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Defining the mechanism and functional consequences of PAI-2-mediated uPA/uPAR endocytosis

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Defining the mechanism and functional consequences of PAI-2- mediated uPA/uPAR endocytosis

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I, David R Croucher, declare that this thesis, submitted in partial fulfillment of the requirements for the award of Doctor of Philosophy, in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

David R Croucher
12th October 2006

List of Publications

Croucher D, Saunders DN, Ranson M. The Urokinase/PAI-2 Complex: A New High Affinity Ligand for the Lipoprotein Receptor-Related Protein. Journal of Biological Chemistry 281: 10206-10213 (2006)

Al Ejeh F, **Croucher D**, Ranson M. Kinetic analysis of plasminogen activator inhibitor type-2:urokinase complex formation and subsequent internalisation by carcinoma cell lines. Experimental Cell Research 297(1):259-271 (2004)

Stutchbury TK, Al-ejeh F, Stillfried G, **Croucher D**, Allen B, Irving D, Andrews J, Links M, Ranson M. Preclinical evaluation of PAI-2-DTTA-^{213}Bi (alpha-PAI-2) in an orthotopic murine xenogenic model of human breast carcinoma. *Manuscript in press - Molecular Cancer Therapeutics*

Samson A, Niego B, Daniel P, Weiss TW, **Croucher D**, Lawrence DA, Medcalf RL. Tissue-type Plasminogen Activator Requires a Co-Receptor to Enhance NMDA receptor function. *Submitted to Journal of Biological Chemistry*

Croucher D, Saunders DN, Stillfried GE, Ranson M. Structural Basis of Differential Signaling by PAI-1 and PAI-2 in Breast Cancer: Implications for Metastatic Potential *Submitted to Cancer Research.*

List of Conference Presentations

Conference Oral Presentations

Croucher D, Saunders D, Ranson M**.**
PAI-2 is internalised by receptor mediated endocytosis.
IX International Workshop on Molecular and Cellular Biology of Plasminogen Activation, Isle of Capri, Italy (2003).

Croucher D, Saunders D, Ranson M.
Characterising the receptor mediated endocytosis of PAI-2.
International Society for Fibrinolysis and Proteolysis, Melbourne, Victoria (2004).

Croucher D, Saunders D, Leung H, Ranson M.
Structural basis of the differential signaling by initiated by PAI-1 and PAI-2: Implications for metastatic potential.
18th International Congress on Fibrinolysis and Proteolysis, San Diego, US (2006).

Samson AL, Niego B, Daniel PB, Weiss TB, **Croucher D,** Lawrence DA, Medcalf RL.
Tissue-type plasminogen activator can promote NMDA-induced neuronal stimulation via LDL receptor and plasmin-dependent mechanisms.
18th International Congress on Fibrinolysis and Proteolysis, San Diego, US (2006).

Conference Poster Presentations

Croucher D, Al-Ejeh F, Ranson M. The binding kinetics and cellular internalisation of PAI-2 by prostate cancer cells: Validating its use for targeted cancer therapy.
15th Lorne Cancer Conference, Lorne, Victoria (2003)

Al Ejeh F, **Croucher D,** Ranson M. Binding and internalisation characteristics of plasminogen activator inhibitor type 2 (PAI-2) on human breast and prostate cancer cell lines. IX International Workshop on Molecular and Cellular Biology of Plasminogen Activation, Isle of Capri, Italy (2003)

Croucher D, Saunders D, Ranson M. The PAI-2/urokinase complex: A new ligand for the low density lipoprotein receptor-related protein X International Workshop on Molecular and Cellular Biology of Plasminogen Activation, Washington DC (2005)

Croucher D, Saunders D, Ranson M. Annexin II, a novel cell surface receptor and avenue of endocytosis for PAI-2. Serpins Conference, Cairns, Australia (2005)

Lobov S, **Croucher D**, and Ranson M. Assessment of known/potential binding sites in the PAI-2 CD-loop for interaction with annexin II and endocytosis receptors. XVIIIth International Congress on Fibrinolysis and Proteolysis, San Diego, USA, (2006)

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Absorbance	A
Amino Terminal Fragment	ATF
Basement Membrane	BM
Bovine Serum Albumin	BSA
Deoxyribonucleic Acid	DNA
Disabled-1	Dab-1
Epidermal Growth Factor	EGF
Epidermal Growth Factor Receptor	EGFR
Ethylenediaminetetraacetic Acid	EDTA
Extracellular Matrix	ECM
Extracellular Signal-Regulated Kinase	ERK
Fluorescein Isothiocyanate	FITC
Foetal Calf Serum	FCS
Glycophosphoinositol	GPI
Gram	g
Gravity	*g*
High Molecular Weight	HMW
Horse Radish Peroxidase	HRP
Hour	h
4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid	HEPES
Immunoglobulin G	IgG
Association Rate	k_a
Dissociation Rate	k_d
Dissociation Constant	K_D

Kilodalton	kDa
Lipopolysaccharide	LPS
Litre	L
Low Density Lipoprotein Receptor	LDLR
Low Density Lipoprotein Receptor-Related Protein	LRP
Low Molecular Weight	LMW
Matrix Metalloprotease	MMP
Metre	m
Micro	μ
Milli	m
Minute	min
Molar	M
Nano	n
N-hydroxysuccinimide	NHS
Para-formaldehyde	PFA
Phenylmethylsulphonylfluoride	PMSF
Phosphate Buffered Saline	PBS
Plasminogen Activator Inhibitor	PAI
Poly-Acrylamide Gel Electrophoresis	PAGE
Propidium Iodide	PI
Reactive Centre Loop	RCL
Receptor Associated Protein	RAP
Retinoblastoma	Rb
Revolutions per Minute	rpm
Sodium Dodecyl Sulphate	SDS

Second	sec
Serine Protease Inhibitor	Serpin
Standard Error of the Mean	SEM
Surface Plasmon Resonance	SPR
Tissue Plasminogen Activator	tPA
Transforming Growth Factor-α	TGF-α
Tris Buffered Saline	TBS
Tumour Necrosis Factor-α	TNF-α
Urokinase Plasminogen Activator	uPA
Urokinase Plasminogen Activator Receptor	uPAR
Very Low density Lipoprotein Receptor	VLDLr
Volts	V

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Abstract

Plasminogen is converted to its active form plasmin by two major serine proteases; the urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA). De-regulated plasmin formation is associated with tumour growth and progression. Whilst tPA is primarily involved in blot clot dissolution, uPA, along with its cell surface receptor uPAR, are commonly over-expressed at the leading edge of a tumour and by the tumour-associated stroma, contributing to plasmin formation, cell proliferation and migration. Soluble and receptor bound uPA is efficiently inhibited by two members of the serine protease inhibitor (serpin) superfamily; the plasminogen activator inhibitors type 1 (PAI-1) and 2 (PAI-2) (Serpin E1 and B2 respectively).

The purpose of this thesis was; **(1)** to examine the fate of cell surface bound PAI-2, a largely un-explored aspect of the plasminogen activation system, with particular focus on the possibility of the internalisation of uPA bound PAI-2; **(2)** to characterise the interaction between PAI-2, uPA:PAI-2 and any putative receptors involved in the internalisation of these proteins; and **(3)** to determine the functional consequences of the process of PAI-2 internalisation, in terms of regulation of uPA/uPAR levels and cell signaling responses.

Confocal microscopy and a novel flow cytometry based internalisation assay were used to both visualise and measure the interaction of PAI-2 with human carcinoma cancer cell lines. This data provided definitive proof that uPA bound PAI-2 was internalised into the endosomes and lysosomes of these cells, mediated through an interaction with endocytosis receptors of the low density lipoprotein receptor (LDLR) family. This finding may lead to the development of a more effective PAI-2 cancer therapeutic utilising the intracellular delivery of cytotoxins to cancer cells.

Surface plasmon resonance and further applications of the flow cytometry based internalisation assay were used to investigate the interactions of uPA:PAI-2 with two receptors of the LDLR family. This lead to the characterisation of the interaction between uPA:PAI-2 and the low density lipoprotein receptor-related protein (LRP) and the very low density lipoprotein receptor (VLDLr). The biochemical analysis of these interactions, in comparison to that of uPA:PAI-1, led to the discovery of a novel difference in the kinetics and affinities of the interactions between uPA:PAI-1, uPA:PAI-2 and these receptors. Differing positive electrostatic potentials and conservation of a putative LDLR binding motif within helix D of these two serpins, specifically surrounding a conserved arginine residue, were implicated in the higher affinity of uPA:PAI-1 for these receptors.

The consequences of this variation in receptor binding were revealed using MCF-7 breast cancer cells. As previously demonstrated, the binding of the high affinity helix D site in uPA:PAI-1 to VLDLr on MCF-7 cells resulted in the propagation of intracellular signaling events and cell proliferation. As uPA:PAI-2 does not contain this high affinity site, these cell signaling events were not induced upon uPA:PAI-2 binding to VLDLr, however the complex was still efficiently endocytosed.

The data presented in this thesis therefore proposes a novel mechanism behind the disparity in patient prognosis associated with tumour expression of PAI-1 and PAI-2. The negative prognostic impact of PAI-1 may be mediated through the mitogenic effects of its high affinity LDLR binding site, whereas the positive prognostic impact of PAI-2 stems from its ability to efficiently inhibit and clear cell surface uPA without inducing the mitogenic effects associated with PAI-1.

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