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Endothelial Cell Regulation by Transforming Growth Factor-Beta

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Abstract Pronounced changes including growth inhibition, increased matrix deposition and suppression of cell-associated proteolytic activity, take place in endothelial cells (EC) upon the application of TGF-β. Interrelationships between these effects have shed some light on the mechanism of action of TGF-β and on its role in regulating EC function vis-a-vis angiogenesis. For instance, preliminary evidence has indicated that increased levels of certain matrix components may be partly responsible for the antiproliferative action of TGF-β. In addition, TGF-β and bFGF have opposing effects on cellular proteolytic balance which may contribute to the antagonistic effect that TGF-β has on bFGF-induced EC growth and possibly to the anti-angiogenic effect exerted by TGF-β under certain circumstances. Of particular interest in this regard is the fact that physical contact between EC and vascular mural cells in EC:mural cell cocultures has been found to generate active TGF-β, thus further implicating TGF-β in the maintenance of the quiescent, differentiated aggregation of EC as found in vascular structures in vivo. While more information is needed to define what, if any, role TGF-β plays in endothelial differentiation, it is to be noted that many of the cellular and biochemical processes affected by TGF-β are linked to differentiation. It is therefore possible that the growth inhibition of EC by TGF-β primes them for differentiation and/or is critical for the maintenance of a differentiated state.

Key words: proliferation, fibroblast growth factor, extracellular matrix, angiogenesis, differentiation, plasminogen activator, plasminogen activator inhibitor

TRANSFORMING GROWTH FACTOR-BETA (TGF-β)

TGF-β was first identified as an activity able to induce anchorage-independent growth of fibroblasts [1]. Since its identification, TGF-β has been shown to influence the growth and differentiation of a variety of cell types, inhibiting cell growth and/or inducing differentiation in some cases and acting as a mitogen in others. The multiple activities of this pleuripotential growth regulator have been recently reviewed and the reader is referred to a chapter by Roberts and Sporn [2].

TGF-β AND ENDOTHELIAL CELL PROLIFERATION

As is the case for many cell types, TGF-β inhibits the growth of endothelial cells (EC) [3–5]. EC have been shown to respond differently to the two best-characterized forms of TGF-β; the ED_{50} for β1 inhibition of aortic EC was 101 ± 34 pg/ml versus 6310 ± 985 for β2 and 10 ± 4 versus 26 ± 2 pg/ml for capillary EC [6]. Another study has reported that TGF-β2 moderately inhibited rat fat pad microvessel EC and had no effect on bovine aortic EC [7]. There are reports of a bifunctional effect of TGF-β on EC proliferation, stimulating growth at low doses (0.02–0.1 ng/ml) and inhibiting at higher concentrations (> 0.5 ng/ml) [8].

Though the mechanism(s) underlying this phenomenon is not clearly understood, there are suggestions that this may occur by the action of TGF-β on other growth factor receptors. Evidence for this comes from Myoken and coworkers [8], who reported a reduction of acidic fibroblast growth factor (aFGF) binding to bovine fetal heart EC treated with inhibitory doses of TGF-β when compared with aFGF binding in the absence of TGF-β or in the presence of a low dose of TGF-β. Human umbilical vein EC, which were not growth inhibited by TGF-β, did not exhibit reduced aFGF binding. Although none of the three classes of TGF-β receptors has been purified, the authors implicate differences in receptor profile in this effect; whereas fetal heart EC displayed 2 classes of receptors (85,000 and

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58,000), the human umbilical vein EC expressed only a single 85,000 dalton receptor. The number of high-affinity EGF receptors was also found to be reduced in TGF-β-treated rat heart EC. However, in keratinocytes, binding of EGF to its receptor was not affected by TGF-β [9].

TGF-β AND PLASMINOGEN ACTIVATION

The bulk of recent data strongly indicates that the activation of plasminogen to the protease plasmin is a complex process that can be regulated by several growth factors. To date, the roles of TGF-β and basic FGF (bFGF) in plasmin level regulation have been described most extensively. Plasmin is generated by the action of plasminogen activators (PA) on the inactive zymogen. Two types of PA exist: the tissue-type PA (tPA) and the urokinase-type PA (uPA), separate gene products that differ both structurally and functionally [10,11]. PA are secreted in an inactive form which have little or no activity. While the initial mechanism of their activation is unknown, it has been shown that plasmin can convert the inactive PA into its active form. This "loop" creates the potential for the uncontrolled production of plasmin; however, such production is restricted both by immobilization of PA to the strict vicinity of the cell (on its surface and in the basement membrane) [12] and by the secretion of plasminogen activator inhibitor-1 (PAI-1) [13–15].

A number of studies have demonstrated that while bFGF increases PA activity, TGF-β decreases PA activity and stimulates PAI production and deposition [16–19]. Using zymographic and reverse zymographic measurements, Saksela et al. [17] showed that a 24 h incubation of bovine capillary EC with bFGF (3 ng/ml) led to increased activity of both uPA (in both cell extracts and conditioned media) and PAI (CM only), but a net increase in proteolytic activity over control cultures. On the other hand, incubation with 1 ng/ml of TGF-β drastically reduced PA activity and substantially increased PAI activity. Similar studies using calf pulmonary artery EC indicated that TGF-β and bFGF have the same effect on the proteolytic balance in large vessel EC; however, in contrast to bovine capillary EC, TGF-β did not stimulate uPA gene transcription at 12 h in calf pulmonary arterial EC. It is interesting to note that Saksela et al. [17] using zymographic assays, did not observe an increase in uPA activity in bovine capillary EC treated with TGF-β for 24 h, despite the increase in uPA mRNA levels as reported by Pepper et al. [16]. This may be due to continued association between the uPA and PAI; alternatively, it may indicate that the uPA mRNA is translated at low levels or in a delayed fashion. If an anomaly between mRNA levels and protein levels for uPA and PAI does exist, it is uncertain to what extent the uPA/PAI mRNA ratios represent an accurate portrayal of the actual proteolytic activity in response to these growth regulators. In another study, bovine aortic EC coincubated with thrombin-activated platelet releasates (i.e., containing proteolytically activated TGF-β) showed a 2-fold increase in secreted PAI-1 (CM and ECM) over cultures coincubated with nonactivated releasates [20]. This effect was almost completely reversed by the addition of neutralizing antibodies to TGF-β, indicating that TGF-β was responsible for the elevated PAI. Similarly, increased levels of PAI-1 production and secretion have also been observed in a human lung fibroblast cell line treated with TGF-β [18]. Consistent with these observations, inhibitors of plasmin and of plasminogen activators inhibit bFGF-induced microvascular EC invasion of explanted acellular amnion membrane [21].

In addition to its matrix-degrading function, plasmin can also convert TGF-β from its latent to its active form in vitro [22]. If plasmin is responsible for the conversion of latent TGF-β (LTGF-β) to TGF-β, then stimulation of PAI activity by TGF-β appears to support the hypothesis by Lyons et al. [23] that TGF-β would regulate its own activation. Further evidence of this self-regulatory role of TGF-β has come from investigations into the activation of TGF-β in cocultures of vascular mural cells (pericytes or smooth muscle cells) and EC. In these cocultures, we [24,25] and others [26] have found that mural cell-EC contact leads to conversion of LTGF-β into active TGF-β, which subsequently inhibits EC growth and migration. Neither CM from pure cell cultures nor contactless presence of both cell types in the same culture has been found to generate active TGF-β. Addition of neutralizing antibodies to PAI-1 in these cocultures resulted in prolonged production of TGF-β, while in control cultures TGF-β production ceased after 12 h [27]. Further, addition of neutralizing anti-TGF-β antibodies greatly reduced PAI-1 levels. These findings indicate that plasmin is indeed involved in the activation of TGF-β in these coculture systems, and it ap-

pears that heterocellular contact results in the conversion of plasminogen into plasmin by some currently unknown mechanism. Activated plasmin then converts LTGF-β into active TGF-β, which autoregulates its production by inducing the antiproteolytic agent PAI-1. The observation that LTGF-β binds to the mannose 6-phosphate receptor [28] and the finding that the activation in cocultures can be blocked by the addition of either mannose 6-phosphate or antibodies against the mannose 6-phosphate receptor [29] indicate a role for this receptor in the activation process. In our initial demonstration of the presence of activated TGF-β in pericyte-capillary EC cocultures, we reported finding long-term (14 day) growth inhibitory activity [24]. This seeming contradiction between our observations and those of Sato et al. [26] may be reconciled by considering this self-regulatory system as a dynamic situation. Undoubtedly, there are a number of variables, such as plasminogen availability, conditions of culture, cell age and density, mural cell:EC ratio, and so forth, which may modulate the magnitude and kinetics of the activation of TGF-β in these cocultures. Given the ability of TGF-β to stimulate both uPA and PAI gene transcription, there may be situations (e.g., low TGF-β concentration) where the uPA/PAI ratio may be shifted to generate a weak net proteolytic effect that may be sufficient to maintain a baseline level of active TGF-β over a prolonged period. If this hypothesis is true, such an effect would probably be restricted only to capillary EC, since Pepper et al. [16] found TGF-β to suppress rather than stimulate uPA gene transcription in calf pulmonary arterial EC.

MECHANISM OF ACTION OF TGF-β

TGF-β and Matrix Synthesis

In most cultured cells, TGF-β treatment has been found to increase both the synthesis and secretion of specific extracellular matrix (ECM) proteins. In cultured fibroblasts, TGF-β increased the synthesis and secretion of fibronectin and collagen [30–32]. Thrombospondin mRNA was also stimulated [32a], although to a lesser extent than the other two. In the case of EC, the cellular substrate appears to influence the response to TGF-β. When EC were cultured on either laminin- or collagen-coated surfaces, TGF-β increased fibronectin production, while the levels of collagen IV and V were enhanced only for EC growing on fibronectin-coated dishes; laminin synthesis was not affected in any case [33]. Large vessel EC migration on a fibronectin substrate was found to be decreased by TGF-β1, which also increased mRNA and protein levels of TGF-β in vitro [34]. These results were consistent with in vivo observations of increased fibronectin on the luminal surface of arteries that had been denuded by endarterectomy. An increase in thrombospondin mRNA has also been observed in TGF-β-treated capillary EC grown on gelatin-coated substrates (RayChaudhury and D'Amore, unpublished observations). In addition, TGF-β has been found to increase the integrin levels for fibronectin, laminin and vitronectin in cultured fibroblasts and various other cell lines [35,36]. This increase is effected by activating transcription, translation, and cellular processing of these receptors. The elevation in the level of vitronectin receptor may be significant in the regulation of some of the antiproteolytic activities induced by TGF-β, since vitronectin has been found to bind and stabilize PAI-1 in the ECM of cultured EC [37]. The increased production of ECM proteins by TGF-β seems to be mediated by two mechanisms: (i) TGF-β increases the mRNA levels for fibronectin and collagen at least partly by stimulating de novo transcription, and (ii) (as described above) TGF-β decreases the degradation of matrix proteins by both attenuating the synthesis of proteases and augmenting the synthesis of PAI and protease inhibitors (Fig. 1).

In one study, EC were shown to be partially growth-inhibited when cultured on ECM that was produced by TGF-β-treated cells [38]. This antiproliferative effect of the matrix derived from TGF-β-treated cells was temporary, however, and after several days the untreated cells growing on this matrix resumed exponential growth. Subsequent analysis of the ECM derived from TGF-β-treated cells revealed a 2-fold increase in the accumulation of various radiolabeled metabolic precursors as compared with untreated matrix, although no qualitative alterations in matrix proteins were observed. This result indicates that the levels of certain ECM components may contribute, at least in part, to the growth inhibition of EC by TGF-β, although no specific component was identified.

TGF-β and *myc*. Investigating the molecular mechanism of TGF-β-induced growth inhibition, Takehara et al. [39] reported that growth

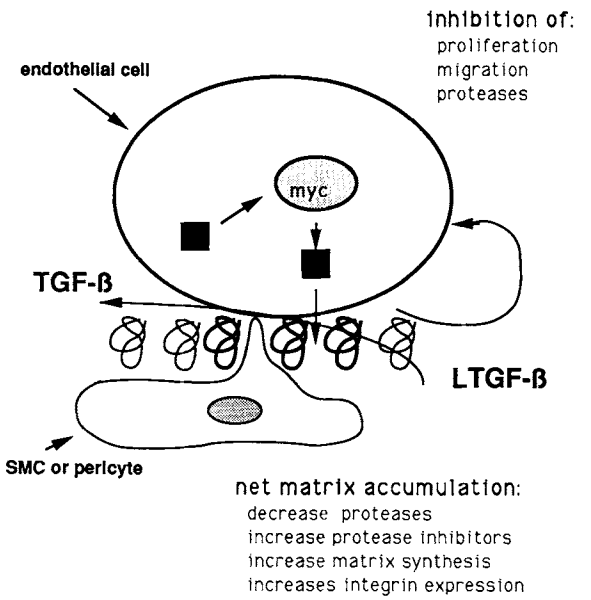

Fig. 1. Schematic diagram of activation and action of TGF-β in the vasculature. Contact between an endothelial cell (EC) and a smooth muscle cell or pericyte leads to the local activation of TGF-β. TGF-β then acts back on the EC, causing changes in the composition and arrangement of the extracellular matrix. The matrix changes, in turn, lead to the inhibition of proliferation, migration, and protease production, and ultimately to the expression of a "differentiated" phenotype.

inhibition of rabbit heart EC by TGF-β was not affected by pretreatment of the cells with cycloheximide or by the simultaneous addition of cycloheximide with TGF-β. These results suggest that in these cells de novo protein synthesis was not required for the growth inhibition and therefore the inhibition was not due to altered matrix production (see discussion below). Instead, they showed that TGF-β dramatically suppressed the stimulation of c-myc transcription by growth stimulatory factors. The suppression of c-*myc* transcription by TGF-β has also been observed in cultured keratinocytes (which also are growth inhibited by TGF-β); in the case of the keratinocytes, however, protein synthesis seems to be necessary for c-*myc* suppression [9,40,41]. It is possible that the two proposed inhibitory mechanisms, increased ECM deposition and c-*myc* suppression, are independent. Alternatively, they may reflect different stages of a single process. These observations have led to the suggestion that TGF-β exerts its antiproliferative effects by thwarting growth stimulators. However, the events following binding of TGF-β to its receptor and ultimately resulting in c-*myc* suppression are not known.

TGF-β AND ANGIOGENESIS

In response to angiogenic stimuli, capillary EC are thought to locally degrade the basement membrane of the parent vessel, invade the interstitial ECM, and commence the process of angiogenesis [42]. The degradation of the ECM that permits cellular invasion is facilitated by localized proteolytic digestion of matrix constituents, due in part to the formation of the serine protease plasmin from the zymogen plasminogen. When EC grown on a collagen gel are stimulated by tumor promoters like phorbol-12-myristate-12-acetate (PMA), or by bFGF, they invade the matrix and form tube-like structures [43,44]. TGF-β is a strong inhibitor of this process [45]. Although TGF-β has been shown to exert a strong growth inhibitory effect on EC [3,4,45], it seems unlikely that the antiproliferative activity of TGF-β is the cause of this inhibition, since mitomycin C-treated EC can still be induced by PMA to form tubular structures [43]. Rather, the behavior of EC treated with TGF-β and/or bFGF has been found to have a striking correlation to the net proteolytic balance as measured by uPA/PAI ratio. In their detailed study of proteolytic regulation by bFGF and TGF-β, Pepper et al. [16] reported that both TGF-β and bFGF stimulate transcription of uPA and PAI; however, the kinetics and magnitude of transcription stimulation in response to these different agents are strikingly different. Whereas bFGF and TGF-β induced a sustained elevation in uPA and PAI mRNA levels, respectively, bFGF caused an ephemeral increase in PAI-1 mRNA levels, reaching a peak at 4 h and decreasing to control levels by 24 h. In contrast, TGF-β yielded a delayed stimulation of PAI transcription (after 8 h). Further, the PAI mRNA stimulation by TGF-β was 39-fold above control levels at maximal stimulation (12 h), while bFGF stimulated PAI transcription only 3.6-fold at 4 h. uPA levels were maximally induced about 12-fold by TGF-β (at 24 h), while bFGF maintained a similar increase of uPA mRNA from 8 h onwards. In effect, bFGF treatment of the capillary EC resulted in a net increase in proteolytic activity and angiogenic stimulation of cells, while TGF-β treatment resulted in a net increase in antiproteolytic activity, peaking at 4 h and inhibiting angiogenesis. Simultaneous addition of bFGF and TGF-β did not change the uPA/PAI mRNA ratio over control levels, thus explaining the inhibitory effect of TGF-β on bFGF-induced in

vitro angiogenesis. In addition to engendering antiproteolytic activity through PAI, TGF-β suppresses the biosynthesis and/or induction and secretion of other proteases, such as transin/stromelysin, a broad-spectrum protease produced in large quantities by various fibroblasts, and increases production of other protease inhibitors, such as tissue inhibitor of metalloproteinase [46]. These effects may further contribute to the ability of TGF-β to antagonize angiogenesis in vitro and perhaps may be physiologically relevant in vivo.

Since ECM components have profound effects on cellular proliferation, migration, and differentiation, it is likely that the TGF-β-induced increased matrix synthesis plays a role in the developmental, wound healing, and growth-regulatory properties of TGF-β. Of particular interest in this regard is angiogenesis, since it involves the proliferation, migration, and multicellular organization of EC. Interestingly, if EC are grown interspersed in 3-dimensional collagen gels, TGF-β does not exert a growth-inhibitory effect on these cells, nor is the production of fibronectin or collagen affected. Indeed, on exposure to 0.5 ng/ml of TGF-β these cells organize to form tube-like structures mimicking angiogenesis [7,33]. This observation has been described by Madri and coworkers to be an in vitro representation of the observed in vivo angiogenic activity of TGF-β, the distribution of EC in the 3-D collagen matrix being configurationally parallel to EC in vivo. It is not clear, however, whether or not the effect of TGF-β in this system is not simply one of stabilizing the tube-like structures (by an indirect matrix effect) rather than direct stimulation of tube formation; the current information offers no means of distinguishing between the two possibilities.

A number of observations indicates that TGF-β is angiogenic in vivo [32,47]. We suspect that the actions of TGF-β in vivo and in vitro are not as inconsistent as they would appear: stimulating angiogenesis in vivo and inhibiting EC proliferation and migration in vitro. It appears likely that the in vivo angiogenic action of TGF-β is not to directly stimulate EC. In their studies on the action of TGF-β on the chicken chorioallantoic membrane, Moses and coworkers [47] concluded that EC are growth-inhibited as a result of TGF-β treatment; however, EC densities are higher around the TGF-β application site due to an apparent chemoattractant action of TGF-β on these cells. The ability of the cells to form tubes may be facilitated by the ECM in the environment and TGF-β may then stabilize these structures (e.g., stabilize the differentiated phenotype). Alternatively, it is possible that the primary action of TGF-β in these conditions is to stimulate the migration of monocytes [48] for which TGF-β acts as a strong chemoattractant [49]. Upon differentiation into macrophages and activation, they secrete known angiogenic agents, like bFGF and TNF-α, which may be the stimulators of angiogenesis.

We also speculate that TGF-β may be an important growth regulator of EC in vivo. Our observations using cocultures of microvessel EC and pericytes (described above) reveal that contact between the two cell types, which is known to occur throughout the microvasculature in vivo [50], leads to the activation of TGF-β, which in turn leads to the inhibition of EC growth. Activation of TGF-β in this manner may thus be important in terminating vessel growth during embryogenesis and wound healing and may even be necessary to maintain the microvasculature in its usually quiescent state [51]. Local increases in stimulatory/angiogenic factors might then act to overcome the suppressive actions of TGF-β in situations such as wound healing. Thus, the growth state of the microvasculature might be determined by a balance in local concentrations of stimulators and inhibitors (in much the same way that Pepper et al. [16] have described for proteolytic activity in their in vitro angiogenesis assay—see above for details).

TGF-β AND ENDOTHELIAL DIFFERENTIATION?

In nearly all systems described, differentiation is temporally correlated with the cessation of cell division. To date, TGF-β has been shown to influence EC growth, migration, and protease production, inhibiting each in a manner that is reminiscent of the characteristics of differentiated, quiescent endothelium in vivo. In one study, however, TGF-β was shown to decrease the density of both spontaneously forming and retinoic acid-stimulated endothelial fenestrations by bovine adrenal cortex EC, a property characteristic of their in vivo phenotype, leading the authors to conclude that TGF-β inhibits EC differentiation [52]. This is in seeming contradiction to the other actions of TGF-β on EC, which, as were described above, are more reflective of the endothelium in quiescent state than a proliferating, migrating, dedifferentiated EC. The explanation for this paradox is not readily apparent. The inhibitory effect of TGF-β on the formation of fenestrations was observed over a

range of times (2–7 days in the presence of 2 ng/ml TGF-β) and concentrations for which growth inhibition is also observed. On the other hand, because of the nature of the assays, investigations examining the effects TGF-β on proliferation and migration usually use subconfluent/sparse cultures of EC, whereas the studies on EC fenestrations used confluent cells. It is possible that the effect of TGF-β on EC may be density-dependent, as has been described for smooth muscle cells [53]. Thus, in spite of the fact that the phenotype of a "differentiated" EC has yet to be described, it is possible that the growth inhibition of EC by TGF-β marks the beginning of a cascade of events that culminates in differentiation.

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