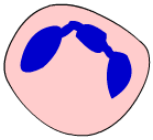
A NOVEL PROINFLAMMATORY ROLE FOR ANNEXIN A1 IN NEUTROPHIL TRANSENDOTHELIAL MIGRATION



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ABSTRACT

Neutrophil extravasation into tissues is an essential process required for the inflammatory response. Upon receiving an inflammatory cue, neutrophils begin accumulating on the luminal surface of the endothelium. Neutrophil recruitment is initiated by selectin-mediated tethering and rolling of neutrophils along the endothelial monolayer, followed by integrin-mediated firm adhesion. Adherent neutrophils then traverse the endothelium in a process known as transendothelial migration. The events mediating the rolling and adhesion steps are well characterised, but research into the molecular mechanisms regulating transendothelial migration is an area of intense focus. A previous study conducted in our laboratory found that the activation of endothelial extracellular signal-regulated kinase (ERK) 1/2 was required for neutrophil transmigration. Furthermore, it was found that endothelial ERK was activated in response to a soluble protein produced by fMLP- or IL-8-stimulated neutrophils.

In the present study, the soluble ERK-activating neutrophil protein was identified as annexin A1, which was selected as a possible candidate following mass spectrometry analysis of proteins secreted from activated neutrophils. Annexin A1 antibodies (Abs) were found to block endothelial ERK activation induced by conditioned medium harvested from stimulated neutrophils. Annexin A1 Abs were additionally able to inhibit neutrophil transmigration across human umbilical vein endothelial cell (HUVEC) monolayers in an *in vitro* transmigration assay. Following the purification of recombinant annexin A1, it was demonstrated that it could activate endothelial ERK in a similar manner to neutrophil conditioned medium. Upon further investigation, ERK activation was found to be induced by a truncated form of annexin A1 present in the protein preparation rather than the full length protein. Calpain I, a calcium dependent protease that is activated upon neutrophil stimulation and is known to cleave annexin A1 within the N-terminal domain, was shown to process full length inactive recombinant annexin A1 into an unidentified product that could activate endothelial ERK. A calpain I inhibitor was also found to prevent stimulated neutrophils from secreting an ERK-activating protein, thus further suggesting a role for calpain I in this process. As full length annexin A1 has been reported to signal through the formyl peptide receptor (FPR) family, a pan-FPR antagonist was incubated with endothelial cells and was found to inhibit ERK activation induced by neutrophil conditioned medium, indicating that pro-inflammatory annexin A1 is also a FPR ligand.

Endothelial projections termed “transmigratory cups” form around neutrophils during extravasation, of which ICAM-1 is a major component. Using an assay that examined transmigratory cups during neutrophil transmigration, it was found that annexin A1 Abs could inhibit neutrophil adhesion and transmigration through HUVEC monolayers by interfering with transmigratory cup formation around neutrophils, as shown by monitoring ICAM-1 during the process. Quantification of transmigrating neutrophils highlighted that the majority of neutrophils were emigrating via a transcellular pathway, which is in opposition to many *in vitro* studies where paracellular transmigration predominates.

The results generated from this study identified a novel pro-inflammatory role for annexin A1 in neutrophil transendothelial migration. Preliminary experiments suggested that the pro-inflammatory annexin A1 responsible for endothelial ERK activation was a truncated form. Calpain I appears to be a likely candidate responsible for the generation of this uncharacterised, truncated annexin A1 product, however further experiments are required to confirm this hypothesis. Pro-inflammatory annexin A1 represents a new target for the treatment of inflammatory disorders.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

Samantha Williams.

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Finally to my husband Craig: Told you I'd finish it! :P 1337 +3 chair buff to thesis writing FTW! Now let teh pwnage begin!!~1!

“When you’re going through hell, keep going.”

-Winston Churchill

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ABBREVIATIONS

Ab	Antibody
Ac2-26	Acetylated N-terminal annexin A1 residues 2-26
ANOVA	Analysis of the variance
AnxA1	Annexin A1
BALF	Bronchoalveolar lavage fluid
Boc2	Boc-Phe-Leu-Phe-Leu-Phe
BSA	Bovine serum albumin
CHO	Chinese Hamster Ovary
CM	Conditioned medium
CMV	Cytomegalovirus
DAPI	4'-6-Diamidino-2-phenylindole
DMSO	Dimethylsulphoxide
EM	Electron microscope
ERK	Extracellular signal-regulated kinase
ERM	Ezrin radixin moesin
ESAM	Endothelial cell-selective adhesion molecule
FBS	Foetal bovine serum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
fMLP	Formyl-Met-Leu-Phe
FPLC	Fast protein liquid chromatography
FPR	Formyl peptide receptor
FPRL	Formyl peptide receptor like
GFP	Green fluorescent protein
HEK293	Human embryonic kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hypothalamic Pituitary Adrenal
HRP	Horse Radish Peroxidise

HSA	Human serum albumin
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular Adhesion Molecule
IL	Interleukin
JAM	Junctional adhesion molecule
KLH	Keyhole limpet hemocyanin
KO	Knock out
LAD	Leukocyte Adhesion Deficiency
LB	Luria Bertani
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
LXA4	Lipoxin A4
mAb	Monoclonal antibody
Mac-1	Macrophage antigen-1
MAPK	Mitogen activated protein kinase
MCS	Multiple cloning site
MEK	Mitogen-activated protein kinase kinase
MLCK	Myosin Light Chain Kinase
MMP	Matrix Metalloprotease
MNEI	Monocyte/Neutrophil Elastase Inhibitor
MOPS	3-(N-morpholino) propanesulfonic acid
MPO	Myeloperoxidase
MQ	Milli Q
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NET	Neutrophil Extracellular Traps
O.D.	Optical density
pAb	Polyclonal antibody
PAF	Platelet activating factor
PBS	Phosphate buffered saline

PBS-T	PBS/0.1% Tween 20
PCR	Polymerase chain reaction
PDZ	Post-synaptic density-95/discs large/zonula occludens-1
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PSGL-1	P-selectin glycoprotein ligand-1
PKC	Protein kinase C
PMA	Phorbyl myristate acetate
PMS	Phenazine methosulfate
PVDF	Polyvinylidene fluoride
ROS	Reactive Oxygen Species
SAA	Serum Amyloid A
SEM	Standard error of the mean
SMP	Skim Milk Powder
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor