PLACENTAL ANGIOGENESIS AND IMMUNE-PRIVILEGE IN NORMAL AND PATHOLOGICAL PREGNANCIES

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Dedicated with love to my dearest wife
and
loving parents

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List of Publications

Peer reviewed papers

1. Rajashekhar G, Loganath A, Roy AC, Wong YC. Expression and Localization of Angiogenin in Placenta: Enhanced Levels at Term over First Trimester Villi. *Mol Reprod Dev* 2002; 62:159-66.
2. Rajashekhar G, Loganath A, Roy AC, Wong YC. Over-expression and secretion of angiogenin in intrauterine growth retardation placenta. *Mol Reprod Dev* 2003; 64:397-404.
3. Rajashekhar G, Loganath A, Roy AC, Wong YC. Expression and Secretion of the Vascular Cell Adhesion Molecule-1 in Human Placenta and its Decrease in Fetal Growth Restriction. *J Soc Gynecol Investig*, 2003a; 10:352-60.
4. Rajashekhar G, Loganath A, Roy AC, Mongelli M. Resistance to Fas-mediated cell death in BeWo and NJG choriocarcinoma cell lines: Implications in immune privilege. *Gynecol Oncol*, 2003; 91:101-111.
5. Rajashekhar G, Loganath A, Roy AC, Mongelli M. Co-Expression of Fas (APO-1, CD95)/Fas Ligand by BeWo and NJG Choriocarcinoma Cell Lines. *Gynecol Oncol*, 2003; 91:89-100.
6. Rajashekhar G, Loganath A, Roy AC, Chong SS, Wong YC. Hypoxia Upregulates Angiogenin Expression and Secretion in Human Placental Trophoblasts. *Placenta* 2003 (Manuscript in preparation).
7. Rajashekhar G, Loganath A, Roy AC, Chong SS, Wong YC. Integrin mediated Angiogenin Expression and Secretion in Human Placental Trophoblasts. *Placenta* 2003 (Manuscript in preparation).

Conference papers

1. Rajashekhar G, Loganath A, Roy AC, Ng SC. Matrix metalloproteinase Genes in Human Placentation: A Bioinformatic Approach. 2nd Combined Annual Scientific meeting, SSBMB-BRETSS-SSMB, Singapore, September 8-9, 2000. Abstract p12.

2. Rajashekhar G, Loganath A, Roy AC, Wong YC. Gestation-dependant Differential Secretion of sICAM-1, sVCAM-1 and Angiogenin by Placental Explants in Culture. 5th NUH-NUS ASM, Singapore, June 29-30. Abstract p61.
3. Rajashekhar G, Loganath A, Roy AC, Wong YC. Localization, expression and secretion of angiogenin in the normal human placenta. First Singapore Angiogenesis/Anti-angiogenesis Symposium, Singapore, November 23-24, 2001. Oral presentation #7, p40.
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List of Abbreviations

A	Absorbance
A/R	Anoxia/Reoxygenation
Ab	Antibody
ANG	Angiogenin
ANOVA	Analysis of variance
AP-1	Activation protein complex-1
APS	Ammonium persulphate
ATCC	American type culture collection
bFGF	Basal fibroblast growth factor
BHK	Baby hamster kidney
BLAST	Basic local alignment search tool
BM	Basement membrane
BMG	Beta-2 microglobulin
bp	Base pairs
Bp	Blood pressure
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
cDNA	Complimentary deoxy ribonucleic acid
CHO	Chinese hamster ovary
CHX	Cycloheximide
CO_2	Carbon dioxide
C_P	Crossing point
CRP	Complement regulatory proteins
CSF-1	Colony stimulating factor-1
CTB	Cytotrophoblast
CTL	Cytotoxic lymphocytes
CV	Coefficient of variation
DAB	3,3-diaminobenzadine
DEPC	Diethyl pyrocarbonate
DISC	death-inducing signalling complex
DMEM	Dulbecos modified eagles medium
DMSO	Dimethyl sulphoxide
dNTP	Deoxy nucleotide tri phosphate
dsDNA	Double stranded deoxy ribonucleic acid
DTT	Dithiothreitol
E/T ratio	Effector to target ratio
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetate
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
Fas	CD-95 or APO-1, Fas receptor
FasL	Fas ligand
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FLIP	Flice like inhibitory protein
FN	Fibronectin
FT	First trimester
g	Gram
g	Relative centrifugal force
G3PDH	Glyceraldehyde 3 phosphate dehydrogenase
GF	Growth factor
GTD	Gestational trophoblastic disease

hCG	Human chorionic gonadotropin
HELLP	Haemolysis elevated liver enzymes and low platelet counts
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HIF-1	Hypoxia inducible factor-1
HIV-1	Human immunodeficiency virus-1
HLA-G	Human leukocyte antigen-G
hPL	Human placental lactogen
HRP	Horse radish peroxidase
HSRRB	The health science research resources bank
HTLV-1	Human T-cell leukaemia virus type 1
HUVEC	Human umbilical vein endothelial cells
Hy	Hypoxia
ICAM-1	Intercellular adhesion molecule
IgCAM	Immunoglobulin cell adhesion molecule
IGF-1	Insulin like growth factor-1
IHC	Immunohistochemistry
IL	Interleukin
INF-γ	Interferon-gamma
IU	International units
IUGR	Intrauterine growth retardation
kDa	Kilo dalton
kg	Kilogram
l	Litre
LFA-1	Leukocyte function-associated antigen-1
M	Molar
mAb	Monoclonal antibody
Mac-1	also known as CD11b/CD18
MFI	Mean fluorescence intensity
mg	Milligram
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility gene complex
mIU	Milli international units
mM	Millimolar
mmHg	Millimeter mercury
mmol	Millimole
MMP	Matrix metallo proteinase
MOPS	[3-(N-morpholino)-propanesulfonic acid]
mRNA	Messenger ribonucleic acid
MTT	(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)
MU	Methlumbelliferone
MUP	4-methylumbelliferyl phosphate
MW	Molecular weight
n	Number
NCAM	Neuronal cell adhesion molecule
NF-κB	Nuclear factor kappa B
ng	Nanogram
NK	Natural killer
NT	Normal term
O_2	Oxygen
^oC	Degree Celsius
P	Probability
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Pre-eclampsia

PECAM	Platelet endothelial cell adhesion molecule
pg	Picogram
PGE_2	Prostaglandin E_2
PI	Propidium iodide
P*l*GF	Placental growth factor
pmol	Picomole
pO2	Partial pressure of oxygen
PS	Phosphotidylserine
RGD	Arg-Gly-Asp
RGDS	Arg-Gly-Asp-Ser
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell park memorial institute
RT	Reverse transcriptase (enzyme)
RT-PCR	Reverse transcription-Polymerase chain reaction
RV	Reaction vessel
SCID	Severe combined immunodeficient
SD	Standard deviation
SDS	Sodium dodesyl sulphate
SEM	Standard error of mean
SGA	Small for gestational age
STB	Syncytiotrophoblast
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-β1	Transforming growth factor-beta 1
TNF-α	Tumor necrosis factor-alpha
tPA	Tissue plasminogen activator
TTBS	Tween-tris buffered saline
U	Units
V	Volts
v/v	Volume/volume
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLA-4	Very Late Antigen-4 (also known as CD49d/CD29)
VN	Vitronectin
w/v	weight per volume
wk	Week
y	Years
β	Beta
μg	Microgram
μl	Microlitre
μM	Micromolar

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Summary

Development of the haemochorial placenta involves the invasion of extravillous trophoblast cells into the uterine wall and subsequent remodelling of the uteroplacental vessels. Although myometrial invasion by cytotrophoblast cells is a crucial step in early placentation, the mechanisms underlying this pivotal process, essential for pregnancy maintenance, are incompletely understood. We hypothesized that fetal cytotrophoblast invasion of the uterus requires the synergistic modulation by both cell adhesion and angiogenesis, two processes which are essential for haemochorial placentation. In this regard, we have demonstrated for the first time presence of a potent angiogenic factor, angiogenin in placenta throughout gestation (Rajashekhar *et al*., 2002). Our finding of the enhanced expression of this pivotal angiogenic factor at term compared in with first trimester (FT) chorionic villi is in accordance with that of the exponential increase in fetal growth during the third trimester when a dramatic rise in the growth of the placental bed occurs (Ahmed and Perkins, 2000). Further to this observation, investigation of the placenta from patients with intrauterine growth retardation (IUGR) revealed over-expression and secretion of this pivotal angiogenic molecule. The enhanced angiogenin production which could be attributed to the ability to circumvent the poor oxygenation resulting from defective fetoplacental blood flow by autoregulation could serve as positive feed back control to induce angiogenesis (Rajashekhar *et al.,* 2003). To understand the molecular mechanism of this over-expression and its association with hypoxia, term placental explants in culture when subjected to hypoxia showed increased expression of angiogenin thus providing a direct evidence for compensatory rise for this angiogenic factor in conditions associated with deficient oxygen supply. Moreover, the extracellular matrix (ECM) proteins *in vitro* amplified the production of angiogenin release from trophoblasts in culture as well as its mRNA transcripts, thus providing evidence that the interactions occur among cell adhesion molecules and angiogenic growth factors. In addition, angiogenin production was reduced by compounds that interfere with integrin

function, such as anti-integrin antibodies, suggesting an important role of ECM binding in the regulation of angiogenin release. This synergy between cell adhesion molecules and angiogenic growth factors could have a key role in cellular invasion and migration, processes that are essential for angiogenesis and subsequent placental growth.

Pre-eclampsia (PE) and IUGR have been attributed to a failure of trophoblast invasion which leads to underperfusion of the uteroplacental bed. The interaction between maternal leukocytes and decidual cells with the invading trophoblast is thought to be established by cell adhesion molecules. In this context, the vascular cell adhesion molecule-1 (VCAM-1), which is a member of the immunoglobulin gene superfamily has been reported to play a proangiogenic role in inducing chemotaxis of human endothelial cells *in vitro* that is essential for angiogenesis and subsequent placental development. We hypothesized that a failure of normal placentation with dysregulation of VCAM-1, the marker of endothelial activation might explain the aetiology of late onset IUGR (>32 weeks of gestation). In this study therefore, using placental explant cultures and RT-PCR, we determined the expression and secretion profiles of this pivotal cell adhesion molecule during the course of gestation and compared it with pregnancies complicated by IUGR. A decrease in mRNA expression and secretion of VCAM-1 in the term chorionic villi over the FT placenta occurred which was further decreased in IUGR placenta, suggesting that the diminished levels of VCAM-1 might contribute to the pathological state.

In addition to the key regulatory agents discussed above, expression profiles of other growth factors including placental growth factor, epidermal growth factor as well as cell adhesion molecules like intercellular adhesion molecules (ICAM-1, -2, -3), P-selectin and VE-cadherin in normal and pathological pregnancies were studied. The results of this study indicated a higher mRNA expression of ICAM-2 and P-selectin at term than FT samples, and their decrease in PE

and IUGR. P*l*GF showed higher levels at FT than term placenta, the concentrations of which were further decreased in PE and IUGR. In conclusion, the pathological pregnancy could be attributed to a defective expression and secretion of cell adhesion molecules and growth factors.

In a bid to understand the hitherto undescribed molecular mechanism(s) for the survival and proliferation of malignant trophoblasts in the gravid uterus (Gestational Trophoblastic Disease, GTD), we have documented the co-expression of Fas and FasL in choriocarcinoma, and hypothesized a role for these malignant trophoblasts in immune-privilege. Using the well-characterized choriocarcinoma cell line, BeWo and the uterine choriocarcinoma cell line, NJG, we provided evidence that the Fas receptors are down regulated and show resistance to Fas-mediated apoptosis. This could be attributed to the presence of a short-lived endogenous inhibitor like cFLIP as demonstrated by the RT-PCR method. In addition, co-culture of these choriocarcinoma cells, which express functional FasL, was noted to induce apoptosis in Fas-sensitive lymphocytic Jurkat cells, suggesting that these tumour cells possess the capacity to evade immune attack thereby imparting immune-privilege. In conclusion, the data suggest that choriocarcinoma cells could evade immune attack by downregulating the Fas receptor and killing the lymphocytes through expression of FasL. Investigations on such molecular mechanisms might provide greater insight into the mechanisms associated with tumour survival and offer possible therapeutic approaches in the treatment of GTD.