Urokinase-activated Stat1 mediates antiproliferative effect in vascular smooth muscle cells cocultured with monocytes

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Master of Science, Sateesh Sreenivasan Kunigal

geboren am 26.11.1969 in Bangalore (Indien)

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Referent:	Prof. Dr. H. -A. Kolb
Korreferent:	Prof. Dr. Luft, Friedrich C.

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To

My Parents

For their intellectual support

My Wife and Daughter

For making everything worthwhile

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ABBREVIATIONS:

% (v/v)	Percentage volume
% (w/v)	Percentage weight
°C	Degree Centigrade (Celsius)
μ	micro (10^{-6})
aa	Amino Acid
APS	Ammonium Per Sulphate
ATF	Amino-terminal (135 residues long) fragment of uPA
BD	Binding Domain
BK	Bradykinin
bp	Base pairs
BrdUrd	5-bromo-2´-deoxyuridine
BSA	Bovine serum albumin
BSF-3	B-cell-stimulating factor-3
CaMKII	Ca2+/calmodulin-dependent kinase II
CBP/p300	CREB (cAMP–response-element-binding protein) binding protein
C/EBPβ	CCAAT/enhancer binding protein
CCD	Coiled-coil domain
Cdk	Cyclin dependent kinase
Ci	Curie
cDNA	Complementary DNA
CIS	Cytokine-induced SH2 protein
CNTF	Ciliary neurotrophic factor
CSF	Colony stimulating factor
CT-1	Cardiotrophin-1
DABCO	1,4-diazabicyclo[2.2.2]ocatne
DBD	DNA binding domain
DNA	Deoxy Ribonuleic Acid
DNTP	Deoxy Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetramine Acetate
EGF	Epidermal Growth Factor
EMSA	Electrophoretic gel-mobility shift assay
EPO	Erythropoietin
ERK	Extracellular-signal regulated kinase
FAK	Focal adhesion kinase

FCS	Fetal calf serum
GAS	Interferon-γ activation site
G-CSF	Granulocyte colony stimulating factor
GDNF	Glial cell line-derived neurotrophic factor
GFD	Growth factor domain
GH	Growth hormone
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPI	Glycosyl Phosphatidyl Inositol
h	Human
HEPES	4-(2-Hydroxyethyl-)-piperazine-1-ehtane sulphonic acid
HMG-I(Y)	'High mobility group' proteins-1
HRPO	Horse raddish peroxidase
IFN-γ	Interferon-gamma
Ig	Imunoglobulin
IgG	Immunoglobulin G
IGF	Insulin like growth factor
IL	Interleukin
IRF	IFN regulatory factor
ISGF-3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response elements
IU	International Units
JAB	Jak-binding protein
Jak	Janus kinase
JH	Jak homology
JNK	c-Jun NH2-terminal kinase
kb	kilobases
kDa	kilo Dalton
kPa	kilo Pascals
LPS	Lipoploysaccharide
M	Molar
mA	milli Amperes
MAPK	Mitogen activated protein kinase
MCM5	Minichromosome maintenance
MHC	Major Histocompatibility Complex
Min.	Minutes
mM	milli Molar

mRNA	Messenger Ribonucleic Acid
mV	milli Volts
MWCO	Molecular weight cut off
n	Number
NES	Nuclear export signals
NF-κB	Necrosis factor-kappa B
NGF-γ	Nerve growth factor-gamma
NLS	Nuclear localization signal
Nmi	N-myc interacting protein
NNT-1	Neurotrophin-1
NPC	Nuclear pore complex
Nup98	Nuclear pore protein 98
OD	Optical Density
OSM	Oncostatin M
ox-LDL	Oxidative Low Density Lippopolysaccharide
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate Buffer Saline
PBS-T	Phosphate Buffer Saline with 0.05% Tween 20
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PH	Pleckstrin homology
PIAS	Protein Inhibitor of Activated Stats
PI3-K	phosphatydilinositol-3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristate ester
PMSF	Phenylmethylsulphonylfluoride
PN-1	Protease nexin-1
PRL	Prolactin
PtdIns3,4,5	Phosphatidylinositol-3,4,5-triphosphate
PTK	Protein tyrosine kinase
PTPase	Protein tyrosine phosphatase
psi	Pounds per Square Inch
PVDF	Polyvinylidenefluoride
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase

rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SH	Src homology
SH2	Src-homology-2 domain
SHP-2	SH2-containing-phosphatase 2
SmGM2	Smooth muscle cells growth medium-2
STAM	Signal transducing adaptor molecule
Stat	Signal transducers and activators of transcription
Stat1si	Stat1 retroviral construct for silencing the Stat1
StIP	Stat3-Interacting Protein
SOCS	Suppressor of cytokine signaling
SSI	Stat-induced stat inhibitors
suPAR	soluble Urokinase type Plasminogen Activator Receptor
TD	Transactivation domain
TNF	Tumor necrosis factor
tPA	Tissue type Plasminogen Activator
TPO	Thrombopoietin
Tyk2	Tyrosine kinase 2
uPA	Urokinase type Plasminogen Activator
uPAR	Urokinase type Plasminogen Activator Receptor
VEGF	Vascular endothelial growth factor
VSMC	Vascular Smooth Muscle Cells

Amino acid	**three letter code**	**single letter code**	**Amino acid**	**three letter code**	**single letter code**
Glycine	Gly	G	Threonine	Thr	T
Alanine	Ala	A	Cysteine	Cys	C
Valine	Val	V	Tyrosine	Tyr	Y
Leucine	Leu	L	Asparagine	Asn	N
Isoleucine	Ile	I	Glutamine	Gln	Q
Methionine	Met	M	Aspartic acid	Asp	D
Phenylalanine	Phe	F	Glutamic acid	Glu	E
Tryptophan	Trp	W	Lysine	Lys	K
Proline	Pro	P	Arginine	Arg	R
Serine	Ser	S	Histidine	His	H

SUMMARY:

Atherosclerosis, Urokinase-type plasminogen activator and receptor (uPA-uPAR) system, Signal transducer and activator of transcription 1 (Stat1), Coculture.

Atherosclerosis is a major progressive disease of the large arteries causing the dysfunction of the heart and the effectiveness of percutaneous vascular therapeutic interventions for symptomatic atherosclerotic lesions is limited by vessel restenosis. Although several theories or hypotheses about atherogenesis have been proposed during the past decades, none can completely explain the whole process of the pathogenesis of atherosclerosis. In spite of this, the concept that atherosclerosis is a specific form of chronic inflammatory process resulting from interactions between plasma lipoproteins, cellular components (monocyte/macrophages, T lymphocytes, endothelial cells and smooth muscle cells) and the extracellular matrix of the arterial wall, is now well accepted. After vascular injury, a remodeling process occurs that features leukocyte migration and infiltration. Loss of endothelial integrity allows the leukocytes to interact with vascular smooth muscle cells (VSMC) and to elicit "marching orders"; however, the signaling processes are poorly understood.

In the present study mechanism involving inhibition of VSMC proliferation by monocytes is elucidated using a direct coculture model. It is shown that monocytes signal the VSMC via the urokinase-type plasminogen activator (uPA). The VSMC uPA receptor (uPAR) receives the signal and activates the transcription factor Stat1 that in turn mediates the antiproliferative effects. Our study for the first time mimics the in vivo events after the loss of endothelial integrity and explains the increased VSMC migratory potential and the absence of VSMC proliferation observed at the early step of a remodeling process of the vessel wall after vascular injury. Additionally they provide an important link between uPA/uPAR-related signaling machinery and human vascular disease which may be a useful strategy to drug discovery

Activation resulting in the nuclear translocation of Stat1 in VSMC upon coculturing with activated monocytes was documented by immunofluorescence. The Stat1 nuclear translocation is as a result of the interaction of uPA from monocytes and uPAR from VSMC. Blockade of uPA and uPAR with specific antibodies led to the inhibition of Stat1 nuclear translocation and further, immunofluorescence experiments using cells from uPA and uPAR knock-out mice in the coculture system demonstrated the importance of the uPA and uPAR system in the activation of Stat1 and involvement of fibrinolytic molecules in the Stat1 induced inhibition of cell growth. The secretion of uPA from monocytes and uPAR from VSMC when cocultured were measured by quantitative ELISA supported our above hypothesis.

The activation of Stat1 involves the phosphorylation of serine and tyrosine residues. With the help of immunoblotting it was demonstrated that the phosphorylated Stat1is predominantly translocated into the nucleus in VSMC cocultured with monocytes compared to monocultured VSMC by using the subcellular fractions and phosphorylation of serine at the position 727 preponderated the phosphorylation of tyrosine residue at 701. Additionally, activation of Stat1 in

VSMC when cocultured with activated monocytes, was shown at the DNA binding level using EMSA and supershift assay.

Upon Stat1 activation, the protein gets dimerized and is translocated to the nucleus, by disrupting the microtubules with nocodazole and by immunofluorescence it was shown that the cytoskeletal elements are not required for Stat1 nuclear translocation.

Furthermore IFN-γ plays a pivotal role in host defence mechanism by activating the JAK-STAT pathway. IFN-γ did not show any additive effect in activating the Stat1 and its nuclear translocation which was documented by immunoblot and EMSA.

The antiproliferative effect of Stat1 as a result of uPA induced activation involving phosphorylation of Stat1 at ser727 residue and its nuclear translocation is an important event involved in this antiproliferative mechanism was shown by blocking the Stat1 at ser727 residue. This was accomplished by the microinjection of the antibody against ser727 Stat1 into the VSMC and using this microinjected cells in coculture setup with monocytes and further performing single cell proliferation assay with these cocultured VSMC. Additionally single cell proliferation assay with different combination of cells from uPA and uPAR knock-out mice, VSMC from Stat1 knock-out mice and Stat1si-VSMC, obtained by RNA silencing for the stable and specific inhibition of Stat1 expression in human VSMC using retroviral RNA interference vector in coculture decisively supported the above results.

Finally, immunocytochemical staining for Stat1 and single cell proliferation assay using VSMC from uPA knock-out mouse and monocytes derived from wild-type mice in the coculture imperatively supported that Stat1 plays an pivotal role in the antiproliferative mechanism and ascertained the activation of Stat1 by uPA via paracrine loop.

The above obtained data supported by the earlier findings led the proposal of a novel mechanism and throw light on vascular remodeling upon vessel injury. uPA derived from monocytes binds to the specific uPAR on VSMC and thereby activates Stat1. This activation leads to the dimerization of Stat1 and translocation to the nucleus where it binds to the DNA and switches on the expression of a particular gene. This finally results in the inhibition of proliferation of VSMC.

Although these identified proteins might already provide new targets in the treatment of vascular disorders, the exact signaling mechanisms from cell surface uPAR activation to the Stat1 activation are still unclear.

ZUSAMMENFASSUNG:

Atherosklerose, Urokinase Plasminogenaktivator und Rezeptor(uPA-uPAR) system, Signal transducer and activator of transcription 1 (Stat1), Kokultur.

Die Atherosklerose ist eine bedeutsame, fortschreitende Erkrankung der großen Arterien, welche zur Funktionsstörung des Herzens führt. Die Wirksamkeit der perkutanen therapeutischen Intervention bei symptomatischen atherosklerotischen Verletzungen der Blutgefäße wird durch die Restenosis begrenzt. Obgleich einige Theorien oder Hypothesen der Entstehung der Atherosklerose in den letzen Jahrzehnten beschrieben worden sind, ist der gesamte Prozess der Pathogenese der Atherosklerose bislang nicht vollständig aufgeklärt. Dennoch ist die Annahme, dass es sich hierbei um eine spezifische Form eines chronischen entzündlichen Prozesses handelt, resultierend aus Interaktionen zwischen Plasmalipoproteinen, zellulären Komponenten (Monozyten/makrophagen, T-Lymphozyten, Endothelzellen und glatten Muskelzellen) und der extrazellulären Matrix der Arterienwand, weitestgehend akzeptiert. Nach innerer Verletzung des Blutgefäßes erfolgt ein umgestaltender Prozess, welcher die veränderte Migration und Infiltration von Leukozyten mit einschließt. Der Verlust der Endothelintegrität ermöglicht Leukozyten mit glatten Muskelzellen (VSMC) zu interagieren,wobei die hierbei ablaufenden Signalprozesse jedoch kaum erforscht sind.

Die vorliegendee Arbeit beschäftigt sich mit der Aufklärung der Mechanismen, die zu einer Hemmung der Zellproliferation glatter Muskelzellen durch Monozyten führen. Die Untersuchungen wurden in einem direkten Kokultur Modell durchgeführt. Es wurde gezeigt, dass Monozyten Urokinase Plasminogenaktivator (uPA) sezernieren und glatte Muskelzellen über deren uPA-Rezeptor (uPAR) das Signal empfangen, welches den Transkriptionsfaktor Stat 1 aktiviert. Die Stat1 Aktivierung wiederum vermittelt die antiproliferativen Effekte in den VSMC. Mit Hilfe des Kokultur Modells reflektiert diese Arbeit zum ersten Mal die in vivo Situation nach Verlust der Integrität des Endothels und erklärt die verstärkte Migration bei gleichzeitigem Fehlen der Proliferation glatter Muskelzellen, wie sie in den frühen Stufen des Umbauprozesses der Gefäßwand nach einer Gefäßverletzung beobachtet wird. Zusätzlich wird ein wichtiger Hinweis auf eine Verbindung des uPA/uPAR Systems mit Gefäßerkrankungen beim Menschen gegeben, welche von Bedeutung für die Entwicklung neuer therapeutischer Ansätze sein kann.

Die Aktivierung von Stat1in VSMC nach Kokultivierung mit aktivierten Monozyten resultiert in der Translokation von Stat1 in den Zellkern und konnte mittels Immunfluoreszenzfärbung dokumentiert werden. Die nukleäre Translokation von Stat1 ist das Ergebnis der Wechselwirkung von Monozyten sezernierten uPAs und dem uPA-Rezeptor glatter Muskelzellen. Die Blockierung von uPA und uPAR mit spezifischen Antikörpern führte zur Hemmung der Translokation von Stat1 in den Zellkern. Weiter zeigte die Verwendung kokultivierter uPA- bzw. uPAR defizienter Mauszellen in der Immunfluoreszenzfärbung die Bedeutung von uPA und uPAR für die Aktivierung von Stat1 und die Beteiligung von Komponenten des fibrinolytischen Systems bei der Stat1 induzierten Hemmung des Zellwachstums auf. Gestützt wird die obere Arbeitshypthese durch Messungen von Monozyten sezernierten uPAs und uPAR von kokultivierten VSMC in einem quantitativen ELISA.

Die Aktivierung von Stat1 bezieht die Phosphorylierung der Serin- und Tyrosinreste mit ein. Mit Hilfe von Immunoblots zytoplasmatischer und nukleärer Fraktionen wurde gezeigt, dass phosphoryliertes Stat1, im Vergleich zu VSMC aus der Monokultur, überwiegend in den Zellkern von mit Monozyten kokultivierten VSMC transloziert wurde. Hierbei überwog die Phosphorylierung von Serinresten in der Position 727 die Phosphorylierung von Tyrosin^{701}-Resten. Weiter wurde die Stat1 Aktivierung in VSMC, welche mit aktivierten Monozyten kokultiviert wurden, auf Protein-DNA Bindungsebene mittels EMSA und Supershift Assay gezeigt.

Nach Aktivierung formt Stat1 Dimere und wandert in den Zellkern. Für die nukleäre Translokation von Stat1 werden keine Zytoskelettelemente benötigt, wie durch den Einsatz von Nocodazol, das Mikrotubuli zerstört, mittels Immunfluoreszenzfärbung gezeigt wurde.

IFN-γ spielt eine zentrale Rolle bei Abwehrmechanismen der Wirtszelle über eine Aktivierung des JAK-Stat Signaltransduktionsweges. IFN-γ zeigte unter unseren Versuchbedingungen keinen additiven Effekt in Bezug auf die Aktivierung von Stat1 und dessen Translokation in den Kern, was mittels Immunoblot und EMSA dokumentiert wurde.

Die uPA induzierten Aktivierung, sprich die Phosphorylierung von Stat1 am Serin^{727}-Rest und dessen Translokation in den Zellkern ist von wichtiger Bedeutung für den beobachteten antiproliferativen Effekt von Stat1, was durch die Blockierug des Serin^{727}-Restes gezeigt werden konnte. Hierzu wurden Antikörper gegen Serin^{727}-Stat1 mittels Mikroinjektion in VSMC eingebracht, die darauf folgend nach Kokultivierung mit Monozyten in einem Einzelze llproliferationsassay untersucht wurden. Die oben genannten Ergebnisse werden bestätigt durch Proliferationsassays mit Zellen von uPA- bzw uPAR-defizienten Mäusen, VSMC von Stat1-Ko-Mäusen sowie Stat1si-VSMC, bei denen durch retroviralen Gentransfer die Stat1 Expression in humanen glatten Muskelzellen spezifisch gehemmt wird,. Im weiteren konnten Immunfluoresze nzfärbungen für Stat1 und Einzelzellproliferationsassays von VSMC aus uPA-Ko-Mäusen und Monozyten präpariert aus Wildtyp-Mäusen, die Schlüsselrolle von Stat1 im antiproliferativen Mechanismus und die parakrine, uPA induzierte Aktivierung von Stat1 klarstellen.

Die oben erhaltenen Daten, gestützt durch frühere Untersuchungen, zeigen einen neuen Signaltransduktionsweges beim Gefäßumbau nach dessen Verletzung auf. Monozytäres uPA aktiviert nach Wechselwirkung mit dem spezifischen Rezeptor (uPAR) glatter Gefäßmuskelzellen den Transkriptionsfaktor Stat1. Diese Aktivierung führt zur Bildung von Stat1-Dimeren, welche in den Zellkern transloziert werden, dort an spezifische DNA Sequenzen binden und die Expression bestimmter Gene anschalten. Letztendlich resultiert eine Hemmung der Zellproliferation VSMC aus diesem Zusammenspiel von Monozyten und VSMC.

Die hier identifizierten Proteine können neue Ansatzpunkte therapeutischer Interventionen sein, wenn auch die genauen Signaltransduktionswege von der Aktivierung des uPAR hin zur Aktivierung des Transkriptionsfaktors Stat1 in einzelnen noch ungeklärt sind.

1 . INTRODUCTION

1.1 Fibrinolytic system in vascular remodeling

Neointima formation after vessel injury is the most well studied model of vascular remodeling, especially accompanied with VSMC migration and proliferation (Figure 1). VSMC migration and proliferation are central to the process of restenosis after angioplasty and are also involved in the formation of atherosclerotic plaque.

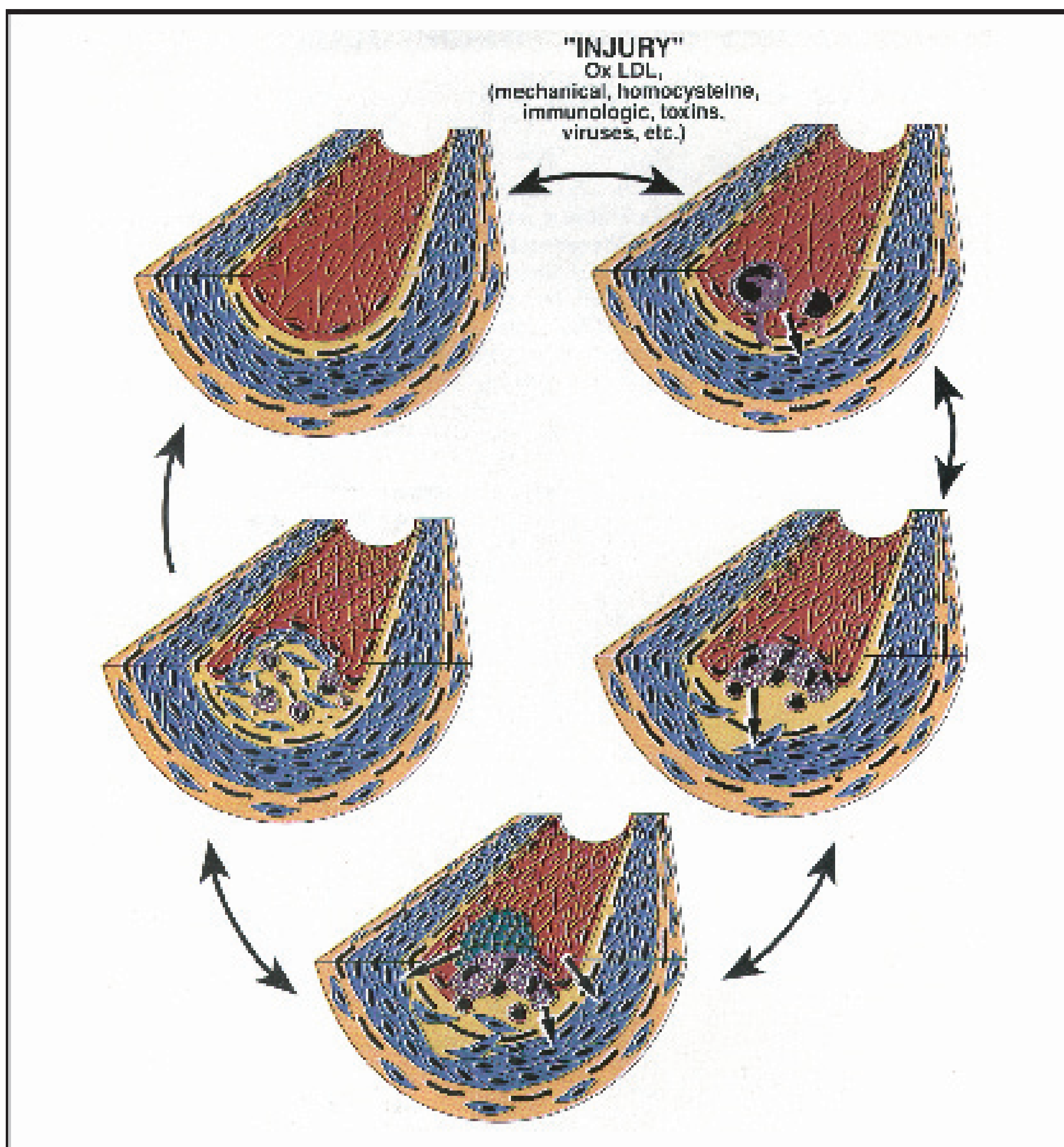

Figure 1. *Diagrammatic representation of the response-to-injury hypothesis of atherosclerosis, delineating the different stages of vascular remodeling involving proliferation and migration of VSMC (blue) (Figure from Ross R. Nature. 1993 Apr 29;362(6423):801-9. Review)*

Upon injury to the vessel wall, there is immediate activation of the coagulation system, which is followed by fibrinolysis. Apart from regulating hemostasis, these systems are also involved in coordinating the cellular responses to injury that involve processes such as cellular adhesion, migration, and proliferation (Esmon, 1995). It has been shown that initial recruitment or infiltration of migratory monocytes is one of the most preliminary determining steps that commands almost all the stages of vascular remodeling. Endothelial denudation at the site of vessel injury leads to infiltration of monocytes which in addition to releasing many biochemical molecules directly interact with medial VSMC and trigger them to migrate into the intima and their proliferation is associated with extracellular matrix production in the neointima (Schwartz et al., 1995b). Several cytokines and growth factors released by activated platelets, infiltrating cells, and damaged vascular cells are thought to play a role in the process that leads to neointima formation in response to vascular injury (Daemen et al., 1991; Lindner and Reidy, 1991; Majesky et al., 1990). In addition, they express components of the fibrinolytic system, namely the urokinase-type plasminogen activator (uPA) and its receptor (uPAR), which are potent chemotactic factors for VSMC . However, it remains poorly understood how the various components initiate and sustain VSMC migration and proliferation resulting in neointima formation. Recent studies in tissue-type plasminogen activator (tPA)–deficient mice (tPA$^{-/-}$ group) and uPA–deficient mice (uPA$^{-/-}$ group) demonstrated that these fibrinolytic factors play a critical role in vascular remodeling, particularly in the pathogenesis of atherosclerosis and restenosis (Carmeliet et al., 1997d; Carmeliet et al., 1997b) It has been suggested that the role of tPA is confined to intravascular clot lysis, whereas uPA mediates cell migration within the vessel wall (Matsuno et al., 1999). Increased expression of the uPAR in atherosclerotic plaques (Noda-Heiny et al., 1995) and knocking-out uPA decreases neointima thickening (Carmeliet et al., 1997d) further strengthened the supposed crutial role for uPA/uPAR in vascular remodeling.

Fibrinolytic activity is balanced by the levels of plasminogen activators and inhibitors that control the formation and action of plasmin. Though primarily mediating fibrin-clot lysis, these components are localized to the cell surface through specific receptors (or bound to specific extracellular molecules) and hence are also able to regulate pericellular proteolysis-related events (Blasi, 1993; Brunner and Preissner, 1994; Bu et al., 1994). The formation of a neointima involves remodeling of the extracellular matrix (Carmeliet and Collen, 1996b) as well as activation of latent growth factors (Koutsilieris et al., 1993; Odekon et al., 1994) through the plasminogen activation system. uPA also induces cellular effects independent of its enzymatic activity, such as stimulation of cell chemotaxis(Gyetko et al., 1994), adhesion of monocytes and neutrophils (Sitrin et al., 1996a; Waltz et al., 1993), release of tumor necrosis factor-α (Sitrin et al., 1996b), superoxide anion production (Cao et al., 1995), and expression of matrix metalloproteinases (Rao et al., 1995), all of

which contribute to neointimal formation. Non-proteolytic effects of uPA include also induction of intracellular signaling events, such as turnover of inositol phosphate, generation of diacylglycerol, phosphorylation of intracellular signaling proteins, and the induction of immediate-early genes such as c-fos (Anichini et al., 1994; Bohuslav et al., 1995; Busso et al., 1994; Del Rosso et al., 1993; Dumler et al., 1993; Dumler et al., 1994; Pacheco et al., 2002; Sitrin et al., 1999).

1.2 uPA/uPAR system

uPA is secreted by cells as a single polypeptide chain of 55 kDa (pro-uPA) that can be converted to active form by proteolytic cleavage by plasmin and other proteases of other classes like cathepsins G and L. Both the single chain and two-chain multidomain uPA, has 3 functionally defined independent regions: an amino or N-terminal growth factor domain, a kringle domain and a carboxy-terminal domain (Figure 2). The first two domains are collectively reffered to as A chain (20 kDa) and C-terminal catalytic serine protease as B chain (34 kDa). The two chains are linked by a single disulphide bond. The growth factor domain (GFD) (A chain) contains the receptor-binding region of the uPA, corresponding to amino acid residues 20-32 (Apella et al., 1987). By cleavage with plasmin a low molecular weight uPA can be isolated, which is endowed with catalytic activity and inhibitor binding activity but lacking the amino-terminal 135 residues.

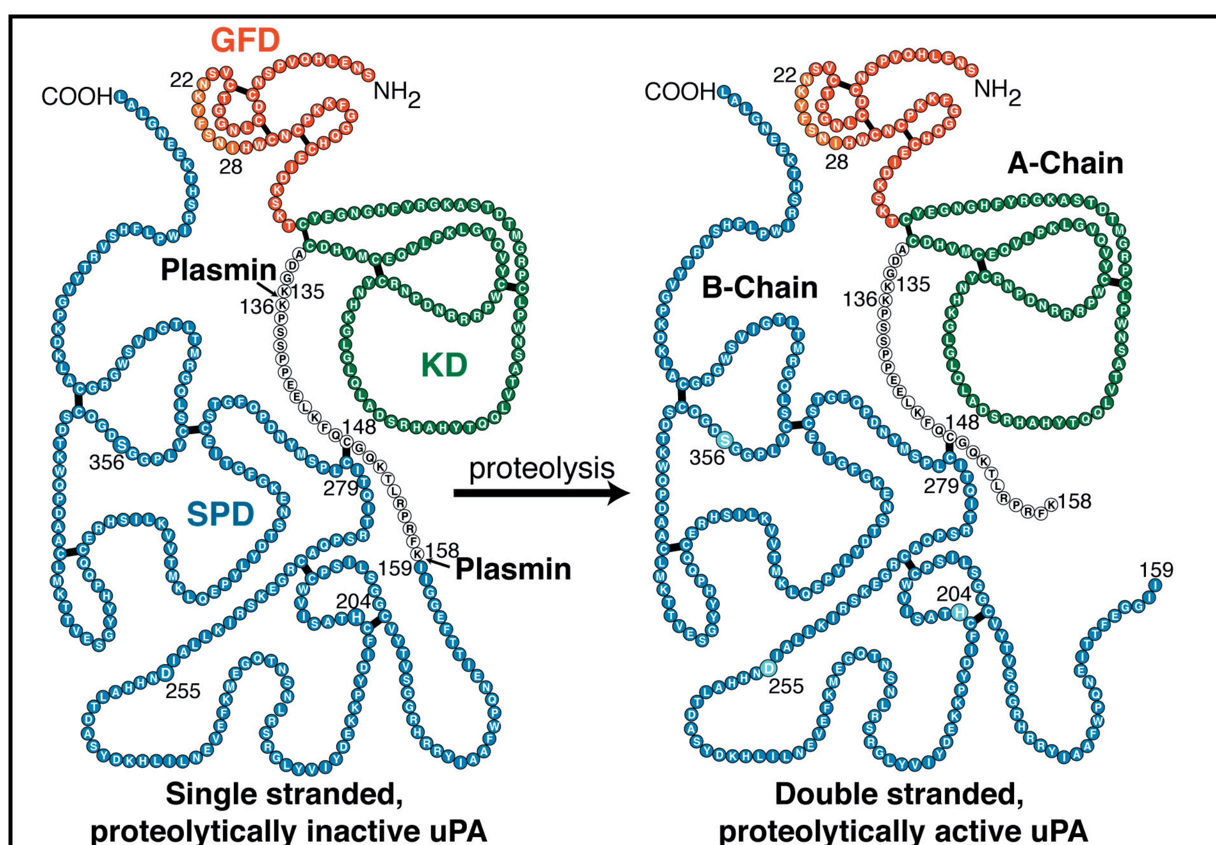

Figure 2. *Structure of single chain and double chain multidomain uPA (modified from Tkachuk et al., 1996)*

Pro-uPA, active uPA and amino-terminal 135 residues long fragment of uPA (ATF) bind to cell surface uPAR with high affinity (Blasi, 1988), while low molecular uPA does not bind to uPAR.

Upon secretion, the pro-uPA binds to specific cellular receptor (uPAR). The in vivo mechanisms by which pro-uPA is converted to active uPA are not completely understood. Findings from the last two decades show that binding of pro-uPA with low intrinsic proteolytic activity (Petersen et al., 1988) to uPAR does stabilize certain molecular conformation allowing the initial activation of plasminogen to plasmin and thus triggering a positive feedback mechanism (Manchanda and Schwartz, 1991). Pro-uPA can be activated by certain other proteases. Plasma kallikrein, trypsin, thermolysin, factor XIIa, cathepsin G, B and L can cleave the Lys158-Ile159 peptide bond and NGF-γ can cleave at the plasmin cleavage site thus forming active uPA (Goretzki et al., 1992; Ichinose et al., 1986; Kobayashi et al., 1991; Koivunen et al., 1989; Learmonth et al., 1992; Marcotte and Henkin, 1993). uPA with little plasminogen activating activity (uPA/T) can be generated by hydrolyses of the bond at Arg156-Phe157 in pro-uPA by thrombin.

Upon receptor binding uPA is also accessible to and its activity inhibited by PAI-1, PN-1 and PAI-2, which belongs to serpin superfamily. When receptor bound uPA is complexed to its above said specific inhibitors, it is internalized and degraded (Conese et al., 1994; Cubellis et al., 1990; Estreicher et al., 1990; Jensen et al., 1990).

The uPAR is a heavily glycosylated, 55-60 kDa monomeric protein (Nielsen et al., 1988) encoded as a 313 amino acid residues polypeptide with an additional 22 residues signal peptide (Roldan et al., 1990) (Figure 3). It consists of three domains D1, D2 and D3 each with approximately 90 residues. It is linked to the cell surface at the carboxy terminus by a glycosylp hosphatidylinositol (GPI) anchor (Ploug et al., 1991) but lacks a cytosolic and a transmembrane domain. The amino terminal domain of uPAR (D1) contains the main uPA-binding-site (Behrendt et al., 1991), whereas the other two domain D2 and D3 bind the extracellular matrix vitronectin (Wei et al., 1994). In addition to membrane bound form, uPAR is released from the plasma membrane by chymotrypsin-cleavage of the GPI anchor and can be found as a soluble molecule which is termed as suPAR. In vitro and in vivo, both uPAR and suPAR are proned to cleavage by several proteases including physiologically relevant enzymes such as neutrophil elastase, plasmin and uPA itself in the region that links domain D1 to domain D2 (amino acids 82-95) to yield a D1 fragment and a D2D3 fragment (Ploug et al., 1994). The D2D3 fragment has direct chemotactic activity (Ploug et al., 1994).

In many pathophysiological conditions such as tumor invasion, angiogenesis, and inflammation, cell migration and invasion are considered to be important steps. These are controlled by the uPA/uPAR system by regulating extracellular proteolysis, cell adhesion and signal transduction.

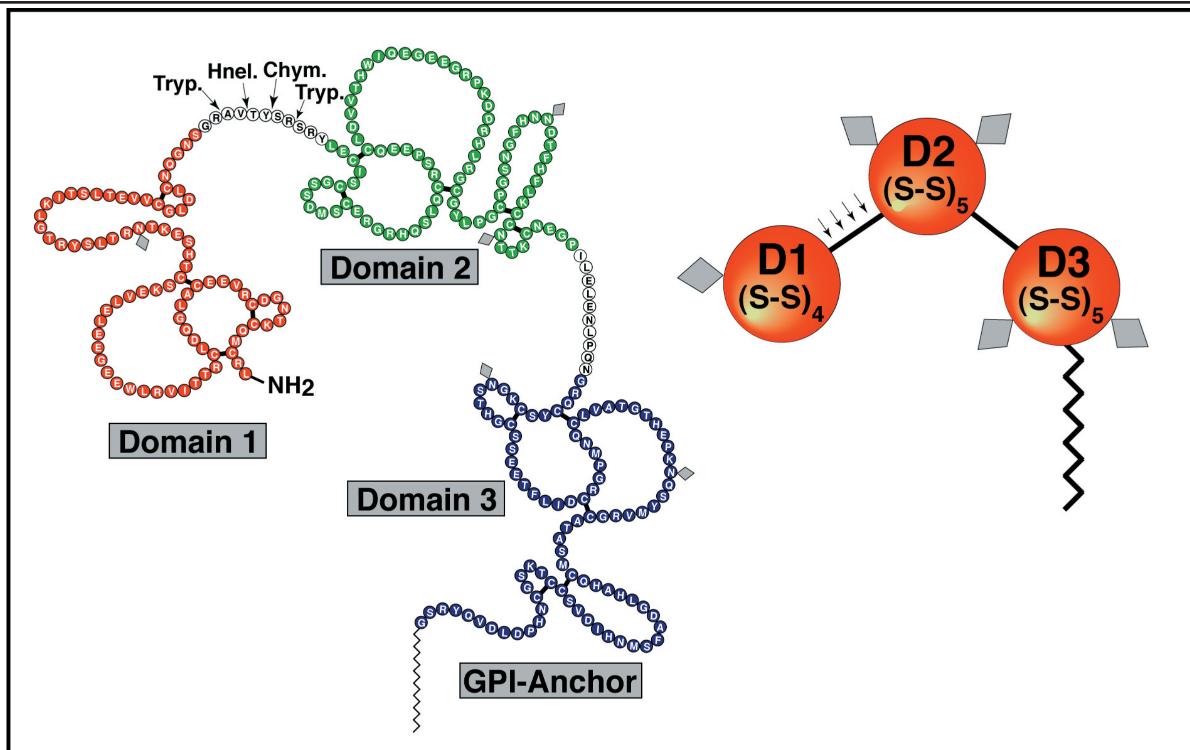

Figure 3. *Structure of uPAR showing the three different domains with the five potential N-Glycosylation sites (modified from Ploug et al.,1994)*

It has been indicated by several datas that the biological properties of uPAR, can be modified by a conformational changes in uPAR. For example, the uPA binding to uPAR causes the appearance of novel binding sites for vitronectin (Higazi et al., 1996b; Hoyer-Hansen et al., 1997b; Sidenius and Blasi, 2000b; Waltz and Chapman, 1994), thrombospondin (Higazi et al., 1996b), uPAR associated protein (Behrendt et al., 2000) and the disappearance of binding site for α_2-macroglobulin receptor (Higazi et al., 1996b). Further, uPA induced chemotaxis can be mimicked by the proteolytic cleavage of uPAR generates uPAR fragments that act as potent inducers of chemotaxis in cells lacking endogenous uPAR (Fazioli et al., 1997; Resnati et al., 1996). Although there are extensive evidence that uPAR is involved in complex interactions with other proteins, very little is known about how these interactions are regulated at the molecular level. In this line of research it has been shown that the ability of uPAR binding to vitronectin which induces cell adhesion (Higazi et al., 1996b; Hoyer-Hansen et al., 1997b; Sidenius and Blasi, 2000b; Waltz and Chapman, 1994) and to change gene expression during the differentiation of human myeloid U937 cells (Rao et al., 1995).

1.3 Jak-Stat signaling pathway

Cells of the immune system communicate with each other to initiate, establish and mediate immune responses. The vascular system, particularly arteries comprise predominantly with smooth muscle cells. The communication of the cells of the immune system with the VSMC occurs through cell-to-cell contact or through a variety of intercellular mediators that include cytokines, chemokines, growth factors, hormones and regulatory proteases which acts as regulators of

important functions. Many of these above said molecules are of biological importance and play central roles in the regulation of a wide array of cellular functions in the lympho-hematopoietic system. These factors stimulate proliferation, differentiation and survival signals in addition to specialized role in host resistance to pathogens. These molecules elicit their effects by activating multiple signaling pathways that together mediate these important cellular functions. The Jak-Stat pathway has been recently elucidated as a remarkably simple pathway which transmits information received from extracellular signals, through receptors having transmembrane domains, directly to target gene promotors in the nucleus providing a mechanism for transcriptional regulation without the aid of a second messengers (Figure 4). Although this pathway was found initially

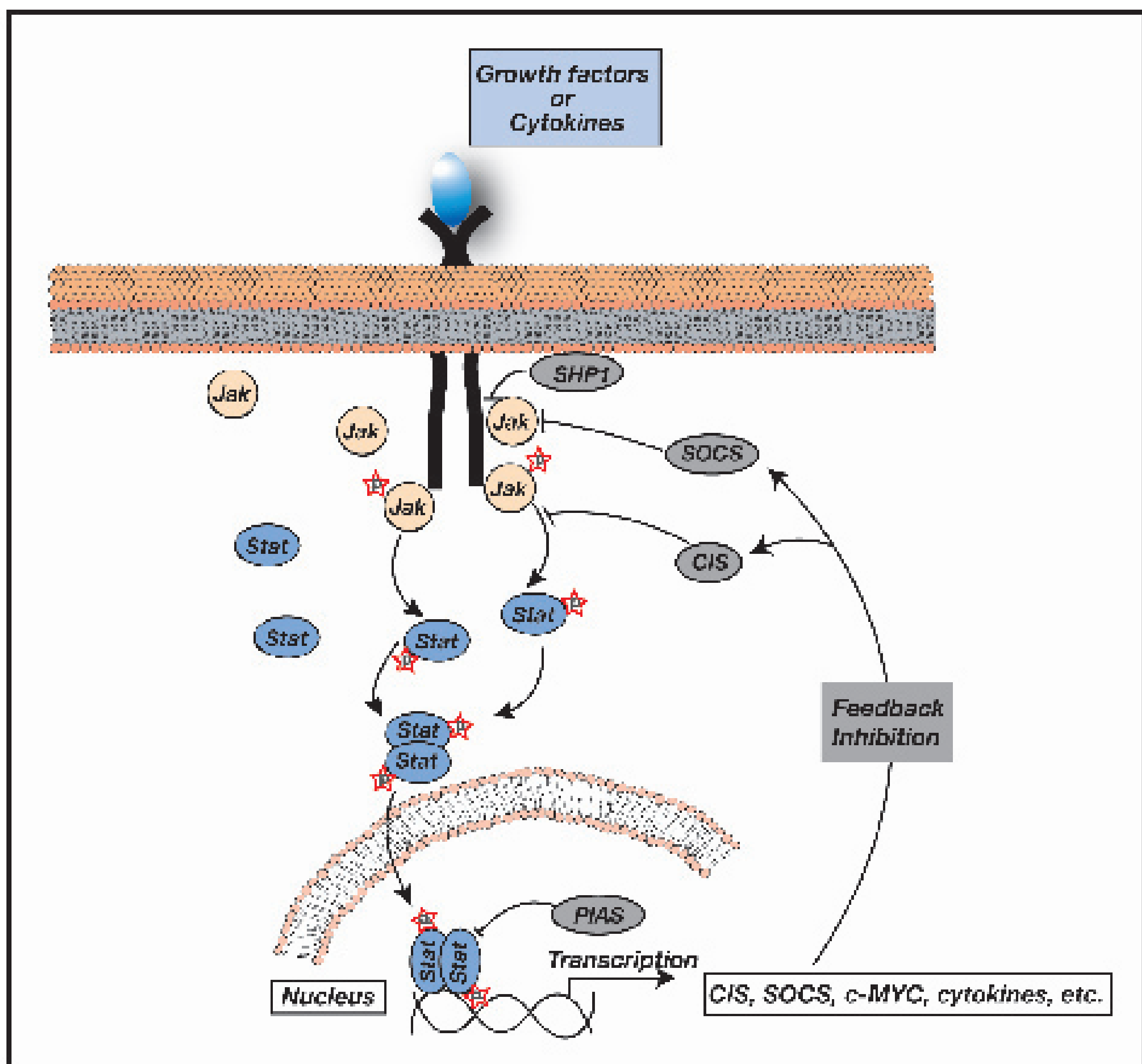

Figure 4. *A flow diagram representing the Jak-Stat pathway: Stimulation with growth factors or cytokines at the cell surface results in receptor activation and subsequent phosphorylation of Stats via phosphorylation of Jaks. Phosphorylation of Stats induces dimerization and translocation to the nucleus, where Stat dimmers bind to specific Stat response elements and directly regulate gene expression. The suppressor of cytokine signaling (SOCS) family of proteins dampen receptor signaling via homologous or heterologous feedback regulation (modified from Sigma-Aldrich on-line catalogue).*

to be activated by interferons, it is now known that a large number of cytokines, growth factors, hormonal factors and even some regulatory proteases like uPA activate Jak and/or Stat proteins. Further more, recent findings have suggested that the interdependence of Jaks and Stats might not be absolute as originally thought.

1.3.1 *The JAK family of protein tyrosine kinases*

In the early 1990s the search for the protein kinases led to the discovery of a novel family of intracellular non-receptor tyrosine kinases, the Janus Kinases or Jaks. At present there are four identified mammalian Janus kinase (Jak) family members : Jak1, Jak2, Jak3 and Tyk2 are highly related proteins of 120-140 kDa. Initially identification of Jak1, Jak2, and Tyk2 through different cDNA-cloning approaches as kinases whose ubiquitous expression gave no hint to their function derived them the names Janus (the Roman God with two heads) or Jak (just another kinase) (Ziemiecki et al., 1994). The fourth mammalian member Jak3 was cloned on the basis of similarity and was found to be predominantly expressed in hematopoietic cells, though low expression was reported recently in normal and transformed human cell types of various origins (Ihle, 1995; Lai et al., 1995; Verbsky et al., 1996). A Drosophila homologue, Hop, was found associated with the Hopscotch mutations (Binari and Perrimon, 1994). Jaks are also known in Xenopus laevis, Caenorhabditis elagans and fish. Amino acid sequence alignment of the Jaks revealed that they possess seven highly conserved domains (JH1-JH7; figure 5). In the recent years pioneering works on interferon signaling and the functional complementation of three mutant cell lines with genomic DNA or cDNAs encoding the Jaks, the role of these enzymes as esential effectors in interferon (IFN) α/β and IFNγ signaling was firmly established (Darnell et al., 1994).

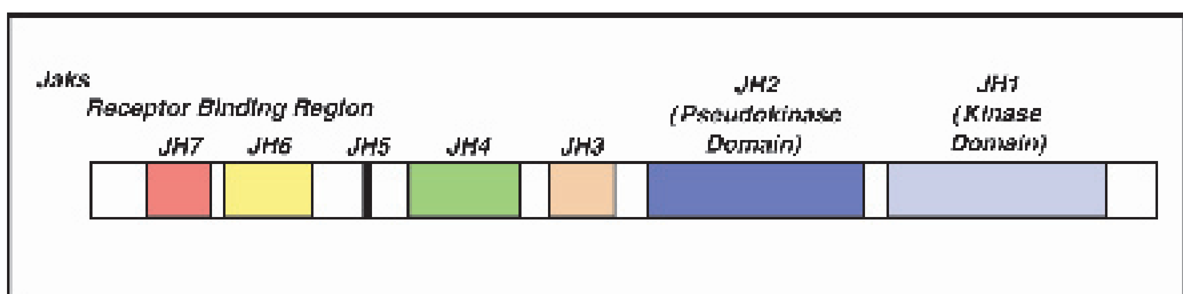

Figure 5. *Structure of Jaks*

Jaks are structurally unique in having tandem kinase and kinase-like domains. The C-terminal kinase domain is the catalytic domain, and the precise function of the kinase-like domain has yet to be determined. Regions of homology shared by Jaks have been termed Jak homology (JH) domains. They are named in a C-terminal-to-N-terminal direction. The JH1 and JH2 domain is the kinase domain and the The remainder of the homology domains are indicated though their functions are not well understood. The N-terminus of the Jaks, however, appears to be important for association with cytokine receptor subunits (modified from Leonard and O´shea, 1998)

Compelling evidence was obtained that dozens of soluble mediators or cytokines, ranging from growth and/or differentiation factors to hormonal polypeptides, pleiotropic interleukins and regulatory proteases like uPA utilised Jaks as immediate intracellular effectors (Briscoe et al., 1994; Ihle, 1995; Taniguchi, 1995). For example in the case of intercellular signaling molecule cytokine, which share a common α-helical structure and utilize a group of structurally related receptors (Nicola, 1994). The cytokine receptor superfamily comprises polypeptides with a single transmembrane domain and common extracellular structural motifs that are important for ligand binding (Bazan, 1990; Thoreau et al., 1991). Since these molecules do not posses intrinsic catalytic activity, they rely on Jaks, which are constitutively associated with their cytoplasmic regions, to transduce the extracellular ligand-binding event to an intracellular signal. Motifs called box 1, usually proline rich and box 2 in the membrane proximal regions of the receptors are important for Jak association. The fundamental role of Jaks in cytokine signaling is evidenced by the inherited immunodeficiencies caused by mutations that block receptor-Jak interactions or the kinase activity of the Jaks. Activation of Jaks occurs within minutes of ligand binding and as a result the kinases are brought together as a result of dimerization/oligomerization or, as ligand-induced conformational changes of pre-existing dimers (Livnah et al., 1999; Remy et al., 1999). In the case of homodimeric receptors, e.g., for growth hormone (GH), prolactin, thrombopoietin (TPO) and erythropoietin (EPO), only Jak2 is activated. In contrast, heteromeric receptors, made up of different subunits, activate distinct combinations of Jaks. Upon activation Jaks proceed to phosphorylate the receptor subunit(s) as well as other substrates. Stat proteins are one of the most intensely studied substrates of Jaks, which will be discussed later in detail and in particular Stat1 is the candidate of this study.

In addition to have a pivotal role in cytokine signaling, Jaks also have accessory roles in other pathways. It has been reported that Jaks get phosphorylated and activated after stimulation with growth factors whose receptors are known to posses intrinsic ligand-inducible tyrosine kinase activity like colony stimulating factor (CSF)-1 (Novak et al., 1996), epidermal growth factor (EGF) (Leaman et al., 1996b), platelet-derived growth factor (PDGF) (Vignais et al., 1996), Insulin and Insulin like growth factor (IGF)-1 (Gaul et al., 1998). Jaks also appear to be involved in signaling by G-protein-coupled receptors, including those for angiotensin (Marrero et al., 1995), serotonin (Guillet-Deniau et al., 1997) and α-melanocyte-stimulating hormone (Buggy, 1998). Recently, it has been reported that Jak2 interacts directly with the cytoplasmic tail of the angiotensin receptor (Ali et al., 1997). Jaks, particularly Tyk2 in association with Stat3 is activated by the peptide hormone bradykinin (BK) signaling (Hong et al., 2000). The Jaks along with Stats can also be activated in response to tumor necrosis factor (TNF) (Guo et al., 1998), osmotic shock (Gatsios et al., 1998) and upon ligation of MHC-I (Skov et al., 1998b) and CD40 proteins (Hanissian and

Geha, 1997). In addition, the Jak-Stat signaling pathways are regulated by a vast array of intrinsic and environmental stimuli, which can add plasticity to the response of a cell or tissue.

Jaks are only part of whole story in the signaling cascade. Upon activation of Jaks, many cytoplasmic proteins get involved and interact and finally signaling cascade effect in gene expression. The knowledge of revelant Jak substrates is quite limited. Aside from kinases themselves being substrates, which is a paradigm, cytokine receptors is another class. Tyrosine phosphorylation of receptors forms docking sites for proteins with phosphotyrosine binding domain, which in turn are also Jak substrates. The Stat (Signal Transducers and Activators of Transcription) family of transcription factors is one such example and the adaptor molecule Shc is another. Grb2, SHP-2, Vav and STAM are other proteins that interact with Jaks (Chauhan et al., 1995; Matsuguchi et al., 1995; Takeshita et al., 1997; Yin et al., 1997). In this direction discovery of Stats provides important insights as to how extracellular signals effect gene expression.

1.3.2 *Stats (Signal transducers and activators of transcription)*

Stats are latent cytosolic transcription factors utilized in signaling triggered by cytokines, chemokines, growth factors, hormones and regulatory proteases which act as regulators of important functions. The seven Stat proteins identified in mammals are denoted as Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6. They range in size from 750-850 amino acids. The chromosomal distribution as well as identification of Stats in more primitive eukaryotes, suggests that this family originated from a single primordial gene. As eukaryotes emerged more and more complex, duplication of this locus appear to reflect an increasing need for cell-to-cell communication. Consistent with this evolutionary pattern, Stats share structurally and functionally conserved domains. This includes the amino-terminal domain (NH2), the coiled-coiled domain (CCD), the DNA binding domain (DBD), the linker domain and the SH2/tyrosine activation domain. In contrast to other domains, the carboxy-terminal transcriptional activation domain (TAD) is quite divergent and contributes to Stat specificity. Though there is limited number of Stats, as they have achieved significant specificity it is possible for over 50 members of hematopoietin family to transduce signals. This is largely because the receptors that transduce signals for these cytokines can be placed into five structurally and functionally related subfamilies, each of which tend to transduce signals through a single Stat protein (refer to the Table 1).

Additionally, tissue specificty and activation of other signaling pathways by these receptors contribute to the specificity. The following section highlights the structural, functional and regulatory properties of the mammalian Stat family.

CYTOKINE / GROWTH FACTOR	JAK KINASE ACTIVATED	STATS ACTIVATED
Type I cytokines		
Cytokines whose receptors share γ_c		
IL-2, IL-7, IL-9, IL-15	Jak1, Jak3	Stat5a, Stat5b, Stat3
IL-4	Jak1, Jak3	Stat6, Stat5a, Stat5b
IL-13	Jak1, Jak2, Tyk2	Stat3, Stat6
IL-21	Jak1, Jak3	Stat1, Stat3, Stat5
TSLP	none	Stat5
Cytokines whose receptors share β_c		
IL-3, IL-5, GM-CSF	Jak2	Stat5a, Stat5b
Cytokines whose receptors share gp 130		
IL-6, IL-11, OSM, CNTF, CT-1	Jak1, Jak2	Stat1, Stat3
IL-12	Tyk2, Jak2	Stat4
Leptin	Jak2	Stat3
IL-23	?	Stat4
NNT-1/BSF-3	Jak1, Jak2	Stat1, Stat3
G-CSF	Jak1, Jak2	Stat3
Cytokines with homodimeric receptors		
GH	Jak2	Stat5a, Stat5b, Stat3
PRL	Jak2	Stat5a, Stat5b
EPO, TPO	Jak2	Stat5a, Stat5b
Type II Cytokines		
IFNγ	Jak1, Jak2	Stat1, Stat2
IFN-αs/β/ω/Limitin	Jak1, Tyk2	Stat1, Stat2, Stats3-6
IL-10	Jak1, Tyk2	Stat3
IL-19, IL-20, IL-24	?	Stat1, Stat3
IL-22	Jak1, Tyk2	Stat1, Stat3, Stat5
Growth factors		
EGF	Jak1, Jak2	Stat1, Stat3, Stat5
PDGF	Jak1, Jak2	Stat1, Stat3, Stat5, Stat6
VEGF	?	Stat1, Stat3, Stat5, Stat6
HGF	?	Stat1, Stat3
CSF-1	Jak1, Tyk2	Stat1, Stat3, Stat5
Seven transmembrane domain receptors		
Angiotensin II AT1	Jak2, Tyk2	Stat1, Stat2

Table 1. *Activatuion of Jaks and Stats. In certain receptors , the Jaks and Stats with essential signaling functions discovered to date are indicated in bold (modified from Leonard and O´shea, 1998; Kisseleva et al., 2002; Schindler, 2002)*

1.3.2.1 *Structure of Stat proteins*

As mentioned above the six conserved domains are identified by structural and functional analysis. The crystal structure of two Stats, Stat1 and Stat3, bound by DNA has been solved, but the structures did not include the amino and carboxy-terminal portions of the molecules reviewed in (Horvath, 2000). Figure 6 shows the crystal structure of Stat1 DNA complex and domains of Stat in general and Stat1 in particular.

1.3.2.2 *N-terminal domain*

The N-terminal domain, comprising approximately 130 amino acids, is conserved among the Stats (51% sequence identity between Stat1 and Stat4; 20% between Stat5 and Stat6). It represents an independently folded and stable moiety, which can be cleaved from the full-length molecule by limited proteolysis (Vinkemeier et al., 1996). The crystal structure of Stat4 (amino acids 1-124) reveals a dimer (Vinkemeier et al., 1998). The interface of this dimer is formed by a ring-shaped element consisting of five short helices. Several studies suggests that this N-terminal dimerization stabilizes binding of the dimers to tandem GAS elements (Vinkemeier et al., 1996; Vinkemeier et al., 1998; Xu et al., 1996). Further, studies have shown that the N-terminal Stat domain promotes interaction with transcriptional coactivator CBP/p300 (Horvath, 2000), the PIAS family (Shuai, 2000), receptor domains (Leung et al., 1996) and that it regulates nuclear translocation (Strehlow and Schindler, 1998).

1.3.2.3 *Coiled-coil domain*

The coiled-coil domain consists of four α-helices comprising approximately 135-315 amino acids. It is linked to the N-terminal domain by a flexible polypeptide chain (24 amino acids for Stat1 and 18 amino acids for Stat3). The crystal structure of Stat1 and Stat3 reveals that this domain protrudes about 80 Å laterally from the core structure (Becker et al., 1998; Chen et al., 1998). This domain is predominant hydrophilic surface, which takes part in specific interactions with other helical proteins. The interacting proteins include p48/IRF9, the transcription factor c-Jun, N-myc interacting protein (Nmi) and StIP (Collum et al., 2000; Horvath et al., 1996; Zhang et al., 1999b; Zhu et al., 1999). Recent studies have implicated the coiled-coil domain in receptor binding, tyrosine phosphorylation and nuclear export (Begitt et al., 2000; Zhang et al., 2000b).

1.3.2.4 *DNA-binding domain*

The DNA binding domain, which occupies approximately between 320-480 amino acid residues is a β-barrel with an immunoglobulin fold and lies carboxy-terminal to the coiled-coil domain. This structure is reminiscent of NF-κB and p53 DNA binding domains (Chen et al., 1998).

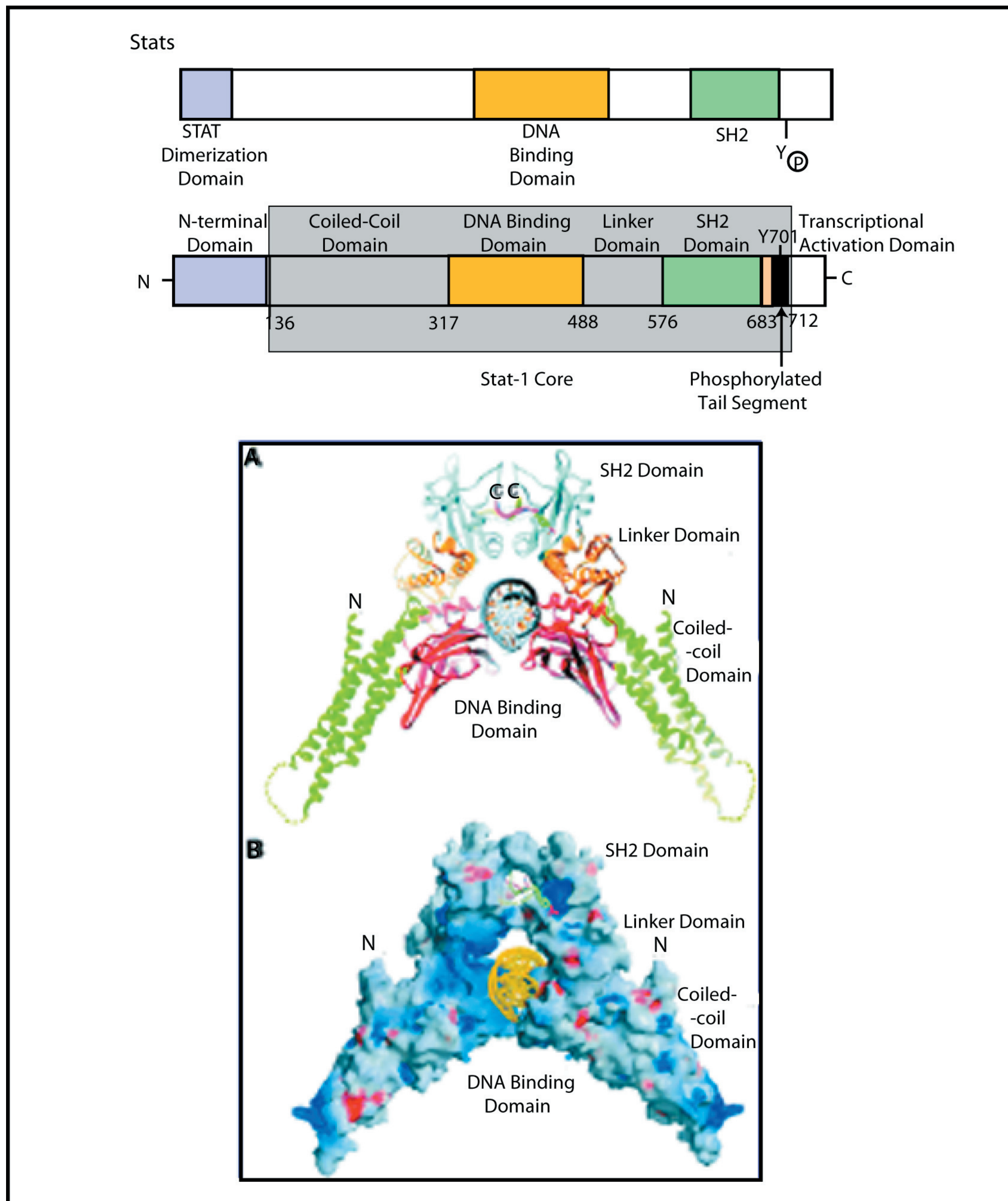

Figure 6. *Structure of Stats*

Schematic diagram showing the domains of Stat in general and Stat1 in particular and additionally the crystal structure of the Stat1 DNA complex (modified from Leonard and O´shea, 1998 and Chen et al., 1998).

The cooperativity in DNA binding is likely to be important in effective transcriptional activity, which is supported by the finding that the number of contact sites between amino acid residues and DNA are moderate. Also the dissociation constant is in nanomolar range. As the DNA binding domain shows different conformation (prior to activation) in the inactive state it is appearing to consider that it may have other additional functions (McBride et al., 2000).

1.3.2.5 *Linker domain*

The linker domain connects the DNA-binding domain with the SH2/dimerization domain. Studies suggest that the linker domain interacts both with the SH2 domain and DNA binding domain (Chen et al., 1998) and in turn mutational studies have also implicated that the transcriptional regulation by the linker domain in Stat1 (Yang et al., 1999).

1.3.2.6 *SH2 domain and tyrosine activation motif*

SH2 domain plays an important role in signaling as they are capable of binding to specific phosphorylated tyrosines. Stat proteins have conserved SH2 domain (approximately residues between 580-680) though they are divergent from other SH2 domains. It consists of an anti-parallel β-sheet flanked by two α-helices, which form a pocket. At the base of this pocket lies a highly conserved arginine (Arg-602 for Stat1, Arg-609 for Stat3), which mediates the interaction with phosphate. The ability of this SH2 domain to recognize specific phosphotyrosine motifs plays an essential role in three Stat signaling events: (1) recruitment to the cytokine receptor through recognition of specific receptor phosphotyrosine motifs, (2) association with the activating JAK (Barahmand-Pour et al., 1998; Gupta et al., 1996) and (3) STAT homo- or heterodimerization (Gupta et al., 1996; Shuai et al., 1994). Interaction between the SH2 domain of one Stat monomer and the tyrosine phosphorylated tail segment of the other monomer results in Stat dimerization. Residues most involved in defining the specificity of the interaction between the SH2 domain and tyrosine motif are located at positions +1, +3 and +5, +6, +7 C-terminal to the phosphotyrosine (Chen et al., 1998). Closely positioned amino acids of the SH2 domain (e.g. Ala-641, Val-642) appear to participate in this interaction. All Stats except Stat2 have been shown to form stable homodimers in vitro and in vivo. Additionally, many STATs, including Stat2, can heterodimerize with other Stats through this reciprocal SH2-phosphotyrosine interaction (Darnell Jr, 1997; Schindler and Darnell, 1995). As each STAT has both an SH2 domain and a phosphorylated tyrosine these dimers are stabilized by bivalent interactions. The bivalent nature of these interactions helps to explain why dimerization of Stats is favored over the monovalent interaction of the Stat SH2 with a phosphorylated receptor.

1.3.2.7 *Transcriptional activation domain (TAD)*

Consistent with its ability to regulate unique transcriptional responses, the carboxy-terminal domain is poorly conserved among the Stats. The first evidence that the carboxy-terminus encodes transcriptional activation domain (TAD) came from a comparative analysis of the full-length Stat1 and an alternatively spliced isoform Stat1β, which lacks the last 38 carboxy-terminal amino acids (Schindler et al., 1992). Well characterized C-terminally truncated isoforms have also been identified for Stat3, Stat4 and Stat5 (Schindler and Strehlow, 2000). They appear to function as dominant-negative regulators. Although a detailed understanding of how the Stat carboxy-terminus regulates transcription remains to be determined, important progress has been made. For example, it has been determined that the transcriptional activity of several Stats can be modulated through serine phosphorylation within the TAD, reviewed in (Decker and Kovarik, 2000). Serine phosphorylation appears to enhance the transcription of some, but not all target genes. It has been suggested that TAD phosphorylation could allow for regulation and crosstalk by different receptors which may involve the recruitment of other transcription factors and coactivators. Serine phosphorylation may alter the affinity for other transcriptional regulators like MCM5, BRCA1, CBP/p300 and c-Jun (Horvath, 2000).

1.4 STAT activation

Structural studies of several hematopoietin receptors from the cytokine family indicate that ligand binding promotes the dimerization of receptors into an active conformation (Wells and de Vos, 1996). In each case activation is believed to lead to close approximation of cytoplasmic receptor tails, enabling the transphosphorylation (i.e. activation) of the receptor-associated Jaks. Activated Jaks then phosphorylate specific tyrosine motifs present in the receptor endodomains, which in turn mediate the recruitment of Stats to their appropriate receptor. This entails the ability of Stat SH2 domains to recognize a phosphotyrosine residue and 4–5 carboxy-proximal amino acids, known as the receptor Stat recruitment motif. Table-1 summarizes functional groups into which hematopoietin receptors can be divided and Jak and Stat family members they activate.

1.5 Nucleocytoplasmic transport of Stats

Stats which are predominantly localized in the cytoplasm, upon activation (phosphorylation and dimerization) rapidly translocate to the nucleus and induce gene expression. After termination of the signal, Stats translocate back to the cytoplasm. This regulated mobilization of Stats is an essential step for signaling and is mediated by the nuclear pore complex (NPC) (Doye and Hurt, 1997). Stats are transported across the NPC in an active bidirectional process that is energy and activation dependent.

Although dimerization presumably unmasks a short amino acid sequence (residues i.e.arginines and lysines) called a nuclear localization signal (NLS), which directs active nuclear import of protein. The residues comprising the Stat NLS are not well defined. The rapid and ligand-dependent nuclear import of Stats was an important feature recognized during the initial characterization of Stat1 and Stat2. Moreover, this was found to be dependent on tyrosine phosphorylation and independent of an association with the cytoskeleton (Lillemeier et al., 2001). Several groups have attempted to locate the elusive Stat NLS through mutagenesis of arginine/ lysine-rich motifs. Recently, the identification of putative NLS elements in cytokine receptors and ligands led to the speculation that Stats may translocate to the nucleus through association with other signaling components (Sekimoto et al., 1997; Subramaniam et al., 2000). It has been reported that Stat1 translocates to the nucleus by a gain-of-function mechanism, it is not anchored in the cytoplasm and does not constitutively shuttle in and out of the nucleus (McBride et al., 2000). Following specific tyrosine phosphorylation, Stat1 dimerizes via intermolecular SH2–phosphotyrosine interactions and this dimerization appears to trigger a conformational change in the Stat1 molecule that reveals a functional NLS. A number of reports together indicate that dimerization is required to facilitate the functional appearance of an NLS on STATs (Bromberg and Darnell, 1999; McBride et al., 2002; Milocco et al., 1999).

Classical NLSs bind to central armadillo repeats in the importin- receptors; however, tyrosine-phosphorylated Stat1 binds to a C-terminal domain that does not overlap with the central armadillo repeats (Conti et al., 1998; Fontes et al., 2000; Sekimoto et al., 1997). STAT1 is the only known imported substrate that interacts with the C-terminus of importin. This finding supports the hypothesis that the Stat1 NLS does not conform to a classical NLS. The DNA binding domain of Stat1 appears to have evolved with NLS and NES motifs that may overlap and serve to direct the Stat1 protein to its proper cellular localization. The position of the NLS and the NES within the DNA binding domain of Stat1 suggests an elegant mechanism to regulate cellular localization of a signal transducer and activator of transcription.

Analogous to import, nuclear export is specified by nuclear export signals (NES), which are characterized by a leucine rich amino acid sequence (Fornerod et al., 1997). In contrast to the poorly understood mechanism of Stat nuclear import, recent studies have provided important insights into how this protein is exported from the nucleus during the period of signal decay that follows stimulation. In summary, more recent progress has been made in understanding how Stats are exported from the nucleus, then imported. Unexpectedly, these studies indicate that in resting cells Stats are continuously exported from the nucleus, raising some intriguing new questions about Stat regulation.

1.6 Stat DNA binding

Once in the nucleus, in general, the Stat homodimers or heterodimers can directly bind to DNA. However, investigation of IFN responses has identified two different transcriptional enhancers within the promoter elements of STAT target genes (Decker et al., 1997b; Kessler et al., 1988; Schindler and Brutsaert, 1999b). Type I IFNs (α, β, ω Limitin) signal through the formation of ISGF-3 complexes, which bind to ISRE sites (IFN stimulated response elements) and initiate gene transcription (Kessler et al., 1988). Comparative analysis of IFN-α response elements revealed an ISRE consensus consisting of two tandem sequences, AGTTTN$_3$TTTCC. Among the proteins that compose the ISGF-3 complex, IRF-9/p48 plays a critical role in recognizing the ISRE site. Stat1 also promotes contact with the additional flanking nucleotides, whereas Stat2 does not directly interact with the DNA (Qureshi et al., 1995).

IFN type II signals (γ) through Stat1 homodimers, which bind to a distinct response element, the GAS (Decker et al., 1997b). This palindromic element has a consensus sequence of TTTCCNGGAAA. Similar responsive elements have been identified in the promoters of other Stat-induced genes. Biochemical studies have determined that TTCN$_{2–4}$GAA consensus defines the optimal binding site for all Stats, with an exception for Stat2, which appears to be defective in DNA binding. This provides an opportunity for most Stat homodimers to exhibit unique DNA binding preferences. In part this is determined by spacing between palindromic half sites. Thus, Stat1 binds to an element with a canonical n=3 spacing, while Stat3 and Stat6 favor elements where n=2 and n=4, respectively (Decker et al., 1997b; Ehret et al., 2001). Additionally, recent studies indicate that the ability of Stats to bind cooperatively to tandem GAS elements (6–10 bp apart) also contributes to DNA binding specificity. Moreover, these tandem sites often include nonconsensus GAS elements (Ehret et al., 2001; Soldaini et al., 2000).

1.7 Regulation of the Jak-Stat signaling pathway

The Jak-Stat pathway is important for many host responses including defense, differentiation, proliferation, and oncogenesis. It is, therefore, not surprising that numerous regulatory layers exist to modulate this signaling pathway. This includes both negative and positive regulation. The effect of these regulatory processes determines the rate at which Stat signals are transduced.

1.8 Negative regulation of Stat signaling

Cells have innate mechanisms to protect themselves from repeated stimulations from external stimuli under certain physiological conditions by down regulating the signals in the signaling cascade. In the case of pathway involving Stat proteins, there are several switch-off points in the signaling cascade at the levels of receptors, Jaks and the Stat molecules themselves.

There are studies reporting receptor endocytosis (Dittrich et al., 1996). Stat proteins and also the receptors have been found to be targets of Ubiquitin-proteosome mediated degradation (Haspel et al., 1996; Kim and Maniatis, 1996). As it is mentioned earlier, Jak activation is dependent on tyrosine phosphorylation so it has been found that the phosphatases containing SH2 domain negatively regulates Jak activity (Jaio et al., 1997; Yi et al., 1993) and in turn Stat dependent signaling. The CIS/SOCS/JAB/SSSI family of proteins have the ability to serve in a more classical 'feedback loop' to down regulate the Stat dependent signaling (Krebs and Hilton, 2003; Nicholson and Hilton, 1998; Starr and Hilton, 1998).

The mechanism by which it acts in down regulating remains to be fully elucidated. Yeast two hybrid screens have identified a number of Stat interacting proteins including the PIAS (Protein Inhibitor of Activated Stats) family, reviewed in (Shuai, 2000). PIAS appears to bind to activated Stat dimers, thereby blocking their ability to bind DNA (Liao et al., 2000). Further intensive studies should help to define the overall significance of PIAS proteins in the regulation of Stat signaling.

1.9 Positive regulation of Stat function

Although initial studies focused on the role tyrosine kinases play in Stat activation, more recent studies have identified other positive regulators. This includes serine kinases and interacting proteins.

1.9.1 *Serine phosphorylation*

The recognition that Stats can be modified by serine phosphorylation raised the possibility of cross-talk between signaling cascades (Decker and Kovarik, 2000). Initial studies demonstrated phosphorylation of serine 727, in a PMSP motif, in both Stat1 and Stat3 (Wen et al., 1995; Zhang et al., 1995). Although there is little sequence homology between the TADs, several TADs have conserved a serine residue at or near position 727 that is phosphorylated in response to ligands and are prerequisites for maximal transcription activity (Wen et al., 1995; Zhang et al., 1998b). The conservation of the PMSP motif in the carboxy-termini of Stat1, Stat3, Stat4, and Stat5, raised the possibility that proline-directed serine kinases (e.g. MAP kinases (Gonzalez et al., 1991)) were responsible for this modification. This issue remains controversial, however, in part because different serine kinases have been found to phosphorylate Stat1 and Stat3 depending on the nature of the stimulus and co-stimulus (Decker and Kovarik, 2000). Recent studies have shown that CaMKII as the downstream serine kinase that phosphorylates S727 in Stat1 critical for IFN-γ-induced gene activation (Jayashree et al., 2002). Reporter gene studies have determined that serine phosphorylation enhances transcriptional activity of Stat1 and Stat3 (Decker and Kovarik, 2000; Wen et al., 1995). More physiological studies in Stat1 deficient cells reconstituted with Stat1 and

Stat1^{S727A} demonstrated that serine phosphorylation only enhances the ability of Stat1 to drive expression of some, but not all target genes (Kovarik et al., 2001). Recent studies also indicate that serine phosphorylation enhances Stat4 transcriptional activity (Visconti et al., 2000). Although both Stat5 and Stat6 can become serine phosphorylated, enhanced transcriptional activity has not convincingly been demonstrated (Yamashita et al., 1998). Rather, for Stat5, serine phosphorylation appears to enhance protein stability (Beuvink et al., 2000).

1.10 Interaction with other transcription factors and cellular proteins

The transcriptional regulation of eukaryotic genes involves the specific and ordered interaction of a large number of proteins including enhancer/promoter specific transcription factors, chromosomal remodeling complexes and components of the basal transcriptional machinery (Maniatis et al., 1998; Pugh, 2000). Consistent with these observations, numerous studies have described a robust interaction between Stats and other transcriptional regulators. The first evidence of an interaction between Stats and other transcription factor was evident when IRF-9 (p48) co-purified with Stat1 and Stat2 in the ISGF-3 complex (Schindler and Strehlow, 2000). Subsequent analysis of Stat dependent promoters provided both functional and biochemical evidence for the interaction with other transcription factors. Stat1 was shown to interact with NF-κB, Sp1, USF-1, PU.1 and the glucocorticoid receptor (Aittomaki et al., 2000; Look et al., 1995; Muhlethaler-Mottet et al., 1998; Ohmori et al., 1997). Stat3 was shown to interact with Sp1, c-Jun and the glucocorticoid receptor (Cantwell et al., 1998; Schaefer et al., 1997; Zhang et al., 1999b). Stat5 was shown to interact with YY-1, Sp1, C/EBPβ and the glucocorticoid receptor (Martino et al., 2001; Meier and Groner, 1994; Stocklin et al., 1996; Wyszomierski and Rosen, 2001). Stat6 has been shown to associate with NF-κB and C/EBPβ (Mikita et al., 1998; Shen, 1998).

Other studies have demonstrated the association between Stats and proteins that facilitate transcription through chromatin modification. Yeast two hybrid screens have played an important role in these studies. Not unexpectedly, the first chromosome modifying proteins shown to interact with a Stat (i.e. Stat2) were CBP/p300 (Bhattacharya et al., 1996). Subsequent studies have demonstrated functional and physical association between CBP/p300 and other STATs (Horvath, 2000). More recently other chromatin modifying proteins have been shown to associate or function with Stats. They include BRAC1, Mcm5, Nmi and HMG-I(Y) (Kim et al., 2001; Ouchi et al., 2000; Zhang et al., 1998b; Zhu et al., 1999). Finally, yeast two hybrid screens have identified non-nuclear proteins that associate with Stats. This includes: StIP1, a novel cytosolic WD40 protein that appears to facilitate the interaction between Jaks and Stats; STAM, a Jak binding protein; and SH2-B, a GH receptor binding protein (Carter-Su et al., 2000; Collum et al., 2000; Takeshita et al., 1997).

Detailed analysis of several eukaryotic promoters suggests that the modest number of interactions outlined in this field may grow to a substantial number as Stat dependent promoters begin to undergo more rigorous scrutiny e.g. (Kim et al., 2001; Zhang et al., 1999b). These studies are also likely to provide insight into the ability of Stat modifications, like serine phosphorylation, to differentially regulate the expression of some genes.

1.11 Biological importance of Stat1

In addition to Stat1being activated by IFNγ, it has been reported recently that it might be activated by many growth factors including IL-6, IL-10, growth hormons, lipopolysaccharides, UV and thrombopoietin.

Exclusively Stat1 and Stat2 are considered important in mediating IFN stimulatory effects. Animal that lack Stat1 or Stat2 are extremely sensitive to microbial infections (Durbin et al., 1996; Meraz et al., 1996; Park et al., 2000). Stat1 is reported to be an important candidate for IFN-dependent signaling pathways, as Stat1 deficient mice is defective in responding in IFN dependent immune responses against viral and microbial pathogen infection (Durbin et al., 1996; Meraz et al., 1996). Studies with Stat1 defecient mice shows that it is primarly important for IFN- dependent signaling. These mice show impairment in responding to IFN dependent immune responses against viral and microbial pathogen infections. But these mice respond to other cytokines and do not have any other developmental abnormalities. Stat1 is known to be a strong antiproliferative agent and plays a pivotal role in ligand mediated growth arrest and apoptosis. Stat1 defecient fibroblasts do not arrest growth in response to IFNα or γ and osteosarcoma-derived cells defecient in Stat1 do not apoptose in response to IFNγ or TNF (Migone et al., 1995; Sahni et al., 1999; Su et al., 1997). In mutant chondroblasts where FGF receptor is constitutively activated shows antiproliferative behavior. This is achieved by persistent Stat1 activation which inturn induces the activation of $p21^{WAF1}$, a cyclin-dependent (Cdk) inhibitor (Sahni et al., 1999; Su et al., 1997).These results supports that Stat1 is an antiproliferative and pro-apoptotic agent and also explains in vivo why the lack of this molecule leads to increased tumor formation. The identification of low levels of Stat1 in nucleus of unstimulated cells might suggests that Stat1 in cooperation with other transcription factors regulate apoptosis by basal expression of responsible genes.

It has been shown that Stat1 is a tumor suppressor (Kaplan et al., 1998) contrastingly when $Stat1^{-/-}$ genotype is coupled with tumor suppressor gene p53, there was increase in tumor formation spontaneously (Kaplan et al., 1998). In response to IFNγ number of human tumors have no effect in terms of Stat1 activation and also growth is retarded in culture (Chin et al., 1997; Kumar et al., 1997). It was reported that tumor surveillance system which is important in preventing tumors is dependent on Stat1 mediated by IFNγ. But the tumor suppression mechanism is not completely

understood but, are in part due to lack of immune mediated surveillance and Stat1 in tumor tissues (Kaplan et al., 1998; Ouchi et al., 2000).

Several viruses target Stat or Jak proteins for degradation or inhibit their activation in an indirect way (Polyak et al., 2001b; Polyak et al., 2001a). Further Stat1 overcomes the inhibition of nuclear translocation of proteins due to vesicular stomatitis virus, by the induction of nuclear pore protein Nup98 (Enninga et al., 2002; Her et al., 1997; von Kobbe et al., 2000). These above findings shows the importance of Stat or in particular Stat1 mediated signals for resistance to infection.

2. AIM OF THE CURRENT RESEARCH

VSMC migration and later proliferation might be governed by several other cell types by interacting with them. Of special importance in this concern are monocytes that infiltrate into the site of injury and promote an inflammatory response with the generation of chemotactic mediators, culminating in the accumulation of intimal VSMC. Our recent findings demonstrate that the uPA/uPAR system is upregulated upon VSMC-monocyte interaction in a coculture model and serves for the increase in VSMC motility. Moreover, VSMC growth in coculture is arrested, although the underlying molecular antiproliferative mechanism remains unclear. These findings imply that the upregulated uPA production by monocytes following vascular injury acts most likely as an endogenous activator of VSMC contributing to the remodeling of vessel wall. A wide array of findings supports the idea that components of fibrinolytic system play a significant role in vascular diseases, including arterial neointima formation after injury. However, little is presently known about how the uPA/uPAR system controls these processes to induce regulated vascular wound healing. uPA is an unusual molecule of a dual function, which switches from the proteolytic enzyme to signal transducing molecule depending on the environmental challenges. There is an increasing body of evidence for the nonproteolytic role for the uPA/uPAR system in vitro and in vivo. These observations suggest that the uPA/uPAR-dependent cellular behavior does not reflect modulation of pericellular proteolysis but rather changes in cellular signaling. One major signaling cascade activated by uPA in human VSMC in vitro is the Jak/Stat pathway. The Janus kinase Tyk2 is associated with uPAR and serves via the downstream phosphatydilinositol-3-kinase (PI3-K)-dependent cascade for the uPA-directed cell migration. Signal transducer and activator of transcription Stat1 is strongly activated in response to uPA in VSMC, although the functional purpose of this activation remains unclear. Cell culture and animal experiments, as well as clinical observations on human cancer cells, suppose that Stat1 is important for growth restraint and can be considered the mediator of antiproliferative effects.

The main purpose of this study was to elucidate molecular mechanisms of antiproliferative effect observed in VSMC cocultured with monocytes. The results provide for the first time the evidence that the uPA expressed by monocytes elicits antiproliferative effect in VSMC achieved by activation of Stat1. Activation of Stat1 is induced by the interaction of monocyte-expressed uPA with its receptor (uPAR) on VSMC. This might also throw light on the migration of VSMC during early stages of remodeling process after vascular injury.

3. MATERIAL & METHODS

3.1 Chemicals and biochemical substances

All used chemicals and solutions were of analytical grade and were purchased from Sigma-Aldrich (Deisenhofen, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) and Roth (Karlsruhe, Germany). All solutions were maintained at the appropriate pH and filter-sterilised where ever necessary.

MACS Large Cell separation columns, anti-CD11b antibody conjugated with magnetic material and the separator were purchased from Miltenyi Biotec GMBH (Bergisch Gladbach, Germany). The CELLocate coverslips (square size 175 μm), Microloaders (finely pulled 10 μl pipette tips) and sterile Femtotips for the purpose of microinjection were purchased from Eppendorf AG (Hamburg, Germany).

3.2 Antibodies

3.2.1 *Primary antibodies*

The monoclonal anti-Stat1 (C-terminus) and polyclonal rabbit anti-Stat1(N-terminus) antibodies were purchased from Transduction Laboratories (Lexington, Kentucky). Polyclonal anti-uPAR (R3 clone) antibody was purchased from Monozyme (Kopenhagen, Denmark) and monoclonal anti-uPA antibody was purchased from American Diagnostica, Inc. (Greenwich, CT). Monoclonal anti-Stat1 (p84/p91) as gel supershift reagent was purchased from Santa Cruz Biotechnology Inc. (SantaCruz, CA). Anti-Stat1 pSer^{727} specific and anti-Stat1 pTyr^{701} polyclonal antibodies were purchased from BioSource International Inc.(Camarillo, California). Monoclonal anti-BrdU antibody was purchased from Becton Dickenson (San Jose, CA).

3.2.2 *Secondary antibodies*

Secondary, Alexa fluor® 546 conjugated, goat anti-mouse and anti-rabbit IgG (H+L) were purchased from Molecular Probes Inc.(Eugene, OR). Peroxidase (HRPO)-conjugated anti-mouse Immunoglobins were from Santa Cruz Biotechnology Inc.(sc-2005, Santa Cruz, CA), Sigma Chemicals Co (#A9917, St. Louis, MO) or Pierce (# 31430. Rockford, IL).

3.3 Cell culture

3.3.1 *Human vascular smooth muscle cells (VSMC)*

Human coronary artery VSMC were obtained from Clonetics (San Diego, CA). The cells were grown in SmGM2 medium (Clonetics) supplemented with 5% fetal bovine serum and were

used between passages 3 and 7. For coculture and cell treatment experiments with nocodazole (5 μg/ml), uPA and uPAR antibodies the cells were cultured for 24 h in serum-free SmGM2 medium and were then treated accordingly.

3.3.2 *Isolation of monocytes from whole blood*

Monocytes were separated from freshly obtained blood from healthy donors using Biocoll (Biochrom KG Seromed, Berlin, Germany) density gradient centrifugation method according to the manufacturer's instruction. Briefly, blood was collected in 50 ml syringe with sodium citrate solution (10 ml of sodium citrate for 40 ml of blood) as anti-coagulant. While drawing the blood into the syringe with the anti-coagulant, the syringe was gently rotated so as to mix the blood with sodium citrate. The collected blood was diluted 1:2, with sterile PBS pH 7.4. The PBS diluted blood was over laid on 12-13 ml of Biocoll solution taken in fresh 50 ml falcon tubes with atmost care. Then the tubes with Biocoll and overlaid diluted blood was centrifuged at 3000 rpm for 30 min at 15 - 20 °C. The leucocytes forms a white ring or boundary between the plasma and Biocoll layer,while erythrocytes and granulocytes formed the sediment. The plasma layer was removed and the leucocytes were carefully pipetted out with pasteur pipette into a fresh 50 ml falcon tube. The cell suspension was washed 2 times (first at 1600 rpm and second at 14000 rpm for 20 min each at 20 °C) in sterile PBS so as to remove traces of Biocoll solution. Finally the cell pellet was resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, Penicillin (100 IU/ml) and Streptomycin (100 μg/ml) for 2-3 h at 37 °C in incubator with 5% CO_2. After the incubation time the medium was removed and the cells were washed gently with sterile PBS so as to remove dead cells. Adhered monocytes were detached with trypsin solution and stimulated with LPS (100 ng/ml) for 2-3 min and pelleted down by centrifugation. A small aliquot of cell suspension was taken before centrifugation and counted using cell counting chamber (Neubauer chamber). The cell pellet was resuspended in SmGM2 medium without supplements and then used for further coculture with VSMC.

3.3.3 *Coculture*

VSMC were seeded on sterile coverslips or culture dishes and cultured till they have reached appropriate density according to the experimental requirements, in SmGM2 medium with supplements. The cells were serum starved for 12-24 h in medium without supplements. Then the freshly prepared, LPS stimulated monocytes, resuspended in SmGM2 medium without supplements, were directly added on to the dish containing VSMC, at a concentration of 1.9 x 10^5 cells per cm^2 area and cocultured for a period of 24-27 h at 37 °C in 5% CO_2 incubator.

In all the experiments involving coculture, monoculture of VSMC are included as control.

3.3.4 *Preparation of VSMC from u-PA and u-PAR knockout mouse respectively*

VSMC were isolated from the aorta of u-PA and u-PAR knock-out mouse respectively, (kindly provided by Prof. Peter Carmeliet and Dr. Mieke Dewerchin, Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology; University of Leuven, Leuven, Belgium) according to the protocol followed by P. Fallier-Becker et al., with certain changes. Briefly, after the excision of the aorta from the mouse, the vessel was made free of the fatty tissues surrounding it and cut longitudinally, above said procedure is carried out under preparation medium (Hank´s/PBS (1:3) solution). Now the intimal layer consisting of endothelium and few intimal smooth muscle cells normally present in the intima was gently scraped with a scalpel. Then the vessel surface was washed with the PBS and fresh preparation medium was poured on to the vessel. Then the vessel was subjected to two steps of enzyme (collagenase + elastase) disaggregation. In the first step, the medial layer was separated from the adventitial layer by incubating the vessel for 8-10 min at 37 °C in the enzyme solution-I, with 1.4 mg/ml of collagenase and 0.5 mg/ml of elastase and in the second step, the media tissue pieces were digested by incubating for 60-90 min in the enzyme solution-II, with 2 mg/ml of collagenase and 0.5 mg/ml of elastase to obtain single VSMC. After complete disaggregation an aliquot of the enzyme suspension was taken to determine the cell number. Then the cell suspension was centrifuged to remove the enzyme. Finally, the pellet was resuspended in culture medium (SmGM2 medium from Clonetics supplemented with 5% fetal bovine serum) and seeded in culture dish. After the primary cells were confluent, it was passaged at 1:2 till the second passage as the cell growth was slow initially and further passaged at 1:6, and used for the experiments passages from 3 – 7.

3.4 Immunofluorescence microscopy

3.4.1 *Fixation and permeabilization*

The method followed was according to the protocol given in Haller et al (1995, 1996). Whether the cells from coculture or monoculture,the VSMC were treated similarly. The cells on the coverslips were washed once with PBS after the said coculture incubation time and fixed in 4% (w/v) paraformaldehyde in PBS for 15 min at RT, followed by washing twice in PBS. Then the cells were permeabilised to analyse the cytoplasmic and nuclear proteins, by incubating the cells in icecold 80% (v/v) methanol at –20 °C for 20 min followed by 2 washes in PBS. Finally the cells were blocked overnight at 4 °C with 1% (w/v) BSA in PBS.

For double staining the paraformaldehyde fixed cells were permeabilised with 0.5% Triton X-100 (v/v) in PBS and proceeded for blocking with BSA/PBS.

3.4.2 *Immunostaining*

Paraformaldehyde fixed, permeabilised and overnight 1% (w/v) BSA/PBS blocked cells were washed thrice with PBS then further incubated in primary antibody solution. The antibody solutions were prepared in 0.2% (w/v) BSA in PBS and centrifuged to remove any precipitates.

The primary antibody solution was prepared at 10-20 μg/ml concentration and for staining the cells on the coverslips 30-40 μl of the antibody solution was added on to each coverslip and incubated for 2 h at RT in humidified chambers followed by three washes with PBS. The primary antibodies on the cell were detected by counter reacting them with a secondary antibody conjugated with fluorescent materials (Alexa 546-,Cy3- or Cy5-) at a concentration of 10 μg/ml at RT in humidified dark chambers for 1h. For double staining the cells were incubated in primary antibody and corresponding secondary antibody as described above followed by three washes with PBS and mounted on to a glass-slide in Mowiol with 2.5% DABCO or Vectashield. The results were documented using fluorescence microscope.

In the case of double staining, particularly staining for the cytoskeleton and nucleocytoplasmic proteins the fixed, permeabilised and 1% (w/v) BSA/PBS blocked cells were stained initially for cytoskeleton with Alexa 488 phalloidin in 1% (w/v) BSA/PBS and then immunostained for nuclear and cytoplasmic proteins.

3.5 Separation of VSMC after coculturing with monocytes using MACS separating system

After the appropriate incubation of VSMC with monocytes in direct coculture as described earlier, medium was removed and the cocultured cells were washed once with the PBS. Cells were detached with 5 mM EDTA and centriuged to remove the EDTA. Before centrifugation an aliquot of cell suspension was taken to estimate the number of cells in cell counting or Neubauer chamber. The cell pellet was resuspended in buffer (PBS supplemented with 2mM EDTA and 0.5% (w/v) BSA) at 80 μl per 10^7 cells. The cell suspension was treated with anti-CD11b antibody conjugated with the magnetic material at 20 μl per 10^7 cells and incubated at 6-12 °C for 15-20 min with repeated gentle shaking. The cells were washed once with 10x-20x the volume of the cell suspension with the buffer to remove free and nonspecifically bound antibodies. The washed cells were gently resuspended avoiding any clumps in the buffer at 500 μl per 10^8 cells. In the mean time the Large Cell column was placed in the separator with the appropriate holder provided and activated by washing with 500 μl of the buffer. Then the cell suspension with free cells and cells coupled with antibody complex was applied to the column. While loading care was taken, that no air bubble was introduced into the column. The flowthrough was collected in fresh tube which contains VSMC whereas the monocytes complexed with the magnetic material conjugated CD11b IgG, are trapped in the column which was in a strong magnetic field. A small aliquot of

the flowthrough was observed under the microscope to check the purity of the cells. If the cell suspension was not pure and retained some amount of monocytes, the above procedure was repeated to get pure VSMC suspension. Pelleted down cells from the flowthrough were further processed to prepare the lysate.

3.6 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from VSMC that were either monocultured or cocultured with freshly isolated monocytes from human blood. Cells were detached by treating with 5 mM EDTA, cocultured VSMC were separated from monocytes using MAC cell separating system as described earlier. After centrifugation, the cell pellet was resuspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT), containing the protease inhibitors (1mM PMSF, 5mM Iodoacetamide, 0.1 mM Quercitin, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin and 300 µM Sodium Vanadate) and incubated on ice for 15 min. After homogenization in a Wheaton 0.1-ml homogenizer, the nuclei were collected by centrifugation. The pellet was resuspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT), containing protease inhibitors, and incubated on ice for 30 min, followed by centrifugation at 13,000 g (5 min, 4 °C). The supernatant was dialyzed against buffer C (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT), containing protease inhibitors for 2 h at 4 °C, followed by centrifugation by 13,000 g for 5 min at 4 °C. The supernatant proteins were used immediately or aliquoted and stored at –80 °C. For lysates from cells stimulated with Interferon γ, the cells were stimulated for 30-45 min, before detachment with EDTA.

Binding reaction was performed for 30 min on ice in a volume of 20 μl, containing 4 μg of nuclear protein extracts, 40 ng of poly(dI-dC), 4 μl of 5x binding buffer (1x binding buffer: 20 mM HEPES, pH 7.9, 50 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 10% glycerol) with or without 20-50 fold excess of cold competitor or of unrelated competitor, and a ^{32}P-labeled probe (3 x 10^4 cpm). In supershift EMSA, protein extracts were incubated with 2 μg of experimental or isotypic control antibody, prior to the addition of ^{32}P-labeled probe. DNA-protein complexes were separated on 5% polyacrylamide gel in Tris/glycine buffer at 4 ^{0}C

The following double-stranded oligonucleotides were purchased from Santa Cruz Biotechnology, Inc. and used in this study:

GAS/ISRE, 5´-AAGTACTTTCAGTTTCATATTACTCTA-3´, 27 bp

AP-1, 5´-CGCTTGATGACTCAGCCGGAA-3´, 21 bp

5´-End-labeled probes were prepared with [γ-^{32}P] ATP using T4 polynucleotide kinase and were gel-purified on NAP-5 Sephadex G-25 DNA-grade columns. The reaction mixture contained

milli Q water, 10x kinase buffer, 1-50 pmol of oligonucleotide (GAS/ISRE), 40 μCi of [γ-^{32}P] ATP and 10 IU/μl of T4 polynucleotidekinase and the reaction volume was set at 20 μl. The reaction was stopped after incubating for 30-60 min at 37 °C with constant mixing with 2.5 μl of 0.5 M EDTA. Then the above mixture was applied on to a NAP-5 Sephadex G-25 DNA grade column, which was washed, in advance with TNE buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) and kept ready for loading. Wash the column once with 600 μl of TNE buffer and elute with TNE buffer by collecting the eluant 100 μl per tube. Normally the fraction 9 shows highest cpm, when an small aliquot is counted using scintillation counter, which is used for the experiments.

3.7 Sodium dodecyl suphate-polyacrylamide gel electrophoresis (SDS-PAGE)

For separation of protein of interest from complex mixtures standard Laemmli (1970), discontinuous, one-dimensional SDS-PAGE was used. Minigel format (9.0 cm x 5.5 cm) was employed for the above purpose and electrophoresis apparatus was from Bio-Rad Laboratories (Modell Mini-Protean II with power supplier Modell 1000/500, Munich, Germany). The gel was run at constant power supply at 30 mA per gel and voltage set to 200 mV, till the bromophenol blue front reached the bottom.

3.8 Western blot analysis

3.8.1 *Semi-dry blotting*

The electrotransfer of proteins from a polyacrylamide gel on to PVDF-membrane is carried out in semi-dry condition using an semi-dry blotting apparatus from Bio-Rad Laboratories (Modell Trans-Blot SD with power supplier Modell 1000/500) at 110 mA and 20 mV for 50 min per one transfer sandwich.

3.8.2 *Immunodetection of the transferred protein on PVDF membrane*

After the complete transfer of the protein from the gel onto the PVDF membrane the membrane was incubated for 1 h at RT in PBS-T with 1% BSA in order to block the nonspecific free binding sites. The membrane was incubated in primary antibody solution in PBS-T with 1% (w/v) BSA for 2 h at RT or overnight at 4 °C. After the membrane is washed thrice, for 10 min each with PBS-T with 1% (w/v) BSA, it was incubated in HRPO conjugated secondary antibody in PBS-T with 1% (w/v) BSA for 1 h at RT. The membrane was washed thrice, 10 min each with, PBS-T. Specifically bound antigens are visualised by incubating the membrabne in peroxidase substrate Luminol (Western Lightning Chemiluminiscence Reagent, PerkinElmer Life Sciences, Inc., Boston, MA). Protein bands appear within few minutes, the output is emitted as blue light

due to the oxidised luminol substrate which was recorded as black images on a X-ray film (CL-xposure™ Film, Pierce, Rockford, IL).

3.9 Enzyme linked immunosorbent assay (ELISA)

Human Interferon-γ, uPA and uPAR were detected from cell culture supernatants using commercially available ELISA kits (human Interferon-γ from R&D Systems, Inc., Minneapolis, MN; human uPA from American Diagnostica, Inc. Greenwich, CT and uPAR from Monozyme, Hoersholm, Denmark), according to the manufacturer´s instructions. Briefly, the assay employs the quantitative sandwich enzyme immunoassay technique, cell coculture supernatants from day-1 till day-4 were collected and PMSF was added to avoid any protein degradation followed by centrifugation to remove any particulate matters. The supernatants were concentrated using Centricon Centrifugal Filter Units (Millipore, Bedford, Massachusetts) cut-off molecular weight at 10,000 kDa.

3.9.1 *For human Interferon-γ and human uPA*

The standards and samples were pipetted 100 µl each, into the wells of the microplate which is precoated with polyclonal antibody, specific for target protein and any target protein present is bound to the immobilized antibody. After the incubation time of 2 h at RT, each well was aspirated and any unbound substances were washed away with washing buffer (solution of buffered surfactants with preservatives) followed by addition of target protein specific enzyme-linked polyclonal antibody to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and incubated for 30 min at RT. Color develops in proportion to the amount of target protein bound in the initial step and it was stopped by addition of stop solution (2 N sulphuric acid) in the case of IFN-γ ELISA The optical density of each well was measured at 450 nm using a microplate reader (MWG-Biotech GmbH, Ebersberg, Germany) with the wavelength correction set at 540 nm or 570 nm. In the case of ELISA for uPA, phosphatase reaction was continuously monitored with the plate in the reader and the reading was recorded at intervals of 10 min. The absorbance was read at 405 nm.

3.9.2 *For uPAR*

Immunoplates MaxiSorp (NUNC, Roskilde, Denmark) wells were coated with antibody by incubating overnight with coating antibody solution provided in the kit at 4 °C. After thorough washing with washing buffer (0.1% (w/v) Tween 20 in PBS) the remaining protein binding sites were blocked with 2% BSA in washing buffer followed by washing. Then provided standard and biological samples were diluted with dilution buffer (1% (w/v) BSA (Sigma, A-7906) in washing

buffer) appropriately and were pipetted 100 μl per well in triplicates and dilution buffer as control and incubated for 1h at 20 °C. After washing with washing buffer, detecting antibody solution, was added at 100 μl, to each well and incubated for 1h at 20 °C followed by washing. Specific antibody-antigen-detecting antibody complex, is then conjugated by an enzyme for the purpose of detection by incubating with phosphatase-conjugate solution for 1h at 20 °C. After washing with washing buffer followed by distilled water, fresh substrate solution (prepared immediately before use by dissolving one p-NPP tablet in 12 ml substrate buffer) was added, 100 μl to each well and incubated for 90 min at 20 °C. The phosphatase reaction was continuously monitored with the plate in the reader and the reading was recorded at intervals of 10 min with the absorbance read at 405 nm.

3.10 Microinjection

VSMC were cultured on CELLocate coverslips till the cell density was around 60-65%. Serum starved cells for 12-14 h, were microinjected with anti-Stat1 $pSer^{727}$ antibody using Nikon DIAPHOT-300 inverted microscope and micromanipulator type MO-8 with hydraulic joystick (Narishige, Tokyo). The Femtotip was attached to microinjector PL-188 (Nikon, melville, New York) preset to constant outflow pressure 0.1-0.4 psi (1 psi=6.89 kPa). The concentration of microinjected antibody was 25 μg/ml in PBS with Mg^{2+} and the antibody solution was centrifuged for 10 min at 13000 rpm at RT to remove precipitates and then loaded into the Femtotip using microloader. The medium was supplemented with penicillin and streptomycin to avoid any infection due to the exposure of cells to the outside environment. Approximately the injection time was around 10-20 min. Microinjected cells were washed twice with fresh medium with the antibiotics and fresh medium is added. After incubation of cells at 37 °C in 5% CO_2 incubator for 2 h, they are cocultured with freshly prepared monocytes as described earlier. Further the cocultured cells were used for proliferation assay. As control nonspecific IgG was microinjected and treated accordingly.

3.11 Single cell proliferation assay

Apart from the above microinjected cells, for proliferation assay cells were plated on coverslips and cultured to a density of 60-65%, the cells were serum starved for 24 h and cocultured with freshly prepared monocytes. A set of controls were included, as follows, monoculture of VSMC without supplements, with 5% FCS and coculture with 5% FCS. After the incubation time the cells were treated with BrdU (Sigma, final, 100 μM) and further incubated for 8-10 h at $37\,^0$C. We washed the cells with PBS and fixed in 4% paraformaldehyde. Further washed thrice in PBS and permeabilised with 0.5% Triton X-100 in PBS at RT and blocked with 1% BSA in PBS overnight

at 4 ^{0}C. Further the cells were incubated with a cocktail containing anti-BrdU monoclonal antibody (Becton Dickinson), 1% BSA, 2x DNAse buffer solution and DNAse solution (to digest DNA) for 1 h at RT. Following washing cells were incubated with Alexa 546- labeled goat anti-mouse IgG (Molecular Probes) for 1 h at room temperature. After washing, DNA was counter-stained with Hoechst 33258, followed by washing with PBS and mounted in Mowiol with 2.5% (w/v) DABCO and documented the results with flourescence microscope.

3.12 Cell treatments

As described under cell culture VSMC were cultured on coverslips or in culture dishes according to the experimental requirements till the density was around 60-70% for preparation of cell lysates and 30-45% confluent for immunocytochemial studies. The cells were serum starved for 12-24 h and treated with nocodazole (5 μg/ml) in medium without supplements and after 90 min of incubation at 37 °C in 5% CO_2 incubator, freshly prepared monocytes were added to initiate the coculture. Whereas in the case of cell treatment with monoclonal anti-uPA (25 μg/ml) and polyclonal anti-uPAR (R3 clone) (100 ng/ml) antibodies and nonspecific IgG (25 μg/ml) serum starved VSMC were incubated in the respective antibody solutions 1 h prior to the addition of monocytes.

In all the above treatments monoculture of VSMC were included as control and treated appropriately.

3.13 General protein chemistry techniques

3.13.1 *Protein estimation by Bradford assay / Coomassie dye binding assay*

The protein estimation was done by Bradford´s method (1976). The assay was carried out in microtiterplates (Macrowell Plate, Nunc, Roskilde, Denmark). The calibration standard used was BSA (4-40 μg/ml) and the volume of the standards and the samples used per well was 50 μl in triplicates. After the addition of sample and standards, 200 μl of the Bradford´s reagent (Protein Assay Dye Concentrate, Bio-Rad Laboratories, Munich, Germany) diluted at 1:5 in water was added. The absorbance was measured at 595 nm with a Microtiterplate-Photometer from Molecular Devices (Modell ThermoMax, Sunnyvale, CA) using a SOFTmax PRO P1.12 Software.

3.13.2 *Dialysis*

The Dialysis of the Protein samples were done in Spectra/Por® Dialysis molecular porous membrane tubing (Spectrum®, Gardena, CA). The membrane used had MWCO of 12-14 kDa. The dialysis was carried out in dialysis buffer 100 fold more than the sample, at 4 °C with constant stirring.

4. RESULTS

4.1 VSMC stop proliferating when cocultured with monocytes

As proliferation is an important functional aspect of cells in tissue remodeling and wound healing it sounded wise to check the proliferative status of VSMC in our coculture model which to a certain extent mimics the in vivo environment in vessel injury. For this purpose, we implemented single cell proliferation assay, where the cells were pulsed with BrdUrd for 8-12 h. After the incubation period, the cells were fixed in paraformaldehyde and further subjected to immunofluorescent-staining. In addition to stain for BrdUrd, the DNA was counterstained with Hoechst. VSMC showed antiproliferative behavior when they were cocultured with freshly isolated monocytes from human blood, even if the cells were cultured in the presence of 5% FCS when compared to the control (Figure 7). These findings are supported by the earlier findings of Proudfoot et al., 1999, where they have reported that monocytes does not stimulate smooth muscle cell growth. It has been reported by numerous authors that uPA/uPAR system is involved in tissue remodeling and wound healing by affecting cell adhesion, migration and proliferation.

Based on the above obtained results and findings, our line of investigation followed two assumptions. First, we supposed that VSMC growth inhibition might be mediated by transcription factor Stat1, which is a known powerful antiproliferative molecule (Bromberg, 2001).

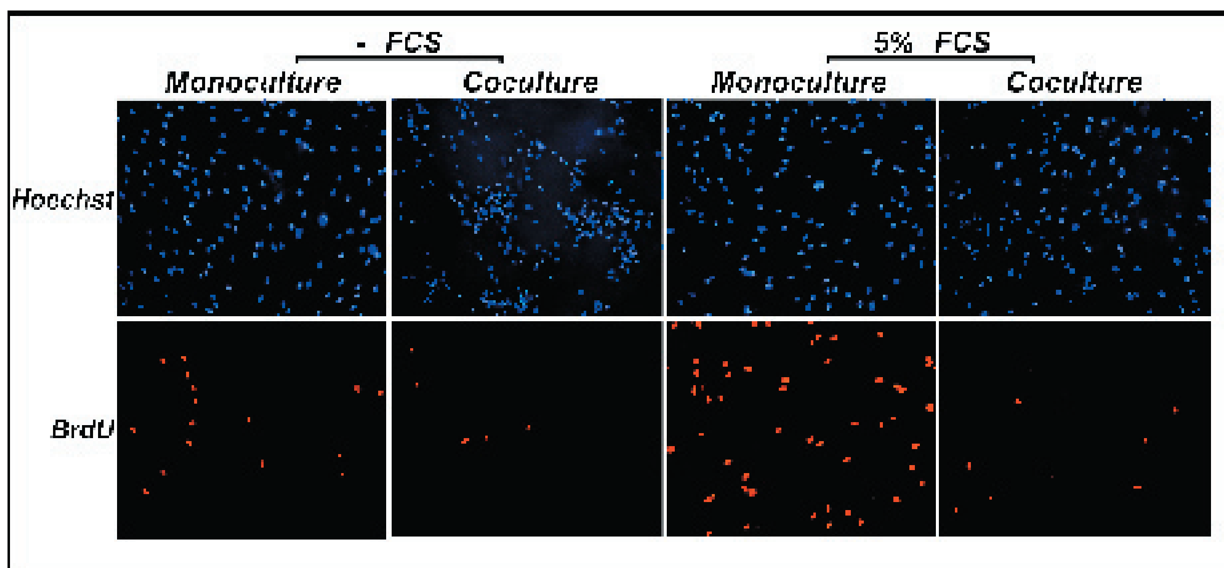

Figure 7. *Inhibition of VSMC proliferation in coculture.*

VSMC were cocultured with LPS-treated monocytes and treated with BrdU to analyze DNA synthesis. Incorporation of BrdU was detected by immunofluorescence using monoclonal antibody against BrdU (lower panel). DNA was counter-stained, with Hoechst 33258 (upper panel). The above stainings were performed in both serum-treated and nontreated samples and VSMC in monoculture was the control.

Second, that the required activation of Stat1 in VSMC might be provided by the monocyte-expressed uPA. This idea was based on our previous studies demonstrated that Stat1 is activated in VSMC in response to exogenous uPA (Dumler et al., 1999a; Dumler et al., 1998) and that the uPA expression in monocytes cocultured with VSMC is strongly upregulated (Kusch et al., 2002).

4.2 Stat1 is upregulated in VSMC cocultured with monocytes

STAT proteins as the name itself indicates are signal transducers in the cytoplasm and activators of transcription in the nucleus. Upon ligand binding to the receptors on the cell surface the Stat proteins get phosphorylated through JAK kinases (Jak1 or Tyk_2). Once the Stat proteins are phosphorylated they form homodimers or heterodimers and gets translocated to the nucleus to activate transcription of genes by binding to the DNA. In order to investigate whether Stat1 in VSMC is affected via the coculture with activated monocytes, we initially performed immunocytochemical studies to visualize the intracellular distribution of Stat1 (Figure 8a). We cocultured VSMC with freshly prepared monocytes from human blood which were activated with LPS before the initiation of coculture. After the incubation period of 24-27h the cells were washed, fixed and stained. Surprisingly, immunofluorescent staining with anti-Stat1 monoclonal antibody revealed that nucleus showed a strong staining for Stat1 protein in coculture. This shows that Stat1 protein was activated after 24-27 h of coculture and translocated to the nucleus leading to a striking accumulation (right panel). In contrast, no Stat1 nuclear translocation occurred when monocytes were absent (left panel).

As activation of Stat1 requires the phosphorylation of tyrosine at around 700 residue (Schindler et al., 1992) and phosphorylation of STAT1 on S727 greatly augments its transcriptional potency (Horvath, 2000; O'Shea, 1997; Stark et al., 1998), we assessed the phosphorylation site of Stat1, activated in coculture. Nuclear and cytosolic fractions were prepared from VSMC cocultured with and then separated from monocytes using MACS separating system. The subcellular fractions were monitored for serine and tyrosine phosphorylation status of Stat1 by Western blotting using phosphospecific polyclonal antibodies (Figure 8b). The antibodies specifically recognized Stat1 phosphorylation on Ser^{727} and Tyr^{701}. VSMC in monoculture served as controls. Serine phosphorylation of Stat1 in cocultured VSMC was promptly elevated (upper panel), whereas activation of Stat1 tyrosine phosphorylation, although reliable, was several folds less and could be observed only after longer exposure time than those used for Stat1-$pSer^{727}$ phosphorylation (middle panel). Although Stat1 phosphorylation on Ser^{727} was increased both in cytosolic and nuclear fractions, the total amount of Stat1 protein in these fractions (lower panel) clearly demonstrates that activated Stat1 was mainly translocated to the nucleus. Whole cell lysates from both the VSMC stimulated with IFNγ and unstimulated were included as positive controls.

The purity of the subcellular fractions was checked by using anti-α-actin antibody.

Further, to distinguish between the Stat1 activation and expression in our experimental model we analysed the mRNA profiles. The results of RT-PCR analysis confirms that there is no change in Stat1 expression on day1 in coculture when compared to the control VSMC monoculture, on day2 though there was increase but not significant and inhibition on day3 of coculture (Figure 9).

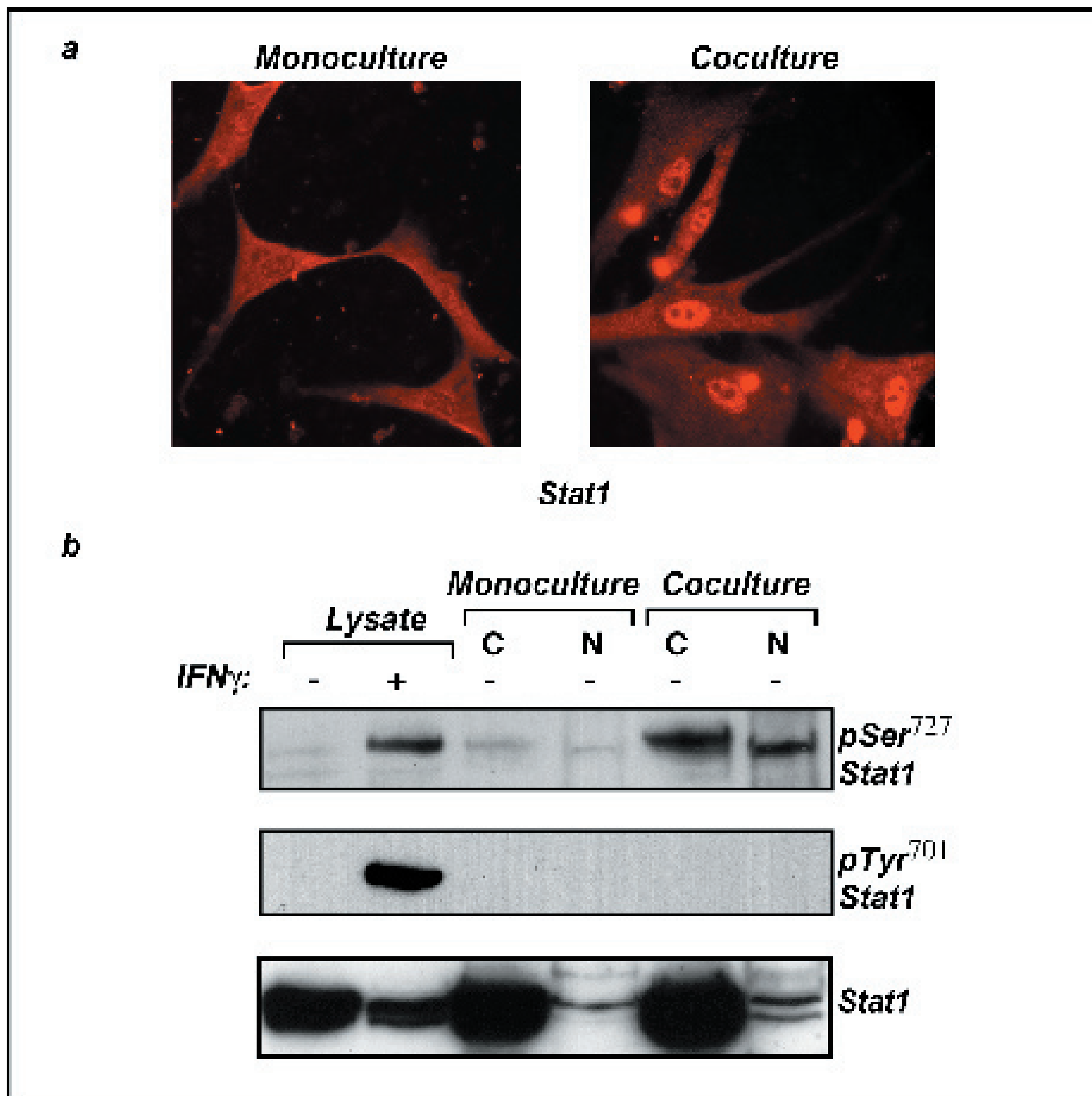

Figure 8. *Stat1 in VSMC is activated in coculture.*

a, Stat1 immunofluorescence in VSMC in coculture with monocytes and monoculture control.

b, VSMC after coculture were separated by MACS separating system and analyzed for pSer^{727}Stat1 and pTyr^{701}Stat1 in whole cell lysate, cytosolic (C) and nuclear (N) fractions by subjecting to SDS-PAGE and Western blotting with anti- pSer^{727}Stat1 and anti- pTyr^{701}Stat1 rabbit polyclonal antibodies respectively. As a control for Stat1 amount, blots were analyzed for Stat1 using anti-Stat1 monoclonal antibody (lower panel).

These above findings demonstrate that monocytes play a pivotal role in the activation of Stat1 and its nulear translocation but not expression.

As far as only activated Stat proteins can bind to the DNA after translocation to the nucleus, a DNA binding assay or EMSA (electrophoretic mobility shift assay) was next addressed, as an

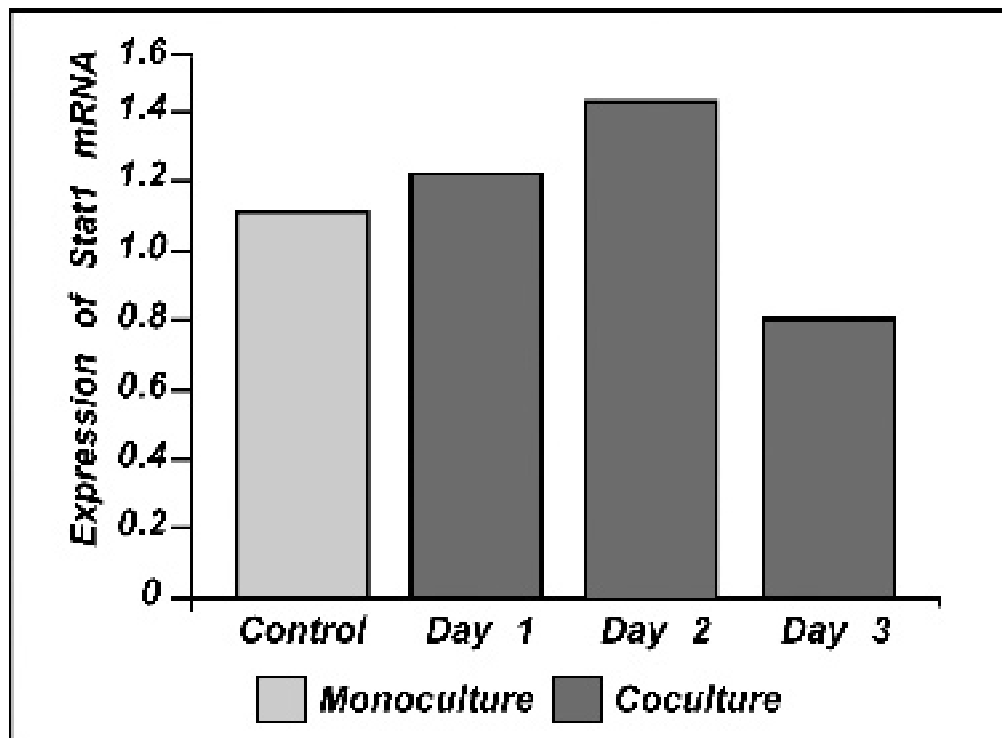

Figure 9. *Stat1 expression in VSMC monocultured and cocultured with monocytes.*
VSMC monocultured and cocultured with monocytes were analysed for Stat1 at mRNA level by RT-PCR using TaqMan analysis and the results graphically represnted.

appropriate tool to check for Stat activation. This assay analyses the mobility of the activated Stat proteins, which get specifically bound to the oligonucleotides. Further to support our above findings, the activation of Stat1 in VSMC when cocultured with monocytes was examined at the DNA-binding level. Nuclear extracts and a ^{32}P-labeled GAS/ISRE oligonecleotide probe containing a consensus binding site specific for Stat1 were used to analyse the nuclear extracts (Figure 10). With coculture, the DNA-protein complex was increased, compared to monoculture controls. In gel supershift assay, with anti-Stat1 specific antibody, the intensity of the DNA binding was reduced as this specific antibody identified Stat1 and inhibited the formation of prominent DNA-protein complex. This mobility shift and supershift assay clearly determined that Stat1 in VSMC was translocated and bound specifically to the GAS/ISRE DNA's sequence upon activation in the presence of monocytes.

Appropriate localization of proteins is crucial for their physiological function and regulation. Once the cells are stimulated, proteins are the main vehicles, which transmit incoming signals. The

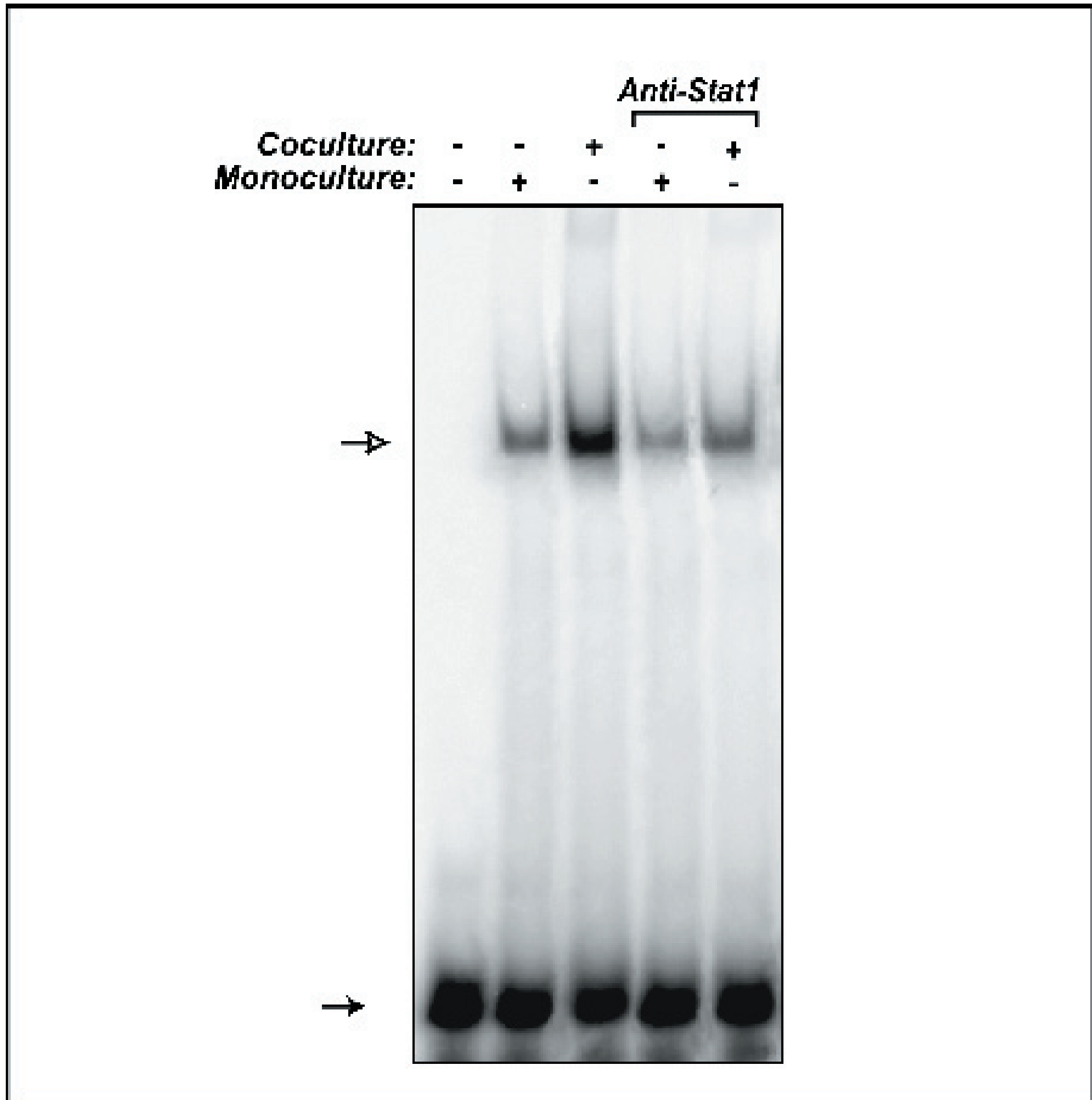

Figure 10. *Binding of Stat1 to GAS/ISRE elements.*

GAS/ISRE binding activity of nuclear extracts prepared from VSMC that were cocultured with monocytes and also from monoculture were analyzed by EMSA. Open arrow indicates the position of protein-^{32}P-GAS/ISRE complex and solid arrow indicates position of free probe.

transmission of the signal is accomplished by interaction with other molecules with or without movement into another location in the cell to the target organelle where the final response is seen. Activation of transcription factors can occur in the cytoplasm or at the cell membrane. Once activated, they translocate to the nucleus through nuclear pores (Kaffman and O'Shea, 1999). However, little is known about the mechanisms of transport to the nuclear pores. Transport through the cytoplasm could, a priori, be active along microtubules as observed for p53 (Giannakakou et al., 2000), or passive, by diffusion, as in a random walk. To determine whether the translocation of Stat1 to the nucleus is dependent on the integrity of the cell or whether this translocation requires the elements of the cytoskeleton as channels, VSMC were treated with the inhibitor of the structural element. The inhibitor nocodazole, which disrupts the formation of microtubules, was used to determine the influence of these structural elements on Stat1 translocation. VSMC were pre-incubated in the absence or presence of the drug for 90 min, before the addition of freshly prepared monocytes and the coculture setting was incubated under continued drug treatment for 24-27 h. The efficacy of the drugs was confirmed by changes in cell morphology. Further, we performed immunofluorescent studies, probing the cells with anti-Stat1 antibody and Alexa 546 conjugated secondary antibody. We found that translocation of Stat1 was not affected though the cytoskeleton was disrupted and comparable to the control (Figure 11). This is in agreement with the earlier finding that nuclear translocation of Stat1 in response to IFNs is not dependent on an intact cytoskeleton (Lillemeier et al., 2001).

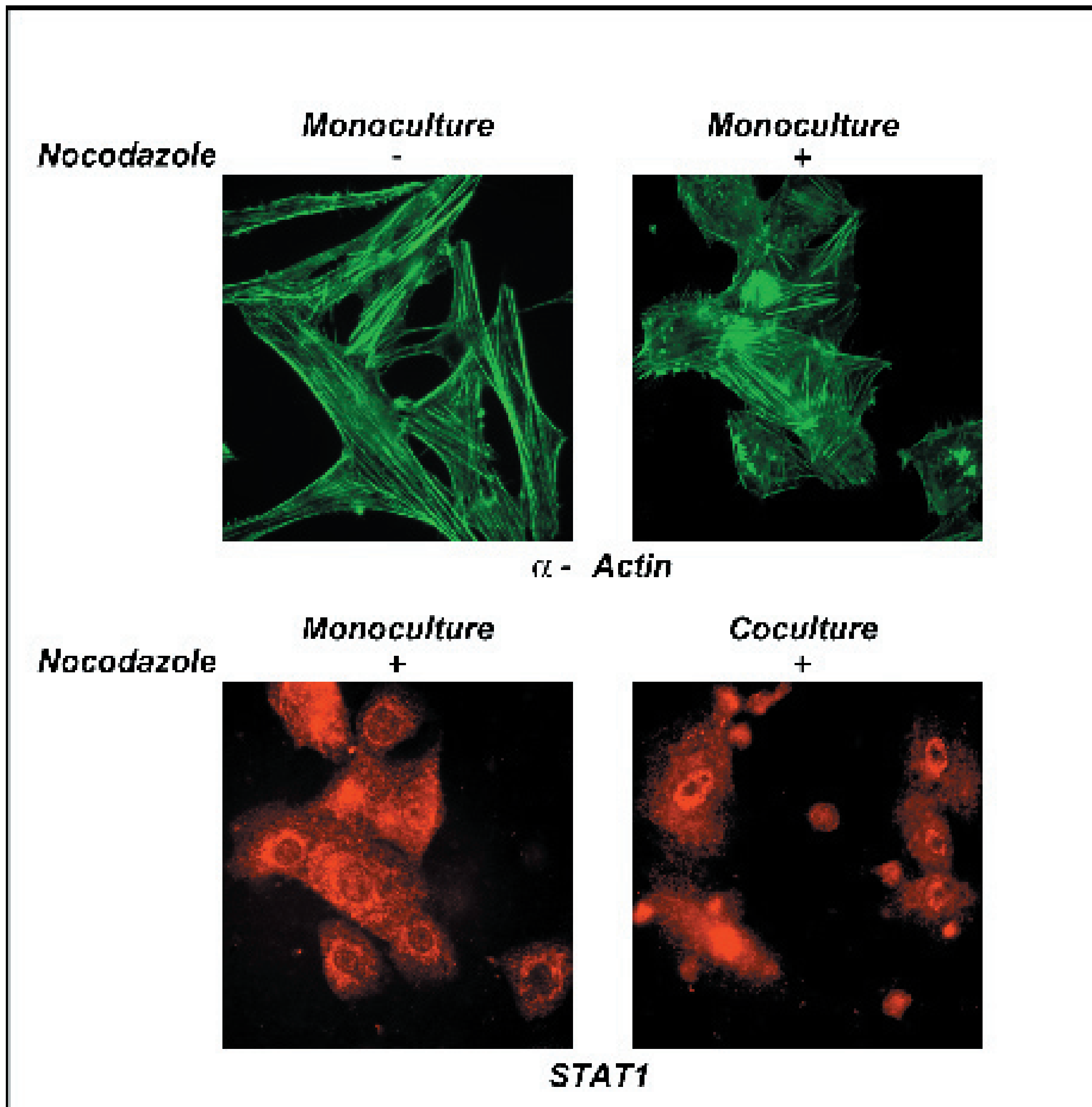

Figure 11. *Stat1 translocation is not dependent on an intact cytoskeleton.*

α-Actin immunofluorescence in VSMC treated and untreated with nocodazole (upper panel) and Stat1 immunofluorescence in VSMC treated with nocodazole prior to coculture with monocytes and monoculture (lower panel).

4.3 VSMC Stat1 activation in coculture requires uPA/uPAR and is interferon-γ-resistant

Activation of Stat1 resulting in dimerization and finally translocation to nucleus in VSMC requires an external stimulus and we supposed that monocytes are a potent inducer of this Stat1 activation in VSMC. Moreover, our recent findings showed that uPA is a known potent activator of Stat1 in VSMC (Dumler et al., 1998), and that uPA expression by monocytes and uPAR expression on VSMC are both upregulated in coculture (Kusch et al., 2002). Based on these above results, it was hypothesized whether this might explain the observed activation of Stat1. To analyse this, inhibiting the interaction of uPA with its specific receptor uPAR in the coculture system sounded promising. VSMC were treated separately with monoclonal anti-uPA and polyclonal anti-uPAR specific antibodies. In separate experiments VSMC were treated with respective antibodies 1h prior to the addition of monocytes and medium was not changed before the addition of monocytes so that the antibody treatment was continued during the incubation time. After the incubation time of 24-27 h the cells were washed, fixed and stained. The immunofluorescent staining with monoclonal anti-Stat1 antibody revealed the localization of Stat1 in VSMC (Figure 12). This treatment abrogated Stat1 nuclear translocation occurred in the coculture system.

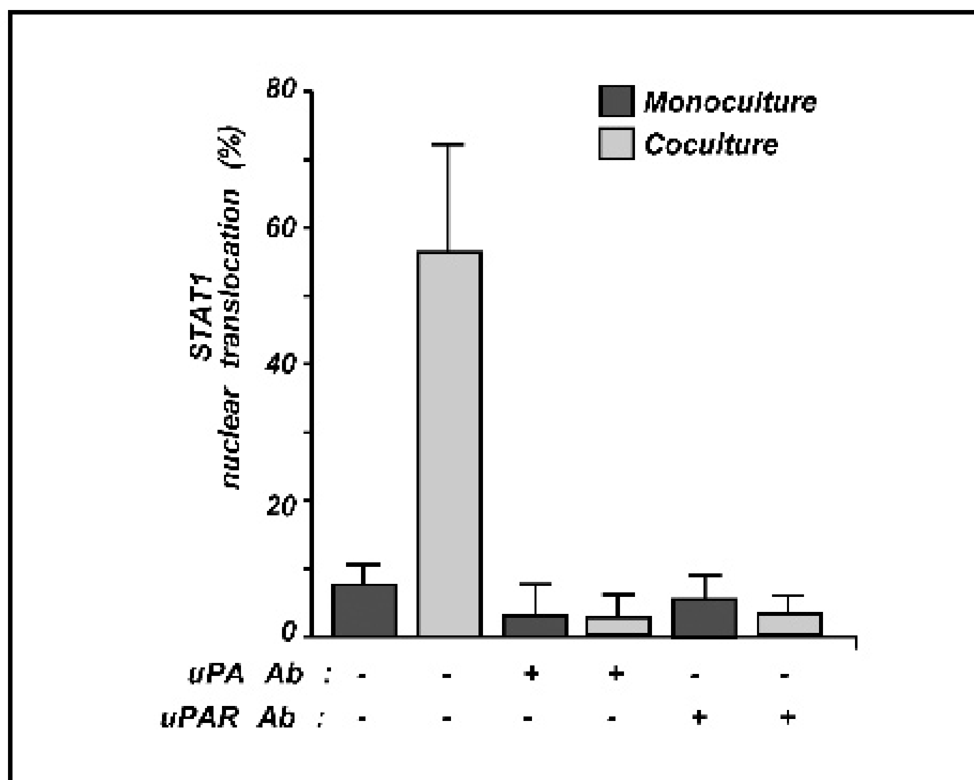

Figure 12. *Stat1 activation in VSMC depends on uPA/uPAR signaling.*

VSMC were treated with anti-uPA and anti-uPAR antibodies separately, before coculture with monocytes, and were analysed for Stat1 by immunofluorescence. Total cell number and the number of cells with translocated Stat1 were counted for each view field. 9-10 view fields have been evaluated in each experiment. The result is a representative of 3 experiments.

Consistent with these data, the EMSA showed Stat1 activation was blocked in coculture when VSMC were treated with anti-uPA antibody (Figure 13). VSMC were treated with anti-uPA antibody prior to the addition of monocytes. After the said incubation time, nuclear extract was prepared from VSMC separated from monocytes by MACS separating system as explained in the methods. In all the above experiments monoculture of VSMC with and without treatments were used as controls.

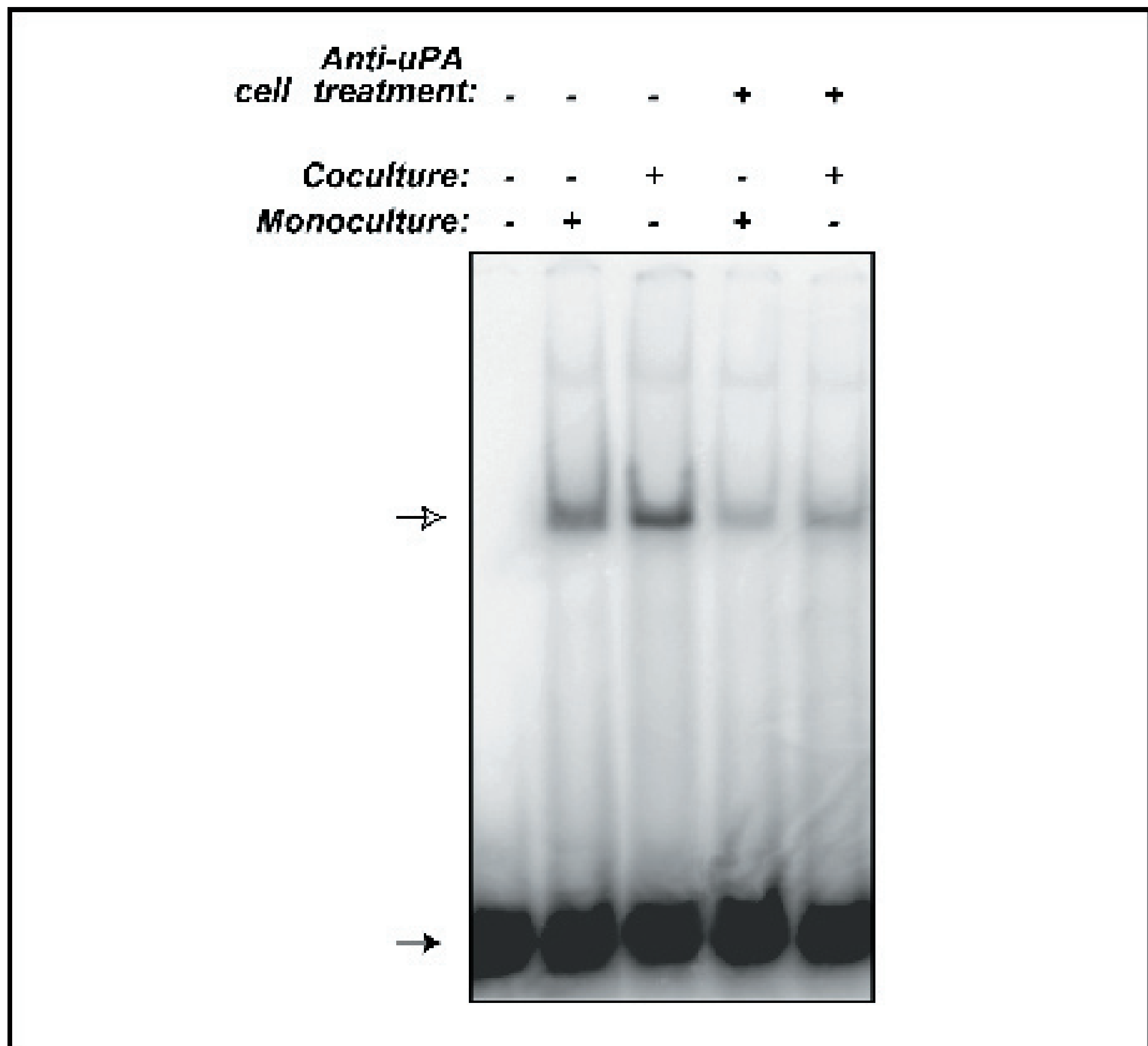

Figure 13. *Inhibition of Stat1 binding to GAS/ISRE elements.*
VSMC were treated with anti-uPA antibody before the addition of monocytes; VSMC in monoculture is control. GAS/ISRE binding activity of nuclear extracts prepared from the above anti-uPA antibody-treated VSMC was analysed by EMSA.

To rule-out any flaw and to verify additionally these findings, uPA- and uPAR-deficient mice were used. The monocytes from uPA$^{-/-}$ mice and VSMC from uPAR$^{-/-}$ mice were isolated accordingly as explained in the methods section and used for coculture setting. Figure 14 shows Stat1 cellular distribution in wild-type VSMC cocultured with the uPA-deficient monocytes (middle panel) and in the uPAR-deficient VSMC cocultured with wild-type monocytes (right panel). As expected, in both the cases, as one of the candidate in the uPA/uPAR system was absent. The required signal for the tyrosine/serine phosphorylation of Stat1 was not conveyed as the ligand-receptor binding was hindered. As a result, in both cases Stat1 was mainly localized in cytoplasm and in the perinuclear space, whereas its nuclear translocation was minimal. In contrast, we observed again pronounced Stat1 nuclear translocation when wild-type VSMC and wild-type monocytes were used in the coculture system (left panel). This indicates that uPA/ uPAR interaction plays a pivotal role in activation of latent cytoplasmic Stat1, dimerization upon activation and nuclear translocation where it accumulates and drives transcription.

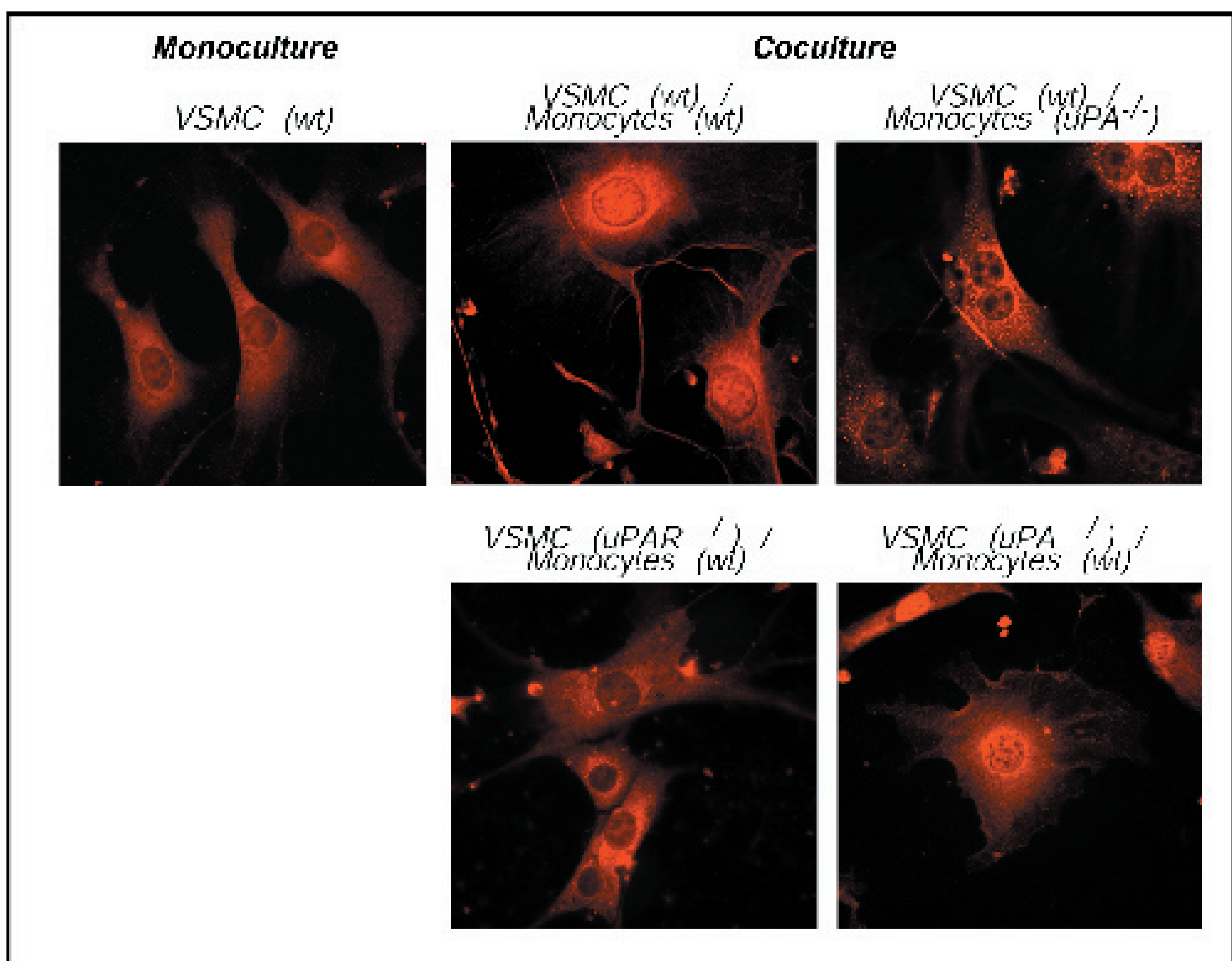

Figure 14. *Stat1 activation in VSMC depends on uPA/uPAR signaling.*

Immunofluorescent staining for Stat1 in mouse VSMC in coculture with monocytes from uPA$^{-/-}$ and in VSMC from uPAR$^{-/-}$ mice cocultured with wild-type monocytes using anti-Stat1 rabbit polyclonal antibody. As a control, wild-type cells and VSMC from uPA$^{-/-}$ are included.

Since interferon-γ (IFN-γ) is a strong activator of Stat1 (Ihle, 2001; Platanias and Fish, 1999), we used IFN-γ as a positive control. Surprisingly, IFN-γ had no or less effect on Stat1 activation when VSMC were cocultured with monocytes. After the stipulated period of 24-27 h of coculture the medium was changed for fresh medium containing IFN-γ and incubated for 30 min. After the said incubation time VSMC were separated from monocytes using MACS separation system. VSMC were lysed and the prepared cell lysate was subjected to Western-blot analysis using anti-Stat1 antibody. Western-blot analysis of proteins from cocultured VSMC stimulated with IFN-γ shows only slight increase in Stat1 phosphorylation, as compared to Stat1 phosphorylation profile in the IFN-γ-stimulated monocultured cells (Figure 15).

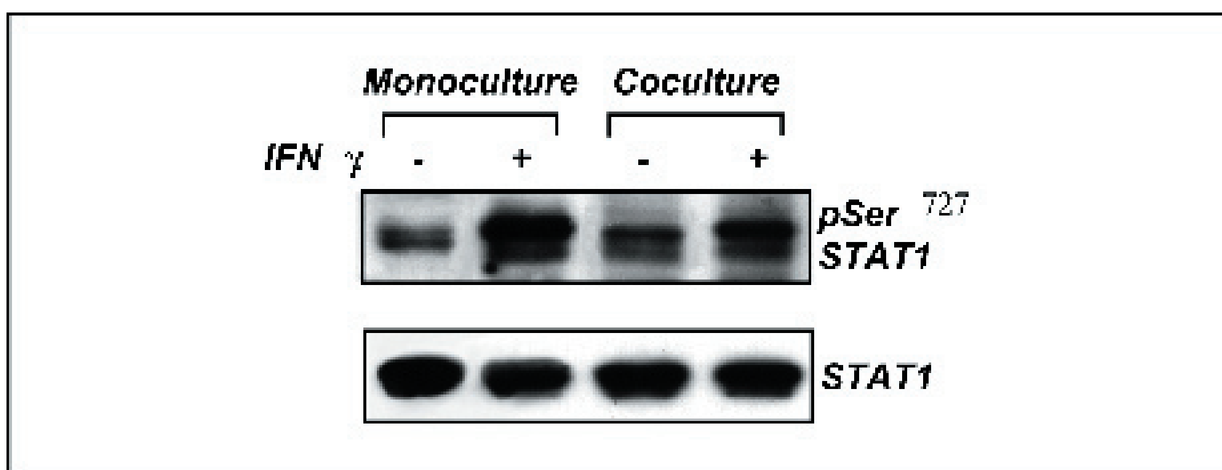

Figure. 15. *VSMC do not respond to IFN-γ stimulation in coculture.*

Lysates were prepared from MACS separated VSMC, upon coculture and stimulated with IFN-γ. Proteins were separated on SDS-PAGE and analyzed by Western blotting with anti-pSer^{727}Stat1 rabbit polyclonal antibody. VSMC in monoculture with and without stimulation are included. As a control for gel loading, anti-Stat1 antibody was used.

Further, it was interesting to check the DNA binding behavior of the Stat1 in IFN-γ stimulated VSMC in coculture. Nuclear extracts was prepared from the IFN-γ stimulated VSMC in coculture and Stat1-DNA binding was monitired by EMSA. Consistent with the data on Western blotting, IFN-γ did not produce any significant increase in the intensity of Stat1-DNA binding in cocultured VSMC (Figure 16). Monoculture of VSMC was used as a control and it was treated appropriately as coculture.

One possible explanation for the observed decreased sensitivity to IFN-γ might be the desensitization of VSMC induced by probable overexpression of IFN-γ in coculture since human leukocytes are capable of synthesizing interferon (Farrar and Schreiber, 1993). To confirm in our hands, we measured the IFN-γ in the supernatants from the coculture system.

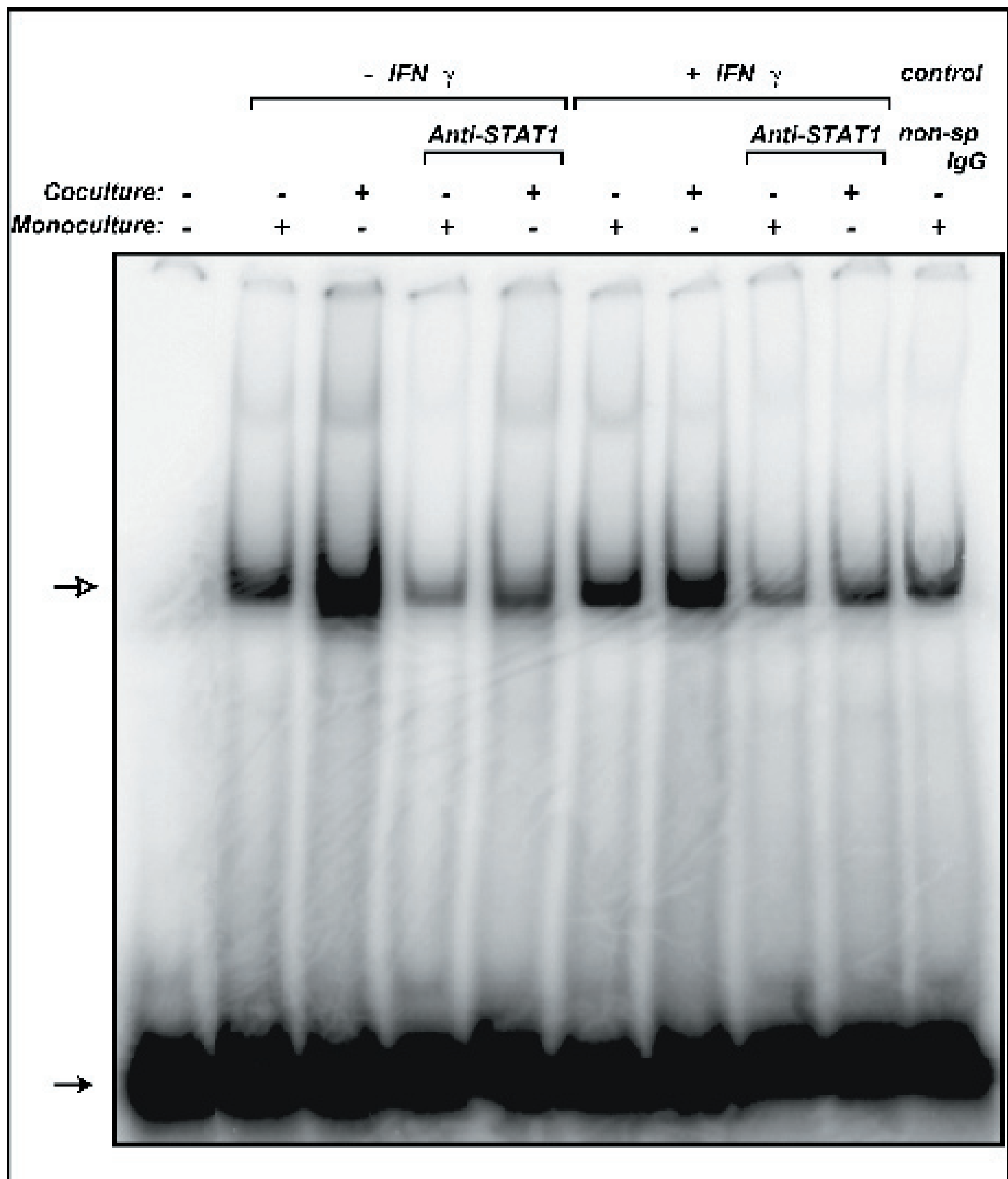

Figure 16. *Binding of Stat1 to GAS/ISRE elements does not increase with IFN-γ stimulation .*
DNA binding activity of nuclear extracts prepared from VSMC treated with or without IFN-γ, from both monoculture and coculture were analysed by EMSA. The presence of stat1 in the observed complex was tested by using anti-Stat1 antibody.

For the purpose of studying the kinetics of IFN-γ secreted in the coculture system, it was incubated for 4 days. Cell culture supernatants were collected from the coculture from day1-day4. To rule out whether the specificity of IFN-γ secretion depends on an individual, monocytes isolated from freshly obtained blood from 4 donors were cocultured separately with VSMC accounting to 4 samples. Cell culture supernatants from monoculture of VSMC and monocytes were included as control. Quantitative ELISA was employed to measure the IFN-γ level using ELISA kit and eventually found a two-fold increase of IFN-γ in our coculture compared to monoculture (Figure 17). The kinetics of this increase reached maximum on day-4 of coculture.

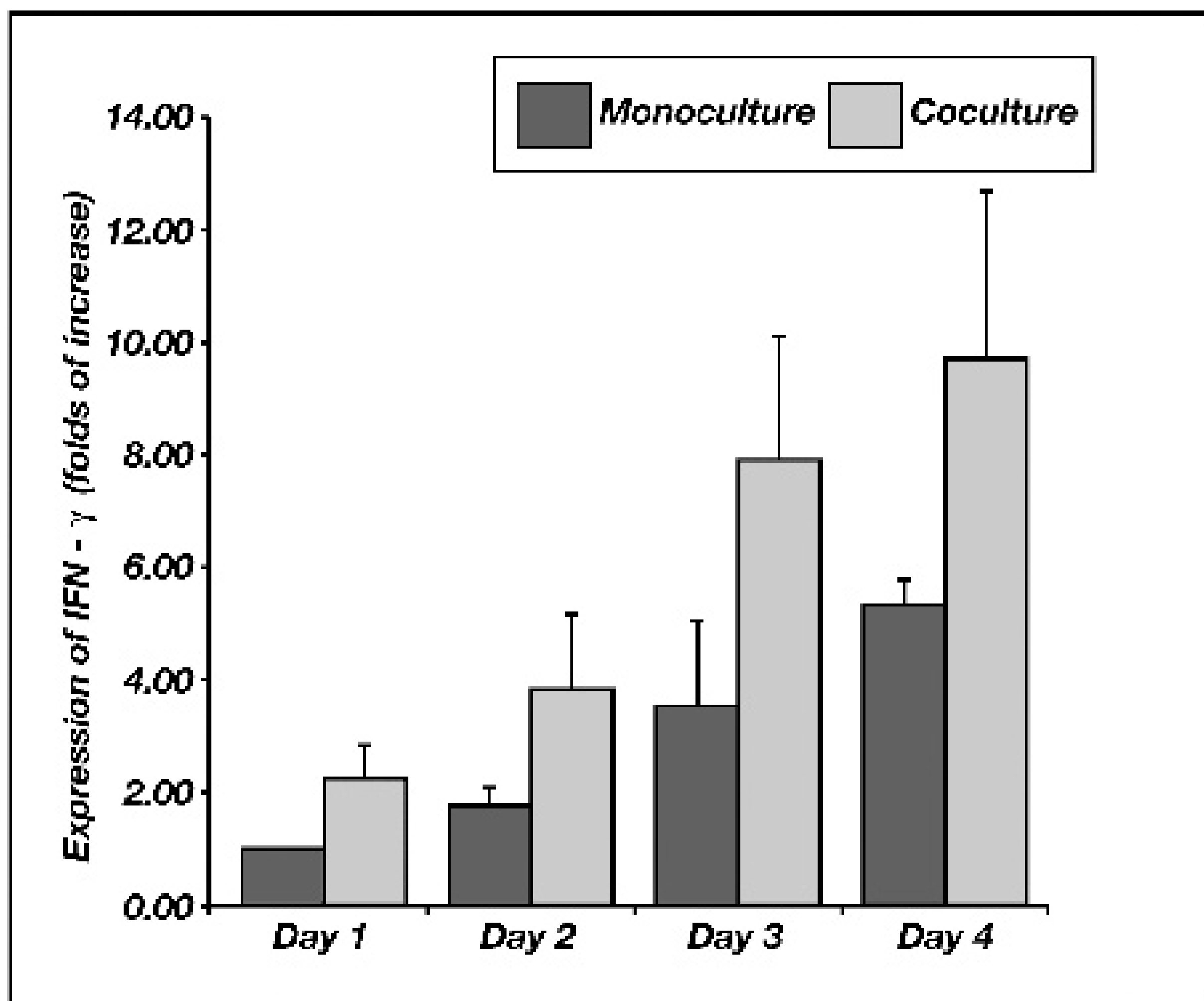

Figure 17. *Upregulation of IFN-γ secretion in coculture.*

ELISA quantification of IFN-γ in the monoculture (monocytes) and coculture supernatants performed using the commercially available kit. Monocytes from 4 different donors were cocultured with VSMC and the cell culture supernatants were quantified for IFN-γ. The value of the monoculture on day1 was considered to be one and accordingly fold of increase was calculated both in monoculture and coculture for different days in each donor. The mean of the values from different donors is shown in the graph (± SD, n=4).

4.4 Stat1 elicits the antiproliferative effect that requires monocyte-expressed uPA and VSMC-expressed uPAR

In the cell cycle progression S phase represents the period in which cellular DNA make its own copy by means of replication. As far as Stat1 is a strong antiproliferative agent, it seems that Stat1 activation and translocation to the nucleus inhibits the replication of DNA or posseses a stop signal for growth of the cell. This activation of Stat1 is achieved by the interaction of uPA with its specific receptor uPAR as supported by our above results. To prove the hypothesis that locally produced uPA by activated monocytes binds to the VSMC cell-surface uPAR and induces Stat1 activation that mediates antiproliferative effect, we relied again on monocytes from uPA$^{-/-}$ and VSMC from uPAR$^{-/-}$ mice. Combination of both cell types was used for coculture, the first by using VSMC from wild type and monocytes from uPA$^{-/-}$ mice. Second, by using VSMC from uPAR$^{-/-}$ and monocytes from wild type mice and third with both cell types from wild type mice as control. Further after the incubation period of coculture, the cells were subjected to proliferation assay using incorporation of 5-bromo-2´-deoxyuridine (BrdUrd). This nucleoside gets incorporated into the cellular DNA in the place of thymidine. The BrdUrd pulsed cells were stained by using antibody against BrdUrd, which positively stains cells that are actively synthesizing DNA during this period. In addition, the DNA was counterstained with Hoechst stain to count the number of cells. The results were documented as described in the methods. With wild-type cells, VSMC in monoculture showed proliferation that was substantially increased when the FCS concentration was raised to 5%. This proliferation was sharply attenuated to low levels in the co-culture system, regardless of the FCS concentration (Figure 18a). With uPA$^{-/-}$ monocytes, coculture increased proliferation in the absence of FCS and had no effect on proliferation in 5% FCS (Figure 18b). With uPAR$^{-/-}$ VSMC, coculture again caused no proliferation inhibition and the results looked identical to those with uPA$^{-/-}$ monocytes (Figure 18c, compared to Figure 18b). To completely exclude the autocrine loop of Stat1 activation a combination of VSMC from uPA$^{-/-}$ and wild-type monocytes were used. In agreement with the other data we observed inhibition of VSMC growth and comparable to the results obtained with wild-type VSMC (Figure 18d, compared to Figure 18a)

To get the evidence that VSMC growth inhibition was mediated by the upregulated Stat1, several experimental approaches were used. Firstly, to show the importance of any given protein, one important and appropriate tool used widely is inhibiting the expression of the said protein by transgenic mode or by knocking out the gene responsible for its expression. So we used the Stat1 knock-out mice. VSMC from Stat1-deficient mice were isolated as described in the methods and used these cells for coculture with wild-type monocytes followed by proliferation assay, as described above.

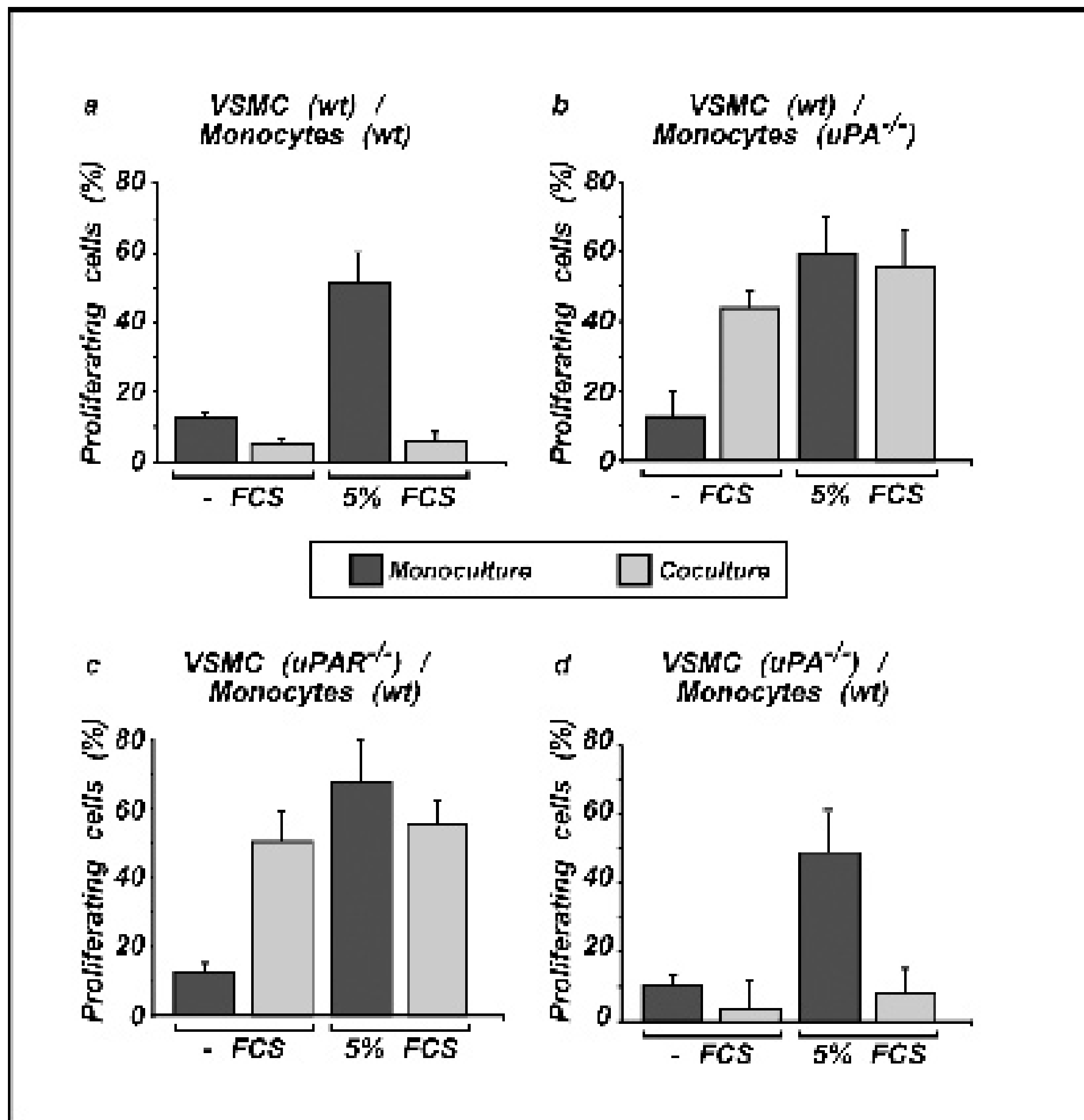

***Figure 18.** Inhibition of VSMC proliferation in coculture requires uPA/uPAR signaling.*

VSMC upon coculture with monocytes were treated with BrdU. Incorporation was detected by immunofluorescence using monoclonal antibody against BrdU. Cells were counted using Hoechst 33258 nuclear staining. Representives of n experiments are shown. a, Result (n=4) using wild-type VSMC and wild-type monocytes. b, uPA$^{-/-}$ monocytes and wild-type VSMC were used for coculture (n=3). c, uPAR$^{-/-}$ VSMC and wild-type monocytes were used (n=3). d, uPA$^{-/-}$ VSMC and wild-type monocytes were used for coculture (n=3).The coverslips were counted in one given field for the number of cells in blue filter for Hoechst 33258 and for number of proliferating cells in red filter for BrdU (Alexa 546). Cells were counted (mean ± S.D. of 5-7 fields).

Monocultured Stat1$^{-/-}$ VSMC showed even in the absence of FCS a higher proliferation level, as compared to wild-type cells, and this proliferation was not further inhibited upon coculture (Figure 19).

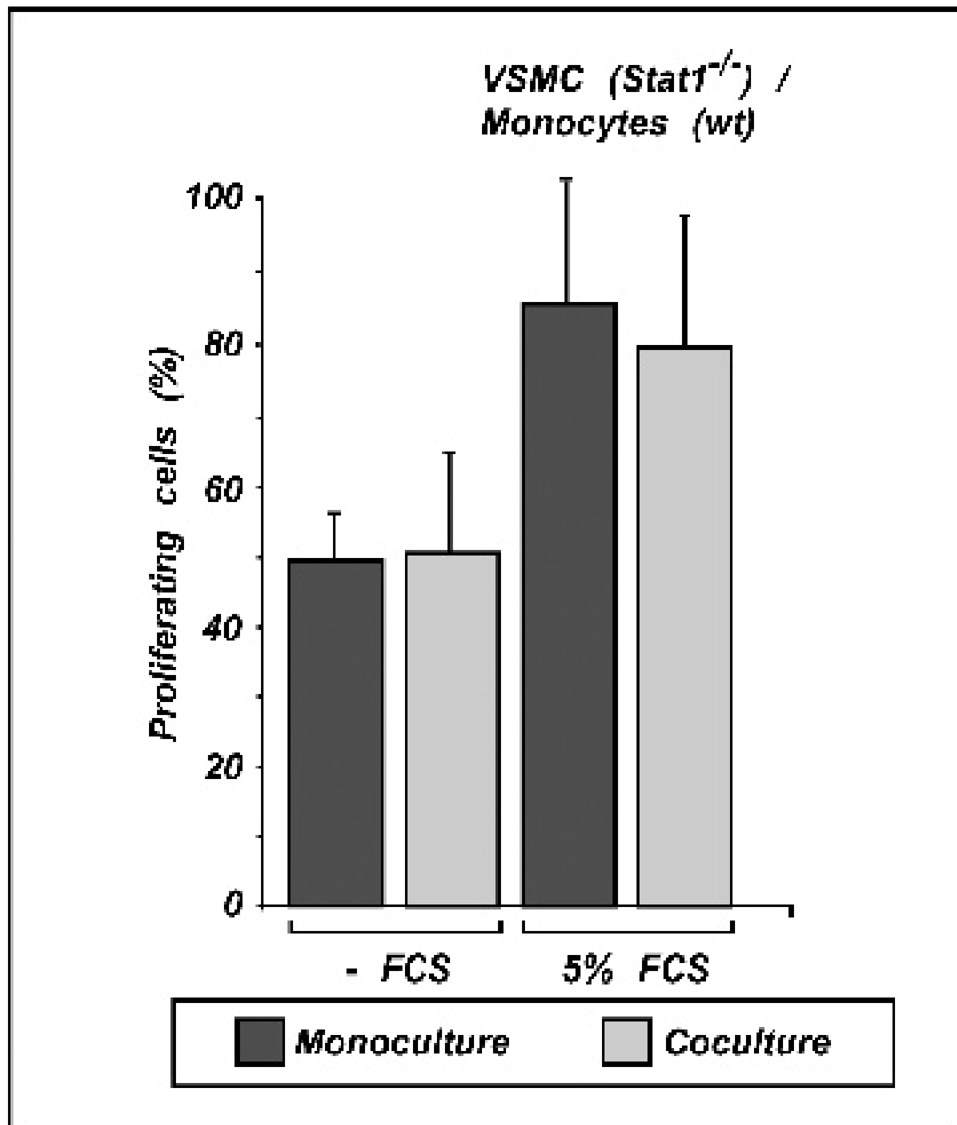

Figure 19. *Stat1 mediates antiproliferative effect in VSMC in coculture.*

VSMC from Stat1$^{-/-}$ and monocytes from wild-type mice were used for coculture and proliferation assay, as indicated in the legend to Fig. 18.

Further, to support the above findings one, more widely used tool to study the importance of a particular protein is to block at the RNA level. In this direction RNA silencing technology for the stable and specific inhibition of Stat1 expression in human VSMC using retroviral RNA interference vector (Cottrell and Doering, 2003) was used. Figure 20 shows that Stat1 expression in these cells (Stat1si-VSMC) was abrogated, as verified by TaqMan RT-PCR (left panel). In proliferation assay, Stat1si-VSMC behaved similar to Stat1$^{-/-}$ VSMC. They displayed increased proliferation level in monoculture that was not affected by coculture with monocytes (right panel, shown for –FCS coculture).

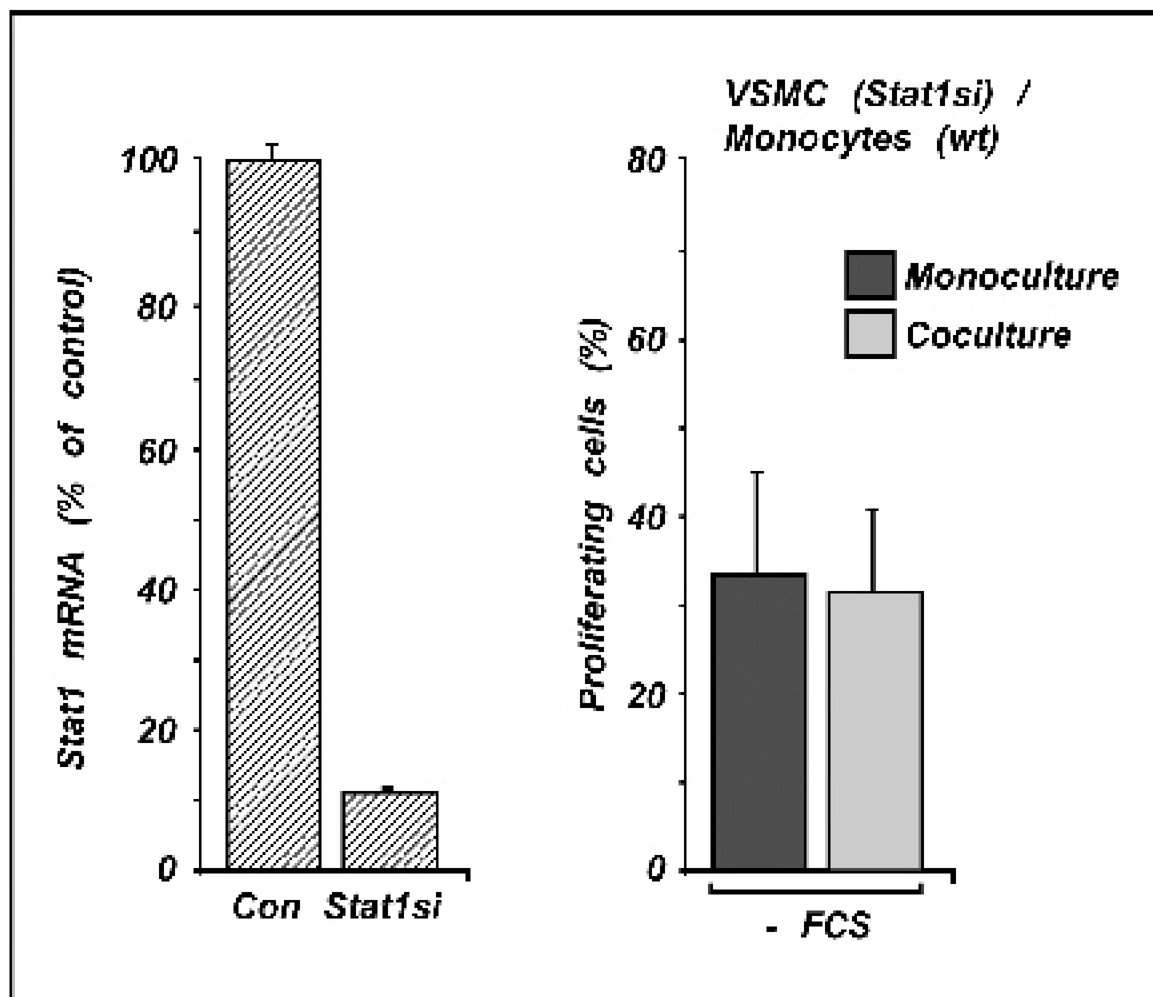

Figure 20. *Downregulation of Stat1 expression restores VSMC proliferation.*
Stat1si-VSMC with downregulated expression of Stat1 (left panel) were used for coculture setting and proliferation assay (right panel).

The above observation was supported by microinjection experiments. As in microinjection one can target a particular protein without disturbing other molecules and tracing the effect of that particular protein of interest, antibody against Stat1 Ser^{727} phosphorylation site was injected to the cytoplasm. Upon injecting the antibody, Stat1 was neutralized in VSMC, blocking particularly Stat1 at Ser^{727} phosphorylation site. These microinjected VSMC were washed and incubated with fresh medium for 1-2 h and further used these cells for coculture setting followed by proliferation assay. As a control VSMC injected with non-specific IgG was treated accordingly as the sample.

The active antibody completely blocked the antiproliferative effect (Figure 21, top panel). Consistent with these data, no Stat1 nuclear translocation was observed in VSMC microinjected with specific antibody, whereas microinjection of control antibody did not affect Stat1 nuclear accumulation (Figure 21, bottom panel).

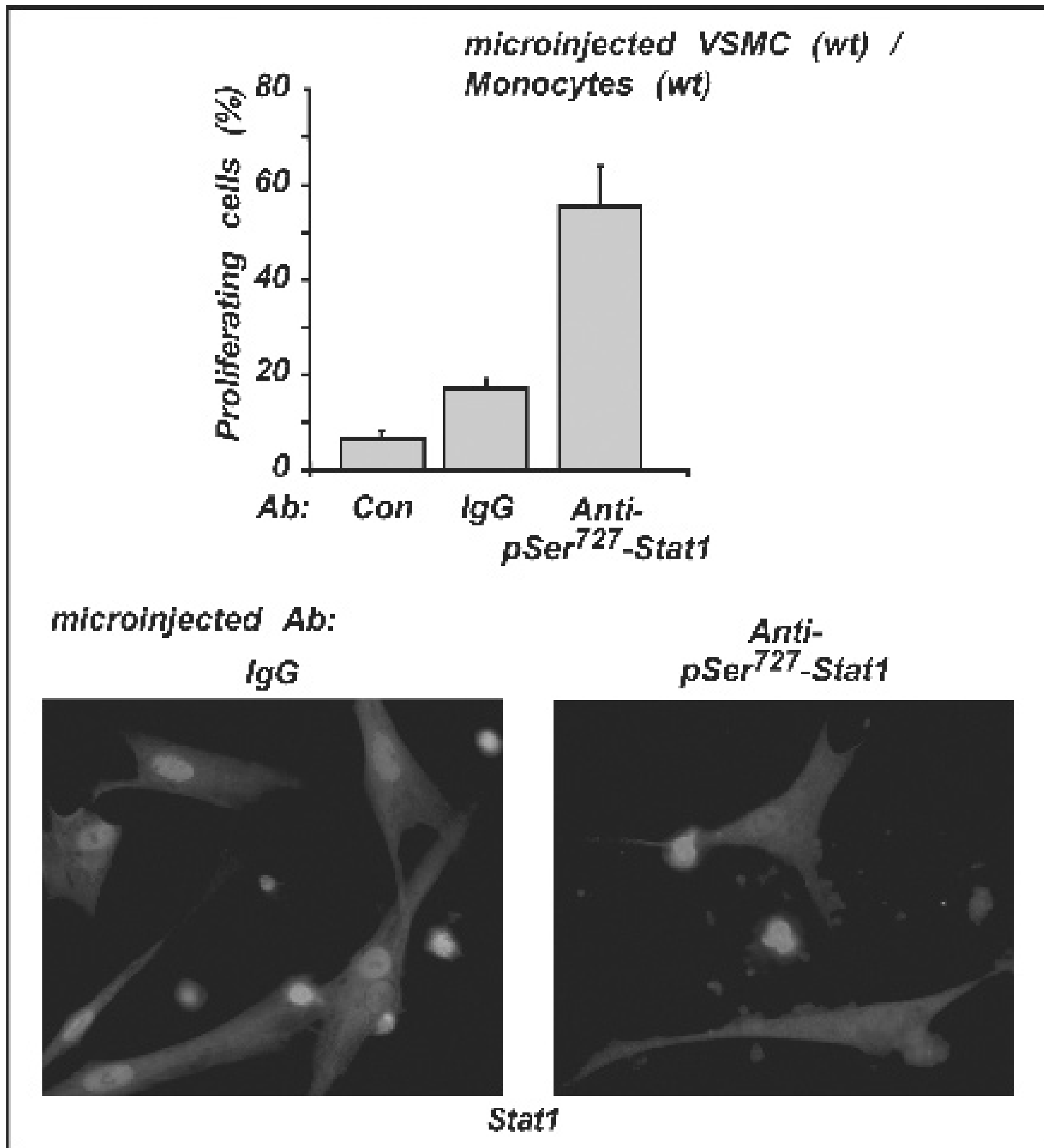

Figure 21. *Inhibition of Stat1 phosphorylation restored VSMC proliferation.*
VSMC were microinjected with pSer^{727}Stat1 antibody and then cocultured. 100-120 cells were microinjected in each experiment and the result shown is a representative of 4 experiments. As a control, non-microinjected and non-specific IgG injected VSMC were included. Microinjected and control cells were used for proliferation assay (top panel), and for immunocytochemistry (bottom panel).

5. DISCUSSION

Arteriosclerosis, the pathological common base of acute and chronic coronary diseases, remains the leading cause of morbidity and mortality. Percutaneous vascular interventions have become successful and widely used treatments for patients with the atherothrombotic diseases. However, chronic restenosis remains one of the major limitations of these procedures leading to the obstruction of dilated arteries (Ellis et al., 1998; Forrester et al., 1991). The major reasons for restenosis development are local changes in medial vascular smooth muscle cell proliferation and migration, followed by increased synthesis of extracellular matrix activated in response to injury during revascularisation. Over several months, these processes result into neointima expansion and plaque formation, finally leading to luminal narrowing (Gallo et al., 1998). Thus, future progress in the prevention of restenosis mainly depends on the development of the therapeutic approaches selectively influencing VSMC activation at the site of vascular intervention.

We show that the interaction of human VSMC with peripheral-blood-derived monocytes in a coculture model results in the inhibition of VSMC growth. The mechanism involves activation of the transcription factor Stat1 in VSMC. This activation is mediated by uPA expressed on the monocytes, which signals the uPAR on the VSMC. The observations imply that monocytes regulate VSMC responses through components of the fibrinolytic system and provide an important link between the uPA/uPAR signaling machinery and VSMC function. The findings may be relevant to VSMC behavior in the face of vascular injury.

The response-to-injury hypothesis proposes that increased endothelial permeability or frank disruption features leukocyte infiltration. By unknown mechanisms, VSMC then migrate from the media into the intima. Here, they proliferate and synthesize an extracellular matrix to form an intimal lesion that may impede flow (Libby et al., 1991; Clowes et al., 1991; Reidy et al., 1992). Several in vivo and in vitro studies suggest that VSMC migration precedes proliferation and that the uPA/uPAR signaling system may be involved (Clowes et al., 1990; Carmeliet et al., 1997; Okada et al., 1998). There is also indirect evidence from humans that inhibition of these early processes can lead to long-term inhibition of the cellular changes (Topol et al., 1994). We asked whether VSMC migration is associated with feedback inhibition of cell proliferation and whether this balance might be controlled by cross-talk of the underlying signaling pathways. We used a coculture model in which human VSMC were directly cocultured with human peripheral-blood-derived monocytes. Although such alternative in vitro models have obvious limitations, they may be helpful for studying communication between 2 or more cell types neighbored in the arterial vessel wall.

Moreover, most data on cell regulation by uPA come from experiments on cultured cells exposed to exogenous purified or recombinant uPA or its peptides, sometimes at fairly high concentrations, making interpretations uncertain. A more specialized in vitro experimental system allowed us to overcome this problem. It is generally considered that the uPA/uPAR system controls the VSMC´s functional behavior mainly via the induction of intracellular signaling. Since uPAR is glycosylphosphatidylinositol (GPI)-anchored and devoid of transmembrane and cytoplasmic domains, it is thought to associate with the transmembrane adaptors of yet incompletely known nature, interacting with uPA and/or uPAR and mediating the uPA-induced intracellular signaling which culminate in cell migration and proliferation (Irigoyen et al., 1999; Rabbani et al., 1992; Wei et al., 1996). The existence of the tandem of transmembrane signaling and GPI-anchored receptors was recently shown for some neurotrophic factors, such as GDNF, neurturin and CNTF (Treanor et al., 1996; Trupp et al., 1995). In the case of uPAR, such a function might be attributed to integrins, G protein-coupled receptors, adaptor protein gp130, which have been demonstrated to interact directly with uPAR with the functional consequences for the cell adhesiveness, migration and signaling (Wei et al., 1996).

The large body of data provide evidence that uPA-induced cell migration and proliferation are supplemented with the rapid and transient activation of intracellular serine and tyrosine phosphorylation (Bohuslav et al., 1995; Busso et al., 1994; Dumler et al., 1993), with activation of Src kinases (Konakova et al., 1998; Resnati et al., 1996), focal adhesion kinase (FAK) and mitogen activated protein kinase (MAPK) (Nguyen et al., 1999; Nguyen et al., 1998; Tang et al., 1998). However, in spite of the availability of such detailed information about uPA-related signaling events, it is not clear how adhesive, migratory, and mitogenic signals eliminating from the uPAR are differentiated. Moreover, numerous studies on uPA-directed signaling cascades in different cell types have figured out that the identified signaling pathways are exclusively cell specific.

In human VSMC, the Jak-Stat pathway is the predominant signaling cascade triggered by uPA/uPAR. It has been demonstrated in our previous studies that uPA activates and uPAR is associated with two Janus kinases, namely Jak1 and Tyk2 (Dumler et al., 1998; Dumler et al., 1998a). Moreover, activation of Stat1, Stat2 and Stat4 in response to physiological concentrations of uPA was additionally shown, although the functional consequences of this activation remained unexplored (Dumler et al., 1999a).

In the present study we have made an attempt to fill up the gaps in the molecular mechanisms involving uPA/uPAR system and Jak-Stat pathway in vascular remodeling using the direct coculture system, where we used VSMC and monocytes both of human origin. This model mimicks to certain extent the in vivo environment of an injured vessel wall, where the monocytes can directly interact with the medial VSMC upon the loss of endothelial integrity. Such alternative

in vitro models might be very helpful to overcome the obvious limitations of animal models and to study communication between two or more cell types, which are neighbored in the arterial vessel wall. Using this coculture system, we targeted some of important molecules in the signaling cascade involving uPA/uPAR at the cell surface and Stat1 as a critical candidate downstream of the cascade within the cell.

We demonstrate here that VSMC in the presence of activated monocytes show an upregulation or activation of Stat1 and its nuclear translocation. By disrupting the microtubule formation using nocodazole, we demonstrated the lack of dependence of Stat1 translocation to the cytoskeleton. The activation of Stat1 is accomplished by the interaction of uPA with its specific receptor. The source of uPA is none other than the monocytes, as our previous studies indicate that expression of uPA was increased in monocytes in coculture with VSMC, whereas the expression of uPAR on VSMC was increased (Kusch et al., 2002). Our finding suggests that monocytes play a decisive role in activation of uPAR-directed signaling in VSMC involving Stat1 as the downstream effector molecule that elicits the antiproliferative effect in VSMC contributing to the pathogenesis of vascular disease.

As it is known from several studies, Stat1 is a strong antiproliferative agent. We have demonstrated the uPA/uPAR-related antiproliferative effect of Stat1 in VSMC by providing several lines of evidence. Thus, blocking uPA and uPAR by antibodies and using VSMC and monocytes from the uPAR- and uPA-deficient mice, we show that uPA/uPAR system plays a pivotal role in activating Stat1 by phosphorylation at ser^{727}, translocation to the nucleus and DNA binding. Further, we demonstrate a functional importance behind this Stat1 activation i.e, growth inhibition. This growth inhibition is evident from the proliferation assay using the cells from the uPA and uPAR knock-out mice and additionally by blocking the Stat1 ser^{727} phosphorylation site using specific antibody in microinjection experiments. In addition it is confirmed that Stat1 is the candidate responsible for this growth inhibition by the experiments with Stat1 knock mouse and by silencing the Stat1 by using Stat1 retroviral construct (Stat1si) where the cells proliferated without any inhibition.

IFN-γ plays a pivotal role in defense mechanisms by switching on the signaling cascade involving Stat proteins and Janus kinases. It seems likely that in different cellular contexts, more than one kinase pathway can converge on Stat1 (Chow et al., 2001). IFN-γ was an unreliable Stat1 activator in our coculture system, although clear-cut Stat1 activation was observed under the monoculture condition. One possible explanation for the observed decreased sensitivity to IFN-γ might be the desensitization of VSMCs induced by the overexpression of IFN-γ in coculture given that leukocytes are capable of synthesizing interferon. ELISA reports in the present study showed

two-fold increase in the IFN-γ secretion in the coculture model. At the same time, the Western analysis showed inhibition of IFN-γ-dependent Stat1 stimulation in VSMC or no effect of IFN-γ on the coculture system. This peculiar behavior can be explained as, in the present case the IFN-γ stimulation may be inhibited where the uPA signaling was dominant and this might have masked the effect of IFN-γ on coculture system. Alternaively, IFN-γ inactivation by limited proteolysis cannot be excluded (Duval-Jobe et al., 1995). Although more work is needed to explore the possibility in greater detail, these data suggest that IFN-γ might use the Stat1-independent pathway in the physiologic situation in which Stat1 activation by uPA is predominant.

Taken together, we suggest a new role for the non-proteolytic function of the fibrinolytic system in the propagation of vascular remodeling following injury through the interplay of VSMC with monocytes (Figure 22). Besides the Jak/Stat pathway, other mediators might contribute to

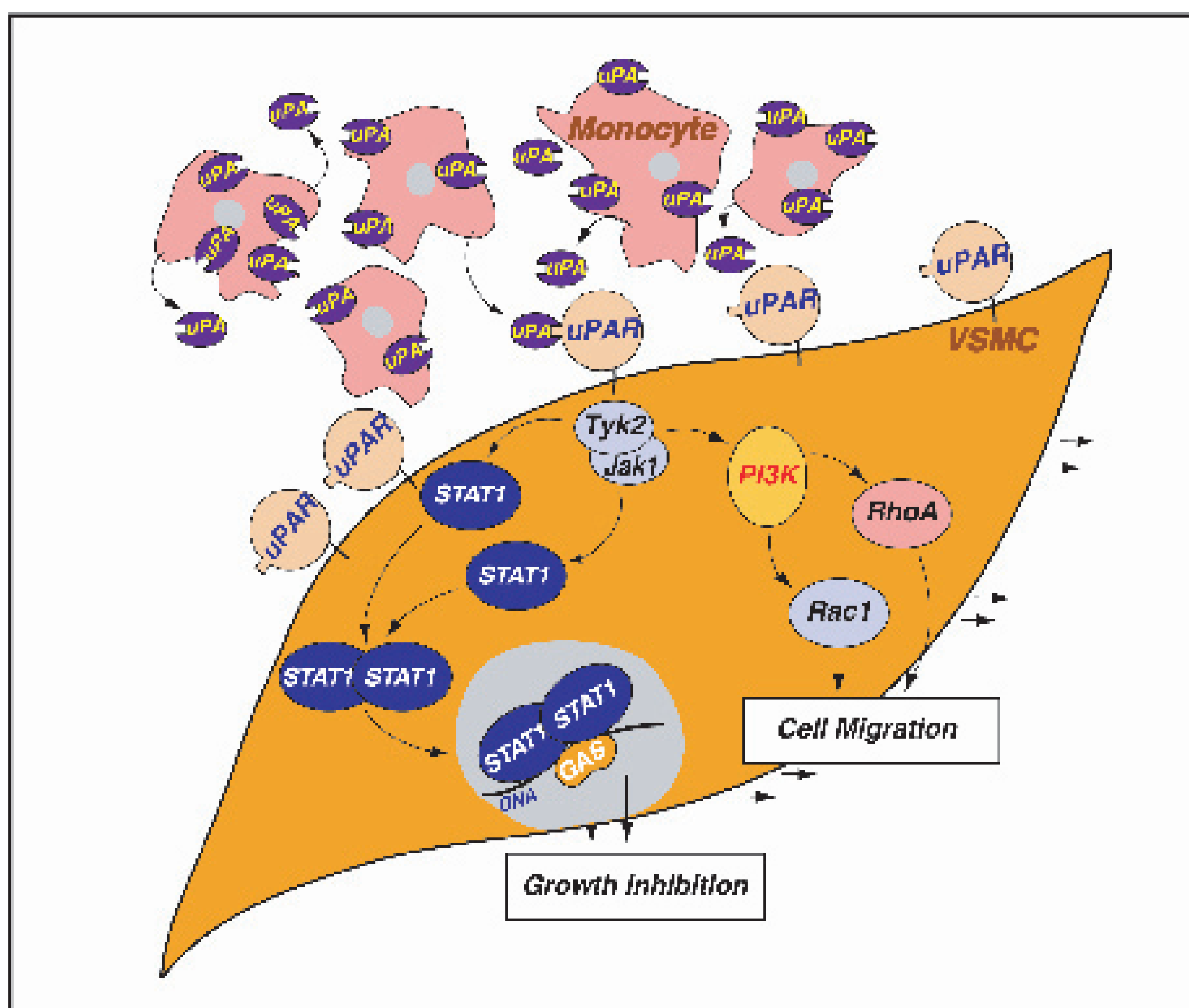

Figure 22.

Schematic representation of the uPA/uPAR-directed Stat1 signaling in coculture.

The study proposes uPA/uPAR signaling at the cell surface results in phosphorylation of Tyk2 and/or Jak1 recruiting Stat1 to activate, dimerize and translocate to the nucleus, leading to gene expression, inhibiting cell proliferation and via a different mechanism promote cell migration involving PI3-K and small GTPases.

VSMC growth inhibition by uPA. Future studies will have to determine whether other signaling proteins play a role in this model. Their elucidation will no doubt provide potential new targets for the treatment and prevention of restenosis.

The precise and rapid propagation of this signaling cascade demands strict and flexible negative regulatory processes that remain unexplored. The nature of the negative regulators involved may have therapeutic implications. Our schematic representation is based on the experimental coculture model and corresponds to in-vivo data showing an increase in VSMC proliferation in uPA-deficient mice (Carmeliet et al., 1997d) and the expression of uPA only in proliferating VSMC in the injured rat carotid artery (Clowes et al., 1990). Why VSMCs use the paracrine and not the autocrine loop for uPA-directed intracellular signaling and how this cell-cell cross-talk remains tightly regulated temporally and spatially in live cells in the face of vascular injury remain unresolved. Many factors other than uPA are involved in the development of vascular disease. However, we have identified a mechanism that suggests how the uPA/uPAR signaling system may contribute to this process through cell-cell communication.

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Ziemiecki, A., Harpur, A. G., and Wilks, A. F. (1994). JAK protein tyosine kinases: their role in cytokine signalling. Trends Cell Biol 4, 207-212.

Curriculum vitae

Sateesh Kunigal Sreenivasan

Date of Birth : November, 26th 1969

Nationality : Indian

Address : Karower Chaussee 67
13125 Berlin
Tel: 9417 2274 (off)
9417 2272 (lab)
9479 5321 (res)
e-mail: kunigal@fvk-berlin.de

Marital Status : Married

Educational Qualification

1998-1991: B,Sc. in Chemistry, Botany and Zoology, Bangalore University, Bangalore, India

1993-1995: M.Sc. in Zoology, Bangalore University, Bangalore, India.

1999: Ph.D., Franz-Volhard-Clinic at Max Delbruck Center for Molecular Medicine, Berlin and Hannover University, Hannover, Germany under the supervision of Prof. Inna Dumler.

Research Experience

May 1994: Summer vacation trainee in 1994 in Prof. N.R. Moudgal´s laboratory in Primate Research Laboratory, Indian Institute of Science , Bangalore India. Standardised the ELISA technique for the measurement of hCG from human urine.

1995-1998: Research assistant in Dr. P.B. Seshagiri´s laboratory in Center for Reproductive Biology and Molecular Endocrinology, Indian Institute of Science, Bangalore, India

1998-1999: Junior research fellow in Dr. D.N. Rao´s laboratory in Department of Biochemistry, Indian Institute of Science, Bangalore, India

Publications

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1. "1st Hannover Seminar on Transplantation and Vascular Biology", 07.-09. December, 2000 held in Hannover.

2. Sateesh Kunigal, Angelika Kusch, Sergey Tkachuk, Julia Kiian, Natalia Tkachuk, Hermann Haller and Inna Dumler. "Urokinase-induced Jak/Stat signaling: molecular link between migration and proliferation of human vascular smooth muscle cells" (Abstract presented at the meeting of the 16th International Society for Fibrinolysis and Proteolysis, held at Munich-Germany from 08-13, 2002

Hiermit versichere ich an Eides statt, daß ich die vorliegende Arbeit selbständig verfaßt und die benutzten Hilfsmittel angegeben habe

Berlin, 9th October 2003

Sateesh S Kunigal