To The Graduate Council:

I am submitting herewith a thesis written by Jayashree Desai entitled "Molecular And Cellular Responses of Normal Human Trophoblast to Vascular Endothelial Growth Factor and Placenta Growth Factor." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Donald Torry, Major Professor

We have read this thesis and
Recommend its acceptance:

Roger Carroll

Joyce Merryman

Accepted for the Council:

Associate Vice Chancellor and Dean
Of The Graduate School

MOLECULAR AND CELLULAR RESPONSES OF NORMAL HUMAN TROPHOBLAST TO VASCULAR ENDOTHELIAL GROWTH FACTOR AND PLACENTA GROWTH FACTOR

A Thesis

Presented for the

Master of Science

Degree

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Jayashree Desai

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DEDICATION

This thesis is dedicated to my grandparents

Mr. Basavantrao Desai

ಬಸವಂತರಾಲ್ ದೇಸಾಯಿ

Mrs. Laxmi Desai

ಲಕ್ಷ್ಮೀ ದೇಸಾಯಿ

and my parents

Mrs. Uma Deshmukh

ಉಮಾ ದೇಶಮುಖ

Mr. Jagadevrao Deshmukh

ಜಗದೇವರಾಲ್ ದೇಶಮುಖ

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LIST OF ABBREVIATIONS

Ab	antibody
AP-1	activating protein-1
Akt	murine retrovirus oncogene (serine/threonine kinase)
Apaf	apoptosis-associated factor
ASK-1	apoptosis signal-regulating kinase
ATF	activating transcription factor
BAE cells	bovine aortic endothelial cells
Bcl-2	an oncogene activated by chromosome translocation in B-cell follicular lymphomas (anti-apoptotic protein)
BH	bcl-2 homology
Crk	oncogene of sarcoma virus CT10
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FGF	fibroblast growth factor
fms	oncogene of McDonough feline sarcoma virus
fos	oncogene of FBJ murine osteogenic sarcoma virus
GAP	GTPase activating protein
GM-CF	granulocyte-macrophage colony-stimulating factor
Grb	growth factor bound protein

GST	gluothione S-transferase
h	hour
hsp	heat shock protein
HUVE	human umbilical vein endothelial cells
IGF	insulin like growth factor
IL-1	interlukin-1
INF	interferon
IP	immunoprecipitation
IRS	insulin receptor substrate
IUGR	intrauterine growth retardation
JEG-3 cells	chorio carcinoma cells
JNK	c-Jun N-terminal kinase
JNKK	JNK kinase (=SEK 1, MKK 4)
Jun	oncogene of avian sarcoma virus
KD	kilodalton
MAPK	mitogen-activated protein kinase
MAPK-APK	MAPK-activated protein kinase
MBP	myelin basic protein
MCF-7 cells	cells from Michigan carcinoma foundation (Breast carcinoma cell line)
MEK	MAP ERK kinase/MAPKK kinase
min	minute

MKK	MAP Kinase Kinase
Myc	oncogene of avian myelocytomatosis virus-29
Nck	adapter molecule with SH2 and SH3 domains that can act as an oncogene
NGF	nerve growth factor
NIH -3T3 cell	non transformed mouse fibroblast cell line
NIK	Nck interacting kinase
PAK	p21-activating kinase
PAE cells	paracrine aortic endothelial cells
PBS	phosphate buffered saline
PC-12	pheochromocytoma cells
PH	pleckstrin-homology
PI-3 kinase	phosphatidylinositol-3 kinase
PKB	protein kinase B
PKC	protein kinase C
cPLA2	cytosolic phospholipase A2
PLC	phospholipase C
PlGF	placenta growth factor
PDGF	platelet derived growth factor
raf	oncogene of 3611 murine sarcoma and MH2 avian carcinoma viruses (ERK kinase kinase)
Ras	oncogene of rat sarcoma virus (GTP binding protein)

RIP	receptor interacting protein
SAPK	stress activated protein kinase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEK-1	SAPK or MAPK kinase (= JNKK, MKK 4)
SH2-SH3 domains	src homology domains
Shc	protein containing SH2 domains that takes part in Ras activation and can function as an oncogene
SOS	son of sevenless (Dorosophila gene), a Ras guanine nucleotide exchange factor
Src	oncogene of Rous sarcoma virus
TGF	transforming growth factor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAF	TNFR-associated factor
Trophoblast	placental cells
UV	ultraviolet
VEGF	vascular endothelial growth factor

PART I.

LITERATURE REVIEW

Implantation and growth of the placenta requires extensive angiogenesis in fetal villi and maternal decidua to form vascular structures involved in placental exchange. Vascular development, cell proliferation, differentiation and invasiveness are required for proper development of the placenta. Many growth factors and receptors are involved in these processes. The human placenta is a rich source of angiogenic growth factors and their receptors, which are known to control vascular changes and control trophoblast function [1, 2]. Within the placenta, trophoblast are a source of angiogenic growth factors like transforming growth factor-α and β (TGF-α and β), epidermal growth factor (EGF), fibroblast growth factor (FGF-1 and 2), vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) [3].

GROWTH FACTORS

Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor is a 40-45 kDa disulfide-linked homodimeric glycoprotein. There are four known isoforms of human VEGF (VEGF121, VEGF165, VEGF189 and VEGF 206) generated by alternative splicing of mRNA [4]. The VEGF gene is organized into eight exons, and is localized to chromosome 6p21.3. VEGF 165 lacks the residues encoded by exon 6, and VEGF 121 lacks the residues encoded by exon 6 and 7. VEGF 189 has a 24 amino acid insertion, while VEGF 206 has an additional insertion of 17 amino acids. VEGF 121 and VEGF 165 are diffusable, while the two larger forms (VEGF 189, VEGF 206) have a heparin binding domain and remain associated with the cell membrane [5].

VEGF is a potent mitogen for endothelial cells, but unlike other angiogenic factors, it does not promote proliferation of fibroblasts, vascular smooth muscle cells, corneal endothelial cells or other epithelial cells [6]. VEGF is also a known vascular permeability factor and it is 50,000 times more potent than histamine in inducing vascular permeability [7]. Several studies indicate that VEGF plays a crucial role in angiogenesis. Two independent gene knockout studies in mice have shown that expression of both alleles of VEGF is required for vasculogenesis/angiogenesis during embryogenesis [8, 9]. Inhibition of VEGF function by VEGF neutralizing antibody [10], anti-sense VEGF expression [11], or expression of a dominant-negative receptor [12] inhibited tumor cell (glioma) growth. In addition, VEGF receptor gene knockout resulted in an embryonic lethal phenotype [13]. Collectively these studies showed that VEGF plays an important role in normal and pathological vascular growth. Recently, several studies have indicated that VEGF acts as a survival factor in that it protects endothelial cells from apoptosis [14, 15].

Several studies showed that VEGF is expressed in small amounts in first trimester cytotrophoblast and syncytiotrophoblast and term syncytiotrophoblast (Fig 1) [16], while others [2, 17, 18] detected VEGF in first trimester villous trophoblast with very little in syncytiotrophoblast. However, some found VEGF expressed at higher levels in syncytiotrophoblast [17,19], while others [2, 18] found cytotrophoblast to express higher levels of VEGF protein. Furthermore, basal expression of VEGF seems to be much lower in comparison to PlGF, but VEGF expression increased greatly in trophoblast cultured under hypoxic conditions [20].

	PlGF	VEGF	flt-1	KDR
HUVE	+++	+/-	+++	+++
1st Trimester Trophoblast	+++	++	+++	–
Term Trophoblast	+++	+/-	+++	–

Figure 1: PlGF/VEGF and KDR/Flt-1 Expression In Trophoblast and HUVE Cells.
+ = low expression, ++ = moderate expression, +++ = high expression

Several factors regulate VEGF gene expression including hypoxia, cytokines, cellular differentiation, and transformation. Among these, oxygen tension plays a major role both in *vitro* and in *vivo* [21]. VEGF expression is up-regulated rapidly and reversibly by hypoxia in many normal and transformed cell types [22, 23]. Several growth factors like EGF, PDGF, TGF-β, FGF-2 and IGF-1 [24-27] and cytokines like TNF-α [28], and IL-1 [29] can up-regulate VEGF expression in certain cell types.

Placenta Growth Factor (PlGF)

Placenta growth factor (PlGF) is a member of the VEGF family of growth factors. The primary sequence of PlGF shows significant homology (53%) to the PDGF-like domain of VEGF. PlGF, a 46-50 kDa protein, like VEGF, is a secreted N-glycosylated homodimeric protein. The PlGF gene is localized to chromosome 14 and has seven exons [30, 31]. There are three known isoforms of PlGF that result from alternative splicing of mRNA, named PlGF-1 (PLGF131), PlGF-2 (PlGF 152) and PlGF-3 (PlGF 221); however recently, a new isoform of PlGF (PlGF-4) has been characterized [32 (unpublished data)]. The PlGF-2 gene consists of all seven exons, while PlGF-1 has six exons (exon six is missing) and PlGF-3 has the same six exons as PlGF-1 plus an additional 72-amino-acid-sequence inserted between exon IV and V. Exon VI encodes a heparin-binding domain and this allows PlGF-2 to bind heparin [33-35]. PlGF-4 has the same exons as PlGF-3, plus exon VI (similar to PlGF-2) [32]. PlGF-1 and PlGF-3 are diffusable, while PlGF-2 remains cell-associated. Biological activity of PlGF-2 is reported to be greater than that of PlGF-1, suggesting heparin binding may facilitate PlGF

activity [35, 36].

In contrast to widespread distribution of VEGF, prominent expression of PlGF is restricted to the placenta and human umbilical vein endothelial cells [33, 37]. Additionally, choriocarcinoma cell lines and some other carcinoma cells have been shown to express PlGF [38, 39]. The major site of synthesis of PlGF in the placenta is the trophoblast. Both normal cytotrophoblast and syncytiotrophoblast express PlGF [20, 1]. Until recently, it was not known which isoform of PlGF was expressed in trophoblast. However, a recent report showed that all three isoforms of PlGF, and a novel isoform (PlGF-4) are expressed by trophoblast (unpublished data). The restricted expression of PlGF suggests that it plays a significant role in the development and/or function of placentae.

PlGF has been shown to have mitogenic activity in endothelial cells [33, 40, 41], and is able to induce angiogenesis and endothelial migration *in vivo* [41]. VEGF and PlGF are known to form VEGF/PlGF heterodimers, which are highly potent endothelial cell mitogens [42].

Regulation of PlGF expression is not very well understood. Unlike VEGF, which is known to be regulated by cytokines and growth factors, it is not known whether cytokines affect PlGF expression. However, a recent study indicated that several cytokines and growth factors regulate PlGF expression; TNF-α seems to down-regulate PlGF expression in trophoblast, while EGF and VEGF up-regulate PlGF expression in human umbilical endothelial cells (HUVE cells) [32 (unpublished data)]. Expression of VEGF and PlGF seems to be differentially regulated. In some thyroid tumor lines, VEGF

expression is up-regulated while PlGF expression is down-regulated [43]. Similarly, in normal cells like cytotrophoblast, syncytiotrophoblast and HUVE cells, basal level of VEGF expression is less than that of PlGF [20, Torry *et al.* (unpublished data)]. Differential expression of both these growth factors seems to be due to regulatory effects of oxygen tension. In normal trophoblast, hypoxia upregulates VEGF and downregulates PlGF [20]. A recent report showed that hypoxia down-regulates PlGF while obstetrical complication like fetal growth restriction upregulates it. This study also showed that PlGF protein levels increase and VEGF protein levels decrease throughout pregnancy, consistent with placental oxygenation [44].

RECEPTORS OF VEGF AND PlGF

KDR and Flt-1

Receptors for the PlGF/VEGF family of growth factors include the fms-like tyrosine kinase Flt-1 (VEGFR-1) [45, 46] and the kinase insert domain containing (split kinase domain) receptor KDR (VEGR-2) [46, 47]. These receptors are homodimeric, tyrosine kinase receptors and have structural homology with PDGF receptors. Both Flt and KDR have seven immunoglobulin (Ig)-like extracellular domains, a single transmembrane region and a split intracellular tyrosine kinase domain [48]. Flt-1 and KDR expression is primarily restricted to vascular endothelial cells [49], although recent evidence shows that a few other cell types express one or both of these receptors. Flt-1 is expressed in trophoblast [1, 20], monocytes [50, 51] and renal mesengial cells [52], while KDR is expressed in hematopoietic stem cells, megakaryocytes and retinal progenitor

cells [53, 54]. Additionally, some tumor cells express Flt-1 or KDR. Flt-1 is expressed in placenta throughout gestation (Fig. 1). It is expressed in first trimester trophoblast, with slight down-regulation during the second trimester, and at low levels in term trophoblast [18]. This finding is further supported by another study, which indicates that term trophoblast express Flt-1 but not KDR [20], while endothelial cells express both Flt-1 and KDR [18, 20].

Flt-1 binds VEGF and PlGF with high affinity, while KDR binds only VEGF [31]. The differences in ligand specificity and the ability of Flt-1 to bind PlGF homodimers and VEGF/PlGF heterodimers [55] suggest that each receptor may convey unique signal transduction responses [56]. Recent studies demonstrated that both Flt-1 and KDR are essential for normal development of embryonic vasculature. Disruption or knockout of Flt-1 and KDR genes result in severe abnormalities of blood vessel formation and embryonic lethal phenotype [12, 13]. The expression of both Flt-1 and KDR seems to be affected by hypoxia. Transcription of both Flt-1 [57] and KDR [58] are enhanced by hypoxia, but the mechanism responsible for KDR up-regulation seems to be post-transcriptional. Up-regulation of these receptors by hypoxia may occur indirectly via VEGF, since VEGF regulates the expression of both of its receptors [59, 60].

Neuropilin

Recently, a new receptor for VEGF and PlGF has been characterized. Neuropilin-1, a 130-135 kDa glycoprotein is a receptor for semaphorin/collapsin family of proteins and is associated with neuronal cell guidance in the developing nervous system. In

addition to neuronal cells, neuropilin is expressed in endothelial cells. Neuropilin binds select isoforms of VEGF and PlGF, VEGF 165 and PlGF-2, and binding of PlGF-2 is heparin-dependent [61, 62]. Several studies indicate that neuropilin receptors have a short intracellular domain and are unlikely to function as independent receptors. Neuropilin receptors alone do not elicit any responses when bound to VEGF 165. KDR binds VEGF 165 more efficiently in cells expressing neuropilin-1, and elicits a stronger response to VEGF 165, suggesting neuropilin-1 serves as a coreceptor for KDR [62]. In contrast, neuropilin did not augment the biological activity of PlGF-2, indicating that neuropilin does not function as a Flt-1 coreceptor [61]. Nevertheless, neuropilin-1 seems to be an important regulator of blood vessel development because gene knockout studies in mice showed that embryos lacking neuropilin-1 gene die because of improper development of the cardiovascular system [63].

VEGF/KDR/Flt-1 and PlGF/Flt-1 Signal Transduction

VEGF/PlGF/KDR/Flt-1 signaling is quite complex. The reasons for this are at least two fold. Firstly, KDR and Flt-1 have individual specificities, where Flt-1 binds both VEGF and PlGF, while KDR binds VEGF only. Secondly, VEGF and PlGF form homo- and heterodimers that can activate different signal transduction pathways. Unlike VEGF/KDR signal transduction, VEGF/ PlGF/Flt signal transduction is not clearly understood.

Flt and KDR appear to have different signal transduction properties. Binding of VEGF to KDR/Flt-1 and PlGF to Flt-1 induces autophosphorylation of both receptors

[64, 65]on tyrosine residues. Both VEGF and PlGF promote association and phosphorylation of SH2-SH3-containing domain proteins. Phospholipase C-γ (PLC-γ), Nck, GTPase-activating protein (GAP), Grb2 (Growth factor-bound protein), Shc and Phosphatidylinositol 3 (PI-3)-kinase are phosphorylated and become associated with KDR in response to VEGF [66-68], while Nck, PLC-γ, and Grb2 seem to be associated with Flt-1 [66, 69, 70]. Biological responses induced by VEGF appear to be different in KDR- and Flt-1-expressing cells. KDR-transfected porcine aortic endothelial cells (PAE cells) exhibited changes in cell morphology, membrane ruffling, chemotaxis, mitogenesis, and MAPK activation in response to VEGF [64, 66, 71]. However, VEGF-induced activation of Flt-1 did not induce chemotaxis or mitogenesis in Flt-1-expressing PAE cells [64]. On the other hand, PlGF activated MAP kinase and stimulated mitogenicity, but did not activate PLC-γ or induce migration in Flt-1-expressing PAE cells [72]. Recent studies showed that both VEGF and PlGF stimulate proliferation but not migration and invasiveness in human first trimester trophoblast cell lines [73, 74], although the signaling pathways for this response are not known. Recently, VEGF has been implicated in protecting endothelial cells against serum withdrawal-induced apoptosis. KDR mediates this effect in endothelial cells through PI-3 kinase/Akt-dependent pathways, while both PlGF and Flt-1 failed to promote cell survival in endothelial cells [15].

SIGNAL TRANSDUCERS

Although many studies have focused on VEGF/KDR signal transduction pathways, Flt-1-mediated transduction is not clearly understood. Approaches to study Flt-1 mediated events in different endothelial cell types are complicated by the presence of KDR receptors. VEGF/KDR- and VEGF/PlGF/Flt-1-mediated signaling seems to be different in endothelial cells. Additionally, despite binding to the same receptors (Flt-1) (in HUVE cells), VEGF and PlGF activate different signaling pathways [75]. This divergence in the signaling pathways upstream of MAPKs suggests that cell type-specific upstream signaling molecules are present. Several potential regulatory molecules, like Nck and PLC-γ among others, may regulate and activate specific MAPK pathways. Furthermore, Nck and PLC-γ are known to associate with Flt-1 and KDR. Nck acts as a mediator in the JNK/p38 pathway, but not in the ERK1/2 pathway [76, 77]. Additionally, Nck seems to be involved in ERK activation in response to VEGF [68]. PLC-γ associates with Flt-1 and KDR receptors upon their activation, but also mediates many biological responses.

Growth factor binding to receptor protein tyrosine kinases (R-PTKs) induces their dimerization, resulting in activation of intrinsic protein tyrosine kinase activity and receptor autophosphorylation [78]. Phosphorylated receptor kinases become docking sites for proteins with SH2 and SH3 domains (Src homology 2 & 3 domains). This interaction appears to be a crucial step by which receptor kinases relay signals to downstream signaling pathways. The majority of SH2 and SH3 domain-containing

proteins possess intrinsic enzymatic activity, but there are other members of this family that lack intrinsic enzymatic activity and are therefore, called adapters. The major functions of these adapters, such as Crk, Grb2, and Nck, is to recruit effector molecules to tyrosine-phosphorylated kinases or their substrates.

Nck

Nck, a 47kDa oncoprotein composed of one SH2 and three SH3 domains is a common target for various cell surface receptors. It is implicated in coordinating various signaling pathways, including those of growth factor and cell adhesion receptors. Nck functions as a linker between receptors, via its SH2 domain, and to downstream SH3-binding effectors. Although Nck is known to mediate the formation of protein-protein complexes during signaling, little is known about its exact function. The SH2 domain of Nck has been known to bind to various growth factor receptors, including PDGF [79], hepatocyte growth factor [80], EGF [78], VEGF [67, 68], as well as Eph [81, 82], insulin receptor substrate, IRS-1 [83], and focal adhesion kinase [84]. There are several studies demonstrating that Nck binds to activated tyrosine kinases via its SH2 domain. However, little is known about the downstream effector pathways regulated by Nck via its SH3 domains. Recent studies have reported that the SH3 domains of Nck interact with several protein kinases, including the Ser/Thr protein kinases PAK (p21-activated kinase) [85], Prk2 [86], casein kinase I [87] and NIK (Nck interacting kinase) [77]. For the most part, the biological significance of the Nck's interactions with these kinases is still not clear. Overexpression of Nck in some cases leads to Ras activation via SOS, but dominant-

negative Nck does not block activation of Ras by cytokines [76]. Nck seems to act as a mediator in JNK/SAPK pathways but not ERK1/2 pathways [76, 77]. Nck is known to be associated with both KDR and Flt-1 receptors [65, 66, 68, 70] and phosphorylated in response to VEGF in endothelial cells [65, 67, 68]. However, PlGF/Flt-1 and Nck interactions are not known.

NIK (Nck interacting kinase), a homologue of sterile 20 kinase (ste 20) binds to Nck and MKKK1 (Mitogen activated protein kinase-kinase-kinase) and activates the JNK/SAPK pathway [76, 77]. Recruitment of Nck to tyrosine kinase receptors seems to couple ligand activation to c-Jun kinase. For example, Nck acts as an intermediary linking Eph A signaling to JNK activation [82]. Nck is also known to mediate activation of another serine /threonine kinase, PAK, with eventual activation of JNK and p38 [88, 89]. Nck is also involved in activation of JNK/SAPK pathway in response to growth hormone [90]. Thus, Nck, through its association with upstream growth factor receptors and downstream effectors such as tyrosine kinases or serine/threonine kinases, seems to be involved in regulation of multiple intracellular signaling events.

Phospholipase C-γ (PLC-γ)

PLC-γ belongs to a family of cellular proteins believed to play a significant role in intracellular pathways utilized by hormones and growth factors. Several growth factors including, EGF, FGF, PDGF and VEGF are known to activate PLC-γ through receptor mediated tyrosine phosphorylation of the specific PLC-γ isozyme [68, 91-94]. PLC-γ, a 145 kDa protein, contains two SH2 domains and one SH3 domain. Unlike Nck, PLC-γ

possesses intrinsic enzymatic activity [95]. Signaling across cell membranes often involves the activation of both phosphatidylinositol-specific PLC-γ and phosphoinositide-3- kinase (PI-3 kinase). PI-3 kinase phosphorylates and PLC-γ hydrolyze phoshotidylinositol-4,5-bis-phosphate to inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). These second messengers induce release of intracellular Ca2+ and activate protein kinase C (PKC) [95, 96]. Tyrosine phosphorylation of PLC-γ is necessary for its activation and this phosphorylation is brought about by its complex formation with growth factor receptors. Tyrosine phosphorylation of PLC-γ is believed to be involved in the mechanism by which tyrosine kinase-dependent growth factors receptors stimulate phosphatidylinositol- 4,5-bis-phosphate hydrolysis [91]. In response to growth factor stimulation, PLC-γ may either act as an enzyme to generate second messengers which in turn activate downstream signaling molecules, or as an adapter molecule by binding to growth factor receptors through SH2 domains and to downstream molecules through its SH3 domains [96].

PLC-γ associates with both KDR and Flt-1 receptors [66, 69, 70, 97], where it may play an important role in KDR/Flt-1-mediated signal transduction. PLC-γ seems to be the major target for KDR. VEGF via KDR induces activation of the ERK pathway and stimulates proliferation in primary endothelial cells [71, 98], in KDR-expressing NIH3T3 fibroblasts [71], and in KDR-expressing PAE cells [67]. Phosphorylation of PLC-γ and PLC-γ-mediated DNA synthesis is inhibited by a KDR receptor antibody [94], and a PLC-γ antibody, respectively [99]. Signaling by VEGF in endothelial cells results in phosphorylation of both of its receptors and PLC-γ, leading to eventual activation of

downstream kinases like ERK and JNK [100].

Like KDR, Flt-1 is also known to serve as a binding site for PLC-γ. There is ample evidence for the involvement of PLC-γ in KDR and Flt-1-mediated signal transduction in response to VEGF. However, the role of PLC-γ in PlGF mediated signaling is not clear [66, 69, 97]. Some reports show that PLC-γ associates with Flt-1 and KDR, and is phosphorylated in response to VEGF, but there is no evidence indicating phosphorylation of Flt-1 in response to PlGF. A recent report showed that PLC-γ was not phosphorylated in PlGF-stimulated, Flt-1-expressing PAE cells,while it was phosphorylated in VEGF-stimulated KDR-expressing PAE cells.

SIGNAL TRANSDUCTION PATHWAYS

Cells respond to extracellular signals by transmitting intracellular instructions to coordinate appropriate responses. Mitogen activated protein kinase (MAPK) cascades are activated in response to various extracellular stimuli, including growth factors/cytokines and environmental stresses, and are mediators of signal transduction from the cell surface to the nucleus. The MAPK signaling pathway comprises three major phosphorylation cascades; the extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinases, and p38 kinases (Fig 2). Each pathway is regulated by distinct stimuli, and each have different as well as overlapping target specificities. Once activated, they translocate to the nucleus to regulate activities of transcription factors and thereby control gene expression. The ERKs are preferentially activated in response to mitogens/growth

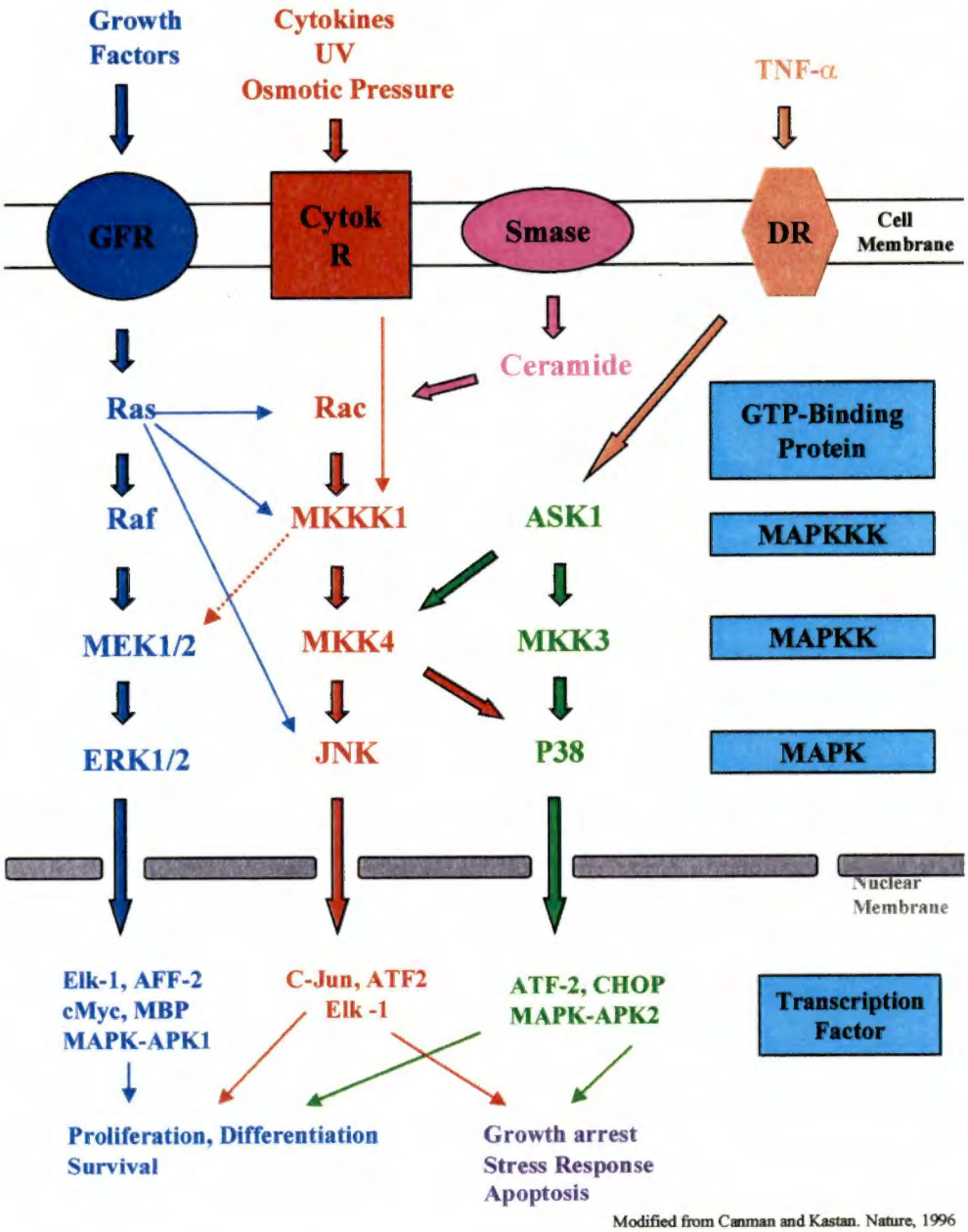


Figure 2: Mitogen Activated Protein Kinase (MAPK) Pathways.
GFR = growth factor; Cytok R = cytokine receptor; Smase = sphingomyeliase; DR = death receptor.

factors, and are mainly associated with cell proliferation or differentiation. JNK and p38 pathways are collectively termed stress-activated protein kinases because they are typically activated by various stress-related stimuli and proinflammatory cytokines [101-103].

c-Jun Amino Terminal Kinase (JNK)

The JNK family of MAPKs consists of JNK1, 2, and 3. Alternative splicing of JNK genes gives rise to at least ten isoforms; JNK-1 (α1, α2, β1, β2), JNK-2 (α1, α2, β1, β2), and JNK-3 (α1, α2). JNK-1 and -2 are expressed in many tissues, while JNK-3 is only expressed in brain, heart and testis [102, 104]. JNKs are activated through dual phosphorylation of Thr and Tyr residues in response to stress. JNK is regulated by the dual specificity sernine/threonine kinases, mitogen activated protein kinase kinase (MKK-4), and mitogen activated protein kinase kinase (MKK-7). MKK-4 activates both JNK and p38, while MKK-7 activates JNK only [105]. MKK-4 is in turn regulated by MKKK-1, apoptosis signal-regulating kinase (ASK-1) and some of the MLK (mixed lineage kinase) family members [106-108]. ASK-1 activates both MKK-3 and MKK-4, leading to activation of p38 and JNK [107]. Further upstream regulators of JNK include members of the Rho family of small GTPases (Ras,Rac1, Rac2, cdc42, Rho) [109] and, p21 Ras activated kinase (PAKs) [89]. JNKs are activated in response to diverse stimuli including DNA damage, UV irradiation, heat shock, IL-1, ceramide, TNF-α, and Fas-L. In addition, JNK is also induced by mitogenic signals, including growth factors, cytokines, Ras, and other signals like CD40 ligation and T cell activation. Activated JNK

in turn regulates activities of transcription factors like ELK-1, SAP-1, ATF-2 and c-Jun (components of dimeric transcription factor AP-1) [102].

Functions of JNK

JNK and Apoptosis

It is generally accepted that activation of JNK and p38 kinases inhibits cell growth or promotes cell death, while ERK is usually activated by growth factors/cytokines and hormones that stimulate cell growth / proliferation [110]. Activated JNK is known to mediate apoptosis in many cell types. JNK-induced apoptosis is observed in neuronal injury and in myocardial infarction [111, 112], and dominant-negative inhibitors of the JNK pathway inhibit JNK-mediated apoptosis [113, 114]. Similarly, JNK pathways also mediate apoptosis following injury induced by UV irradiation and heat [115], and expression of dominant-negative mutants of JNK block γ-radiation and UVC-induced cell death [116]. A specific JNK pathway inhibitor, CEP-1347/KT7515, protects chick neurons [117] and embryonic neurons [118] from apoptosis. In the absence of growth factors and serum, ceramide-mediated JNK activity induces apoptosis in pheochromocytoma (PC12) cells [119]. Several studies have implicated JNK in TNF- induced apoptosis [102, 107, 120-122]. However, several reports show that activation of JNK can be mediated through a non-cytotoxic TRAF-2-dependent pathway initiated by TNF-α, which is not linked to apoptosis [123]. Additionally there is a recent report in which TNF-α-induced ASK-1 regulates JNK activity, which is instrumental in executing the apoptotic signal of TNF-α [107, 122]. These studies provide strong evidence for a JNK-stimulated apoptotic pathway.

Although many stimuli seem to induce apoptosis via JNK activation, whether JNK activation is required for apoptosis still remains controversial.

JNK and Survival

JNKs are also involved in other cellular events beside apoptosis, including cellular growth, differentiation and transformation. A recent study showed that activation of JNK in Il-3-dependent BAF_3 (pre-B cells) was not involved in apoptosis, but rather mediated proliferation of these cells in response to IL-3. IL-3-stimulated proliferation of BAF_3 was inhibited when JNK activity was specifically inhibited by $M_3/6$ (JNK inhibitor, a phosphatase) [124, 125]. Selective activation of JNK by CD40 on B cells rescues these cells from anti-IgM (receptor cross-linking) mediated apoptosis [126]. Activation of JNK and subsequent phosphorylation of c-Jun are required for hepatocyte proliferation in hepatic regeneration after partial hepatectomy [127]. SEK-1, a direct activator of JNK, protects thymocytes from Fas- and CD3- mediated apoptosis [128].

Other Functions of JNK

The JNK pathway also is involved in cell activation and induction of other genes. JNK is specifically involved in T cell activation and IL-2 induction [129]. A recent report also showed that JNK is required for IL-1-induced IL-6 (which promotes cell growth) and IL-8 (which plays a role in inflammation) gene expression in the keratinocyte cell line KB; inhibition of JNK activation in these cells inhibits IL-6 and IL-8 gene expression [130]. JNK is activated during co-stimulation of T cells [131] and may contribute to the secretion of IL-2, and the proliferation of thymocytes [132]. In addition, JNK is activated during the differentiation of Th1 and Th2 cells, and this may help in the

differentiation process [133]. These observations confirm that JNK plays a role in regulation of normal cell functions.

p38 Kinases

The p38 mitogen-activated protein kinases are mammalian homologues of the HOG-1 MAP kinase of *S. cerevisiae* which is necessary for their growth under hyperosmolaric conditions. p38 kinase is activated by both physical and chemical stresses including UV irradiation, osmotic stress, heat, bacterial lipopolysachariide and the pro-inflammatory cytokines, TNF-α, IL-1 [134-136]. The p38 family of MAP kinase consists of p38α, p38β, p38γ and p38δ [134]. p38 kinase is activated by dual specificity MAP kinase kinases, including MKK3, MKK6, and MKK4, that phosphorylate p38 on threonine 180 and tyrosine 182 residues [137]. Activated p38 in turn regulates activities of several transcription factors including ATF2, CHOP, ElK-1, MEF-2C, and other protein kinases such as mitogen-activated protein kinase-activated protein-2 (MAPKAP-2) and MAPKAPK-3, both of which activate heat shock proteins (HSP) 25/27 [138].

Many stimuli that activate JNK also activate the p38 kinase pathway. However, the biological responses elicited by activation of these kinases are distinct, and in many cases, only one of the pathways is activated. These pathways may also have different biological effects on the same cell type [139-141].

Functions of p38 Kinase

p38 kinase and Apoptosis

Like JNK, p38 kinase is implicated in neuronal apoptosis [119], B lymphocyte

apoptosis [142], TNF-α induced apoptosis [122], Fas-induced apoptosis [143], apoptosis induced by NGF withdrawal [144], and UV-induced apoptosis [145].

p38 Kinase and Survival

Like JNK, p38 inhibits apoptosis in certain situations. p38 kinase inhibits apoptosis in cardiac myocytes [146, 147], Jurkat T cells [148], mast cells [149] and WEH-23 cells (B lymphoma cell line) [150].

Other Functions of p38 Kinase

p38 kinase is also involved in other cellular functions like growth, differentiation and gene activation. p38 is required for serum-stimulated growth [151]. TNF-α induced activation of p38 leads to gene induction but not cytotoxicity in L929 cells [152]. Heat shock protein-27 (HSP-27), which is involved in growth and differentiation in many cell types is regulated by p38 kinase. p38 kinase is also involved in differentiation of pheochromocytoma (PC12) cells [153], and proliferation and differentiation in breast carcinoma cells (MCF-7) cells [154]. VEGF-induced activation of p38 is known to mediate actin reorganization and cell migration in endothelial cells [155]. Additionally, hematopoietic growth factors regulate the development and function of hematopoietic cells through activation of p38 kinases [149].

Extracellular Signal Regulated Kinase (ERK)

ERK-1 and ERK-2 are expressed in almost all cells. Both kinases are activated by phosphorylation of Thr/Tyr residues by MEK-1. MEK-1 is in turn activated by other upstream kinases like Raf [156], and MKKK-1 [106]. Activated ERK-1 and -2 regulate

activities of various transcription factors such as, ELK-1 [157], c-Jun [158], c-Fos [159], c-Myc , p53 [160] and other signaling proteins, such as MAPK-APKs [161], cPLA2, SOS-1[162]. ERKs are activated by growth factors/cytokines and hormones. They are mainly involved in mediating biological responses like cellular growth, proliferation and cell survival in response to various stimuli.

Some cytokines/growth factors and serum inhibit apoptosis by either down regulating the stress-activated protein kinases, JNK/p38 kinase, or by up-regulating ERK pathways. Many growth factors inhibit apoptosis in various cell types by activating ERK pathways. IGF-1 inhibits apoptosis in PC-12 cells by activating ERK pathways [163]. Serum and NGF protect HeLa carcinoma cells and PC-12 (pheochromocytoma) cells from apoptosis by up-regulating the ERK pathway and down-regulating the p38 kinase pathway [114, 139]. IGF-1 rescues neuroblastoma cells from apoptosis by inhibiting the JNK pathway, and up-regulating the ERK pathway [140]. Insulin protects fetal neurons from apoptosis by inhibiting p38 kinase [164]. Thus, cell survival and death are dictated by a balance between ERK, JNK, and p38 kinase signaling.

APOPTOSIS

Two common forms of cell death have been described in vertebrate tissue. Necrosis refers to the morphology seen when cells die from severe and sudden injury, such as ischemia, sustained hyperthermia, or physical or chemical trauma. The earliest changes include swelling of the cytoplasm and organelles, especially the mitochondria. The changes ultimately lead to organelle dissolution and rupture of the plasma

membranes, allowing cellular contents to leak out into the extracellular space, provoking an inflammatory response [165].

In contrast to necrosis, apoptosis is a morphologically distinct, energy dependent, suicidal process, involving a series of well-regulated synthetic events. This form of cell death occurs in many different tissues under various conditions and is the major mode of cell death [166].

The growth, development and function of most tissues and organs require the appropriate balance between cell growth and death. Apoptosis is essential for normal cell growth, development of tissues and organs, and is required for maintaining tissue homeostasis. Apoptosis is an active process of programmed cell death in multicellular organisms, characterized by cytoplasmic shrinkage, nuclear condensation and DNA fragmentation in 200-base pair fragments [167, 168].

Mechanism of Apoptosis

Apoptosis can be triggered in many cell types by various stumuli. Induction of apoptosis can be divided into two groups.

1. Deprivation of growth factors or serum causes apoptosis in growth factor-dependent cells [169, 170]. These growth factors and cytokines mediate survival signals and protect cells from apoptosis. The survival and differentiation of neurons depend on specific neurotrophic factors like NGF and insulin [164]. Serum deprivation induces apoptosis in various cell types, like endothelial cells [15, 171], PC12 cells [172], epithelial cells [173],

fibroblasts [174], porcine granulosa cells [175], and vascular smooth muscle cells [176].

2. The second type includes apoptosis in response to physical, chemical, or biological assaults such as ionizing radiation, oxidants, free radicals, heat shock, exposure to inflammatory cytokines like TNF-α and IFN-γ [177], bacterial toxins or toxins like ethanol and anti-cancer drugs.

Cytokine Induced Apoptosis

Inflammatory cytokines like TNF-α and INF-γ induce apoptosis in many cell types and cell lines [107, 178-181]. TNF-α -induced responses are mediated through two distinct cell surface receptors, TNFR-1/p55 or TNFR-2/ p75. TNF induces apoptosis through several pathways. TNFR-1 activation results in generation of ceramide by Smase (sphingomyelinase) which is responsible for mediating the cytotoxic effect of TNF-α. Ceramide -activated JNK is known to induce apoptosis in some cells [120]. However, there is evidence showing that JNK activation is not involved in TNF-α mediated-apoptosis [123]. TNF-α-mediated apoptosis may be due to activation of the caspase cascade, through RIP mediated apoptosis, or ASK-1-mediated activation of SAPKs. However, the role of SAPKs in TNF-α induced-apoptosis remains controversial.

Apoptosis in the Female Reproductive Tract

Apoptosis plays a major role in embryonic development, pregnancy and normal development and maintenance of reproductive organs. Throughout the menstrual cycle,

cell death and proliferation occur in the female reproductive tract in a regulated fashion. The process of follicular atresia, luteolysis, and cyclic shedding of the endometrium involves apoptosis [182, 183].

Apoptosis in the placenta is also observed during normal pregnancy and pregnancies complicated by infections or other pathologies. Discontinuities in the integrity of the syncytiotrophoblast may be due to apoptosis and apoptosis is responsible for the progressive disappearance of trophoblast in the chorionic laeve as pregnancy approaches term in normal pregnancies. Apoptosis has been demonstrated in normal placentae from both first and third trimesters and the rate of apoptosis increases significantly as pregnancy progresses [184-187]. Recent studies have shown that apoptosis occurs in the conceptus during the first trimester of normal pregnancy [188]. These observations suggest that apoptosis plays an important role in the normal development and aging of placentae.

In addition to its role in normal pregnancy, trophoblast apoptosis has been implicated in several obstetrical complications. Apoptosis is known to increase greatly in cases of spontaneous abortions [188]. Apoptosis in the placenta leads to fetal growth retardation in rats [189] and the incidence of placental apoptosis is significantly greater in human pregnancies complicated with IUGR (intrauterine growth restriction). Placental bed hypoxia is generally thought to occur in preeclampsia and IUGR, and is known to induce apoptosis in many cell types [190]. A recent study also indicated that pre-eclampsia is associated with widespread apoptosis of the cytotrophoblast that invade the uterus [191]. Our present study indicated that angiogenic growth factors like PlGF and

VEGF may protect trophoblast from growth factor withdrawal-induced apoptosis by activation of the SAPKs, JNK and p38 kinase [75]. Placental bed hypoxia in preeclampsia and IUGR may down-regulate PlGF and increase trophoblast susceptibility to stress-induced apoptosis. In addition, decreased serum levels of PlGF observed during preeclampsia [192] contribute to increased trophoblast apoptosis, and which in turn may have a significant role in the pathophysiology of the disease.

Tumor necroses factor-alpha (TNF-α) and gamma interferon (IFN-γ) are expressed in human placental villi and uterine cells in early and late gestation [193, 194]. Studies have shown that TNF- α and IFN-γ induce apoptosis in primary trophoblast *in vitro* [180, 195, 196], and that EGF protects trophoblast from TNF-α- and IFN-γ-induced apoptosis [180]. In addition, a recent study showed that VEGF inhibits TNF- α-induced apoptosis in endothelial cells [197]. Several studies have shown that EGF is able to protect from pro-inflammatory cytokine-induced apoptosis in trophoblast [75, 180].

All of the above reports suggest that regulation of apoptosis is quite complex and multifactorial. The decision to die or live depends on the dynamic balance between growth factor activated-MAPKs and stress activated-JNK/p38 pathways, expression of anti-apoptotic and pro-apoptotic proteins, apoptotic signals and anti-apoptotic signals, generated by various extracellular and intracellular stimuli.

Oncogenes Modulating Apoptosis

Apoptosis can be induced by two ways: **i.** When the cells are exposed to apoptotic inducing agents (cytokines/or growth factors, environmental stresses, genotoxic

substances etc.), or **ii.** when cells lose apoptotic suppression signals, i.e., withdrawal of serum/cytokines/growth factors, etc.

Serum and growth factors/cytokines regulate cell survival through different mechanisms, including activation or inhibition of different signal transduction pathways, by up-regulating anti-apoptotic proteins or downregulating expression of anti-apoptotic proteins.

Akt

The serine /threonine kinase Akt (Protein Kinase B), a proto-oncogene, may play a central role in growth factor-dependent cell survival. Many growth factors utilize PI-3 kinase/Akt signal transduction pathways to inhibit apoptosis. Vascular endothelial growth factor protects endothelial cells from serum-deprivation-induced apoptosis through the PI-3 kinase/Akt signaling pathway, and Akt activation is essential for VEGF-mediated induction of Bcl-2 [14, 198]. Similarly, many growth factors, like IGF, NGF [199, 200], insulin [201] and serum [139, 199] mediate their survival effects in various cell types in a PI-3 kinase-dependent fashion. In addition, inhibition of Akt activity with wortmannin abrogates the protective effect of serum/growth factors on cellular survival.

Briefly, when receptors bind cytokines/growth factors, they are phosphorylated and become docking sites for SH2 domain-containing proteins like PI-3 kinase. Activated PI-3 kinase at the cell membrane phosphorylates the 3'-position of the inositol ring in phosphatidylinositol 4,5-bisphosphate to generate phosphatidylinositol-3,4,5-triphosphate (PIP_3). The phosphorylated product binds to Akt, translocates it to membrane and activates it [202]. Activated Akt then phosphorylates the pro-apoptotic

protein Bad, resulting in sequestration of Bad by 14-3-3 proteins. This prevents Bad from heterodimerizing with Bcl-2. Thus, the Bcl-2 or Bcl-X_L /Apaf-1 (apoptosis-associated factor) /procaspase interaction remains intact. On the other hand, in the absence of cytokines/growth factors, Bad is dephosphorylated and dissociates from 14-3-3 proteins and heterodimerizes with Bcl-2/or Bcl-X_L. This heterodimerization disrupts the Bcl-2/Bcl-X_L/Apaf-1/pro-caspase interaction, setting off the caspase cascade leading to apoptosis [203, 204]. Akt also can inactivate caspase-9 directly by phosphorylation [205].

Bcl-2

Some growth factor/cytokines or serum rescue cells from apoptosis by either up-regulating anti-apoptotic proteins, or down-regulating anti-apoptotic proteins. Growth factors like IGF, EGF [206, 207], FGF, and NGF [208, 209] inhibit apoptosis either by up-regulating the expression of Bcl-2 and Bcl-X_L, or down-regulating Bcl-X_S.

The proto-oncogene Bcl-2 and its related proteins comprise a family of apoptosis regulatory gene products, which may be either pro-apoptotic or anti-apoptotic. Bcl-2 is an antiapoptotic intracellular protein that is predominantly localized in the outer mitochondrial membrane, nuclear membrane and endoplasmic reticulum (ER) [210]. All the members of Bcl-2 family possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4). Most death-inhibitory members contain at least BH1 and BH2, and those most similar to Bcl-2 have all four BH domains. Bcl-2 homology domains control the ability of these proteins to dimerize and function as regulators of apoptosis [210]. Pro- and anti-apoptotic Bcl-2 family members regulate

each other's functions, and they also heterodimerize. Their ratio seems to determine whether a cell will respond to an apoptotic signal [211].

Bcl-2 is known to protect cells against the effects of hypoxia, diverse cytotoxic insults like cytokine withdrawal, dexamethasone, staurosporine and cytotoxic drugs [212-216].

The Bcl-2 family of proteins plays a major role in preventing apoptosis caused by withdrawal of growth factors/cytokines. Viability of certain cytokine-dependent cells depends on expression of Bcl-2. Both VEGF (in HUVE cells) and EGF (in keratinocytes) are known to increase Bcl-2 expression, and protect cells from apoptosis under serum withdrawal conditions [14, 207]. Cytokine-bound receptors prevent apoptosis by inducing phosphorylation of apoptosis-inducing Bad, making it unavailable for dimerization with Bcl-2 or Bcl-X_L. In the absence of cytokines and growth factors, dephosphorylated Bad dimerizes with Bcl-2, disrupting Bcl-2-Apaf-1-inactive cystine-protease interaction, there by activating a cystine protease (caspase-9) inducing apoptosis [217].

The link between receptor-mediated signal transduction and Bcl-2 function is not clearly defined. Bcl-2 is phosphorylated on serine residues in response to many stimuli, like growth factors/cytokines or cytotoxic drugs [218-220]. The functional role of Bcl-2 phosphorylation in the promotion [218, 221] or inhibition [219, 222] of apoptosis is controversial. However, there are several studies showing that phosphorylation of Bcl-2 inhibits apoptosis in some cell types [219, 222]. Activation of JNK/SAPK in other cell types has been shown to phosphorylate Bcl-2 and regulate its functional activity [223].

In a hematopoietic cell line, Bcl-2 is phosphorylated on serine residues, following exposure to IL-3 [220], but it is also hyperphosphorylated on serine residues following treatment with Taxol [218]. Furthermore, the biological relevance of Bcl-2 phosphorylation is not as clear as that of BAD. It remains uncertain whether phosphorylation of Bcl-2 leads to its activation or deactivation. Additionally, it has been suggested that since phosphorylation sites of Bcl-2 have not been detected in all systems, it may be possible that distinct phosphorylation sites may regulate Bcl-2 function differentially [224].

Many growth factors like IGF, NGF, IL-3, and GM-CSF among others have been shown to facilitate survival in different cell types. However, their function seems to be independent of Bcl-2, because these growth factors/cytokines do not regulate expression of Bcl-2 [225, 226]. On the other hand, there are several growth factors like VEGF (in endothelial cells) and equine chorionic gonadotropin (eCG) (in granulosa cells) that up-regulate Bcl-2 expression [14, 227].

Regulation of Bcl-2 function is multifactorial. Thus, along with cytokines, relative levels of antiapoptotic Bcl-2 and proapoptotic (Bad) proteins, post-transcriptional modification of Bcl-2 or related proteins, can influence the propensity of a cell to live or die [211].

Bcl-2 is expressed in syncytiotrophoblast throughout pregnancy [228-230]. A rise in Bcl-2 protein levels is evident in placental extracts from ten weeks of gestation to term. There is a differentiation-dependent pattern of Bcl-2 expression in trophoblast, in that cytotrophoblast and JEG-3 choriocarcinoma cells express very low to no Bcl-2, while

terminally differentiated syncytiotrophoblast express abundant amounts of Bcl-2 protein. Expression of Bcl-2 in syncytiotrophoblast suggest that it may protect trophoblast from apoptosis [231-233]. Treatment of JEG-3 cells with 8-Br-cAMP induces genes characteristic of the syncytiotrophoblast and raises levels of Bcl-2 protein [231]. This differentiation-dependent pattern of Bcl-2 expression in the placenta suggests that Bcl-2 may protect trophoblast from apoptosis, and is necessary for the preservation of placental function during gestation.

PART II.

FLT-1 MEDIATED SIGNAL TRANSDUCTION AND FUNCTIONS OF VEGF AND PlGF IN NORMAL HUMAN TROPHOBLAST

ABSTRACT

Trophoblast function and vascular development in the placenta and endometrium during pregnancy are critical to successful gestation. Although growth factors and cytokines are thought to govern trophoblast function, little is known about the specific responses they elicit in trophoblast. This study was conducted to investigate VEGF and PlGF as potential regulators of placental growth and development. In addition to their role as paracrine angiogenic factors within the placenta and endometrium, presence of their receptor, Flt-1, on trophoblast suggests that both VEGF and PlGF may have autocrine role(s) in regulating trophoblast function. To elucidate their roles in trophoblast, the signal transduction and functional responses of primary human term trophoblast to VEGF and PlGF were investigated. Exogenous PlGF and VEGF induced specific activation of the stress-activated protein kinase (SAPK) pathways, c-Jun-NH_2 terminal kinase and p38 kinase, in primary trophoblast, with little to no induction of the extracellular signal regulated kinases (ERK1- and -2). In contrast, both PlGF and VEGF induced significant ERK1 and 2 activities, but not SAPK activity, in human umbilical vein endothelial (HUVE) cells. VEGF and PlGF both phosphorylated/activated SH2-SH3 -domain containing proteins like Nck and PLC-γ in endothelial cells and trophoblast.

Functionally, PlGF and VEGF protected trophoblast from growth factor withdrawal-induced apoptosis, but were unable to protect trophoblast from apoptosis induced by the proinflammatory cytokines, tumor necrosis factor -α (TNF-α) and interferon- γ (INF-γ). VEGF phosphorylated/activated Akt (protein kinase B) in endothelial cells and

trophoblast, while PlGF had no effect on Akt phosphorylation/activation in either endothelial cells or trophoblast. VEGF, but not PlGF upregulated the antiapoptotic protein Bcl-2 in endothelial cells, while neither factor modulated Bcl-2 protein levels in trophoblast. These findings suggested that VEGF and PlGF protect trophoblast from stress induced apoptosis by Bcl-2- independent pathway (s). Collectively, these results provide the first direct evidence of a biochemical and functional role for PlGF/VEGF/Flt-1 in normal trophoblast and suggest that aberrant PlGF and VEGF expression during pregnancy may impact trophoblast function as well as vascularity within the placental bed.

INTRODUCTION

Successful pregnancy is dependent upon adequate vascular growth, permeability and remodeling in the placenta and the endometrium. Vascular and placental insufficiencies are associated with many obstetrical complications, notably spontaneous abortions, preeclampsia and intrauterine growth retardation (IUGR). During normal implantation and placentation, fetal trophoblast invade maternal endometrium, myometrium and remodel spiral arteries, converting them into low resistance high capacity vessels. This process is necessary to enhance uterine and placental blood flow in pregnancy. However, shallow invasion of trophoblast and inadequate conversion of maternal arteries leads to poor placental perfusion and placental hypoxia and may eventually result in fetal and placental growth retardation and preeclampsia [234, 235].

Many growth factors and cytokines are known to govern trophoblast function. However, modes of their action or specific functional responses elicited by these growth factors in trophoblast are not well understood.

Expression of two angiogenic growth factors, vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) in normal trophoblast has been characterized [20]. Receptors for the VEGF family of growth factors include fms-like tyrosine kinase, Flt-1 [45, 236]) and a kinase insert domain-containing receptor, KDR [47]. Flt-1 binds VEGF and PlGF with high affinity, while KDR binds only VEGF. Flt-1 and KDR expression is primarily restricted to vascular endothelial cells, [49] although recent evidence shows that trophoblast express Flt-1 [1, 20] (Fig.1). Isolated term trophoblast express high levels of PlGF and low levels of VEGF when cultured in normoxia (21% O_2). However, strong induction of expression of VEGF and down regulation of PlGF occurs when trophoblast are cultured under hypoxic conditions (1% O_2). Studies have shown that both Flt-1 and KDR are also up-regulated by hypoxia [57, 58]). Trophoblast expression of both VEGF and PlGF is modulated by oxygen tension, suggesting that trophoblast may directly influence local vascular growth, permeability, stability, and remodeling during pregnancy. The current research involves investigation of VEGF and PlGF and their receptors, Flt-1 and KDR, as potential regulators of placental growth and development. Both VEGF and PlGF are known to be mitogenic to endothelial cells *in vitro* and are able to induce all aspects of angiogenesis *in vivo*. Even though the biological functions of VEGF in endothelial cells are well documented, its effects on trophoblast are not known. Furthermore, the biological functions of Flt-1 are not known.

Since biological activities mediated by Flt-1 and KDR receptors seem to be different [64], it is important to determine and compare signaling pathways mediated by Flt-1 receptors in term trophoblast, and KDR- and Flt-1-mediated signaling in endothelial cells.

The production of PlGF and VEGF by trophoblast and the presence of Flt-1 receptors on trophoblast raises the possibility that both VEGF and PlGF may have active roles in regulating trophoblast functions through autocrine mechanisms, in addition to their angiogenic roles in placenta and endometrium. However, the biochemical responses and physiological significance of Flt-1/VEGF/PlGF interactions in normal trophoblast are not known.

The long-term goal of this project is to determine the molecular and cellular responses of normal trophoblast to VEGF and PlGF. Results of these studies may elucidate novel functions of these angiogenic factors in pregnancy. Given that trophoblast function and vascular development during pregnancy are critical to successful gestation, these studies may also provide insights in to new therapeutic avenues for reproductive failures.

The long term goal of this project will be attained by the following specific aims.

Specific Aims:

1. Define the molecular responses of normal trophoblast to PlGF and VEGF stimulation.
   1 a. Determine whether exogenous PlGF and VEGF induce MAPK (SAPKs and ERK) responses in trophoblast.

1 b. Compare VEGF- and PlGF-induced ERK and SAPK responses in trophoblast and human umbilical vein endothelial cells (HUVE).

1 c. Characterize the upstream regulatory signal transduction components responsible for disparate ERK and SAPK signal transduction responses in trophoblast and endothelial cells.

2. Determine the cellular function of PlGF and VEGF in trophoblast.

2 a. Determine the ability of PlGF and VEGF to protect trophoblast from apoptosis.

2 b. Determine the molecular mechanisms responsible for anti-apoptotic effects of VEGF and PlGF in trophoblast.

MATERIALS AND METHODS

Reagents

Keratinocyte growth media (KGM), keratinocyte basal media (KBM), bovine pituitary extract (BPE) were purchased from Clonetics Corp. (San Diego, CA) and RPMI 1640 from Bio-Whitaker (Walkersville,MD); Dulbeccos Modified Eagles-Medium (DMEM) - high glucose (HG), 3-4,5-dimethylthizol-2,5-diphenyl-tetrazolium bromide (MTT), heparin, wortmannin, and LY-294002 were from Sigma Chemical Co. (St. Louis, MO). rhVEGF, TNF-α and INF-γ were from R&D Systems (Minneapolis, MN); rhepidermal growth factor (EGF), myelin basic protein (MPB), anti-bovine Nck were from Upstate Biotechnology (Lake Placid, NY). Anti-c-Jun-N-terminal kinase (JNK-1), extra-cellular signal regulated kinase 2 (ERK-2), p38, goat anti-rabbit horseradish

peroxidase (HRP)-conjugated antibodies, and glutathione-S-transferase (GST)-c-Jun fusion protein were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse horseradish peroxidase HRP-conjugated antibody was from Jackson Immuno Research laboratories (West Grove, PA). Rabbit anti-phosphotyrosine antibody was purchased from Transduction laboratory (Lexington, KY). Mouse anti-human Bcl-2 antibody was purchased from DAKO Corporation (Carpinteria, CA). Chemiluminescence reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Percoll was obtained from Pharmacia and Upjohn (Kalamazoo, MI). Anti-phospho Akt and Akt antibodies were from New England BioLabs (Beverly, MA).

Isolation and Cell Culture

Human term trophoblast were isolated and cultured as follows. 24h prior to collection of placenta, digestion medium was made up with 25 mM HEPES and 1X HBSS (Ca++ and Mg++ free). Two Percoll gradients were prepared (ranging from 25 % - 50 %) in 5 % increments for every 40-50 gm of tissue. Immediately prior to the collection of the placenta, trypsin was weighed out in its three digestion aliquots, but was not added to the digestion media (0.19 gm, 0.25 gm and 0.19 gm added to 150 ml, 100 ml and 75 ml of digestion media, respectively). Fetal calf serum was aliquoted into 50 ml tubes (5 ml/tube). Percoll gradients were layered slowly starting with 50 % without mixing. Immediately after delivery, the placenta was placed on ice. 3-5 cotyledons (depending on the size) were removed from the placenta, large blood clots and calcium deposits were removed and the tissue was finely minced with sterile scissors. The minced

tissue was thoroughly washed with ice-cold PBS through sterile gauge until the PBS washed through clearly with minimal blood. Approximately 50 g was placed in a large flask and warmed digestion media containing 0.19 gm of trypsin and 150 µl (300 µg) of DNase was added. The tissue and digestion media were placed in a shaking incubator (37°C) for 30 min. This was repeated for two more times with different amounts of trypsin (0.25 gm trypsin + 100 µl (200 µg) DNase /100 ml of digestion media for second digestion, and 0.19 gm trypsin + 75 µl (150 µg) DNase /75 ml of digestion media for the third digestion). After each digestion step, the supernatant was collected and passed through a Falcon cell strainer and layered over 5 ml of FCS preloaded in 50 ml tubes. Tubes were centrifuged 1000 x g (1700 rpms) for 5 min. The supernatant was aspirated and the cell pellet was resuspended in 8 ml of DMEM-HG and stored at 37°C. After the third digestion, cells were pooled, additional media was added to help 'wash' the cells, and the cells were centrifuged again for 5 min. The pellet was resuspended in 10 ml DMEM-HG and equal amounts (5 ml each) was layered carefully over the two Percoll gradients and centrifuged at 1200 x g for 20-25 min. Trophoblast banded at 34 %-42 % Percoll, RBCs fall below 25 %, and cell digestion debris collected on the top gradient. Cell debris was aspirated and layers containing trophoblast were collected and washed with fresh DMEM-HG and centrifuged at 1000 x g for 10 min. Wash media was removed and the cell pellet was resuspended in DMEM-HG. An aliquot of cells was diluted in DMEM-HG 1:10 and counted on a hemocytometer.

This procedure yields ~ 90 % pure population of trophoblast as assessed by antibodies to vimentin and cytokeratin [2]. Out of 10 % vimentin-positive cells, ~ 7.5 %

were macrophages, and the rest were other contaminants like fibroblasts or cellular debris. There were no endothelial cells in the trophoblast preparation. To detect contaminating macrophages and endothelial cells, trophoblast cultures were analyzed with monoclonal antibody to CD 68 (clone EBM 11) and CD 31 (clone JCI 70 A), respectively.

Freshly isolated cytotrophoblast were plated in KGM containing insulin, 5 μg/ml; EGF, 0.1 μg/ml; hydrocortisone, 0.5 μg /ml; and 10 % fetal calf serum (FCS). By 48 h, mononuclear trophoblast aggregate in to multinuclear colonies and fuse to form multinucleated syncytiotrophoblast. Syncytiotrophoblast were rendered quiescent by incubation in KBM without FCS or mitogens for 18-24 h before treatment with growth factors. Endothelial cells collected from human umbilical veins [237] were cultured in RPMI-1640 with L-glutamine (25 mM) and HEPES (25 mM) supplemented with bovine pituitary extract (30 μg/ml), 10 % FCS, 219 μg/ml L-glutamine, 90 μg/ml heparin. Human umbilical vein endothelial (HUVE) cells were cultured to 80 % confluency and used before passage 8. They were serum-starved in serum free RPMI for 18-24 h before growth factor treatment.

***In Vitro* Kinase Assays for JNK and ERK-1 and -2**

All the growth factors used in this study were prepared in sterile PBS and 10 % BSA. Trophoblast and HUVE cells were treated with 10 ng/ml of VEGF or 10 ng/ml PlGF or 20 ng/ml EGF along with 1μg/ml heparin for 2.5, 5, 10, and 20 min. Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.1 % NP-40, 1 mM

PMSF, 1 mM Na_3VO_4, and 10 nM NaF) and the lysates were cleared of cellular debris by centrifugation. Cell lysates were stored at -80°C until ready to use. Protein concentrations were determined by Bradford assay. Cell lysates (100 μg of protein for JNK assay and 50 μg protein for ERK assay) were immunoprecipitated overnight at 4°C with 0.5 μg/sample JNK or 0.5 μg/sample ERK-2 antibodies conjugated to protein A/G agrose beads. The beads were washed three times in lysis buffer and twice in kinase buffer (25 mM HEPES pH 7.5, 25 mM $NaCl_2$, 25 mM glycerophosphate). The kinase reaction for JNK or ERK was performed by incubating the immunoprecipitated proteins with kinase buffer containing 1 mM DTT, 0.1 mM Na_3VO_4, 10 mM ATP, 5 μCi/sample of (γ ^{32}P) ATP, and 0.2 μg GST-c-Jun or MBP at room temperature for 30 min. Kinase reactions were terminated by addition of Laemmli sample buffer and samples were resolved on 12 % (JNK) or 14 % (ERK) SDS polyacrylamide gels (SDS/PAGE). The radiolabeled substrate bands were quantified by laser densitometry.

Immunoprecipitation and Immunoblotting

Trophoblast and HUVE cells were treated with 10 ng/ml VEGF or 10 ng/ml PlGF or 20 ng/ml EGF along with 1 μg/ml heparin for 5 and 10 min. Cells were lysed in lysis buffer (20 mM Tris-Hcl, pH 7.4, 150 mM NaCl, 1 % Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 10 μg leupeptin, and 1 mM Na_3Vo_4). For immunoprecipitation with antisera to Nck, cell lysates were precleared with protein-A beads for 2 h at 4°C. Precleared lysates (250-300 μg of protein) were immunoprecipitated with 1-1.5 μg/sample Nck antibody and protein-A/G beads overnight

at 4°C. The beads were washed three times in lysis buffer. After the third wash, 2X Laemmli sample buffer was added to samples and samples were boiled for 5 min. Immunoprecipitated proteins were separated on 12 % SDS-polyacrylamide gels and blotted onto nitrocellulose membranes by electrotransfer in transfer buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5 % methanol) at 75 volts for 1.30 h. The membranes were blocked with 1 % BSA in TBST (Tris-buffered saline with 0.1 % Tween-20) for 1 h and incubated with rabbit anti-phosphotyrosine antibody (1:500, 250 μg/ml) in blocking buffer (TBST with 1 % BSA) overnight at 4°C. The membranes were washed several times with TBST and then incubated with anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (1:5000, Jackson Immuno Research Labs) for 30 min and washed several times with TBST. The enhanced chemiluminescent detection system (ECL) was used for protein detection. The membrane was incubated in chemiluminescent reagents for 5 min, covered with plastic wrap and exposed to X-ray film.

For ERK-2 and p38 kinase activity, equal quantities of cell lysates (30-40 μg for ERK and 80-90 μg for p38 kinase; protein concentrations were determined by Bradford assay) were separated on 12-14 % SDS-polyacrylamide gels and were transferred to nitrocellulose membranes. The membranes were blocked with 0.5 % casein in TBST (Tris buffered saline with 0.1 % Tween-20) for 30 min, incubated with anti-active p38 kinase antibody (1:2000, Promega) or with anti active MAPK antibody (25 ng/ml) overnight at 4°C. Membranes were washed in TBST several times, and then incubated with goat anti-rabbit HRP-conjugated antibody (1.5 μg/ml) for 30 min, and washed

several times with TBST. The proteins were detected with chemiluminescent reagents. The protein bands were quantitated by laser densitometry.

For the Akt phosphorylation assay, HUVE cells and trophoblast were grown as described previously. Before start of serum starvation, cells were washed with PBS, and serum-free RPMI medium complemented with 0.1 % bovine serum albumin for HUVE cells, or KBM without serum for trophoblast, was added. HUVE cells are highly sensitive to serum starvation and they withstand this stress better if they are supplemented with BSA. 9-12 h after starvation, cells were washed with PBS and fresh starvation medium was added with or without 200 nM wortmannin for 30 min or 30 μM 2-(4-mormpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002, (Sigma)) for 20 min. Cells were treated with growth factors at indicated concentrations for 15 min. Cells were lysed in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF, 1 mM NaF. Equal quantities of total cell lysates (80-100 μg of protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 0.5 % casein in TBST and Akt phosphorylation/activation was detected by incubating the membrane with rabbit anti-phospho-Akt antibody (1:1000, New England BioLabs) overnight at 4°C. Membranes were washed with TBST several times and incubated with anti-rabbit HRP-conjugated antibody (1.5 μg/ml) for 30 min. Membranes were washed with TBST and the immune complexes were detected with the enhanced chemiluminescence detection system as described earlier.

To control for protein loading, the membranes were stripped with buffer containing 0.2 M glycine, pH 2.2, 0.1 % SDS, and 10 % Tween 20, for 20 min, washed with TBST and reblocked with blocking buffer for 30 min. The membranes were probed with anti ERK-2 antibody (0.5 μg/sample) or p38 antibody (0.5 μg/sample), or Akt antibody (1:1000, New England BioLabs) overnight and developed as above.

For detection of Bcl-2 protein levels, trophoblast and HUVE cells were maintained in KGM and RPMI-1640 with 10 % FCS and mitogens, respectively. Before the experiment, complete media was replaced with serum- and -mitogen free basal medium (complemented with 0.01% bovine serum albumin for HUVE cells) or supplemented with 75 ng/ml VEGF or 75 ng/ml PlGF or 75 ng/ml EGF for the indicated periods of time. Equal amounts of cell lysates (50 μg-100 μg) were loaded on to 10 % SDS-polyacrylamide gels and western blotting was done with mouse anti-human Bcl-2 antibody as described above. Anti-mouse HRP-conjugated antibody (1:5000, Jackson Immuno Research Labs) was used to detect Bcl-2 by chemiluminescent reagents.

Apoptosis Assays

Trophoblast apoptosis was monitored with DNA fragmentation and MTT assays. Cultured trophoblast were plated in KGM/10 % FCS and incubated for 4-6 h at 37°C to allow attachment. Complete media was replaced with DMEM-HG with or without 10 ng/ml VEGF, or 10 ng/ml PlGF, or 10 ng/ml EGF, or 10 % FCS. For experiments involving cytokines, 10 ng/ml tumor necrosis factor-α (TNF-α) and 100 U/ml interferon-γ (IFN-γ) were used to induce apoptosis. For DNA fragmentation analyses, both attached

and floating cells were collected after 72h and incubated in digestion buffer (50 mM Tris pH 8, 0.5 % sodium lauryl sarcosine, 0.5 mg/ml Proteinase K, and 10 mM EDTA) for 3 h at 55°C. Digested cells were treated with DNase-free RNase for 1h at 55°C, and DNA was extracted twice with phenol/chloroform and once with chloroform. Extracted DNA was precipitated with ethanol overnight at -80°C. Ten μg of DNA from each treatment group was resolved on 1.6 % agarose gels, stained with 2 μl of ethidium bromide (1 mg/ml), and photographed.

For the MTT assay, trophoblast were plated in 96 well plates (600,000 cells / well). Media from quadruplicate cultures were replaced with DMEM-HG containing 0.5 mg/ml MTT(3-4,5-dimethylthizol-2,5-diphenyl-tetrazolium bromide) and incubated at 37°C for 3.5-4 h. Intracellular formazan was extracted with isopropanol, and absorbance of each well was measured at 570 and 650 nm. Percentage of apoptosis was calculated after normalizing to control cultures containing 10 % FCS.

RESULTS

PlGF Induces JNK and p38 Kinase Activities, but Not ERK-1 and -2 Activities in Term Trophoblast

Our previous studies documented that Flt-1 receptors expressed in normal human trophoblast are functional and that VEGF induces JNK activity [20]. To extend these initial findings, and to determine whether PlGF induces similar activation of the Flt-1 receptor, isolated normal syncytiotrophoblast were treated with rhPlGF, and time-course

induction of JNK, p38 and ERK-1/2 activities were determined. Exogenous rhPlGF rapidly induced transient JNK activity (Fig 3 A). Induction of JNK activity was evident at 5 min. and the response peaked (mean 6.1 ± 0.5 fold) at 10 min before diminishing at 20 min Longer time course experiments showed that JNK activity levels returned to background levels by 40 min (data not shown). Activation of JNK by PlGF was qualitatively and quantitatively similar to the increase in JNK induced within 10 min by UV treatment, a known activator of JNK [238]. Addition of exogenous rhPlGF to trophoblast also resulted in a time-dependent induction of p38 kinase activity (mean 7.5 ± 0.5 fold at 10 min.; Fig 3 B) ($p = 0.0007$, ANOVA). In contrast to these SAPK results, PlGF did not induce significant ($p>0.14$, ANOVA) ERK-1 and -2 activity in syncytiotrophoblast (mean 2.2 ± 0.53 fold at 10 min.; Fig 3 C). Treatment of the syncytiotrophoblast with EGF resulted in a large increase in ERK-1 and -2 activity, indicating that the ERK-1 and -2 pathway was functional in the cultured trophoblast.

PlGF Induces ERK-1 and-2 Kinase Activities, but not JNK Kinase Activity in Endothelial Cells

Since endothelial cells also express Flt-1 receptor, signal transduction responses to PlGF was determined in HUVE cells. The ability of PlGF to induce JNK and p38 kinase, but not ERK-1 and -2, in syncytiotrophoblast is in sharp contrast to the effects elicited in HUVE cells. Exogenous rhPlGF induced large, transient increases in ERK-1 and -2 activities in HUVE cells (Fig 4 A). PlGF induced a peak activation (mean 20 ± 2.8-fold) of both ERK-1 (p44) and ERK-2 (p42) in HUVE cells by 5min, which returned

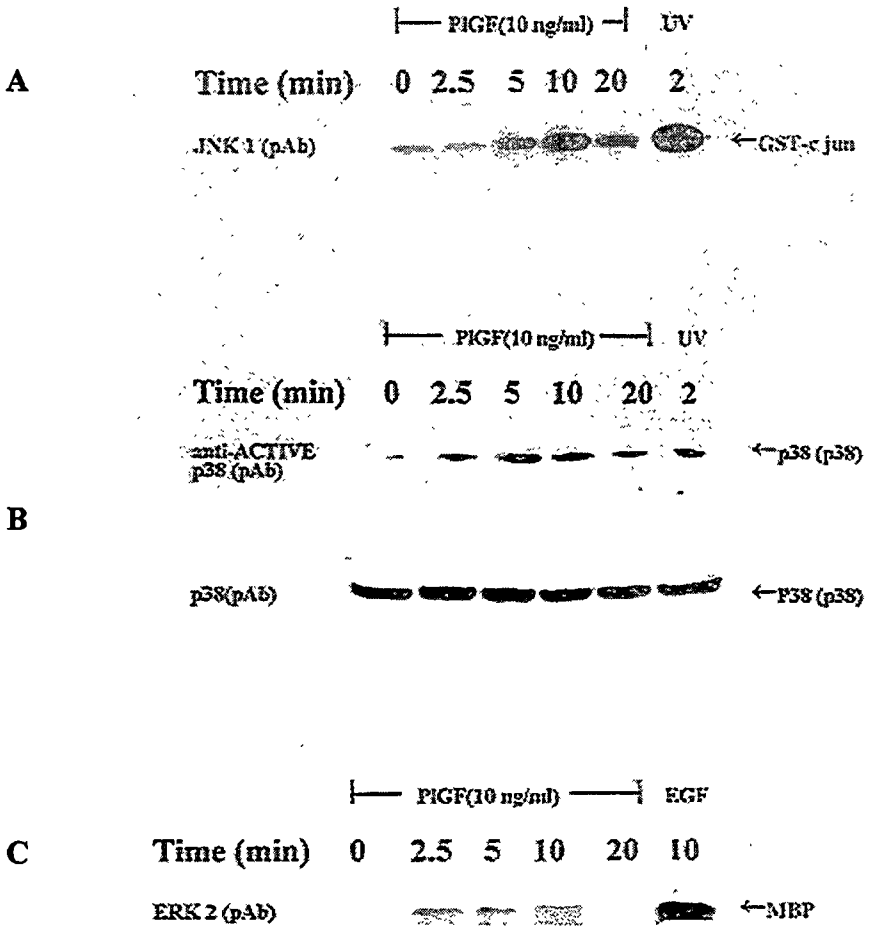
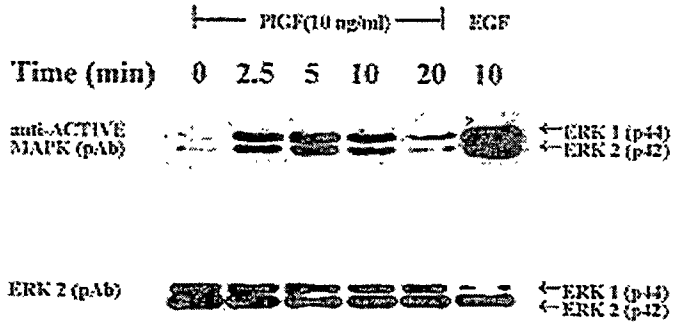


Figure 3: PlGF Induces JNK/SAPK and p38 Kinase Activities But Little ERK-1 and- 2 Activities In Trophoblast. *in vitro* kinase assays/western blot analysis demonstrating PlGF induction of JNK activity (A), p38 kinase activity (B), and ERK1 & 2 activities (C) in trophoblast. Syncytiotrophoblast were treated with rhPlGF (10 ng/ml) for the indicated time intervals. PlGF rapidly induced peak JNK activity (mean 6.1 ± 0.5-fold by 10 min), peak p38 activity (mean 7.5 ± 0.5- fold) by 5 min, but not significant ERK1 & 2 activities (mean 2.2 ± 0.5- fold by 2.5 min) ($p>0.14$). Results are from representative experiments that were repeated four times with comparable results ($p = 0.0007$, ANOVA). pAb, polyclonal antibody.

A



B

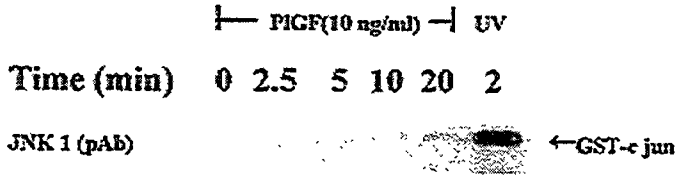


Figure 4: PlGF induces ERK-1 and-2 Activities but Not JNK/SAPK Activity In Human Umbilical Vein Endothelial Cells (HUVECs). HUVECs were treated with rhPlGF (10 ng/ml) for the indicated time points. (A); western blot analysis (with anti active MAPK) demonstrating ERK1 & 2 activities, (B); *in vitro* kinase assay demonstrating induction of JNK/SAPK activity. PlGF (A) induces ERK1 & 2 activities (mean20 ± 2.8-fold at 5 min) ($p < 0.03$, ANOVA) but not JNK/SAPK activity. Results are from representative experiments that were repeated three times with comparable results. pAb, polyclonal antibody.

to background levels by 20 min ($p < 0.03$). This response was similar to that invoked by the known endothelial cell mitogen, EGF. PlGF did not induce JNK activity (Fig 4 B) in HUVE cells.

VEGF Induces JNK and p38 kinase Activities, but not ERK-1 and -2 Activities In Term Trophoblast

Like PlGF, VEGF binds to Flt-1. To determine the signal transduction properties of VEGF in term trophoblast, similar experiments as shown in Fig 3 were conducted. Normal syncytiotrophoblast were treated with rhVEGF and time-course induction of JNK, p38, and ERK-1 and -2 activities was determined. Exogenous rhVEGF rapidly induced transient JNK activity (Fig 5 A). JNK activity peaked (mean 3 ± 0.6-fold) at 10 min. before diminishing at 20 min and returned to basal level at 40 min (data not shown) ($p < 0.01$). UV treatment was used as a positive control for JNK activation. Like PlGF VEGF also induced p38 kinase activity (mean 7 ± 0.5-fold) at 10 min. (Fig 5 B) ($p < 0.04$). Again like PlGF, VEGF did not induce significant ERK-1 and 2 activities in syncytiotrophoblast (mean 2 ± 0.5-fold at 5 min; Fig 5 C).

VEGF Induced ERK-1 and -2 Kinase Activities, but not JNK Kinase Activity in Endothelial Cells

Kinase activity induced by VEGF in HUVE cells was very similar to that of PlGF. In contrast to the effects elicited by VEGF in trophoblast, VEGF induced a robust transient increase in ERK-1 and -2 activities in HUVE cells (Fig 6 A). VEGF induced peak activation of both ERK-1 and -2 (mean 9 ± 1.4-fold) in HUVE cells by 10 min

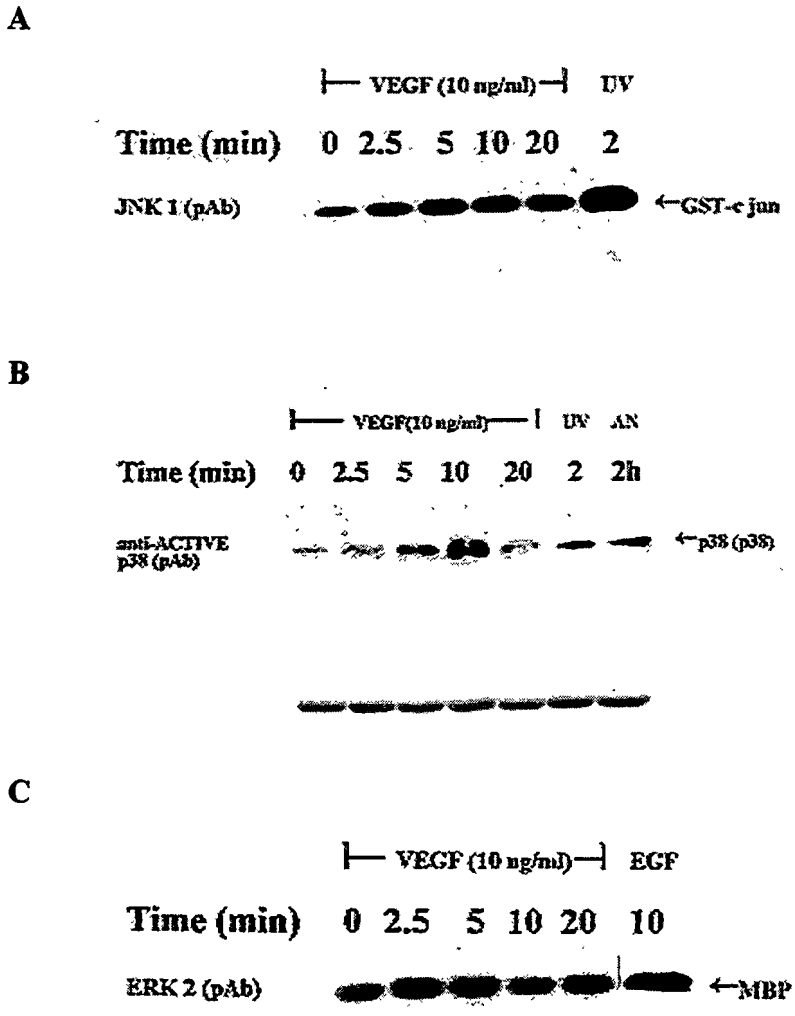
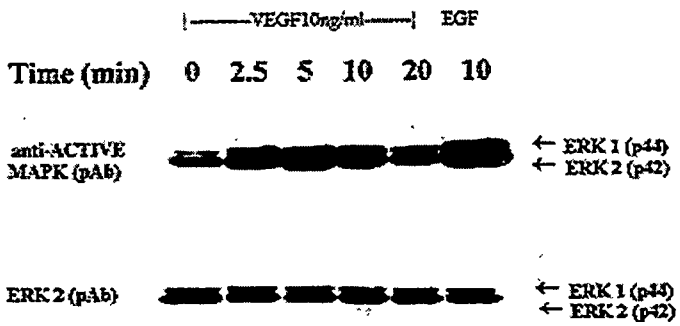


Figure 5: VEGF Induces JNK/SAPK and p38 Kinase Activities but Little ERK-1 and-2 Activities In Trophoblast. Syncytiotrophoblast were treated with rhPlGF (10ng/ml) for the indicated time intervals. *in vitro* kinase assays/Western blot analysis demonstrating VEGF induction of JNK activity (A), p38 kinase activity (B), and ERK1 & 2 activities (C) in trophoblast. VEGF rapidly induces peak JNK/SAPK activity (mean 3 ± 0.6 fold) by 10 min ($p < 0.01$), p38 peak activity (mean 10 ± 0.5 fold) by 5 min ($p < 0.04$, ANOVA) but not significant ERK1 & 2 activities. Results are from representative experiments that were repeated four times with comparable results. pAb, polyclonal antibody.

A



B

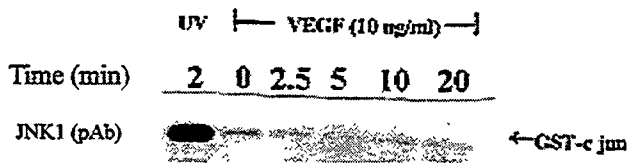


Figure 6: VEGF Induces ERK-1 and-2 Activities But Not JNK/SAPK Activity In Human Umbilical Vein Endothelial Cells (HUVE cells). HUVE cells were treated with rhVEGF (10 ng/ml) for indicated time points. (A); Western blot analysis (with anti active MAPK) demonstrating ERK1 & 2 activities, (b); *in vitro* kinase assay demonstrating induction of JNK/SAPK activity. VEGF (A) induces ERK1 & 2 activity (mean 9 ± 1.4- fold) by 5min ($p < 0.03$, ANOVA) but not JNK/SAPK activity. Results are from representative experiments that were repeated two times with comparable results. pAb, polyclonal antibody.

which returned to basal levels by 20 min ($p < 0.03$) VEGF, like PlGF, did not induce JNK activity in endothelial cells (Fig 6B).

Collectively, the above data show that although PlGF and VEGF bind Flt-1 receptors on trophoblast and endothelial cells, the signal transduction pathways utilized by these cells are clearly different. In term trophoblast, Flt-1-mediated activation of the JNK/SAPK pathway may be the major pathway for signaling in response to PlGF and VEGF, while in endothelial cells, ERK-1 and-2 may be the major pathway for signal transduction. Alternatively, these disparate signal transduction responses of PlGF/Flt-1 and VEGF/Flt-1/KDR in trophoblast and endothelial cells may reflect differences in proliferation potentials between the cell types, or possibly signaling through KDR in HUVE cells.

Phosphorylation and Association of PLC-γ and Nck in Endothelial Cells

Nck and PLC-γ are known to associate with both Flt-1 and KDR in Flt-1- and -KDR expressing Sf9 insect cells, the endothelial cell line MS1, rat sinusoidal endothelial cells and KDR- expressing fibroblasts [66, 70, 71]. Nck acts as a mediator in the JNK pathway [77]. Several growth factors like EGF, FGF and VEGF are known to stimulate PLC-γ activity, and in turn activate ERK pathways and induce proliferation [68, 91, 98]. Furthermore, in bovine aortic endothelial cells, Nck and PLC-γ are phosphorylated in response to VEGF [98]. To determine whether signal transducer or adapter proteins are responsible for the observed disparate signal responses of VEGF/PlGF/Flt-1 in trophoblast and HUVE cells, phosphoproteins from VEGF - or PlGF- stimulated HUVE

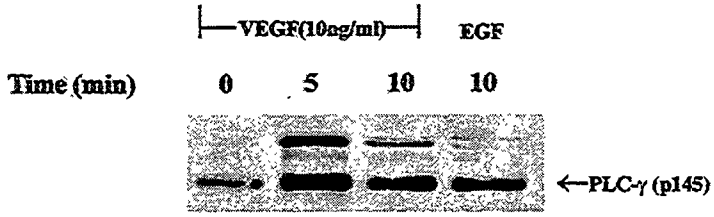
cells were immunoprecipitated with anti- Nck antibody and analyzed by western blotting with antiphosphotyrosine antibody. The anti- Nck antibody used is against an epitope which is common to both PLC-γ and Nck, and thus recognizes both PLC-γ and Nck. Both PLC-γ (mean 3 ± 0.8- fold at 5 min) (Fig 7 A) and Nck (mean 3.2 ± 1.0- fold at 10 min) (Fig 7 B) were phosphorylated in response to VEGF in endothelial cells. This suggests that PLC-γ and Nck may be involved in VEGF- induced signal transduction in these cells. These results are consistent with the study by Guo *et al* [68] in which VEGF induced phosphorylation of both PLC-γ and Nck.

Treatment of endothelial cells with PlGF resulted in phosphorylation of PLC-γ (mean 3.2 ± 0.8- fold at 10 min) (Fig 8 A) and Nck (mean 3.3 ± 0.6-fold at 10 min) (Fig 8 B). These results differ from other studies in which PlGF/VEGF did not activate these signal transducers in transfected PAE/KDR and PAE/Flt-1 cells [72]. PAE cells do not express KDR and Flt-1 while these receptors are expressed costitutively in HUVE cells. Thus, cells lacking endogenous receptors may not activate certain signaling molecules. PLC-γ and Nck are known to be downstream targets of KDR, while association of PLC-γ and Nck with Flt-1 is not very well understood.

Phosphorylation and Association of PLC-γ and Nck in Trophoblast

Similarly to the results observed in endothelial cells, exogenous VEGF induced peak phosphorylation of PLC-γ (mean 3 ± 1.0- fold at 5 min) (Fig 9 A) and Nck (mean 3 ± 1.0- fold 10 min) (Fig 9 B) in trophoblast. Exogenous PlGF also induced phosphorylation of both PLC-γ (mean 3.1 ± 0.8- fold at 10 min) (Fig 10 A) and Nck

A



B

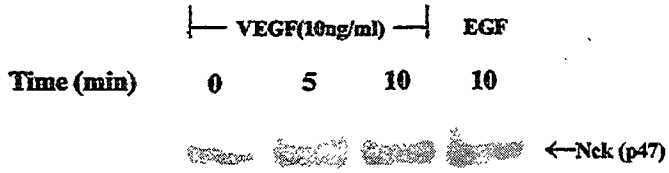
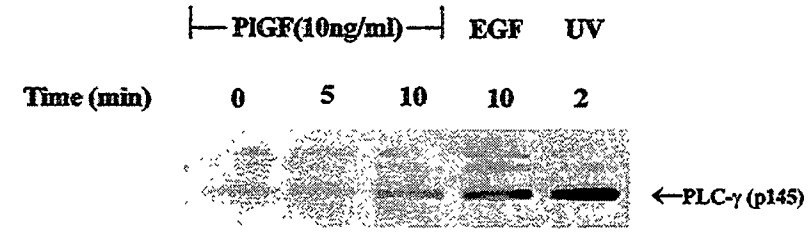


Figure 7: VEGF Induces Phosphorylation of PLC-γ And Nck In Human Umbilical Vein Endothelial Cells (HUVE Cells). HUVE cells were treated with rhVEGF (10 ng/ml) for indicated time points. Proteins were immunoprecipitated with anti-Nck Ab and western blotted with anti-pTyr Ab. Representative western bolts demonstrating VEGF induced phosphorylation of PLC-γ (A) and Nck (B). VEGF rapidly induced phosphorylation of PLC-γ (mean 3 ± 0.8-fold) by 5 min and induced phosphorylation of Nck (mean 3.2 ± 1.0- fold) by 10 min. Results are from representative experiments that Were repeated three times with comparable results.

A



B

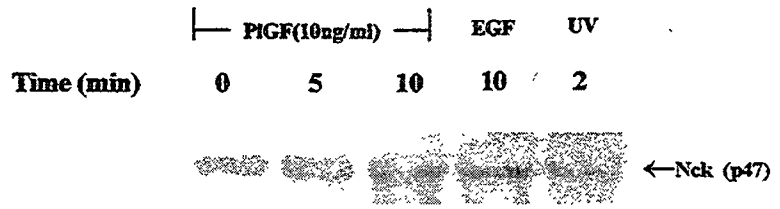
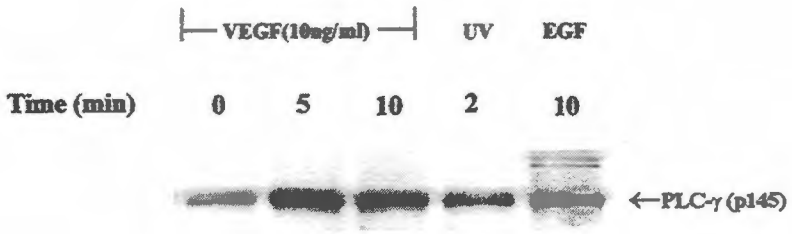


Figure 8: PlGF Induces Phosphorylation of PLC-γ and Nck in Human Umbilical V ein Endothelial Cells (HUVE cells). HUVE cells were treated with rhVEGF (10 ng/ml) for indicated time points. Proteins were immunoprecipitated with anti-Nck Ab and western blotted with anti-pTyr Ab. Representative western bolts demonstrating PlGF induced phosphorylation of PLC-γ (A) and Nck (B). PlGF rapidly induced phosphorylation of PLC-γ (mean 3.2 ±0.8 fold) by 10 min, and induced phosphorylation of Nck (mean 3.3 ±0.6-fold) by 10 min. Results are from representative experiments that were repeated three times with comparable results.

A



B

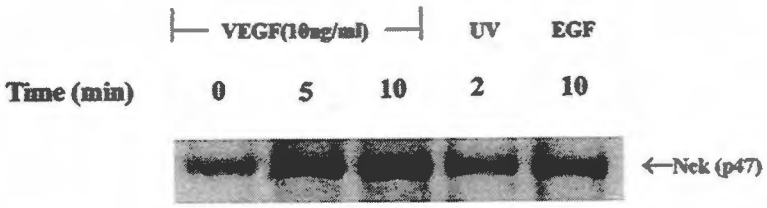
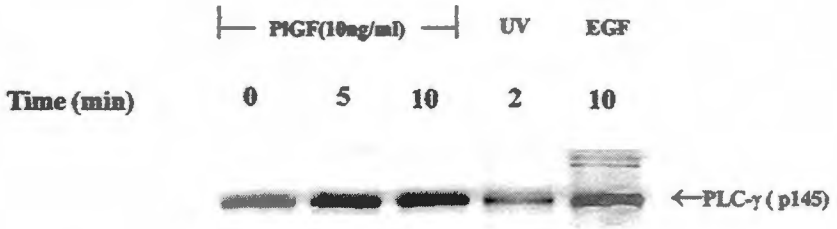


Figure 9: VEGF Induces Phosphorylation of PLC-γ and Nck in Trophoblast. Trophoblast cells were treated with rhVEGF (10 ng/ml) for indicated time points. Proteins were immunoprecipitated with ant-Nck Ab and western blotted with anti-pTyr Ab. Representative western blots demonstrating VEGF induced phosphorylation of PLC-γ (A) and Nck (B). VEGF rapidly induced phosphorylation of PLC-γ (mean 3 ± 1.0-fold) by 5 min, and induced phosphorylation of Nck (mean 3 ± 1.0-fold by 10 min) by 5 min. Results are from representative experiments that were repeated two times with comparable results.

A



B

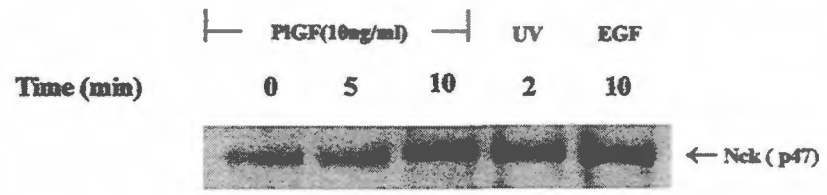


Figure 10: PlGF Induces Phosphorylation of PLC-γ and Nck In Trophoblast. Trophoblast were treated with rhPlGF (10ng/ml) for indicated time points. Proteins were immunoprecipitated with anti-Nck Ab and Western blotted with anti-pTyr Ab. Representative western blots demonstrating PlGF induced phosphorylation of PLC-γ (A) and Nck (B). PlGF rapidly induced phosphorylation of PLC-γ (mean 3.1 ± 0.8-fold) by 10 min, and induced phosphorylation of Nck (mean 3.5 ± 1.0 fold) by 10 min. Results are from representative experiments that were repeated two times with comparable results.

(mean 3.5 ± 1.0-fold at 10 min) (Fig 10 B). EGF was used as a positive control for phosphorylation of PLC-γ and Nck. EGF induced phosphorylation of PLC-γ and Nck (mean 2.7 ± 0.5-fold). VEGF/PlGF induced phosphorylation of PLC-γ and Nck was as strong as EGF induced phosphorylation of these molecules. These findings in trophoblast are different from other reports in which ectopically expressed Flt-1 receptors in PAE cells and fibroblasts did not activate PLC-γ and other signaling molecules. Effects of both VEGF and PlGF on Nck activation in endogenously expressing Flt-1 cells are not known. Phosphorylation of Nck usually leads to its activation. This study shows that PLC-γ and Nck are phosphorylated in response to VEGF/PlGF and they may be involved Flt-1 mediated signal transduction.

PlGF and VEGF Protect Trophoblast from Apoptosis Induced by Growth Factor Withdrawal

Activation of JNK and p38 kinase has been shown to protect some cell types from apoptosis [129]. In order to determine whether PlGF and VEGF function similarly in trophoblast, their effects on the fragmentation of DNA and metabolic activity of trophoblast were examined. Syncytiotrophoblast maintained in 10 % FCS showed little DNA fragmentation even after 72 h; however, cultures subjected to serum deprivation (72 h) demonstrated significant DNA fragmentation. EGF, a known inducer of ERK-1 and -2 in trophoblast did not prevent apoptosis. However, exogenous PlGF (10 ng/ml) (Fig 11 A) and VEGF (10 ng/ml) (Fig 11 B) significantly protected the cells maintained in serum-free media for 72 h from apoptosis as shown by no DNA fragmentation.

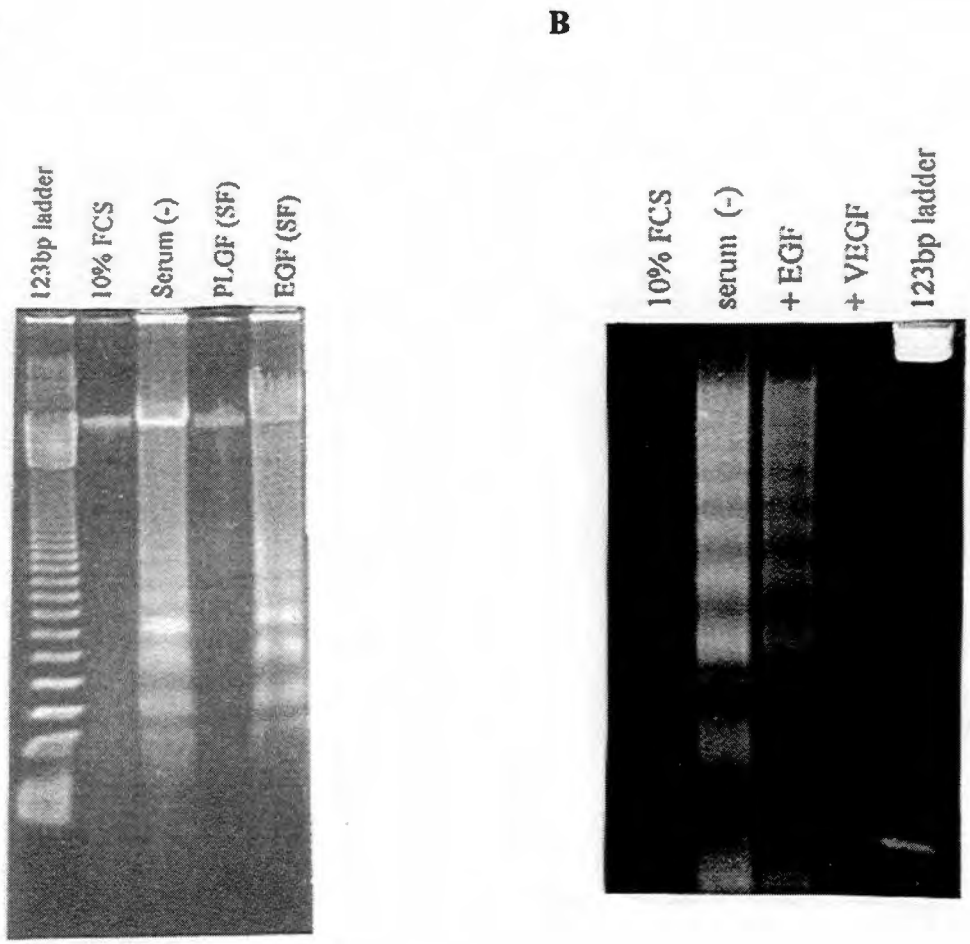


Figure 11: PlGF and VEGF Inhibit Growth Factor Withdrawal-induced Apoptosis in Trophoblast. Trophoblast were cultured for 4h and complete media was replaced with serum free media containing either no supplements, 10 ng/ml PlGF, 10 ng/ml VEG, 20 ng/ml EGF, or 10 % FCS. After 72 h, samples were analyzed for DNA fragmentation. PlGF (A) and VEGF (B) protected the trophoblast from apoptosis, while EGF had only a marginal effect.

The DNA fragmentation results were confirmed by measuring the loss of trophoblast viability as measured by their metabolic capacity to reduce MTT (Yui *et al.* [196]) (Fig 12). In four separate experiments, serum deprivation significantly ($p < 0.0005$, ANOVA) reduced MTT activity as compared with control trophoblast cultured in 10 % FCS. Furthermore, cultures receiving rhPlGF significantly increased MTT reduction to 186 % of that found under serum-free culture conditions ($p < 0.005$). The addition of PlGF to serum-free cultures increased MTT activity to 145 % of cultures containing 10 % FCS ($p = 0.05$). In parallel experiments, the addition of rhVEGF significantly increased MTT reduction to 112 % of that levels found under serum-free culture conditions ($p < 0.01$), and serum deprivation resulted in significant reduction in MTT activity compared to trophoblast cultured in 10 % FCS ($p < 0.05$). In contrast, the addition of VEGF to serum- free cultures increased MTT activity to 130 % of cultures containing 10 % serum (Fig 13). Thus, exogenous PlGF and VEGF protect trophoblast from serum- deprivation induced apoptosis, and suggest that PlGF/VEGF- mediated activation of JNK and p38 pathways in term trophoblast may facilitate cell survival.

PlGF and VEGF Do Not Protect Trophoblast From Cytokine Induced Apoptosis

Tumor necrosis factor-alpha (TNF-α) and gamma interferon (IFN-γ) are expressed by cytotrophoblast within human placental villi during normal pregnancy [193, 196]. EGF is known to protect trophoblast from TNF-α- and IFN-γ - induced apoptosis *in vitro* [180]. In addition, a recent study showed that VEGF inhibits TNF-α- induced apoptosis in endothelial cells [197]. Therefore, the next experiment was designed to

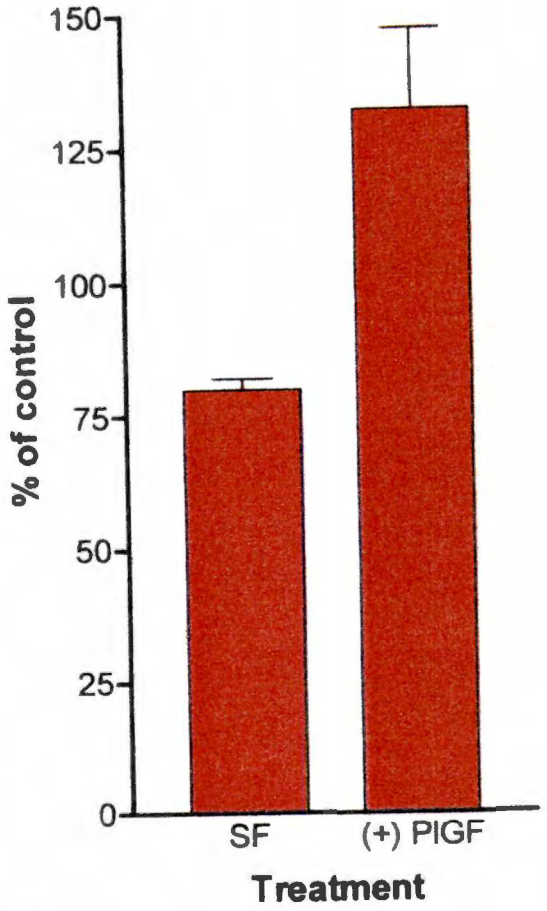


Figure 12: PlGF Protects Trophoblast From Growth Factor Withdrawal-Induced Apoptosis. Trophoblast were isolated and cultured for 4h and complete media was replaced with serum free media containing either no supplements or 10 ng/ml PlGF or 20 ng/ml EGF or 10 % serum. Cell viability was measured by MTT reduction. MTT reductive capacities were determined after 48 hours and normalized to control cultures (10 % serum). Serum depravation significantly ($p < 0.0005$) reduced MTT activity, while addition of PlGF significantly ($p < 0.005$, ANOVA) increased MTT activity. Addition of PlGF protects trophoblast from apoptosis. Results shown are mean % ± SEM of quadruplicate wells of 4 independent experiments.

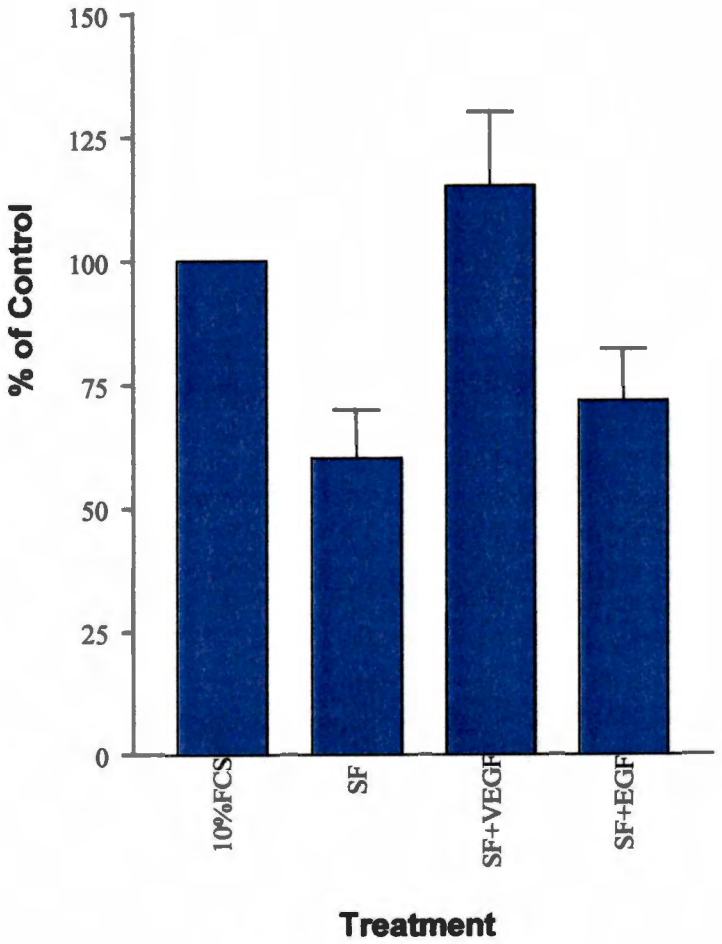


Figure 13: VEGF Protects Trophoblast from Growth Factor Withdrawal-Induced Apoptosis. Trophoblast were isolated and cultured for 4 h and complete media was replaced with serum free media containing either no supplements, or 10 ng/ml VEGF or 20 ng/ml EGF or 10 % serum (FCS). Cell viability was measured by MTT reduction. MTT reductive capacities were determined after 48 hours and normalized to control cultures (10 % serum). Serum deprivation significantly ($p < 0.0005$) reduced MTT activity, while addition of VEGF significantly ($p<0.01$, ANOVA) increased MTT activity. Addition of VEGF protects trophoblast from apoptosis. Results shown are mean % ± SEM of quadruplicate wells of 4 independent experiments. SF = serum free.

determine if VEGF and PlGF provided similar protection from TNF- α- and - IFN- γ induced apoptosis in trophoblast.

The MTT assay was used to measure TNF-α- and IFN-γ - induced apoptosis. As shown previously [196], significant ($P < 0.005$) apoptosis was induced in trophoblast with TNF-α and IFN-γ [180] and this apoptosis was inhibited with exogenous EGF ($p < 0.01$). In contrast, neither exogenous VEGF ($p > 0.90$) (Fig 15) nor PlGF ($p > 0.80$) (Fig 14) was able to inhibit significantly the cytokine-induced apoptosis. Thus, the mechanism by which PlGF/VEGF protects trophoblast from apoptosis appears to be different than that of EGF, and this difference may be due to different signal transduction pathways that each activates. Moreover, PlGF and VEGF may only provide protection against stress-induced apoptosis by SAPK activation, but not against cytokine-induced apoptosis, while EGF, in contrast, may provide protection against cytokine-induced apoptosis by ERK activation, but may not affect trophoblast survival in the absence of growth factors.

Statistical Analysis

Normalized data were compared by one-sample t-test against control values (100 %), differences between multiple treatment groups were analyzed by ANOVA, and significance between two treatments groups determined by Tukey's honest significant difference post-hoc comparison. Statistics were calculated with STATISTICA (StatSoft, Inc., Tulsa, OK), and significance $p<0.05$.

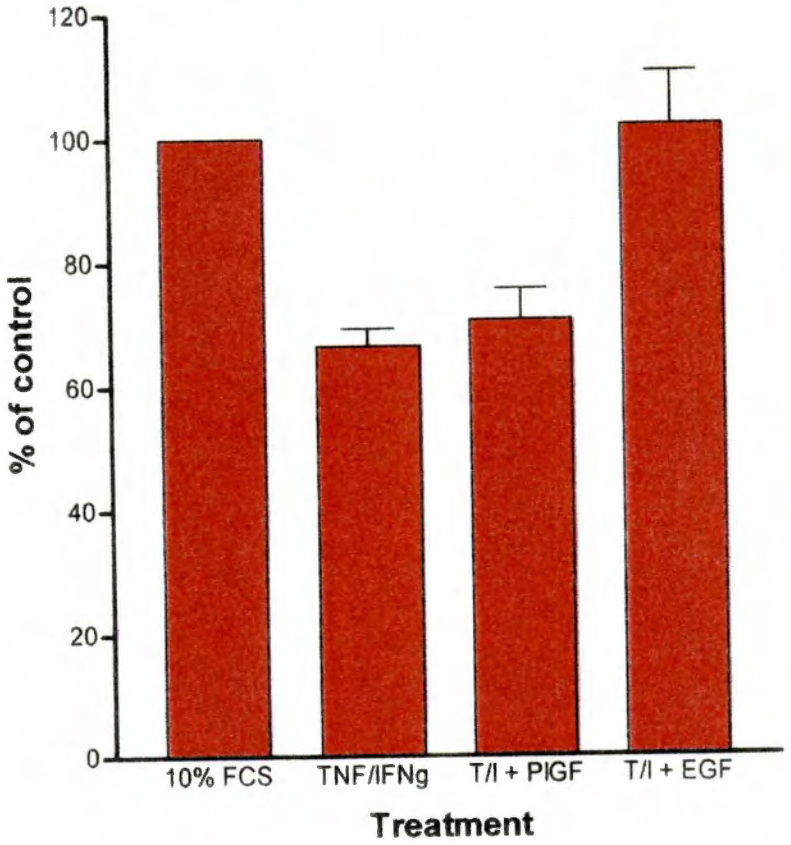


Figure 14: PlGF Does Not Inhibit Pro-inflammatory Cytokine Induced Apoptosis in Trophoblast. Trophoblast were cultured for 4h and complete media was replaced with serum free media containing either no supplements, or 10 % serum or 10 ng/ml TNF- α + 100 U/ml IFN-γ or TNF-α + IFN-γ + 10 ng/ml PlGF or TNF-α + IFN-γ + 20 ng/ml EGF. Cell viability was measured by MTT reduction. MTT reductive capacities were determined after 48 h and normalized to control cultures (10 % serum). TNF-α and IFN-γ significantly ($p < 0.005$) reduced MTT activity. Addition of PlGF did not protect cells from TNF- α/IFN- γ induced apoptosis ($p > 0.80$). However, addition of EGF significantly ($p < 0.01$, ANOVA) inhibited cytokine induced apoptosis. Results shown are mean % ± SEM of quadruplicate wells of 4 independent experiments.

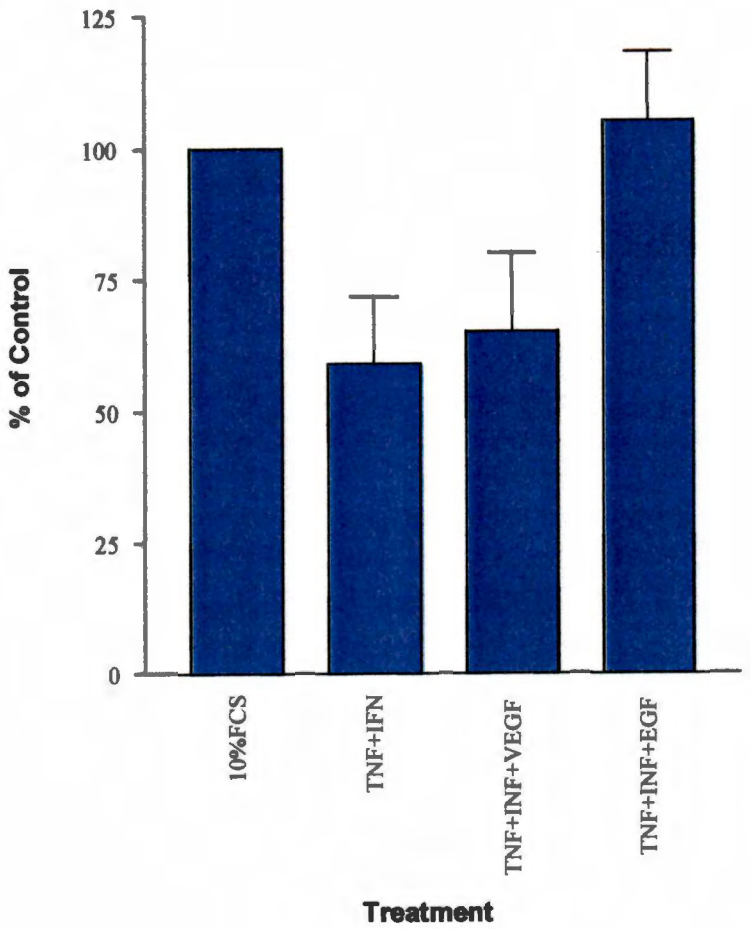


Figure 15: VEGF Does Not Inhibit Pro-Inflammatory Cytokine- Induced Apoptosis in Trophoblast. Trophoblast were cultured for 4h and complete media was replaced with serum free media containing either no supplements, or 10 % serum (FCS) or 10 ng/ml TNF- α + 100 U/ml IFN-γ or TNF-α + IFN-γ + 10 ng/ml VEGF, or TNF-α + IFN-γ + 20 ng/ml EGF. Cell viability was measured by MTT reduction. MTT reductive capacities were determined after 48 h and normalized to control cultures (10 % serum). TNF-α and IFN-γ significantly ($p < 0.005$) reduced MTT activity. Addition of VEGF did not protect cells from TNF- α/IFN- γ– induced apoptosis ($p > 0.90$). However, addition of EGF significantly ($p < 0.01$, ANOVA) inhibited cytokine-induced apoptosis. Results shown are mean % ± SEM of quadruplicate wells of 4 independent experiments.

VEGF but Not PlGF Activates Akt Kinase in Endothelial Cells

The apoptosis studies indicated that both PlGF and VEGF may act as survival factors for term trophoblast as they both protect trophoblast from stress (growth factor withdrawal)- induced apoptosis. It has been recently shown that the survival signals mediated by various growth factors and cytokines may be dependent upon the PI3-kinase/Akt signal transduction pathway. Akt phosphorylation/activation was monitored to determine if VEGF and PlGF mediate their survival effects in endothelial cells through PI3-Kinase/Akt. Akt is activated by phosphorylation within the activation loop at Thr308 and within the C-terminus at Ser473. The PhosphoPlus Akt antibody, used in this experiment recognizes Akt only when phosphorylated at Ser473 and it does not cross react with related family members such as PKC or p70 S6 kinase (New England BioLabs, product list). Thus, phosphorylation/ activation of Akt detected by western blot analysis reflects its activated state. Additionally, phosphorylation/activation of Akt in response to VEGF/PlGF was confirmed by Akt kinase assay (data not shown). HUVE cells were treated with VEGF or PlGF (50 ng/ml) with or without the PI-3 Kinase inhibitors wortmannin or LY294002 compound. Activation of Akt was determined by western blot analysis with anti-phospho Akt antibody. VEGF induced Akt kinase activity at 15 min (mean 2.5 ± 0.5- fold at 5 min) in HUVE cells (Fig 16 A). A 30 min preincubation with 200 nM wortmannin or 20 min with LY294002 blocked Akt phosphorylation /activation induced by VEGF. Wortmannin is also known to inhibit myosin light chain kinase and phospholipase D, while LY 294002 specifically inhibits PI-3 kinase. Thus, in combination, these two PI-3 kinase inhibitors show specific inhibition of PI-3 kinase

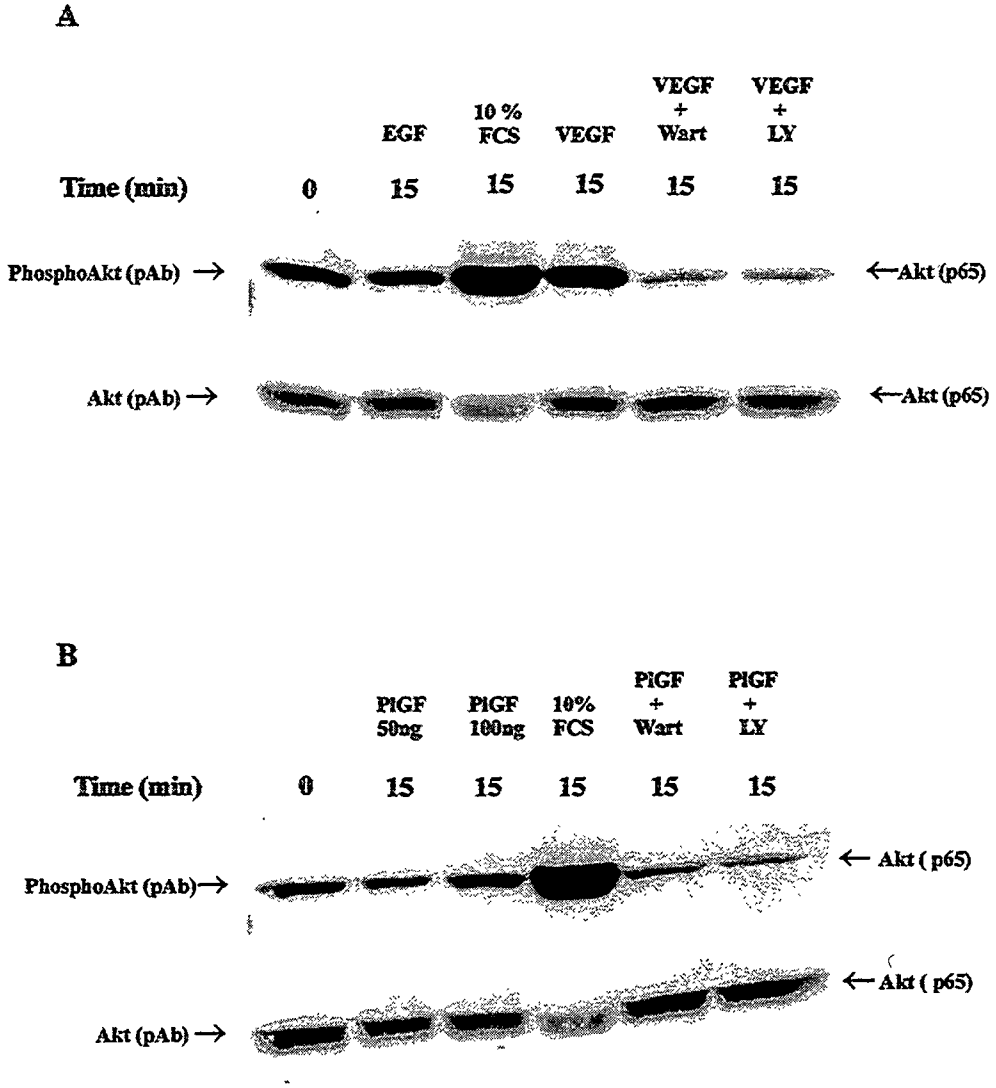


Figure 16: VEGF but Not PlGF Activates/Phosphorylates Akt Kinase in Human Umbilical Vein Endothelial Cells (HUVE cells). HUVE cells were treated with VEGF (50 ng/ml) or rhPlGF (50-100 ng/ml) with or without wortmannin (200 nM for 30 min) or LY294002 (30 μM for 20 min) for indicated time points. Western blots (with anti-phospho Akt antibody) demonstrating VEGF induced Akt phosphorylation/activity. VEGF (A) induced Akt phosphorylation/activity (mean 2.7 ± 0.9- fold) at 15 min while PlGF (B) did not induce Akt activity. Results are from representative experiments that were repeated two times with comparable results. pAb, polyclonal antibody.

mediated Akt phosphorylation/activation. However, PlGF had no effect on Akt phosphorylation/activation (Fig 16 B). Treatments with PlGF for shorter time points (5 and 10 min) did not elicit any Akt activity in these cells. These results further suggested that the survival effect of VEGF in endothelial cells is mediated through KDR but not Flt-1. PlGF, which binds Flt-1 only, did not induce phosphorylation/activation of Akt.

VEGF but not PlGF Activates Akt Kinase in Trophoblast.

Similarly to the endothelial cells, VEGF induced phosphorylation/activation of Akt kinase (mean 3 ± 0.5- fold at 15 min) in trophoblast (Fig 17) and this activation was PI-3 kinase dependent. Pre-treating trophoblast with wortmannin abolished VEGF-induced Akt activity. In contrast, PlGF did not induce phosphorylation/activation of Akt kinase in trophoblast (Fig 17). These results suggested that Akt may play a role in mediating survival effects of VEGF in trophoblast. The mechanism by which Akt protects trophoblast from apoptosis is not known. Akt is known to exert its protective effect in several ways. Akt phosphorylates the anti-apoptotic protein Bad, making it unavailable to heterodimerize with Bcl-2 [202]. Additionally, Akt has been reported to translocate to the nucleus following the stimulation of cells with growth factors, suggesting that it may have nuclear targets that could inhibit apoptosis [239]. Thus, there are several ways in which VEGF-activated Akt could protect trophoblast from apoptosis. Survival effects of PlGF in trophoblast may be mediated through PI3-kinase/Akt-independent signaling.

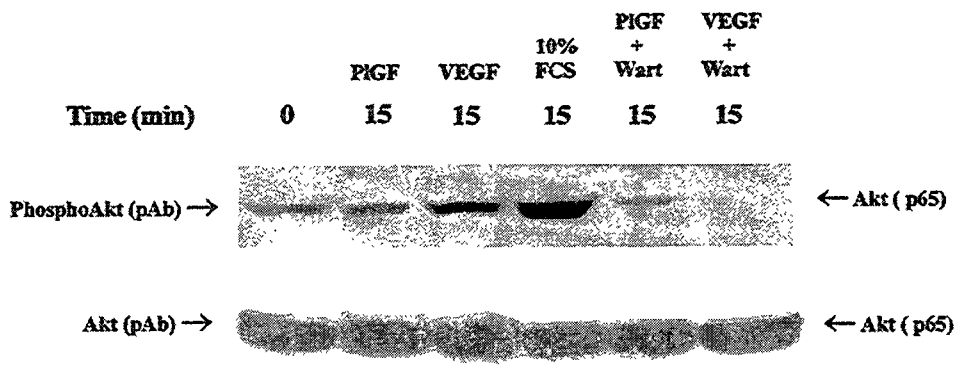


Figure 17: VEGF but Not PlGF Activates/Phosphorylates Akt Kinase in Trophoblast. Trophoblast were treated with VEGF (50 ng/ml) or rhPlGF (50 ng/ml) with or without wortmannin (200 nM for 30 min) or LY294002 (30 μM for 20 min) for indicated time points. Western blots (with anti- phospho Akt antibody) demonstrating phosphorylation Akt activity. VEGF induces phosphorylation/Akt activity (mean 3-fold) at 15 min (A), but PlGF did not induce phosphorylation/Akt activity (B). Results are from representative experiments that were repeated two times with comparable results. pAb, polyclonal antibody.

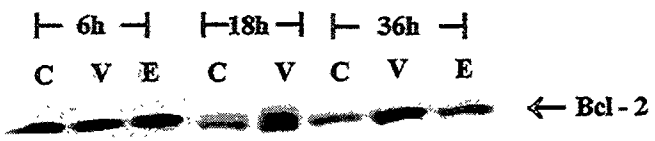
VEGF but not PlGF Induces Expression of Anti-apoptotic Protein Bcl-2 in Endothelial Cells

Many cells resist apoptosis by regulating apoptotic proteins like Bcl-2 or Bcl-X_L. Overexpression of Bcl-2 in some cell types is known to protect them from apoptosis [240]. These findings suggest that the viability of certain cytokine -dependent cells depends on expression of Bcl-2. Some growth factors like VEGF and EGF are known to increase Bcl-2 expression in endothelial cells and keratinocytes, respectively, under serum withdrawal conditions [14, 207]. To determine if VEGF/PlGF modulate expression of anti-apoptotic proteins, we looked at protein levels of Bcl-2 by western blot analysis in HUVE cells treated with VEGF or PlGF. Bcl-2 protein levels increased 18 h – 36 h after the addition of VEGF (mean 3 ± 0.5- fold) as detected by western blot analysis with anti-Bcl-2 antibody (Fig 18 A). In contrast, PlGF had no effect on Bcl-2 protein levels (Fig 18 B). This observation, along with the VEGF induced- activation of Akt, suggested that VEGF may mediate its survival activity in HUVE cells through anti-apoptotic proteins like Bcl-2, while PlGF may not act as a survival factor for endothelial cells. Alternatively, observed survival effects in response to growth factors in HUVE cells may be mediated only through KDR and not Flt-1. Since PlGF binds only to Flt-1, it may not have any effect on endothelial cell survival.

VEGF and PlGF do not Affect Bcl-2 Protein Levels In Term Trophoblast

Bcl-2 is expressed in syncytiotrophoblast throughout pregnancy [228-230]. Expression of Bcl-2 in syncytiotrophoblast suggests that it may protect trophoblast from

A



B

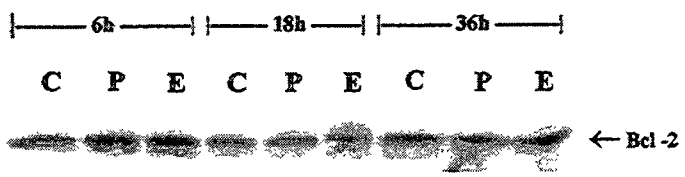


Figure 18: VEGF but Not PlGF Induces Expression of Bcl-2 Protein in Human Umbilical Vein Endothelial Cells (HUVE cells). HUVE cells were treated with VEGF (50 ng/ml) or PlGF (50 ng/ml) or EGF (50 ng/ml) 18 h, 36 h, and 36 h. Western blots (with anti-Bcl-2 Ab) as shown demonstrating Bcl-2 protein levels. VEGF up-regulated Bcl-2 protein levels by 18h in serum deprived HUVE cells (mean 3.2 ± 0.5-fold) ($p < 0.01$) (A), while PlGF had no effect on Bcl-2 protein levels (B). Results are from representative experiments that were repeated two times with comparable results. C = control (no treatment), V = VEGF, P = PlGF, E = EGF.

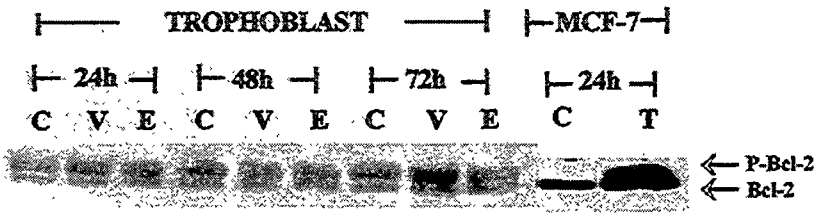
apoptosis [231, 232]. To determine if VEGF and PlGF regulate Bcl-2 expression and activity in trophoblast I looked at Bcl-2 protein levels. In contrast to HUVE cells, VEGF did not affect Bcl-2 protein levels in trophoblast. Furthermore, western blot analysis with Bcl-2 antibody detected Bcl-2 in both the phosphorylated and unphosphorylated states in term trophoblast (Fig 19 A & B). Like VEGF, PlGF did not upregulate Bcl-2 (Fig 19 B), and neither VEGF nor PlGF affected phosphorylation of Bcl-2. The anti-cancer drug, Taxol, is known to induce phosphorylation of Bcl-2 in some cells. Taxol treated MCF-7 cells (breast carcinoma cells) were used as a positive control for Bcl-2 phosphorylation. No change in Bcl-2 protein levels observed in trophoblast are consistent with the assumption that upregulation of Bcl-2 requires signaling through KDR but not Flt-1 [15].

DISCUSSION

Collectively, results of this study demonstrated that both VEGF and PlGF have active roles in regulating trophoblast function. Additionally, this study contributes to further understanding of Flt-1- mediated signal transduction responses in general (Fig 20 and 21).

Expression of both VEGF and PlGF and their receptor Flt-1 on trophoblast suggests that both of these growth factors may act in an autocrine manner to regulate trophoblast function. However, to date there is no biochemical or physiological evidence for such a role. This study shows that PlGF induced transitory activation of the SAPKs, JNK and p38 kinase, with little or no induction of the ERK-1 and -2 pathways. Similarly, VEGF also induced the activation of JNK and p38 kinase with little activation of ERK-1

A



B

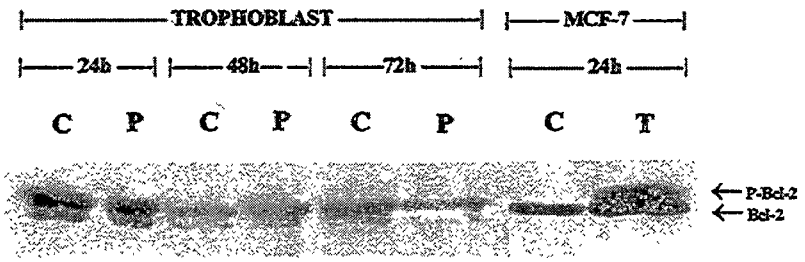


Figure 19: VEGF and PlGF do Not Affect Bcl-2 Protein Levels in Term Trophoblast. Trophoblast were treated with VEGF (50 ng/ml) or PlGF (50 ng/ml) or EGF (50 ng/ml) for 24 h, 48 h, and 72 h. MCF-7 cells (used as positive control) were treated with Taxol. Western blots (with anti-Bcl-2 Ab) as shown demonstrating Bcl-2 protein levels. Both VEGF (A) and PlGF (B) had no effect on Bcl-2 protein levels but Bcl-2 was detected in both the unphosphorylated and phosphorylated states in trophoblast. Results are from representative experiments that were repeated two times with comparable results. C = control (no treatment), V = VEGF, P = PlGF, E = EGF, T = Taxol.

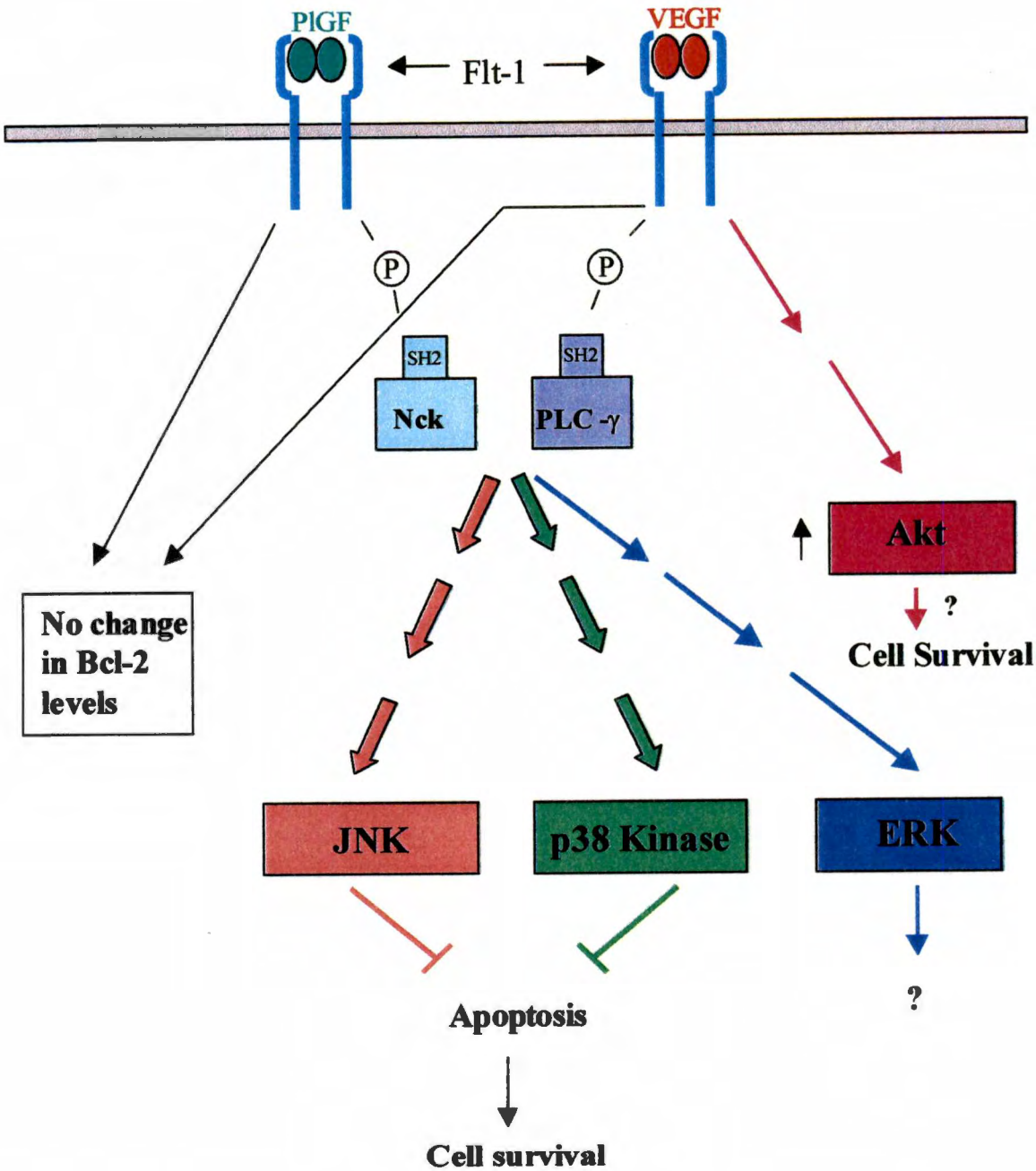


Figure 20: Flt-1 Mediated Signal Transduction Pathways in Trophoblast.
Flt-1 = receptor; VEGF = vascular endothelial growth factor; PlGF = placenta growth Factor; Nck and PLC-γ = adapter proteins; JNK = c-Jun-amino terminal kinase; p38 = p38 kinase; ERK = extra cellular signal regulated kinase; Akt = Akt kinase; Bcl-2 = anti-apopototic protein.

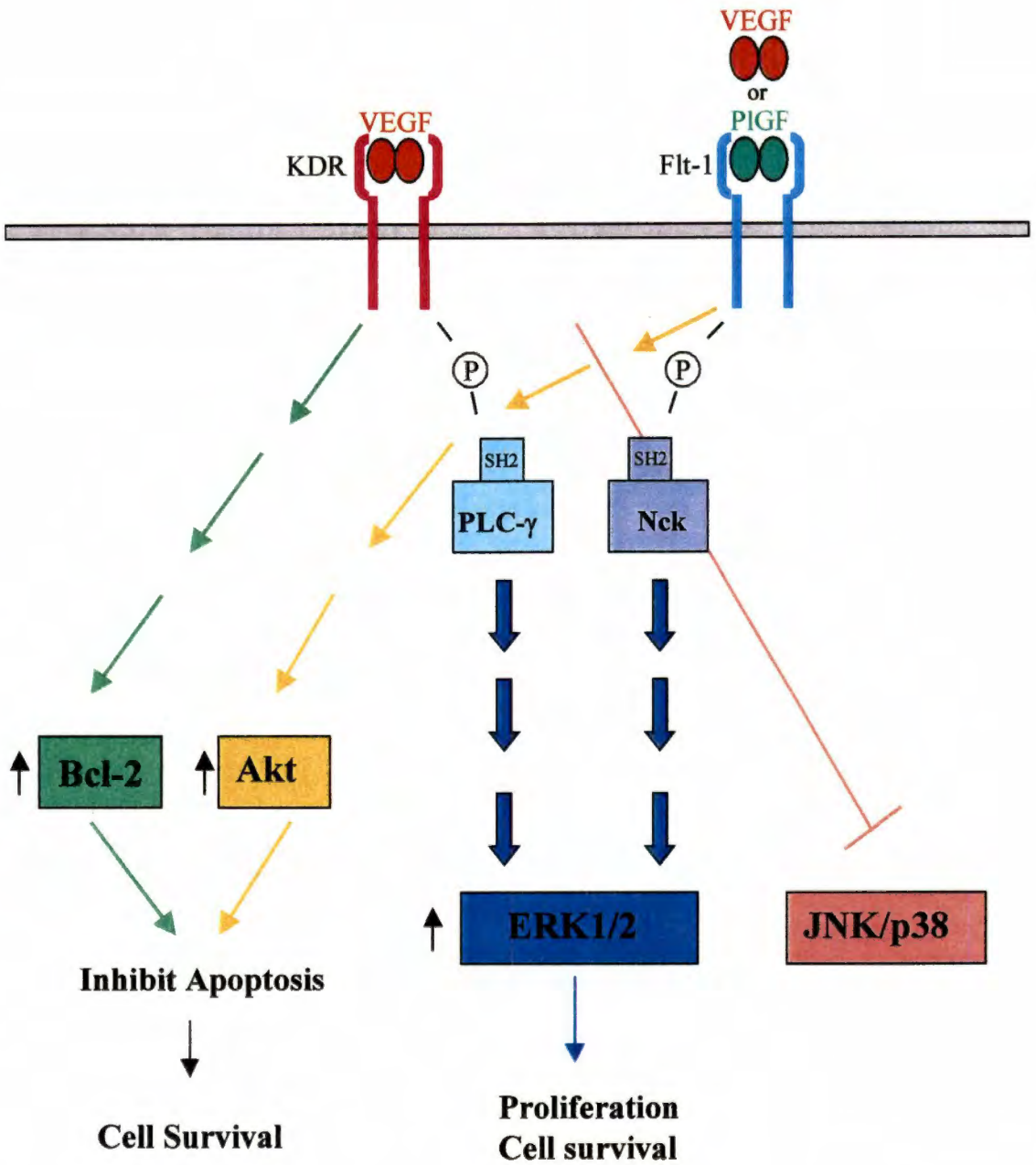


Figure 21: Flt-1/KDR Mediated Signal Transduction Pathways in Human Umbilical Vein Endothelial Cells.

Flt-1 = receptor; KDR = receptor; VEGF = vascular endothelial growth factor; PlGF = placenta growth factor; Nck and PLC-γ = adapter proteins; JNK = c-Jun-amino terminal kinase; p38 = p38 kinase; ERK = extra cellular signal regulated kinase; Akt = Akt kinase; Bcl-2 = anti-apopototic protein

and -2 kinase. In contrast, PlGF and VEGF induced strong ERK-1 and ERK-2 activities, with no induction of JNK pathway in human umbilical vein endothelial cells (HUVE cells). Additionally, both VEGF and PlGF phosphorylated activated signal transducers, like PLC-γ and Nck, upstream of MAPKs in endothelial cells. Similarly, both these adapter proteins, PLC-γ and Nck were phosphorylated in trophoblast in response to VEGF and PlGF (Fig 22). VEGF and PlGF- mediated activation of JNK and p38 pathways may protect the trophoblast from apoptosis induced by growth factor withdrawal.

VEGF, but not PlGF phosphorylated/activated Akt in trophoblast. While, Akt kinase activity/ phosphorylation was not induced in response to PlGF in trophoblast. The antiapoptotic protein Bcl-2 was detected in both phosphorylated and unphosphorylated forms in trophoblast, but PlGF and VEGF had no effect on either expression or phosphorylation of Bcl-2 in these cells (Fig 22). These results confirm that VEGF and PlGF function as survival factors for term trophoblast and suggest that they may act in a Bcl-2- independent manner. These data are the first direct evidence for a functional role of PlGF/Flt-1 and VEGF/Flt-1 in mediating trophoblast survival.

In spite of recent attempts to characterize the biochemical functions of Flt-1 in both endothelial cells and nonendothelial cells, functions of Flt-1 are still not clearly understood. Monocytes [50] and trophoblast [1, 20] are the only normal non-endothelial cell types that express Flt-1 receptors. However, there are no studies characterizing the molecular functions of Flt-1 in these nonendothelial cells. Approaches to study Flt-1-mediated events in endothelial cells are complicated by the co-presence of KDR

Cells			Trophoblast		Endothelial Cells	
Growth Factors			PlGF	VEGF	PlGF	VEGF
Mitogen Activated Protein Kinases	JNK		+++	++	–	–
	p38		+++	++		
	ERK		+/–	+/–	+++	+++
Adapter Molecules	PLC-γ		++	++	++	++
	Nck		++	++	++	++
Apoptosis Assays	Growth Factor Withdrawal Induced [a]	DNA Fragmentation	–	–		
		MTT	+++	+++		
	Cytokine Induced [b]	MTT	–	–		
Oncogenes Modulating Apoptosis	AKT		–	++	–	++
	Bcl-2 protein [c]		–	–	–	++

Figure 22: Summary Chart of Results.
+++ = significant activation or expression; ++ = moderate activation or expression; + = low activation or expression; a = growth factor induced apoptosis (+++ = increase in MTT activity (increased in cell survival)); - = no DNA fragmentation (no apoptosis)/no increase in MTT activity; b = cytokine induced apoptosis (- = no increase in MTT activity, no effect on cell survival); c = Bcl-2 protein levels (++ = up-regulation of Bcl-2 protein levels, - = no effect on Bcl-2 protein levels).

receptors. There are several studies which report significant responses to VEGF in endothelial cells. However, activation of the Flt-1 receptor by either PlGF or VEGF have been reported to have little or no effect in some studies [31, 55, 64, 65, 241, 242], while other studies have shown significant biological responses, such as angiogenesis, chemotaxis, and proliferation [64, 67, 243-246]. The reason for these discrepant findings are not clear, but they may be due to different cell types or assay end points [35]. Known cellular functions of PlGF/Flt-1 or VEGF/Flt-1 are limited to monocytes and first trimester trophoblast cell line. Both VEGF and PlGF act as mitogens for human first trimester extravillous trophoblast cell line [50, 73, 74]. PlGF is also known to stimulate ERK and mitogenicity in Flt-1-expressing porcine aortic endothelial cells [72]. Additionally, PlGF has been reported to have weak to moderate proliferative effects on HUVE cells [36, 41]. In monocytes, both VEGF and PlGF induce chemotaxsis and tissue factor production. Endogenously expressed Flt-1 in these cells mediates the cellular responses to VEGF and PlGF [50].

The present study showed that PlGF and VEGF, despite binding to the similar Flt-1 receptors on trophoblast and HUVE cells, produced different biochemical responses. In primary trophoblast, PlGF and VEGF induced rapid activation of JNK and p38 kinase activities. In contrast, both PlGF and VEGF induced strong activation of ERK-1 and -2, but not JNK in endothelial cells. This PlGF- induced activation of ERK activity in endothelial cells is consistent with a recent study in which PlGF induced ERK activity in porcine aortic endothelial cells overexpressing Flt-1 [72]. Activation of ERK-1 and 2 in response to VEGF in endothelial cells has been well documented. However, this

response may be mediated through the KDR receptor, because activation of Flt-1 by VEGF induced little or no cellular responses in some studies [64]. VEGF- induced activation of Flt-1 did not stimulate cellular responses like ERK activation, mitogenicity or migration. However, VEGF induced all these cellular responses in KDR-expressing PAE cells [72].

The upstream regulatory factors controlling the disparate signal transduction responses mediated by Flt-1 receptor in HUVE cells and trophoblast are not known. However, they may reflect differences in proliferative potentials between cell types or possible signal facilitation by KDR in HUVE cells, or the presence or absence of yet-to-be defined signaling molecules in trophoblast. To begin to characterize the potential differences in signal transduction in these cell types, signaling molecules upstream of MAP kinases were investigated.

Signal transducers or adapter proteins are known to impart specificity in signaling. The present data showed that both PLC-γ and Nck are phosphorylated/activated in response to exogenous VEGF or PlGF in HUVE cells. Similarly, as in endothelial cells, both VEGF and PlGF induce phosphorylation/activation of PLC-γ and Nck in trophoblast. PLC-γ is a known target for KDR, and is implicated in inducing cellular proliferation [71, 93]. Other reports showed that VEGF did not induce phosphorylation of PLC-γ in either PAE/KDR -or PAE/Flt-1- expressing cells, and PlGF did not phosphorylate/activate PLC-γ in Flt-1 expressing PAE cells [64, 72]. One explanation for the different PLC-γ activation patterns seen in the HUVE cells used in the present study and transfected PAE cells used in the Waltenbeger and Landgren studies is

that the latter cells did not express endogenous VEGF/PlGF receptors, and thus may lack certain downstream signaling molecules. The present data showed that both PLC-γ and Nck are phosphorylated in response to VEGF/PlGF, implicating their involvement in Flt-1 mediated signaling in HUVE cells and trophoblast. Additionally, this data also suggested that signal transducers other than PLC-γ and Nck may be responsible for the observed disparate signal transduction responses of VEGF/PlGF/Flt-1 in trophoblast and HUVE cells.

Activation of SAPK responses has been shown in other cell types to regulate apoptosis [247]. In certain cell types, JNK is activated in response to stress stimuli, but it may not necessarily lead to apoptosis. SAPKs are also known to be involved in other events beside apoptosis, including cellular growth, differentiation and transformation [124, 130, 133]. Furthermore, there is lack of correlation between activation of JNK in Fas-induced apoptosis, JNK- mediated induction of apoptosis in anoikis (apoptosis in cells after detachment from ECM), and involvement of JNK in Fas-induced apoptosis in T cells [248, 249]. The present study showed that culturing primary term trophoblast in serum-free conditions, which induces apoptosis in many cell types [195], resulted in significant apoptosis. Stress-induced apoptosis in this study was inhibited by exogenous PlGF or VEGF. However, EGF, an inducer of ERK-1 and -2 kinase activities in trophoblast failed to protect trophoblast from growth factor withdrawal- induced apoptosis. These results further support the role played by SAPKs in inhibiting apoptosis in some cell types, but they also raise an interesting question.

How can JNK mediate both pro-apoptotic and anti-apoptotic signals? The

duration and the time of JNK activation seems to be the determining factor for cell proliferation verses apoptosis. Transient JNK induction leads to cell proliferation, where as sustained JNK activation causes cell death. The induction of JNK in T cell activation and a subsequent apoptosis are due to different activation patterns, i.e., transient verses persistent [116]. Early and transient activation of JNK regulates cell survival in response to TNF-α, while late and persistent activation of JNK leads to apoptosis [178, 179]. Another possibility is that apoptosis may depend on the cell type. However, JNK-dependent and -independent apoptotic pathways have been observed in the same cell type. In addition, different JNK isoforms seem to mediate different signals in lung cancer cells; dominant- negative JNK-1 inhibits UV-induced apoptosis, while dominant-negative JNK-2 is ineffective. The other possibility is that the biological effects of JNK depend on other factors present in signaling pathways that are active within the cells [250]. The results of this present study demonstrated that in trophoblast, SAPKs are transiently activated in response to VEGF and PlGF, and that VEGF- and PlGF- induced transient activation of SAPKs may protect trophoblast from stess -induced apoptosis.

Studies have shown that TNF-α and IFN-γ can induce apoptosis in primary trophoblast *in vitro* [180, 196, 251]. In this present study, exogenous EGF inhibited pro-inflammatory cytokine- induced trophoblast apoptosis, similarly to the anti-apoptotic activity exhibited by EGF in studies done by others [180]. However, even relatively high levels of exogenous PlGF or VEGF did not protect trophoblast against cytokine -induced apoptosis. These results further suggest that mechanisms by which PlGF and VEGF protect trophoblast from apoptosis are distinct from those mediated by EGF. This

functional difference is probably due to different signal transduction pathways activated by each growth factor. The EGF- induced ERK pathway seems to protect trophoblast from pro-inflammatory cytokine- induced apoptosis, while PlGF- and- VEGF induced SAPK pathways may protect trophoblast from stress- induced apoptosis in trophoblast.

One potential target for growth factor- induced survival effects of VEGF and PlGF may be the PI-3/Akt signal transduction pathway [15, 139, 200, 201, 252]. In this study, VEGF induced phosphorylation/activation of Akt in endothelial cells, an effect that was completely abolished by wortmannin. This suggested that VEGF induces Akt phosphorylation/activation in PI-3 kinase -dependent manner in endothelial cells. However, PlGF did not have any effect on Akt phosphorylation/activation. These results are consistent with other studies [15, 198], suggesting that VEGF but not PlGF phosphorylates/activates Akt kinase in endothelial cells. The divergent effects of VEGF and PlGF on Akt activation in endothelial cells may be due to their differential receptor (KDR or Flt-1) ligand specificity. The above results suggest that VEGF, but not PlGF, may act as a survival factor for endothelial cells. Similar to the results in endothelial cells, VEGF but not PlGF phosphorylated/activated Akt in trophoblast. Collectively, these results suggested that VEGF may act as a survival factor for trophoblast and HUVE cells, and this survival effect may be mediated through PI-3 kinase/ Akt- dependent pathways. However, survival effects of PlGF in trophoblast seem to be independent of Akt activation. Several possible mechanisms have been elucidated regarding the mode of action of Akt in protecting cells from apoptosis. Akt phosphorylates the anti-apoptotic protein Bad and prevents its heterodimerization with Bcl-2 [202]. Akt is known to

synergise with the Raf/MAPK pathway to transform some cells [253]. In addition, Ras-induced survival effects are known to be mediated through the PI-3 kinase/Akt pathway [254]. Additionally, Akt has been reported to translocate to the nucleus following the stimulation of cells with growth factors, suggesting that it may have nuclear targets that could inhibit apoptosis [239]; Akt is also known to induce E2F transcriptional activity [255]; Akt also inactivates caspase-9 directly by phosphorylation [205].

Viability of certain cell types depends on the expression of Bcl-2, and this antiapoptotic protein and/or some of its family members are known to play major roles in preventing apoptosis caused by withdrawal of growth factors/cytokines. The present study showed that VEGF up-regulates Bcl-2 in serum -deprived endothelial cells, while PlGF had no affect on Bcl-2 levels. These results in part are consistent with other studies. VEGF protects endothelial cells from serumdeprivation- induced apoptosis by inducing expression of Bcl-2 [14]. Furthermore, Akt activation was found to be essential for VEGF- mediated up-regulation of Bcl-2 in endothelial cells [198]. Up-regulation of Bcl-2 protein by VEGF and its effect on Akt phosphorylation/activation together suggest that VEGF may mediate survival effects through Akt/Bcl-2 pathways in endothelial cells. The inability of PlGF to up-regulate Bcl-2 in HUVE cells suggested that PlGF might not act as a survival factor for endothelial cells. It may be that the survival effects of growth factors in HUVE cells are receptor- specific, i.e., survival responses may be restricted to signaling through KDR. In contrast to HUVE cells, neither VEGF nor PlGF up-regulated Bcl-2 protein levels in trophoblast. However, Bcl-2 was detected in both the phosphorylated and unphosphorylated states in trophoblast, and neither VEGF nor PlGF

seemed to affect phosphorylation of Bcl-2. Phosphorylation is known to regulate Bcl-2 activity, although the precise role of Bcl-2 phosphorylation remains controversial. Some reports indicate that Bcl-2 becomes activated upon phosphorylation [219, 222] while others indicate that phosphorylation of Bcl-2 leads to its inactivation and eventual apoptosis [218, 219, 221]. If phosphorylation augments Bcl-2 function, the observed phosphorylation status of Bcl-2 (in the present study) may protect term trophoblast from apoptosis and maintain viability of the placenta during the late stages of gestation. Activation of JNK in other cells has been shown to regulate phosphorylation and functional activity of Bcl-2 [223]. However, both PlGF and VEGF had no effect on phosphorylation of Bcl-2. Thus, PlGF and VEGF may exert their observed protective effect against apoptosis in a Bcl-2- independent manner in term trophoblast.

The possible pathways mediating this survival effect are not known. However, several growth factors, like IGF-1 [225] and neurotrophic factor (NTF) [226] are known to exert their protective effects in fibroblast and ciliary neurons without altering Bcl-2 levels. There are several studies which show there is no link between Bcl-2 phosphorylation and apoptosis. Bcl-2 phosphorylation may occur only in the cells arrested at G2/M phase of the cell cycle [256]. A recent study also indicated that Bcl-2 phosphorylation as a marker of M phase of the cell cycle, and argues against its role as a determinant of apoptosis [257]. Irrespective of the molecular mechanism(s), this study suggested that PlGF/VEGF/Flt-1- mediated SAPK activation provide protection against stress-induced apoptosis whereas EGF/EGFR- mediated ERK activation does not increase trophoblast survival in the absence of growth factors.

The ability of PlGF and VEGF to modulate apoptotic events is clinically important because trophoblast apoptosis occurs both in normal pregnancies as well as pregnancies complicated by infections or other pathologies. Apoptosis has been demonstrated in the normal placenta throughout pregnancy. Discontinuities in the integrity of the syncytiotrophoblast may be due to apoptosis, and apoptosis is also responsible for the progressive disappearance of trophoblast in normal pregnancies in the chorion laeve as pregnancy approaches term [258, 259]. Apoptosis has been demonstrated in normal placentae from both the first and the third trimesters, and the rate of apoptosis appears to increase significantly as pregnancy progresses [185-188, 260]. These observations suggest that apoptosis plays an important role in the normal development and aging of placentae.

The mechanisms regulating trophoblast apoptosis during gestation are not known. Increased placental apoptosis during the first and third trimester in normal pregnancies [186] may be due to the low PlGF serum levels found during these trimesters [192]. Thus, it may be that low levels of PlGF increase trophoblast susceptibility to apoptosis in early and late stages of pregnancy. VEGF is produced by trophoblast throughout pregnancy and certain levels of VEGF may be needed to maintain vessel integrity and permeability in the placenta [261], along with conferring protection on trophoblast against apoptosis.

In addition to its potential role in normal pregnancy, trophoblast apoptosis has been implicated in several obstetrical complications. Apoptosis in the placenta leads to fetal growth retardation in rats [189], and the incidence of placental apoptosis is

significantly greater in human pregnancies complicated with intrauterine growth restriction (IUGR) [262]. A recent report also showed that preeclampsia is associated with widespread apoptosis of cytotrophoblast [191]. Placental bed hypoxia is generally thought to occur in preeclampsia and IUGR, and it is known to induce apoptosis in many cell types [190], as well as to decrease PlGF expression in primary trophoblast [20]. Decreased expression of PlGF in hypoxia in trophoblast *in vitro,* along with deceased levels of PlGF in preeclampsia [20, 192] suggest that decreased PlGF levels may contribute to increased trophoblast apoptosis. Thus, it is possible that relative placental bed hypoxia may cause decreased production of PlGF by trophoblast, increasing trophoblast susceptibility to stress-induced apoptosis.

Reports on maternal serum levels and immunoreactivity of VEGF in preeclampsia are conflicting. Studies by Baker *et al* [263] and Sharkey *et al* [264] showed increased serum levels of VEGF while other studies [265, 266] reported decreased serum levels and immunoreactivity in preeclamptic patients. However, serum levels of VEGF in preeclampsia may not be a true measure of local VEGF levels within the placental bed. Many sources other than the placenta contribute to VEGF levels found in preeclamptic sera [192]. Decreased VEGF mRNA levels were found in preeclamptic placenta [234, 267]. Thus, it is likely that placental VEGF levels in preeclampsia are reduced. Conceivably, the significantly decreased serum levels of PlGF and decreased local levels of VEGF during preeclampsia contribute to increased trophoblast apoptosis, which in turn have a significant role in the pathophysiology of the disease.

Collectively, results of this study suggested that trophoblast expression of PlGF

and VEGF could significantly influence the maternal-fetal interface. VEGF and PlGF may control decidua and villous vascular function via paracrine mechanisms, and influence trophoblast function directly via autocrine mechanisms. Consequently, aberrant trophoblast expression of PlGF and VEGF may contribute significantly to both the vascular and the placental pathologies commonly noted in perfusion -compromised pregnancies.

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VITA

Jayashree Desai was born in Belgaum, India. She received a Bachelor of Science degree in Microbiology in 1994 from the University of Tennessee at Knoxville TN. She then worked in Dr. Donald Torry's laboratory, Department of Obstetrics and Gynecology, Graduate School of Medicine, University of Tennessee Medical Center, Knoxville, TN. In August 1997, she reentered the University of Tennessee to pursue the Master of Science degree in Comparative and experimental Medicine. The Master of Science degree was received in December 1999.

She is presently working as senior Research Assistant in Dr. Donald Torry's laboratory, Department of OB/GYN, Graduate School of Medicine, Knoxville, TN.

Publications:

1. **Jaya Desai, Vicky Holt-Shore, Ronald J. Torry, Michael Caudle and Donald S. Torry.** Signal Transduction and biological functions of placenta growth factor in primary human trophoblast. Biology of Reproduction (1999), **60:** 887-892.
2. **Jaya Desai, Vicky Holt-Shore, Tzu-Hao Wang, Ronald Torry, Michael Caudle and Donald Torry.** Signal Transduction and functions of VEGF/Flt-1 in normal human trophoblast (manuscript in preparation).

Abstracts Published:

1. J. B. Desai, V.H. Shore, R.T. Torry, M.R. Caudle, D.S. Torry. Signal transduction and functions of VEGF/Flt-1 in normal human trophoblast. FASEB Journal. 13 (4): A469, March 1999.
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