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

David R. Croucher
University of Wollongong
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Defining the mechanism and functional consequences of PAI-2-mediated uPA/uPAR endocytosis

A thesis submitted in partial fulfillment of the requirements for the award of the degree

Doctor of Philosophy

from

University of Wollongong

by

David R Croucher
Bachelor of Biotechnology (Honours 1st Class)

School of Biological Sciences
University of Wollongong
2006
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


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*

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IX International Workshop on Molecular and Cellular Biology of Plasminogen Activation, Isle of Capri, Italy (2003).

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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.

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.

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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)


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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<tbody>
<tr>
<td>Absorbance</td>
<td>A</td>
</tr>
<tr>
<td>Amino Terminal Fragment</td>
<td>ATF</td>
</tr>
<tr>
<td>Basement Membrane</td>
<td>BM</td>
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<tr>
<td>Bovine Serum Albumin</td>
<td>BSA</td>
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<tr>
<td>Deoxyribonucleic Acid</td>
<td>DNA</td>
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<tr>
<td>Disabled-1</td>
<td>Dab-1</td>
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<tr>
<td>Epidermal Growth Factor</td>
<td>EGF</td>
</tr>
<tr>
<td>Epidermal Growth Factor Receptor</td>
<td>EGFR</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic Acid</td>
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</tr>
<tr>
<td>Extracellular Matrix</td>
<td>ECM</td>
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<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid</td>
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<td>Dissociation Constant</td>
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<td>Term</td>
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<tr>
<td>------------------------------------------</td>
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<tr>
<td>Kilodalton</td>
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<td>Low Density Lipoprotein Receptor</td>
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<tr>
<td>Plasminogen Activator Inhibitor</td>
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<tr>
<td>Poly-Acrylamide Gel Electrophoresis</td>
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<tr>
<td>Propidium Iodide</td>
<td>PI</td>
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<tr>
<td>Reactive Centre Loop</td>
<td>RCL</td>
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<tr>
<td>Receptor Associated Protein</td>
<td>RAP</td>
</tr>
<tr>
<td>Retinoblastoma</td>
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<td>Revolutions per Minute</td>
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<td>Sodium Dodecyl Sulphate</td>
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<td>Serine Protease Inhibitor</td>
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<tr>
<td>Standard Error of the Mean</td>
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<td>uPAR</td>
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<tr>
<td>Very Low density Lipoprotein Receptor</td>
<td>VLDLr</td>
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<td>Volts</td>
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Acknowledgements

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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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