. Sci. USA*; 86: 7260-7264.

Ehrnsperger, M., Buchner, J., and Gaestel, M. (1997) Molecular chaperones in the life cycle of proteins. Structure, function and mode of action. (Fink, A. L. & Goto, Y., Eds.), Marcel Dekker, New York.

Fadok V. A., Voelker D. R., Campbell P. A., Cohen J. J., Bratton D. L. and Henson P. M. (1992 a) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* **148,** 2207-2216.

Fadok V. A., Savill J. S., Haslett C., Bratton D. L., Doherty D. E., Campbell P. A. and Henson P. M. (1992 b) Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J. Immunol.* **149,** 4029-4035.

Fady C., Gardner A., Jacoby F., Briskin K., Tu Y. P., Schmid I. and Lichtenstein A. (1995) Atypical apoptotic cell death induced in L929 targets by exposure to tumor necrosis factor. *J. Interferon Cytok. Res.* **15,** 71-80.

Farahbakhsh, Z.T., Huang, Q.L., Ding, L.L., Altenbach, C., Steinhoff, H.J., Horwitz, J. and Hubbell, W.L. (1995) Interaction of alpha-crystallin with spin-labeled peptides. *Biochem.* **43,** 509-516.

Fiers W., Beyaert R., Boone E., Cornelis S., Declercq W., Decoster E., Denecker G., Depuydt B., Devalck D., Dewilde G., Goossens V., Grooten J., Haegeman G., Heyninck K., Penning L., Plaisance S., Vancompernolle K., Vancriekinge W., Vandenabeele P., Vandenberghe W., Vandecraen M., Vandevoorde V. and Vercammen D. (1996) TNF-induced intracellular signaling leading to gene induction or to cytotoxicity by necrosis or by apoptosis. *J. Inflam.* **47,** 67-75.

Fink T. M., Zimmer M., Tschopp J., Etienne J., Jenne D. E. and Lichter P. (1993)

Human clusterin (CLI) maps to 8p21 in proximity to the lipoprotein lipase (LPL) gene. *Genomics* **16,** 526-528.

Fischer-Colbrie R., Zangerle R., Frischenschlager I., Weber A., Winkler H. (1984) Isolation and immnuological characterization of a glycoprotein from adrenal chromaffin granules. *J. Neurochem.* **42,** 1008-1016.

Flick D. A. and Gifford G. E. (1984) Comparison of in vitro cell cytotoxic assays for tumor necrosis factor. *J. Immunol. Meth.* **68,** 167-175.

Fournel S., Genestier L., Roualt J. P., Lizard G., Flacher M., Assossou O. and Revillard J. P. (1995) Apoptosis Without Decrease Of Cell DNA Content. *FEBS Let.* **367,** 188-192.

French L., Tschopp J. and Schifferli J. A. (1994 a) Clusterin in renal tissue: preferential localization within the terminal complement and immunoglobulin deposits within glomeruli. *Clin. Exp. Immunol.* **88,** 389-393.

French L. E., Wohlwend A., Sappino A. P., Tschopp J. and Schifferli J. A. (1994 b) Human clusterin gene expression is confined to surviving cells during *in vitro* programmed cell death. *J. Clin. Invest.* **93,** 877-884.

Fritz I.B. (1995) Introduction to clusterin: history and perspectives: In "Clusterin: role in vertebrate development, function and adaptation." R G Landes. Austin Texas, USA.

Fritz I. B. and Burdzy K. (1989) Novel action of carnitine: Inhibition of aggregation of dispersed cells elicited by clusterin in vitro. *J. Cell. Physiol.* **140,** 18-28.

Fritz I. B., Burdzy K., Setchll B., Blaschuk O. (1983) Ram rete testis fluid contains a protein (clusterin) which influences cell-cell interactions in vitro. *Biol. Reprod.* **28,** 1173-1188.

Fuqua S.A.W., Blum-Salingaros M., McGuir W.L. (1989) Induction of the estogen-regulated "24K" protein by heat shock. *Cancer Res.* **49,** 4126-4129.

Garden G. A., Bothwell M. and Rubel E. W. (1991) Lack of correspondence between mRNA expression for a putative cell death molecule (SGP-2) and neuronal cell death in the central nervous system. *J. Neurobiol.*, 590-604.

Gardner A. M. and Johnson G. L. (1996) Fibroblast growth factor-2 suppression of Tumor necrosis factor alpha-mediated apoptosis requires Ras and the activation of mitogen-activated protein kinase. *J. Biol. Chem.* **271,** 14560-14566.

Gelissen I.C., Hochgrebe T., Wilson M.R., Easterbrook-Smith S.B., Jessup W., Dean R.T., Brown A.J. (1998) Apolipoprotein J (clusterin) induces cholesterol export from macrophage-foam cells: a potential anti-atherogenic function? *Biochem. J.* **331,** 231-237.

Ghiso J., Matsubara E., Koudinov A., Choimiura N. H., Tomita M., Wisniewski T. and Frangione B. (1993) The cerebrospinal-fluid soluble form of alzheimers amyloid-beta is complexed to SP-40,40 (apolipoprotein-J), an inhibitor of the complement membrane-attack complex. *Biochem. J.* **293,** 27-30.

Glogauer M., McCulloch C.A.G. (1992) Introduction of large molecules into viable fibroblasts by electroporation: Optimization of loading and identification of laeled cellular compartments. *Exp. Cell Res.* **200,** 227-234.

Glomset J.A., (1968) The plasma lectithins:cholesterol acyltransferase reaction. *J. Lipid Res.* **9,** 155-167.

Goossens V., Grooten J., De Vos K. and Fiers W. (1995) Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc. Natl. Acad. Sci. U S A* **92,** 8115-8119.

Goossens V., Grooten J. and Fiers W. (1996) The oxidative metabolism of glutamine. A modulator of reactive oxygen intermediate-mediated cytotoxicity of tumor necrosis factor in L929 fibrosarcoma cells. *J. Biol. Chem.* **271,** 192-196.

Gorman A., McCarthy J., Finucane D., Reville W. and Cotter T. (1994): Morphological

assessment of apoptosis. In "Techniques in apoptosis, a user's guide." Cotter TG, Martin SJ (eds). Portland Press Ltd. London.

Griswold M. D., Roberts K. and Bishop P. (1986) Purification and characterization of a sulfated glycoprotein secreted by Sertoli cells. *Biochem.* **25,** 7265-7270.

Grooten J., Goossens V., Vanhaesebroeck B. and Fiers W. (1993) Cell membrane permeabilization and cellular collapse, followed by loss of dehydrogenase activity: early events in tumour necrosis factor-induced cytotoxicity. *Cytokine* **5,** 546-555.

Harding M. A., Chadwick L. J., Gattone V. H., II and Calvet J. P. (1991) The SGP-2 gene is developmentally regulated in the mouse kidney and abnormally expressed in collecting duct cysts in polycystic kidney disease. *Develop. Biol.* **146,** 483-490.

Hartmann K., Rauch J., Urban J., Parczyk K., Diel P., Pilarsky C., Appel D., Haase W., Koch-Brandt C. and Al E. (1991) Molecular cloning of gp **80,** a glycoprotein complex secreted by kidney cells in vitro and in vivo: A link to the reproductive system and to the complement cascade. *J. Biol. Chem.* **266,** 9924-9931.

Hayakawa M., Jayadev S., Tsujimoto M., Hannun Y. A. and Ito F. (1996) Role of ceramide in stimulation of the transcription of cytosolic phospholipase α(2) and cyclooxygenase 2. *Biochem. Biophys. Res. Comm.* **220,** 681-686.

Hennessy L. K., Kunitake S. T., Jarvis M., Hamilton R. L., Endeman G., Protter A. and Kane J. P. (1997) Isolation of subpopulations of high density lipoproteins - three particle species containing apoE and two species devoid of apoE that have affinity for heparin. *J. Lip. Res.* **38,** 1859-1868.

Hennet T., Richter C., Peterhans E. (1993) Tumour necrosis factor-α induces superoxide anion generation in mitochondria in L929 cells. *Biochem J.* **289,** 587-592.

Heuer K. H., Mackay J. P., Podzebenko P., Bains N. P., Weiss A. S., King G. F. and Easterbrook Smith S. B. (1996) Development of a sensitive peptide-based immunoassay: application to detection of the Jun and Fos oncoproteins. *Biochem.* **35,**

9069-9075.

Hjalm G., Murray E., Crumley G., Harazim W., Lundgren S., Onyango I., Ek B., Larsson M., Juhlin C., Hellman P., Davis H., Akerstrom G., Rask L. and Morse B. (1996) Cloning and sequencing of human gp**330,** a Ca(2+)-binding receptor with potential intracellular signaling properties. *Eur. J. Biochem.* **239,** 132-137.

Hochstrasser A. C., James R. W., Martin B. M., Harrington M., Hochstrasser D., Pometta D. and Merril C. R. (1988) HDL particle associated proteins in plasma and cerebrospinal fluid: identification and partial sequencing. *Appl. Theor. Electrophor.* **1,** 73-76.

Hochgrebe T.T, Humphreys D.T., Wilson M.R., Easterbrook-Smith S.B. (1999) A re-examination of the role of clusterin as a complement regulator. *Exp. Cell Res. In press.*

Hook, D. W. A., and Harding, J. J. (1997) Molecular chaperones protect catalase during thermal stress. *Eur. J. Biochem.* **247,** 380-385.

Horwitz, J. (1992) Alpha-crystallin can function as a molecular chaperone. *Proc. Natl. Acad. Sci. USA* **89,** 10449-10453.

Humphreys D.T. (1994) A study of clusterin's interaction with apolipoprotein A-I and its involvement in apoptosis. Wollongong University Honours Thesis.

Humphreys D.T., Carver J.A., Easterbrook-Smith S.B., Wilson M.R. (1999). Clusterin has chaperone like activity similar to that of small heat shock proteins. *J. Biol. Chem.* **274,** 6875-6881.

Humphreys D., Hochgrebe T. T., Easterbrooksmith S. B., Tenniswood M. P. and Wilson M. R. (1997) Effects of clusterin overexpression on TNF-α- and TGF-β-mediated death of L929 cells. *Biochemistry* **36,** 15233-15243.

Humphreys D.T., andWilson M.R. (1999) Modes of L929 cell death induced by TNFα and other cytotoxic agents. *Cytokine. In press.*

Huovinen R., Warri A., Collan Y. (1993) Mitotic activity, apoptosis and TRPM-2 mRNA expression in DMBA-induced rat mammary carcinoma treated with anti-estrogen toremifene. *Int. J. Cancer* **55,**685-691.

Ide H., Yeldandi A. V., Reddy J. K. and Rao M. S. (1994) Increased expression of sulfated glycoprotein-2 and DNA fragmentation in the pancreas of copper-deficient rats. *Toxicol. & Appl. Pharm.* **126,** 174-177.

Idziorek T., Estaquier J., Debels F. and Ameisen J. C. (1995) YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *J. Immunol. Meth.* **185,** 249-258.

Igdoura S. A., Hermo L. and Morales C. R. (1994) Sulfated glycoprotein-2 synthesized by nonciliated cells of the efferent ducts is targeted to the lysosomal compartment. *Microscopy Res. Tech.* **29,** 468-480.

Izawa M. (1998) Identification of a transcript predicting an alternative form of sulfated glycoprotein-2 (clusterin) in rat tissues. *Biochem. Mol. Biol. Int.* **44,** 9-18.

Jakob U., Gaestel M., Engel K., Buchner J. (1993) Small heat shock protein are molecular chaperones. *J. Biol. Chem.* **268,** 1517-1520.

James R.W., "Clusterin as a lipid transport protein" *In* Clusterin: role in vertebrate development function and adaptation (1995) Edited by Harmony J.A.K. R.G. Landes, Austin, Texas, U.S.A.

James R.W., Hochstrasser A-C., Borghini I., Martin B., Pometta D., Hochstrasser D. (1991) Characterization of a human high density lipoprotein-associated protein, NA1/NA2. Identiy with SP-40,40, an inhibitor of complement-mediated cytolysis. *Arterioscler. Thromb.* **11,** 645-652.

James R. W., Hochstrasser D., Tissot J. D., Funk M., Appel R., Barja F., Pellegrini C., Muller A. F. and Pometta D. (1988) Protein heterogeneity of lipoprotein particles

containing apolipoprotein A-I without apolipoprotein A-II and apolipoprotein A-I with apolipoprotein A-II isolated from human plasma. *J. Lipid Res.* **29,** 1557-1571.

Jayadev S., Linardic C. M. and Hannun Y. (1994) Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor alpha. *J. Biol. Chem.* **269,** 5757-5763.

Jayadev S., Hayter H. L., Andrieu N., Gamard C. J., Liu B., Balu R., Hayakawa M., Ito F. and Hannun Y. A. (1997) Phospholipase a(2) is necessary for tumor necrosis factor alpha-induced ceramide generation in L929 cells. *J. Biol. Chem.* **272,** 17196-17203.

Jenne D. E. and Tschopp J. (1989) Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: Identity to sulfated glycoprotein **2,** a constituent of rat testis fluid. *Proc. Natl. Acad. Sci, USA* **86,** 7123-7127.

Jenne D. E., Lowin B., Peitsch M. C., Boettcher A., Schmitz G. and Tschopp J. (1991) Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-I in human plasma. *J Biol Chem.* **266,** 11030-11036.

Jenne D. E. and Tschopp J. (1992) Clusterin: The intriguing guises of a widely expressed glycoprotein. *Trends Biochem. Sci.* **17,** 154-159.

Jin G. and Howe P. H. (1997) Regulation of clusterin gene expression by transforming growth factor beta. *J. Biol. Chem.* **272,** 26620-26626.

Jomary C., Ahir A., Agarwal N., Neal M.J., Jones S.E., (1995) Spatio-temperal patern of ocular clusterin mRNA expression in the rd mouse. *Mol. Brain Res.* **29,** 172-176.

Jomary C., Murphy B. F., Neal M. J. and Jones S. E. (1993) Abnormal distribution of retinal clusterin in retinitis-pigmentosa. *Mol. Brain Res.* **20,** 274-278.

Jomary C., Neal M. J. and Jones S. E. (1993) Comparison of clusterin gene expression in normal and dystrophic human retinas. *Mol. Brain Res.* **20,** 279-284.

Jones S. E., Meerabux J. M. A., Yeats D. A. and Neal M. J. (1992) Analysis of differentially expressed genes in retinitis pigmentosa retinas: Altered expression of clusterin mRNA. *FEBS lett.* **300,** 279-282.

Jordan-Starck T. C., Witte D. P., Aronow B. J. and Harmony J. A. K. (1992) Apolipoprotein J: a membrane policeman? *Curr. Opin. Lipidol.* **3,** 75-85.

Kapron J. T., Hilliard G. M., Lakins J. N., Tenniswood M. P. R., West K. A., Carr S. A. and Crabb J. W. (1997) Identification and characterization of glycosylation sites in human serum clusterin. *Prot Sci.* **6,** 2120-2133.

Kerjaschki D., Farquhar M.G. (1983) Immunocytochemical localization of the Heymann nephritis antigen (GP330) in glomerular epithelial cells of normal Lewis rats. *J Exp. Med.* **157,** 667-686.

Kelso G. J., Stuart W. D., Richter R. J., Furlong C. E., Jordanstarck T. C. and Harmony J. A. K. (1994) Apolipoprotein-J is associated with paraoxonase in human plasma. *Biochemistry.* **33,** 832-839.

Kessel R. G. and Katow H. (1984) Effects of prolonged antitubulin culture on annulate lamellae in mouse alpha L929 fibroblasts. *J. Morphol.* **179,** 291-304.

Kida E., Choimiura N. H. and Wisniewski K. E. (1995) Deposition of apolipoproteins E and J in senile plaques is topographically determined in both alzheimers disease and downs syndrome brain. *Brain Res.* **685,** 211-216.

Kida E., Pluta R., Lossinsky A. S., Golabek A. A., Choimiura N. H., Wisniewski H. M. and Mossakowski M. J. (1995) Complete cerebral ischemia with short-term survival in rat induced by cardiac arrest .2. extracellular and intracellular accumulation of apolipoproteins E and J in the brain. *Brain Res.* **674,** 341-346.

Kimura K. and Yamamoto M. (1996) Modification of the alternative splicing process of testosterone-repressed prostate message-2 (TRPM-2) gene by protein synthesis inhibitors and heat shock treatment. *Biochim. Biophys. Acta* **1307,** 83-88.

Kimura K., Asami K. and Yamamoto M. (1997) Effect of heat shock treatment on the production of variant testosterone-repressed prostate message-2 (TRPM-2) mRNA in culture cells. *Cell Biochem. Funct.* **15,** 251-257.

Kirszbaum L., Sharpe J. A., Murphy B., d'Apice A. J. F., Classon B., Hudson P. and Walker I. D. (1989) Molecular cloning and characterization of the novel, human complement-associated protein, SP-40,40: A link between the complement and reproductive systems. *EMBO J.* **8,** 711-718.

Koopman G., Reutelingsperger C. P. M., Kuijten G. A. M., Keehnen R. M. J., Pals S. T. and Vanoers M. H. J. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84,** 1415-1420.

Kounnas M. Z., Loukinova E. B., Stefansson S., Harmony J. A. K., Brewer B. H., Strickland D. K. and Argraves W. S. (1995) Identification of glycoprotein 330 as an endocytic receptor for apolipoprotein J/clusterin. *J. Biol. Chem.* **270,** 13070-13075.

Kroemer G., Zamzami N., Santos A.S., (1997) Mitochondrial control of apoptosis. *Immunol Today* **18,** 44-51.

Kronke M., Schutze S., Wiegmann K., Machleidt T. (1996) Sphingomyelinases and TNF induced apoptosis. *Cell Physiol. Biochem.* **6,** 337-344.

Kunitake S.T., Carilli C.T., Lau K., Protter A.A., Naya-Vine J., Kane J.P. (1994) Identification of proteins associated with apolipoprotein A-I-containing lipoproteins purified by selected affinity immunosorption. *Biochemistry.* **33,** 1988-1993.

Kuwajima, K., Ikeguchi, M., Sugawara, T., Hiraoka, Y., and Sugai, S. (1990) Kinetics of disulphide cond reduction in alpha-lactalbumin by dithiothreitol and molecular basis of superreactivity of the cys6-cys120 disulphide bond. *Biochemistry.* **29,** 8240-8249.

Kyprianou N., Alexander R. B. and Isaacs J. T. (1991) Activation of programmed cell death by recombinant human tumor necrosis factor plus topoisomerase II-targeted drugs in L929 tumor cells. *J. Natl. Cancer Inst.* **83,** 346-350.

La Du B.N., (1992) Human serum paraoxonase areylesterase: *In* "Pharmacologenetics of drug metabolism" (Kaow W., editor). Pergamon Press Inc., New York.

Lampert-Etchells M., McNeill T. H., Laping N. J., Zarow C., Finch C. E. and May P. C. (1991) Sulfated glycoprotein-2 is increased in rat hippocampus following entorhinal cortex lesioning. *Brain Res.* **563,** 101-106.

Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., and Hartl, F. U. (1992) Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature* **356,** 683-689.

Laping N. J., Morgan T. E., Nichols N. R., Rozovsky I., Youngchan C. S., Zarow C. and Finch C. E. (1994) Transforming growth factor-beta-1 induces neuronal and astrocyte genes - Tubulin alpha-1, Glial fibrillary acidic protein and clusterin. *Neuroscie.* **58,** 563-572.

Laster S. M., Wood J. G. and Gooding L. R. (1988) Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. *J. Immunol.* **141,** 2629-2634.

Lavoie J.N., Gingras-Breton G., Tanguay R.M., Landry J. (1993) Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization *J. Biol. Chem.* **268,** 3420-3429.

Lee K. H., Ji Y. M., Lim H. M., Lee S. C. and You K. H. (1993) Molecular cloning and sequencing of sulfated glycoprotein-2 cDNA from testis of mouse - implications of two different messenger RNAs of SGP-2. *Biochem. Biophys. Res. Com.* **194,** 1175-1180.

Leger J.G., Montpetit M.L., Tenniswood M.P., (1987) Characterization and cloning of androgen-repressed mRNAs from rat ventral prostate. *Biochem. Biophys. Res. Comm.* **147,** 196-203.

Leroux, M. R., Melki, R., Gordon, B., Batelier, G., and Candido, E. P. M. (1997) Structure-function studies on small heat shock protein oligomeric assembly and interaction with unfolded polypeptides. *J. Biol. Chem.* **272,** 24646-24656.

Levesque A., Paquet A. and Page M. (1995) Measurement of tumor necrosis factor activity by flow cytometry. *Cytometry* **20,** 181-184.

Levesque A., Paquet A. and Page M. (1995) Improved fluorescent bioassay for the detection of tumor necrosis factor activity. *J. Immunol. Meth.* **178,** 71-76.

Lewis M., Tartaglia L. A., Lee A., Bennett G. L., Rice G. C., Wong G. H., Chen E. Y. and Goeddel D. V. (1991) Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. *Proc. Natl. Acad. Sci. U S A* **88,** 2830-2834.

Li W. F., Furlong C. E. and Costa L. G. (1995) Paraoxonase protects against chlorpyrifos toxicity in mice. *Toxicol. Lett.* **76,** 219-226.

Lizard G., Fournel S., Genestier L., Dhedin N., Chaput C., Flacher M., Mutin M., Panaye G. and Revillard J. P. (1995) Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis. *Cytometry* **21,** 275-283.

Loetscher H., Pan YCE., Lahm HW., Gentz R., Brockhaus M., Tabuchi H., Lesslauer W. (1990) Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. *Cell* **61,** 351-359.

Lyons A. B., Samuel, K, Sanderson, A and Maddy, A H (1992) Simultaneous analysis of immunophenotype and apoptosis of murine thymocytes by single laser flow cytometry. *Cytometry* **13,** 809-821.

Mackness M.I., Arrol S., Durrington P.N., (1991). Paraoxonase prevents accumulation of lipoperoxides in low density lipoprotein. FEBS Lett. **286,** 152-154.

Mackness B., Hunt R., Durrington P. N. and Mackness M. I. (1997) Increased immunolocalization of paraoxonase, clusterin, and apolipoprotein A-I in the human artery wall with the progression of atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **17,** 1233-1238.

Mahon M.G., Lindstedt K.A., Hermann M., Nimpf J., Schneider W.J. (1999) Multiple involvement of clusterin in chicken ovarian follicle development. *J. Biol. Chem.* **274,** 4036-4044.

Martin S. J., Reutelingsperger C. P. M., McGahon A. J., Rader J. A., Vanschie R., Laface D. M. and Green D. R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus - inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* **182,** 1545-1556.

Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L., and Hartl, F. U. (1991) Chaperonin-mediated protein folding at the surface of GroEL through a molten globule-like intermediate. *Nature* **352,** 36-42.

Matsubara E., Frangione B. and Ghiso J. (1995) Characterization of apolipoprotein J-alzheimers α-beta interaction. *J. Biol. Chem.* **270,** 7563-7567.

May P. C. and Finch C. E. (1992) Sulfated glycoprotein 2: New relationships of this multifunctional protein to neurodegeneration. *Trends Neuroscie.* **15,** 391-396.

May P.C., Gitter B.D., Waters D.C., Simmons L.K., Becker G.W., Small J.S., and Robison P.M. (1992 a) β-amyloid *in vitro* toxicity: lot to lot variability. *Neurobiol. Aging* **13,** 605-607.

May P. C., Robison P., Fuson K., Smalstig B., Stephenson D. and Clemens J. A. (1992 b) Sulfated glycoprotein-2 expression increases in rodent brain after transient global ischemia. *Mol. Brain Res.* **15,** 33-39.

McDonald J.F., and Nelsestuen G.L. (1997) Potent inhibition of terminal complement assembly by clusterin: characterization of its impact on C9 polymerization. *Biochemistry.* **36,** 7464-7473.

McGeer P. L., Kawamata T. and Walker D. G. (1992) Distribution of clusterin in Alzheimer brain tissue. *Brain Res.* **579,** 337-341.

McQuilkin W. T. and Earle W. R. (1961) Cinemicrographic analysis of cell populations in vitro. *J. Nat. Canc. Inst.* **28,** 763.

Mehlen P., Mehlen A., Guillet D., Preville X. and Arrigo A. P. (1995) Tumor necrosis factor-alpha induces changes in the phosphorylation, cellular localization, and oligomerization of human HSP27, a stress protein that confers cellular resistance to this cytokine. *J. Cell. Biochem.* **58,** 248-259.

Michel D., Chatelain G., North S. and Brun G. (1997) Stress-induced transcription of the clusterin/apoJ gene. *Biochem. J.* **328,** 45-50.

Michel D., Gillet G., Volovitch M., Pessac B., Calothy G., Brun G. (1989) Expression of a novel gene encoding a 51.5 kD precursor protein is induced by different retroviral oncogenes in quail neuroretinal cells. *Oncogene Res* **4,** 127-136.

Michel, D., Moyse, E., Trembleau, A., Jourdan, F., and Brun, G. (1997) Clusterin/Apo J expression is associated with neuronal apoptosis in the olfactory mucosa of the adult mouse. *J. Cell Sci.* **110,** 1635-1645.

Mishra S. (1995) A spin decay assay for tumor necrosis factor cytotoxicity. *Ind. J. Biochem. Biophys.* **32,** 254-260.

Moestrup S.K., Holtet T.L., Etzerodt M., Thogersen H.C., Nykjaer A., Andreasen P.A., Rasmussen H.H., Sottrup-Jensen L., Gliemann J. (1993) α_2-Macroglobulin-proteinase complexes, plasminogen activator inhibitor type-1-plasminogen ativator complexes, and receptor associated protein bind to a region of the α_2-macroglobulin receptor containing a cluster of eight complement-type repeats. *J. Biol. Chem.* **268,** 13691-13696.

Montpetit M. L., Lawless K. R. and Tenniswood M. P. R. (1986) Androgen-repressed messages in the rat ventral prostate. *Prostate* **8,** 25-36.

Morales C. R., Igdoura S. A., Wosu U. A., Boman J. and Argraves W. S. (1996) Low density lipoprotein receptor-related protein-2 expression in efferent duct and epididymal epithelia: evidence in rats for its in vivo role in endocytosis of apolipoprotein

J/clusterin. *Biol. Reprod.* **55,** 676-683.

Morgan T. E., Laping N. J., Rozovsky I., Oda T., Hogan T. H., Finch C. E. and Pasinetti G. M. (1995) Clusterin expression by astrocytes is influenced by transforming growth factor beta-1 and heterotypic cell interactions. *J. Neuroimmunol.* **58,** 101-110.

Moskalewski S. and Thyberg J. (1988) On the role of the nucleus in the structural organization of the cell: dispersion and rearrangement of the Golgi complex in cytoplasts treated with antimicrotubular drugs. *J. Submicrosc. Cytol. Pathol.* **20,** 305-316.

Murphy B., Kirszbaum L., Walker I. D. and d'Apice J. F. (1988) SP-40,40, a newly identified normal human serum protein found in the SC5b-9 complex of complement and in the immune deposits in glomerulonephritis. *J. Clin. Inves.* **81,** 1858-1864.

Murphy B. F., Saunders J. R., O'Bryan M. K., Kirszbaum L., Walker I. D. and d'Apice A. J. F. (1989) SP-40,40 is an inhibitor of C5b-6-initiated haemolysis. *Int. Immunol.* **1,** 551-554.

Muzio M., Chinnaiyan A.M., Kischkel F.C., O'Rourke K., Shevchenko A., Ni J., Scaffidi C., Bretz J.D., Zhang M., Gentz R. *et al.* (1996) FLICE, a novel FADD homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/Apo-1) death inducing signaling complex. *Cell* 85:817-827.

Nakamura M., Yagi H., Kayaba S., Ishii T., Ohtsu S., Gotoh T. and Itoh T. (1995) Most thymocytes die in the absence of DNA fragmentation. *Arch. Histol. Cytol.* **58,** 249-256.

Navab M., Hamalevy S., Vanlenten B. J., Fonarow G. C., Cardinez C. J., Castellani L. W., Brennan M. L., Lusis A. J. and Fogelman A. M. (1997) Mildly oxidized LDL induces an increased apolipoprotein J paraoxonase ratio. *J. Clin. Inves.* **99,** 2005-2019.

Nicholson D.W., Thornberry N.A. (1997) Caspases: Killer proteases. *Trends Biochem. Sci.* **22,**299-306.

Nicoletti I., Migliorati, G, Grignani, F. and Riccardi, C. (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Meth.* **139,** 271-279.

Nover L., Sharf K.D., Nuemann D. (1989) Cytoplasmic heat shock granules are formed from precursor particles and are associated with a specific set of mRNAs. *Mol. Cell. Biol.* **9,** 1298-1308.

O'Bryan M. K., Baker H. W. G., Saunders J. R., Kirszbaum L., Walker I. D., Hudson P., Liu D. Y., Glew M. D., Apice A. J. F. and Murphy B. F. (1990) Human seminal clusterin (SP-40,40). Isolation and characterization. *J. Clin. Inv.* **85,** 1477-1486.

O'Bryan M. K., Murphy B. F., Liu D. Y., Clarke G. N. and Baker H. W. G. (1994) The use of anticlusterin monoclonal antibodies for the combined assessment of human sperm morphology and acrosome integrity. *Hum. Rep.* **9,** 1490-1496.

Oberhammer F., Wilson J. W., Dive C., Morris I. D., Hickman J. A., Wakeling A. E., Walker P. R. and Sikorska M. (1993) Apoptotic death in epithelial cells - cleavage of DNA to 300 and or 50 Kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J.* **12,** 3679-3684.

Oconnell M. A., Kelleher D., Liskamp R. M. J., Hall N., Oneill L. A. J. and Long A. (1997) TNF-mediated cytotoxicity of L929 cells - role of staurosporine in enhancement of cytotoxicity and translocation of protein kinase C isozymes. *Cytokine.* **9,** 83-92.

Oda T., Pasinetti G. M., Osterburg H. H., Anderson C., Johnson S. A. and Finch C. E. (1994) Purification and characterization of brain clusterin. *Biochem. Biophys. Res. Comm.* **204,** 1131-1136.

Oda T., Wals P., Osterburg H. H., Johnson S. A., Pasinetti G. M., Morgan T. E., Rozovsky I., Stine W. B., Snyder S. W., Holzman T. F., Krafft G. A. and Finch C. E. (1995) Clusterin (apo J) alters the aggregation of amyloid beta-peptide (α-Beta(1-42)) and forms slowly sedimenting α-beta complexes that cause oxidative stress. *Exp. Neurol.* **136,** 22-31.

Ostrove J. M. and Gifford G. E. (1979) Stimulation of RNA synthesis in L-929 cells by rabbit tumor necrosis factor. *Proc. Soc. Exp. Biol. Med.* **160,** 354-358.

Palmer D. J. and Christie D. L. (1990) The primary structure of glycoprotein III from bovine adrenal medullary chromaffin granules. *J. Biol. Chem.* **265,** 6617-6623.

Pankhurst G.J., Bennett C.A., Easterbrook-Smith S.B. (1998) Characterisation of the heparin binding properties of human clusterin. *Biochemistry.* **37,** 4823-4830.

Park Y. M., Han M. Y., Blackburn R. V. and Lee Y. J. (1998) Overexpression of HSP25 reduces the level of TNF-alpha-induced oxidative DNA damage biomarker, 8-Hydroxy-2'-deoxyguanosine, In L929 Cells. *J. Cell. Physiol.* **174,** 27-34.

Partridge S.R., Baker M.S., Walker M.J., Wilson M.R. (1996) Clusterin, a putative complement regulator, binds to the cell surface of *Staphylococcus aureus* clinical isolates. *Infect. Immun.* **64,** 4324-4329.

Peitsch M.C., Mannhertz H.G., Tschopp J. (1994) The apoptosis endonucleases: cleaning up after after cell death?. *Trends Cell Biol.* **4,** 37-41.

Pilarsky C., Haase W. and Kochbrandt C. (1993) Stable Expression Of Gp80 (TRPM-2, Clusterin), a secretory protein implicated in programmed cell death, in transfected BHK-21 cells. *Biochim. Biophys. Acta* **1179,** 306-310.

Popper P., Farber D. B., Micevych P. E., Minoofar K. and Bronstein J. M. (1997) TRPM-2 expression and tunel staining in neurodegenerative diseases: studies in wobbler and rd mice. *Exp. Neurol.* **143,** 246-254.

Primo-Parmo S. L., Sorenson R. C., Teiber J. and La Du B. N. (1996) The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. *Genomics* **33,** 498-507.

Ptitsyn, O. B., Pain, R. H., Semisotnov, G. V., Zeovnik, E., and Razgulyaev, O. I.

(1990) Evidence for a molten globule state as a general intermediate in protein folding. *FEBS Lett.* **262,** 20-24.

Purrello M., Bettuzzi S., Di Pietro C., Mirabile E., Di Blasi M., Rimini R., Grzeschik K.-H., Ingletti C., Corti A. and Sichel G. (1991) The gene for SP-40,40, human homolog of rat sulfated glycoprotein 2, rat clusterin, and rat testosterone-repressed prostate message 2, maps to chromosome 8. *Genomics* **10,** 151-156.

Rao, P. V., Horwitz, J., and Zigler, J. S. (1993) Alpha-crystallin, a molecular chaperone, forms a stable complex with carbonic anhydrase upon heat denaturation. *Bioch. Biophys. Res. Comm.* **190,** 786-793.

Randall, L. L. (1992) Peptide binding by chaperone SecB: implications for recognition of nonnative structure. *Science* **257,** 241-245.

Reddy K.B., Karode M.C., Harmony J.A.K., Howe P. (1996a) Interaction of transforming growth factor β receptors with apolipoprotein J/clusterin. *Biochemistry.* **35,** 309-314.

Reddy K.B., Jin G., Karode M.C., Harmony J.A., Howe P.H. (1996b) Transforming growth factor beta (TGFβ)-induced nuclear localization of apolipoprotein J/clusterin in epithelial cells. *Biochemistry* **35,** 6157-6163.

Roeth P. J. and Easterbrooksmith S. B. (1996) C1q is a nucleotide binding protein and is responsible for the ability of clusterin preparations to promote immune complex formation. *Biochim. Biophys. Acta.* **1297,** 159-166.

Rosenberg M. E., Dvergsten J. and Correa-Rotter R. (1993) Clusterin: an enigmatic protein recruited by diverse stimuli. *J. Lab. Clin. Med.* **121,** 205-214.

Rosenberg M. E. and Silkensen J. (1995) Clusterin - physiologic and pathophysiologic considerations. *Int. J. Biochem. Cell Biol.* **27,** 633-645.

Rossi J., Lindquist S. (1989) The celllulaer location of yeast heat shock protein 26

varies with metabolism *J. Cell. Biol.* **108,** 425-439.

Russel S. W., Rosenau W. and Lee J. C. (1972) Cytolysis induced by human lymphotoxin:cinemicrographic and electron microscope observations. *Amer. J. Path.* **69,** 103.

Savessen G.S., Dixit V.M. (1997) Caspases: intracellular signalling by proteolysis. *Cell* **91,** 443-446.

Sanchezalcazar J. A., Ruizcabello J., Hernandezmunoz I., Pobre P. S., Delatorre P., Silesrivas E., Garcia I., Kaplan O., Munozyague M. T. and Solisherruzo J. A. (1997) Tumor necrosis factor-alpha increases ATP content in metabolically inhibited L929 cells preceding cell death. *J. Biol. Chem* **272,** 30167-30177.

Sarraf E. A. (1994) Tumour necrosis factor and cell death in tumours. *Int. J. Oncology* **5,** 1333-1339.

Saunders J. R., Aminian A., McRae J. L., Ofarrell K. A., Adam W. R. and Murphy B. F. (1994) Clusterin depletion enhances immune glomerular injury in the isolated perfused kidney. *Kidney Int.* **45,** 817-827.

Savill J., Fadok V., Henson P., Haslett C. (1993) Phagocyte Recognition of cells undergoing apoptosis. *Immunol. Today* **14,**131-136.

Schall TJ., Lewis M., Koller KJ., Lee A., Rice GC., Wong GHW., Gatanaga T., Granger T., Lentz R., Raab H., Kohr WJ., Goeddel DV. (1990) Molecular cloning and expression of a receptor for human tumor necrosis factor. *Cell* **61,** 361-370.

Schmid I., Uittenbogaart C. H., Keld B. and Giorgi J. V. (1994) A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. *J. Immunol. Meth.* **170,** 145-157.

Schulze Osthoff K., Bakker A. C., Vanhaesebroeck B., Beyaert R., Jacob W. A. and Fiers W. (1992) Cytotoxic activity of tumor necrosis factor is mediated by early damage

of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. *J. Biol. Chem.* **267,** 5317-5323.

Schulze Osthoff K., Beyaert R., Vandevoorde V., Haegeman G. and Fiers W. (1993) Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *EMBO J.* **12,** 3095-3104.

Schulze Osthoff K., Walczak H., Droge W. and Krammer P. H. (1994) Cell nucleus and DNA fragmentation are not required for apoptosis. *J. Cell Biol.* **127,** 15-20.

Schwochau G.B., Nath K.A., Rosenberg M.E. (1998) Clusterin protects against oxidative stress in vitro through aggregative and nonaggregative properties. *Kidney Int.* **53,** 1647-1653.

Seiberg M., and Marthinuss J., (1995) Clusterin expression within skin correlates with hair growth. *Devel. Dynam.* 202:294-301.

Sensibar J. A., Sutkowski D. M., Raffo A., Buttyan R., Griswold M. D., Sylvester S. R., Kozlowski J. M. and Lee C. (1995) Prevention of cell death induced by tumor necrosis factor alpha in LNCaP cells by overexpression of sulfated glycoprotein-2 (clusterin). *Cancer Res.* **55,** 2431-2437.

Shanmugaratnam J., Berg E., Kimerer L., Johnson R. J., Amaratunga A., Schreiber B. M. and Fine R. E. (1997) Retinal muller glia secrete apolipoproteins E and J which are efficiently assembled into lipoprotein particles. *Brain Res. Mol. Brain Res.* **50,** 113-120.

Shayo M., McLay R. N., Kastin A. J. and Banks W. A. (1997) The putative blood-brain barrier transporter for the beta-amyloid binding protein apolipoprotein J is saturated at physiological concentrations. *Life Scie.* **60,** 115-118.

Shimizumatsumoto A., Adachi W., Mizuno K., Inazawa J., Nishida K., Kinoshita S., Matsubara K. and Okubo K. (1997) An expression profile of genes in human retina and isolation of a complementary DNA for a novel rod photoreceptor protein. *Invest. Ophthal. Vis. Science* **38,** 2576-2585.

Shin, W. S., and Hirose, M. (1995) Thiol-dependent gelation of the domain I-truncated fragment of bovine serum albumin. *Biosci. Biotech. Biochem.* **59,** 817-821.

Shoji Y., Uedono Y., Ishikura H., Takeyama N. and Tanaka T. (1995) DNA damage induced by tumour necrosis factor-alpha in L929 cells is mediated by mitochondrial oxygen radical formation. *Immunol.* **84,** 543-548.

Silkensen J. R., Schwochau G. B. and Rosenberg M. E. (1994) The role of clusterin in tissue injury. *Biochem. Cell Biol.* **72,** 483-488.

Silkensen J.R., Skubitz M., Skubitz A.P.N., Chmielewski D.H., Manivel J.C., Dvergsten J.A., Rosenberg M.E. (1995) Clusterin promotes the aggregation and adhersion of renal porcine epithelial cells. *J. Clin. Invest.* **96,** 2646-2653.

Silkensen J. R., Hirsch A. T., Lunzer M. M., Chmielewski D., Manivel J. C., Muellerleile M. R. and Rosenberg M. E. (1998) Temporal induction of clusterin in the peri-infarct zone after experimental myocardial infarction in the rat. *J. Lab. Clin. Med.* **131,** 28-35.

Simboli-Campbell M., Narvaez C.J., Tenniswood M., Welsh J. (1996) **1,**25-Dihydroxyvitamin D induces morphological and biochemical markers of apoptosis in MCF-7 breast cancer cells. *J. Steroid Biochem. Molec. Biol.* **58,** 367-376.

Simm A., Bertsch G., Frank H., Zimmermann U. and Hoppe J. (1997) Cell death of Akr-Sb fibroblasts after serum removal - a process between apoptosis and necrosis. *J. Cell Scie.* **110,** 819-828.

Sisodia S. S. and Price D. L. (1995) Role of the beta-amyloid protein in alzheimers disease. *FASEB J.* **9,** 366-370.

Smith S. B., Bora N., McCool D., Kutty G., Wong P., Kutty R. K. and Wiggert B. (1995) Photoreceptor cells in the Vitiligo mouse die by apoptosis - TRPM-2 clusterin expression is increased in the neural retina and in the retinal pigment epithelium. *Invest. Ophthal. Vis. Science* **36,** 2193-2201.

Smulders, R. H. P. H., Carver, J. A., Lindner, R. A., van Bockel, M. A. M., Bloemendal, H., and de Jong, W. W. (1996) Immobilization of the C-terminal extension of bovine alphaA-crystallin reduces chaperone-like activity. *J. Biol. Chem.* **271,** 29060-29066.

Song H. Y., Dunbar J. D. and Donner D. B. (1994) Aggregation of the intracellular domain of the type 1 tumor necrosis factor receptor defined by the two-hybrid system. *Biol. Chem.* **269,** 22492-22495.

Stadtmann E.R., (1992) Protein oxidation and againg. *Science* **257,** 1220-124.

Steinberg J., Oyasu R., Lang S., Sintich S., Rademaker A., Lee C., Kozlowski J. M. and Sensibar J. A. (1997) Intracellular levels of SGP-2 (clusterin) correlate with tumor grade in prostate cancer. *Clin. Cancer Res.* **3,** 1707-1711.

Stryer L. (1988) "Biochemistry." 3rd edition. W.H. Freeman. New york, USA.

Stuart W. D., Krol B., Jenkins S. H. and Harmony J. A. K. (1992) Structure and stability of apolipoprotein J-containing high-density lipoproteins. *Biochemistry.* **31,** 8552-8559.

Sun X., Cohen G.M. (1994) Mg2+ dependent cleavage of DNA into kilobase fragments is responsible for the initial degradation of DNA in apoptosis. *J. Biol. Chem.* **26,** 19857-60.

Susan S.A., Zamzami N., Casteo M., Hirsch T., Marchetti P., Macho A., Daugas E., Geuskens M., Kroemer G. (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *Exp. Med.* **184,** 1331-1334.

Sylvester S., Griswold M. (1995): The reproductive bioloy of clusterin. In "Clusterin: role in vertebrate development, function and adaptation." R G Landes. Austin, Texas, USA.

Sylvester S.R., Morales C., Oko R., Griswold M.D. (1991) Localization of sulfated glycoprotein-2 (clusterin) on spermatozoa and in the reproductive tract of the male rat. *Biol. Reprod.* **45,** 195-207.

Sylvester S.R., Skinner M.K., Griswold M.D. (1984) A sulfated glycoprotein synthesized by sertoli cells and by epidymal cells is a component of the sperm membrane. *Biol. Reprod.* **31,** 11087-101.

Thomas-Salgar S. and Millis A. J. T. (1994) Clusterin expression in differentiating smooth muscle cells. *J. Biol. Chem.* **269,** 17879-17885.

Tomasovic S. P., Vasey T. A., Story M. D., Stephens L. C. and Klostergaard J. (1994) Cytotoxic manifestations of the interaction between hyperthermia and TNF: DNA fragmentation. *Int. J. Hyper.* **10,** 247-262.

Trost L. C. and Lemasters J. J. (1994) A cytotoxicity assay for tumor necrosis factor employing a multiwell fluorescence scanner. *Anal. Biochem.* **220,** 149-153.

Tschopp J., Chonn A., Hertig S. and French L. E. (1993 a) Clusterin, the human apolipoprotein and complement inhibitor, binds to complement-C7, C8-beta, and the B-domain of C9. *J. Immunol.* **151,** 2159-2165.

Tschopp J., Jenne D. E., Hertig S., Preissner K. T., Morgenstern H., Sapino A. P. and French L. (1993) Human megakaryocytes express clusterin and package it without apolipoprotein-A-1 into alpha-granules. *Blood* **82,** 118-125.

Tsuruta J.K., Wong K., Fritz I.B., Griswold M.D., (1990) Structural analysis of sulfated glycoprotein 2 from amino acid sequence. Relationship to clusterin and sperm protein 40, 40. *Biochem. J.* **268,** 571-578.

Urban J., Parczyk K., Leutz A., Kayne M., Kondor-Koch C. (1987) Constitutive apical secretion of an 80-kD sulfated glycoprotein complex in the polarized epithelial Madin-Darby canine kidney cell line. *J. Cell Biol.* **105,** 2735-2743.

Vakeva A., Laurila P. and Meri S. (1993) Co-deposition of clusterin with the complement membrane attack complex in myocardial infarction. *Immunol.* **80,** 177-182.

Van Lint J., Agostinis P., Vandevoorde V., Haegeman G., Fiers W., Merlevede W. and Vandenheede J. R. (1992) Tumor necrosis factor stimulates multiple serine/threonine protein kinases in Swiss 3T3 and L929 cells. Implication of casein kinase-2 and extracellular signal-regulated kinases in the tumor necrosis factor signal transduction pathway. *J. Biol. Chem.* **267,** 25916-25921.

Vaux D.L., Strasser A. (1996) The molecular biology of apoptosis. *Proc Natl. Acad. Sci. USA.* **93,** 2239-2244.

Vermes I., Haanen C., Steffensnakken H. and Reutelingsperger C. (1995) A novel assay for apoptosis - flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J. Immunol. Meth.* **184,** 39-51.

Vincenz C., Dixit V.M., (1997). Fas associated death domain protein interleukin-1β-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. *J. Biol. Chem.* 272:6578-6583.

Ware C.F., Vanarsdale S., Vanarsdale T.L. (1996) Apoptosis mediated by the TNF-related cytokine and receptor families. *J. Cell Biochem.* **60,** 47-55.

Watson A. D., Berliner J. A., Hama S. Y., Ladu B. N., Faull K. F., Fogelman A. M. and Navab M. (1995) Protective effect of high density lipoprotein associated paraoxonase - inhibition of the biological activity of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **96,** 2882-2891.

Weisman H. F., Bartow T., Leppo M. K., Marsh H. C., Jr., Carson G. R., Concino M. F., Boyle M. P., Roux K. H., Weisfeldt M. L. and Fearon D. T. (1990) Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science* **249,** 146-151.

Westwood J.T., Clos J., Wu C. (1991) Stress-induced oligomerization and chromosomal relocalisation of heat shock factor. *Nature* **353,** 822-827.

Willnow T.E., Goldstein J.L., Orth K., Brown M.S., Herz J. (1992) Low desity lipoprotein receptor-related protein and gp330 bind similar ligands, including

plasminogen activator-inhibitor complexes and lactoferrin, an inhibitor of chylomicron remnant clearance. *J. Biol. Chem.* **267,** 26172-26180.

Wilson M. R., Roeth P. J. and Easterbrook-Smith S. B. (1991) Clusterin enhances the formation of insoluble immune complexes. *Biochem. Biophys. Res. Comm.* **177,** 985-990.

Wilson M. R. and Easterbrook-Smith S. B. (1992) Clusterin binds by a multivalent mechanism to the Fc and Fab regions of IgG. *Biochim. Biophys. Acta.* **1159,** 319-326.

Wilson M. R., Easterbrook-Smith S. B., Lakins J. and Tenniswood M. P. R. (1995): Mechanisms of induction and function of clusterin at sites of cell death: In "Clusterin: role in vertebrate development, function and adaptation." R G Landes. Austin, Texas, USA.

Witztum J.L., Steinberg D. (1991). The role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* **88,** 1785-1792.

Wong P., Pineault J., Lakins J., Taillefer D., Leger J., Wang C. and Tenniswood M. (1993) Genomic organization and expression of the rat TRPM-2 (clusterin) gene, a gene implicated in apoptosis. *J. Biol Chem.* **268,** 5021-5031.

Wong P., Taillefer D., Lakins J., Pineault J., Chader G. and Tenniswood M. (1994 a) Molecular characterization of human TRPM-2/Clusterin, a gene associated with sperm maturation, apoptosis and neurodegeneration. *Eur.. J. Biochem.* **221,** 917-925.

Wong P., Kutty R. K., Darrow R. M., Shivaram S., Kutty G., Fletcher R. T., Wiggert B., Chader G. and Organisciak D. T. (1994) Changes in clusterin expression associated with light-induced retinal damage in rats. *Biochem. Cell Biol.* **72,** 499-503.

Wylie A.H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284,** 555-556.

Xu J., Xu Y., Nguyen Q., Novikoff P. M. and Czaja M. J. (1996) Induction of hepatoma

cell apoptosis by c-myc requires zinc and occurs in the absence of DNA fragmentation. *Am. J. Physiol.* **270,** 60-70.

Yang Z., Costanzo M., Golde D. W. and Kolesnick R. N. (1993) Tumor necrosis factor activation of the sphingomyelin pathway signals nuclear factor kappa B translocation in intact HL-60 cells. *J. Biol. Chem.* **268,** 20520-20523.

Yankner B. A., Duffy L. K., and Kirschner D. A. (1990) Neurotrophic and nuerotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides. *Science* **250,** 279-282.

Zamai L., Falcieri E., Marhefka G. and Vitale M. (1996) Supravital exposure to propidium iodide identifies apoptotic cells in the absence of nucleosomal DNA fragmentation. *Cytometry* **23,** 303-311.

Zamzami N., Marchetti P., Castedo M., Zanin C., Vayssier J.L., Petit P.X., Kroemer G., (1995) Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo *J. Exp. Med.* **181,** 1661-1672.

Zamzami N., Susin S.A., Marchetti P., Hirsch T., Gomez-Monterrey I., Costeldo M., Kroemer G., (1996) Mitochondrial control of apoptosis. *J. Exp. Med.* **183,** 1533-154.

Zavialov, A., Benndorf, R., Ehrnsperger, M., Zav'yalov, V., Dudich, I., Buchner, J., and Gaestel, M. (1998) The effect of the intersubunit disulfide bond on the structural and functional proerties of the small heat shock protein HSP25. *Int. J. Biol. Macromol.* **22,** 163-173.

Zheng G., Marino M., Zhao J. and McCluskey R. T. (1998) Megalin (gp330): a putative endocytic receptor for thyroglobulin (Tg). *Endocrin.* **139,** 1462-1465.

Zlokovic B. V., Martel C. L., Mackic J. B., Matsubara E., Wisniewski T., McComb J. G., Frangione B. and Ghiso J. (1994) Brain uptake of circulating apolipoproteins J and E complexed to alzheimers amyloid beta. *Biochem. Biophy. Res. Comm.* **205,** 1431-1437.

Zlokovic B. V., Martel C. L., Matsubara E., McComb J. G., Zheng G., McCluskey R. T., Frangione B. and Ghiso J. (1996) Glycoprotein 330/Megalin - probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid beta at the blood-brain and blood-cerebrospinal fluid barriers. *Proc. Natl. Acad. Sci. U.S.A* **93,** 4229-4234.

APPENDIX

Publications containing material from this thesis

Articles below removed for copyright reasons.

Please refer to citation.

Humphreys D., Hochgrebe T. T., Easterbrooksmith S. B., Tenniswood M. P. And Wilson M . R. (1997) Effects of clusterin overexpression on TNF-α- and TGF-β-mediated death of L929 cells. *Biochemistry* 36, 15233-15243.

Humphreys D.T., Carver J.A., Easterbrook-Smith S.B., Wilson M.R. (1999). Clusterin has chaperone like activity similar to that of small heat shock proteins.
J. Biol. Chem. 274,6875-6881.

Publications in press:-

Humphreys D.T., and Wilson M.R. (1999) Modes of L929 cell death induced by TNFα and other cytotoxic agents. *Cytokine.*

Hochgrebe T.T., Humphreys D.T., Wilson M.R., Easterbrook-Smith S.B. (1999) A reexamination of the role of clusterin as a complement regulator. *Exp. Cell. Res.* **249**, 13-21.