Regulation of Smooth Muscle Cell Growth

by Endothelium-Derived Factors

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*The endothelium is a source of molecules that either stimulate or inhibit the prolifera-
tion of the underlying smooth muscle cells. In the normal, healthy vessel wall the smooth
muscle cells are quiescent, but they proliferate when damage to the endothelium oc-
curs. The implication of such observations is that although the endothelium provides a
source of growth factors, their stimulatory activity on smooth muscle cells is countered
by endothelium-derived growth inhibitors. The inhibitors appear to comprise at least 3
distinct types of molecules: heparin/heparan sulfate; transforming growth factor β; and
nitric oxide. Each molecule inhibits growth of cultured smooth muscle cells by mecha-
nisms that remain to be elucidated and are discussed in this communication. Heparin/
heparan sulfate is the most thoroughly characterized of the 3, and has been used for
clinical intervention to prevent restenosis. Transforming growth factor β exhibits bimo-
dal activity on growth, acting as a stimulant at low levels and as an inhibitor at elevated
concentrations. Nitric oxide mediated vasorelaxation is dependent upon activation of
soluble guanylate cyclase. Because elevation of cyclic guanosine monophosphate in
smooth muscle cells depresses their proliferation, nitric oxide would appear to possess
the properties necessary to inhibit vascular smooth muscle cell proliferation.* **(Texas
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Contractile smooth muscle cells in the walls of adult blood vessels retain their ability to revert to a less well differentiated phenotype, which exhibits both high proliferative and synthetic capacity.[1,2] During embryogenesis, this last capability is important for the structural development of the blood vessel wall. This unique property of mature smooth muscle cells may account for their apparent sensitivity to stimulation by growth factors and for their subsequent inappropriate proliferation associated with vascular pathologies.[3,4] The proliferative responses that ensue from such stimuli are accompanied by increased synthesis of extracellular matrix molecules and, in combination with the increased cellular mass, new "tissue" is formed. Restenosis following balloon angioplasty is but 1 example of such behavior of smooth muscle cells; there are many other instances that lead to change in vascular structure.[5,6] These changes in vascular structure result in local or more general intimal thickening and lead to increased peripheral resistance. This in turn aggravates the vascular system, and the proliferative response of the smooth muscle cells may be accompanied by an inflammatory response and by the recruitment of cells associated with such an event.[5,7]

The "response to injury" hypothesis and the role of growth factors derived from platelets have been invoked as the events that initiate the change in behavior of adult, contractile smooth muscle cells.[1,2] This hypothesis has been the stimulus for a large amount of experimentation that has identified a number of growth factors that appear to be key to the whole process of smooth muscle cell "activation."[8] However, in the classical models of experimental vascular injury, wherein denudation of endothelium is accomplished, regrowth of the endothelium does not restore the cells in the neointima to the quiescent state. This suggests that although the endothelium clearly plays an important role in the regulation of smooth muscle proliferative behavior, once the latter has been activated the endothelium is unable to restore the status quo. There are a number of possible explanations for this apparent lack of effectiveness of the reformed endothelium. One of these may be an impaired production of growth inhibitors by the new endothelial cells (Fig.

1). Additionally, the extracellular matrix elaborated by the intimal smooth muscle cells may be "permissive" for their growth. The concept of the extracellular matrix's acting as a long-term signaling system for the cells that it surrounds has been proposed for more than a decade.[8] A limited number of studies suggest that the extracellular matrix produced by proliferative "intimal" smooth muscle cells may consist of a blend of molecules typically associated with the developing blood vessel wall. This premise has its basis in observations of elevated expression of certain matrix molecule genes by intimal cells.[6] Since the extracellular matrix is turned over slowly, any alteration in matrix composition will persist for a prolonged period.

The identification of growth inhibitors in the vessel wall has received little attention, compared to that focused on the characterization of promoters of smooth muscle cell growth. Additionally, expression of a particular agonist (either inhibitory or stimulatory to growth) will not always ensure that it exerts an effect, since there has to be expression of the specific cell-surface receptor for the agonist. Three molecules produced by the endothelium have been identified as possible growth inhibitors. They are
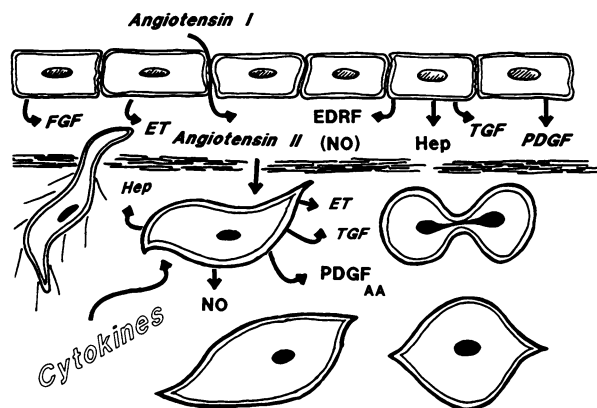

Fig. 1 *Endothelium-derived factors and smooth muscle growth modulation. The schematic illustrates some of the many factors that arise from the endothelium and that either stimulate growth (hypertrophy or hyperplasia) and migration or inhibit it. This may occur through direct interaction of the endothelium-derived factors with smooth muscle or through their induction of additional factors. Angiotensin II, for example, induces the expression of a number of growth factors by the smooth muscle cells themselves. Additionally, cytokine production by vessel-wall cells can induce expression of NO synthase in smooth muscle cells.*

Endothelium-derived growth stimulators include: FGF (fibroblast growth factor); ET (endothelin); TGFβ (transforming growth factor β); and PDGF (platelet-derived growth factor). Endothelium-derived growth inhibitors include: EDRF-NO (endothelium-derived relaxing factor); and Hep (heparin/heparan sulfate). TGFβ may act as either a growth promoter or inhibitor (see text). NO = nitric oxide; PDGF$_{AA}$ = A chain homodimeric form of platelet-derived growth factor.

very different from one another, and their mechanisms of action remain to be clearly elucidated. The most well characterized of these are the heparan sulfate or heparin proteoglycans, and, in spite of a great deal of elegant work, the cellular effects of these matrix molecules remain largely enigmatic.[9,10] Several studies have indicated that the peptidic transforming growth factor β (TGFβ) family may be growth inhibitory to smooth muscle cells.[11,12] The 3rd type of molecule implicated as a growth inhibitor is endothelium-derived relaxing factor or nitric oxide (NO). A number of studies have demonstrated that elevation of intracellular cyclic guanosine monophosphate (GMP) in smooth muscle leads to inhibition of proliferation.[13,14] Binding of NO to its intracellular receptor, guanylate cyclase, results in the elevation of intracellular cyclic GMP.[15] In addition to these compounds, it has been proposed that growth-inhibitory prostanoids may be produced by the endothelium.[16]

Several reviews have appeared on the growth-inhibitory actions of heparin or heparan sulfate[17,18] and TGFβ.[11,19] Therefore, the major emphasis of this communication will be on the role of NO as a growth inhibitor and on the importance of NO production by inducible forms of NO synthase.[20,21] The paucity of information on growth-inhibitory prostanoids renders their discussion invalid at this time. It is important to understand at the outset that most of the studies from which the information is gleaned have employed cultured smooth muscle cells, and only a few of these studies have used human cells. Because such cells usually express the "proliferative/synthetic" phenotype[16] in culture, it is difficult to extrapolate the data to the in vivo situation. However, the fact that inhibition of proliferation of such cells by the 3 classes of compounds mentioned above has been reported does provide some grounds for optimism, since in vivo it is the *proliferative* phenotype that is responsible for pathogenic activity.

Heparin/Heparan Sulfate

Chemical Composition and Structural Features. Heparin and heparan sulfate are similar molecules that are generally associated with the extracellular milieu.[22] They belong to a large family of compounds known as proteoglycans and are synthesized by both endothelial and smooth muscle cells. Heparin and heparan sulfate share structural features common to all proteoglycan molecules, such as linear carbohydrate (glycosaminoglycan) chains made up of disaccharide repeats covalently linked to a peptide core. However, in the case of the heparinoids, the complexity of the modifications to the glycosaminoglycan (GAG) components is more extensive than in other proteoglycans.[17,22] The degree of N- and O-sulfation of individual disaccharide repeat units is more variable with heparin and heparan sulfate, and

this leads to domains of high-charge density. The recognition of these structural features of the heparinoids has led to research to develop similar molecules to act both as anticoagulants and as inhibitors of smooth muscle proliferation. Since, in general, compounds that lack -N-SO$_4$ groups also appear to lack anti-proliferative activity,[17] it is this component of the heparin and heparan sulfate structure that is believed to be important for biological activity.

Inhibition of Proliferation by Heparin/Heparan Sulfate. The inhibition of smooth muscle cell proliferation by heparin in injured arteries was demonstrated by Clowes and Karnovsky[10] in 1977, and the production of heparin/heparan sulfate by endothelial cells in co-culture with smooth muscle cells followed about a decade later.[16] The co-culture of primary smooth muscle cells with endothelial cells has been shown to retard the former's dedifferentiation to the "proliferative phenotype."[16] Studies with isolated smooth muscle cells have indicated that there were changes in the structure of heparan sulfate when cells changed from the "contractile" to the "proliferative" phenotype.[23,24] Also, extracellular matrix material produced by endothelial cells inhibited growth of smooth muscle cells when they were plated on such matrix material.[25] The matrices produced by endothelial cells contain 3 major components: glycopeptides (e.g., laminin and fibronectin); type IV collagen; and heparan sulfate. Treatment of matrices with heparinase to degrade the GAG component suppressed its smooth muscle growth-inhibitory properties.[25] In a comparative study of the effects upon growth of extracellular matrices elaborated by smooth muscle cells derived either from spontaneously hypertensive rats or from normotensive rats, the former extracellular matrices were found to be more growth stimulatory and to contain less heparan sulfate.[26] The conclusions from the studies mentioned and numerous others of similar nature are that matrix-associated heparin/heparan sulfate plays an important role in the regulation of smooth muscle cell growth. It remains unclear whether matrix heparinoids interact while associated with their environment, or after release by degradative enzymes.[27]

Mechanism of Heparinoid Anti-Proliferative Action. Cultured smooth muscle cells express receptors for heparin/heparan sulfate, and saturable uptake of radiolabeled molecules has been demonstrated by a number of laboratories.[28,29] Internalized molecules become associated with the peri-nuclear region of cells, and this may be indicative of direct action on gene expression. Heparin/heparan sulfate binds competitively to inositol trisphosphate receptors on the sarcolemma, and thus modulates release of Ca^{2+} from intracellular stores following activation of phospholipase C.[30]

Heparin/heparan sulfate may have both direct and indirect action on peptide growth factors. Treatment of cultured smooth muscle cells with heparin downregulates the expression of epidermal growth factor (EGF) receptors and consequently the responses to the growth factor.[29,31] This action on EGF-mediated signal transduction is not apparent for other growth factors such as platelet-derived growth factor (PDGF) or insulin-like growth factor.[31] The importance of EGF in the vessel wall has yet to be demonstrated, since apart from the generation of EGF-like peptides during the turnover of extracellular matrix, no evidence of EGF production by vessel-wall cells has been documented. Heparinoids bind a number of peptide growth factors, in particular fibroblast growth factor (FGF), and this modulates the actions of these molecules.[32] Heparin infusion has been shown to remove vessel-wall-bound FGF following balloon catheter injury.[32] Degradation of matrix-associated heparin/heparan sulfate leads to the release of bound FGF,[32] and this may act as a local stimulus to smooth muscle cell growth, but it may also increase the lability of the growth factor, making it more susceptible to proteolytic degradation. Heparin also binds TGFβ, and this prevents the growth factor's interaction with α2-macroglobulin. This complex is the latent form of TGFβ, and most of the peptide circulating in plasma and secreted from cells is in this form.[33] One consequence of this interaction with heparin appears to be an increase in the efficacy of TGFβ, which may be a mechanism for the indirect growth-inhibitory action of heparinoids.

Transforming Growth Factor β

Multiplicity of Actions of TGFβ. Because platelet α-granules contain at least as much TGFβ as PDGF,[11] the release of their contents yields both a potent smooth muscle mitogen (PDGF) and an apparent inhibitor (TGFβ) of this activity. There is a rapid induction of TGFβ expression following balloon denudation of rat carotid arteries.[34] The inhibitory action of TGFβ on smooth muscle cells is complex and is dependent upon the expression of receptor subtypes.[35] Since TGFβ has a profound effect on extracellular matrix synthesis,[34,36] it is conceivable that the actions of the growth factor are mediated through cellular interaction with matrix molecules. The binding of TGFβ to heparin/heparan sulfate has been mentioned, but in addition it binds to the matrix glycopeptides thrombospondin[37] and fibronectin.[38] Induction of PDGF-A chain expression by smooth muscle cells exposed to TGFβ may account for some of its apparent anomalous action, but TGFβ also elicits down-regulation of PDGF receptor α-subunit expression.[39] The levels at which these events are evoked appear to be different, and this may be an

explanation for the apparent bimodal action of the growth factor.[39]

Both endothelial and smooth muscle cells produce TGFβ in response to a number of agonists, including angiotensin II.[4,40] The proliferative response to angiotensin II observed in smooth muscle cells isolated from spontaneously hypertensive rats has been ascribed to TGFβ production.[41] In contrast, angiotensin II-induced TGFβ production by smooth muscle cells isolated from normotensive rats results in growth inhibition.[41] Clearly a great deal more experimentation will be required to clarify the ambiguous behavior of TGFβ.

Mechanism of Actions of TGFβ. The purpose of this communication is to discuss the repression of smooth muscle cell growth by endothelial cell derived factors such as TGFβ; therefore, the mechanisms of growth stimulation will be ignored. The investigation of the mechanism of growth inhibition by TGFβ has been studied in fibroblast cell lines.[42,43] The ability of TGFβ to inhibit FGF-induced mitogenesis of growth-arrested CCL39 fibroblasts decreased, the longer the addition of the transforming growth factor was delayed after stimulation of cells by FGF.[42] However, many early events that were initiated by FGF stimulation, such as phosphoinositide turnover, S6 kinase activation, c-*fos* and c-*myc* induction, and the induction of ornithine decarboxylase activity, were unaffected by the presence of TGFβ.[42] Therefore, in spite of a strong indication that TGFβ's presence early in the cell cycle was required for it to exert its inhibitory action, none of the events commonly associated with early signal transduction was affected. However, later events—like induction of thymidine kinase—were inhibited as long as TGFβ was present at the start of the stimulation period.[42]

In attempts to elucidate this apparent contradiction between the observed actions of TGFβ and its effects on established early pathways of signal transduction, agents that uncoupled G-protein-linked events were employed.[43] Although treatment of AKR-2B fibroblasts with TGFβ elicits rapid expression of a number of cellular oncogenes (among them c-*sis* and c-*myc*), this reaction was markedly reduced in cells exposed to pertussis toxin (PT) prior to treatment with TGFβ.[43] However, TGFβ stimulation of extracellular matrix molecule gene expression in AKR-2B cells was unaffected by prior treatment with PT.[43] These observations indicate not only that there are at least 2 distinct pathways of gene induction by TGFβ, but that they presumably are mediated by different receptor subtypes. Three receptor subtypes (type I, II and III) have been identified in confluent cultures of smooth muscle cells, on the basis of their molecular size. Some evidence suggests that types I and II mediate the signal transduction events associated with inhibition of proliferation.[44]

Endothelium-Derived Relaxing Factor (NO)

Regulation of NO Production. A clear distinction between 2 isoenzyme families responsible for the production of NO is apparent when the regulation of this process is considered. A number of cell types, including endothelial cells, produce NO by a rapid response to a variety of agonists, such as acetyl choline, thrombin, bradykinin, platelet activating factor (PAF), and electrical stimulation. The enzyme that catalyzes this production is referred to as constitutive NO synthase (cNOS), since the rapidity of this event precludes enzyme induction.[45] In contrast, inducible NO synthase (iNOS) is associated with a variety of cells that respond to cytokines and lipopolysaccharides to produce large quantities of NO after a time lag of several hours; both macrophages and smooth muscle cells are examples of this cell type.[46,47]

Invariably, the actions of the agonists associated with cNOS stimulation elicit a rapid elevation in intracellular Ca^{2+} and the interaction of calmodulin with the enzyme. The production of NO is transient and is accompanied by the return of intracellular $[Ca^{2+}]$ to basal levels and by the dissociation of calmodulin from cNOS.[46] Production of NO by inducible enzyme systems may also be dependent upon calmodulin binding, but this appears to be a more stable association, since samples of purified enzyme contain tightly bound cofactor.[47] Once induced, iNOS activity appears to be long-lived and levels of NO production are at least 10-fold higher than that of cNOS systems.[45]

An additional regulatory mechanism for NO production by inducible enzyme may be the supply of tetrahydrobiopterin (BH4), a cofactor for conversion of L-arginine to citrulline. Production of NO from the guanidino-nitrogens of L-arginine occurs by oxidation requiring a 5-electron donation, 2 of which are provided by BH4.[48] Therefore, NO production is limited effectively by the level of intracellular BH4, and this is regulated by the enzyme GTP:cyclohydrolase I (GTP:CH), which catalyses the 1st step in the de novo synthesis of the cofactor (Fig. 2). Cytokines such as interleukin-1β (IL-1β) that induce expression of iNOS also elicit strong induction of GTP:CH, and this is at least a superficial explanation for the efficient production of NO by cells exposed to this cytokine. Activation of GTP:CH appears to be dependent upon elevation of intracellular cyclic adenosine monophosphate (AMP) levels.[49] Consequently, signal transduction elicited by IL-1β, which includes elevation of cyclic AMP, may account for the activation of the BH4 biosynthetic pathway.[50]

The presence of bound BH4 in preparations of cNOS and the demonstration of enhanced NO production in the presence of added exogenous pterin cofactor may be indicative of another mechanism for
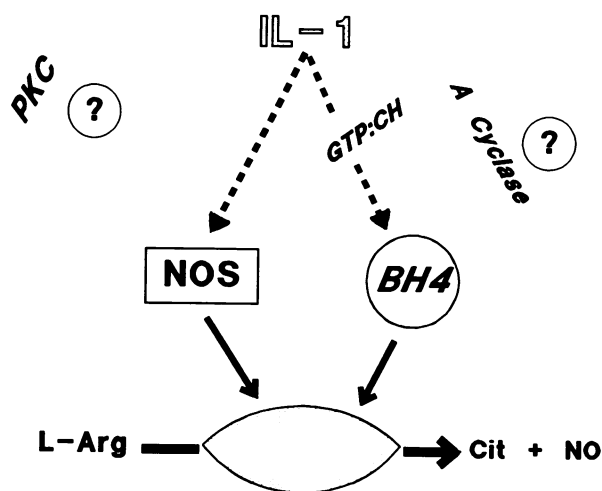
Fig. 2 *Production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) requires the synthesis of tetrahydrobiopterin (BH4). Production of NO by smooth muscle iNOS is dependent upon an adequate supply of tetrahydrobiopterin. Interleukin-1β (IL-1β) stimulates both the expression of NOS and GTP:cyclohydrolase (GTP:CH), the regulatory enzyme for the biosynthesis of BH4. The individual pathways appear to be sensitive to stimulation by protein kinase C (PKC for NOS) activation and adenylate cyclase (A cyclase for GTP:CH) activation. NOS in the presence of BH4 converts L-arginine (L-Arg) to citrulline (cit) and NO.*

the regulation of NO production. Constitutive expression of GTP:CH by cells having the cNOS isoenzyme may allow for a very rapid increase in BH4 production in response to elevation of intracellular cyclic AMP levels. This may explain an early observation that elevation of intracellular cyclic AMP leads to increased NO production by endothelial cells.[51]

Evidence That NO Inhibits Growth. The circumstantial evidence that NO is both an important growth inhibitor of smooth muscle and a regulator of muscle tone has been discussed. Growth arrest of cultured smooth muscle cells by NO has been demonstrated;[46] and since elevated levels of NO are cytotoxic, this action of the molecule may account for its effect on the growth of cultured cells. Experimental growth inhibition by cellular NO, in contrast to that derived from nitrovasodilator drugs, has been demonstrated most often using cells that have been stimulated to express high levels of iNOS. There have been few demonstrations using systems where NO production arises through the actions of the constitutive enzyme associated with the endothelium.[52] A correlation between the amount of NO to which smooth muscle cells are exposed and the degree to which they are growth inhibited has been demonstrated, and is indicative of the role of the compound in the depression of growth.[46] Furthermore, under the conditions used in these studies, there was no indication that NO was cytotoxic.[46]

Mechanism(s) of Growth Inhibition by NO. Some of the circumstantial evidence for considering NO as a growth inhibitor relates to the observation that elevation of cyclic GMP results in depressed proliferation of smooth muscle cells, and one recognized cellular action of NO is the elevation of cyclic GMP.[15] This led to the initial assumption that growth inhibition by NO was due to its interaction with soluble guanylate cyclase; however, a more direct role for NO has been proposed recently. Chemical modification of peptides by NO can result in their loss of biological activity, an example of which is the s-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase by NO.[53] The enzyme subsequently undergoes further modification by ADP-ribosylation, which results in inactivation of this key enzyme in cellular energy metabolism.[53] Levels of nitrite anions (an indirect measure of NO production) in conditioned media can be correlated with lactate production as a consequence of inhibition of mitochondrial respiration.[54] These findings suggest that although activation of guanylate cyclase may lead to actions mediated by cyclic GMP-dependent kinase(s), other actions of NO need to be investigated.

Recently, the s-nitrosylation of serum peptides has prompted suggestions that such modification of proteins may be a mechanism for the actions of NO.[55] Within the extracellular matrix, there are a number of molecules containing sulfhydryl-rich domains, and these may be accessible to chemical modification by NO. This type of interaction may stabilize NO and be important for its biological activity in the vessel wall.

Conclusions

The endothelium clearly produces a number of factors capable of inhibiting the proliferative activity of the underlying smooth muscle cells. Although the molecules discussed here take very different molecular forms, they eventually must mediate their activity through common pathways, in order to control growth. The most likely candidates for regulation of such common pathways are the protein kinases; and as more information is obtained on the actions of molecules like p34^{CDC2} kinase,[56] which control the cell cycle, it is hoped that this will lead to the development of agents that can effectively prevent the "pathogenic" growth of smooth muscle cells.

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