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Control of smooth muscle cell growth by components of the extracellular matrix: Autocrine role for thrombospondin

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ABSTRACT **Addition of platelet-derived growth factor (PDGF) to growth-arrested cultured smooth muscle cells (SMC) induces the synthesis and secretion of thrombospondin (TS), a glycoprotein component of the SMC extracellular matrix *in vitro*. This induction occurs at PDGF concentrations that are suboptimal for a mitogenic response. In this study we examined the effect of TS on the proliferation of SMC, using a serum-free mitogenesis assay. Addition of either epidermal growth factor (EGF) or purified human platelet TS to quiescent rat vascular SMC did not substantially stimulate mitogenesis; the 30-hr nuclear labeling index increased from a mean of 7% in control cells to 20% for EGF-treated SMC and 17% for cells exposed to TS alone. However, TS and EGF acted synergistically to stimulate DNA synthesis by SMC, increasing the labeling index to 47%. The facilitative effect of TS on EGF-mediated mitogenesis was inhibited by heparin, a known inhibitor of SMC growth and migration that also blocks incorporation of TS into the SMC extracellular matrix. The effect was specific for EGF; TS did not augment the response of cells to insulin or insulin-like growth factor 1. These data establish a functional role for cell-derived TS and provide evidence for the presence of an autocrine, growth-supportive mechanism involving the extracellular matrix. In addition, our experiments support the existence of a novel, heparin-sensitive SMC mitogenic pathway and suggest a mechanism whereby heparin-like molecules may inhibit SMC proliferation.**

Unscheduled proliferation of vascular smooth muscle cells (SMC) in response to arterial injury accounts in large part for the development of arteriosclerotic lesions (1) and for the failure of a significant number of vascular reconstructions (2). While the mechanisms underlying SMC growth control *in vivo* remain unclear, the response of cultured SMC to a wide variety of growth promoters (3–8), growth inhibitors (9, 10), and matrix influences (11) suggests that a number of independent or interrelated pathways controlling SMC replication may be operative.

We are interested in defining the molecular mechanism underlying the control of vascular SMC growth, with particular emphasis on the role played by heparin-like growth inhibitors and other components of the extracellular matrix. We have recently reported (12) that production of thrombospondin (TS), a glycoprotein component of the SMC extracellular matrix *in vitro* (12, 13), was induced by platelet-derived growth factor (PDGF) in a rapid and transient manner. The induction was dose dependent, occurred early in the G_1 phase of the cell cycle, was sensitive to inhibition by actinomycin D, and resulted in the deposition of TS into the extracellular matrix. Heparin, a known inhibitor of SMC growth (9, 10, 14, 15) and migration (16), markedly reduced the incorporation of TS into the Triton X-100-insoluble matrix (12). On the basis of these data, we speculated that a mitogen-induced, TS-rich matrix may facilitate the optimal growth and/or migration of SMC. This hypothesis was supported by additional data demonstrating that affinity-purified antibodies to TS inhibited SMC migration and mitogenesis *in vitro* (17).

In this report we provide direct evidence that TS facilitates the SMC mitogenic response to epidermal growth factor (EGF). This TS-mediated activity appears different from that of other known growth promoters and is sensitive to inhibition by heparin. These data provide support for the concept that the extracellular matrix, whose character may be modified by known growth factors (12, 18, 19) or growth inhibitors (20, 21), can exert functional control over cellular proliferation. In addition, the data establish an autocrine, growth-promoting function for cell-derived, matrix TS, define a novel SMC mitogenic pathway, and suggest a mechanism for the growth-inhibitory action of heparin-like glycosaminoglycans.

MATERIALS AND METHODS

Cell Culture. Rat aortic SMC were grown from explants as described (16). Cells were subcultured in Waymouth's medium containing 10% fetal calf serum and antibiotics. SMC from frozen stocks were used for experimentation between the fourth and eighth passage.

Materials. All cell culture materials, except where indicated, were from GIBCO. PDGF, prepared from human platelets (22), and monospecific goat antisera to human PDGF were gifts of E. Raines and R. Ross (Univ. of Washington). EGF (receptor grade), transferrin, and insulin were from Collaborative Research (Waltham, MA). Recombinant insulin-like growth factor 1 (IGF-1), purchased from Amgen Biologicals (Thousand Oaks, CA), was a gift of M. Majesky and S. Schwartz (Univ. of Washington). Heparin (type I, 169 USP units/mg) was from Sigma. Monospecific rabbit antiserum to human platelet TS was previously prepared and characterized in this laboratory (13). Lab-Tek four-chamber tissue culture slides were from Miles Scientific (Naperville, IL), and [*methyl*-^{3}H]thymidine was from Amersham.

Preparation of TS. Calcium-replete TS was prepared from fresh human platelets obtained from the Puget Sound Blood Center as described by Raugi *et al.* (23). Essentially, the protocol involves activation of platelets with α-thrombin, separation of high molecular weight proteins on a Sepharose CL-4B column, and further purification of a TS-rich CL-4B peak on a heparin-Sepharose CL-6B column. TS was eluted in 50 mM Tris·HCl/600 mM NaCl/0.5 mM $CaCl_2$. All buffers used contained at least 0.5 mM $CaCl_2$ to maintain the native Ca^{2+}-dependent conformation of TS (24). The final concentration of our TS preparations was 600–800 μg of protein per ml.

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Abbreviations: PDGF, platelet-derived growth factor; SMC, smooth muscle cells; TS, thrombospondin; EGF, epidermal growth factor; IGF, insulin-like growth factor.

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Mitogenesis Assays. SMC were plated at 1×10^4 cells per chamber on glass four-well chamber slides (plating density 0.5×10^3 cells per cm^2), in Waymouth's medium containing 10% fetal calf serum and antibiotics. After 24 hr to allow efficient cell attachment and spreading, the culture medium was changed to 0.5% fetal calf serum in Waymouth's medium for 72 hr. Cells were then incubated for 24 hr in serum-free medium consisting of a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's medium and F-12 medium containing 1.0 mg of bovine serum albumin and 5 μg of transferrin per ml (25). Labeling of cells with 1.0 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine per ml was initiated at the time of addition of test substances and was terminated after 30 hr by fixation with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) after several washes in cold PBS. Slides were washed for 30 min in 0.1 M glycine, air-dried, and coated with Kodak NT2B liquid emulsion. After a 3-day exposure at 4°C, slides were developed in Kodak D-19 developer, fixed, washed, and dried prior to assay for radioactivity. The labeling index of control cells not exposed to mitogens averaged <10%; addition of 10% fetal calf serum increased the labeling index in each experiment to >95%.

RESULTS

Response of SMC to Mitogens and TS. The role of various mitogens and TS in the SMC growth response was examined by using a serum-free mitogenesis assay. The experiments were designed to investigate the functional role of specific mitogens and matrix components (TS and heparin) in the control of G_1 progression. As shown in Fig. 1, a substantial (9-fold) mitogenic response was elicited by addition of PDGF alone; addition of 5 ng of EGF per ml did not potentiate PDGF-mediated mitogenesis over a concentration range (0.1–25 ng/ml) of PDGF. This lack of requirement for "progression" factors (26) during PDGF-directed mitogenesis very likely reflects the ability of PDGF to stimulate SMC to secrete its own progression factor, IGF-1 (27). Addition of purified platelet TS at 20 μg/ml increased the labeling index only slightly above the control level. TS did not augment or potentiate the response of SMC to PDGF, even at suboptimal concentrations of PDGF. EGF alone was not a potent mitogen for SMC in our assay system. However, when EGF and TS were added together, a substantial mitogenic response was observed (Fig. 1). This response (46.5% labeled nuclei vs. control levels of 7.3%) represents an additional recruitment into the cell cycle of 39.2 cells per 100 by TS and EGF. The magnitude of this response, compared to the mitogenic responses of SMC to EGF alone (+ 12.5 cells per 100) or to TS alone (+ 9.3 cells per 100) suggests that EGF and TS act synergistically to stimulate SMC mitogenesis. The response to the combination of TS and EGF was significantly greater ($P < 0.001$, when compared by one-way analysis of variance) than the response to EGF or TS alone or to the calculated value expected if the effect were additive rather than synergistic (Fig. 1).

Synergistic Effects of EGF and TS. SMC responded weakly to increasing concentrations of EGF (Fig. 2), even at 50 or 100 ng/ml (not shown). In the presence of 20 μg of TS per ml, a significant mitogenic response was observed at EGF concentrations above 1 ng/ml. SMC did not respond mitogenically to low concentrations of TS alone (Fig. 3), although some mitogenic activity was noted at higher concentrations (20 μg/ml). The SMC response to EGF was facilitated at concentrations of TS > 5 μg/ml. The levels of TS required to facilitate SMC growth are consistent with recent binding data, which demonstrate maximal, saturable binding of TS to endothelium and fibroblasts at concentrations of TS > 10 μg/ml (28). Laminin, another growth-facilitative matrix component, is active only at much higher concentrations (50–100 μg/ml) (29). The relatively high concentration of TS required to promote SMC growth (i.e., compared to a true mitogen)

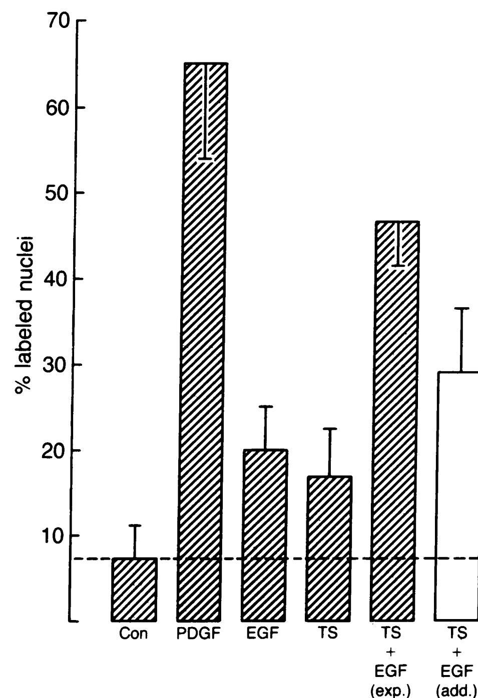

FIG. 1. Response of vascular SMC to mitogens and TS under serum-free conditions. Rat aortic SMC were plated onto glass four-well chamber slides and allowed to attach for 24 hr. Cells were then growth-arrested by maintaining the cultures in 0.5% fetal calf serum for 72 hr, followed by an additional 24 hr in serum-free medium. Labeling was initiated by adding 1 μCi of [^{3}H]thymidine per ml in fresh serum-free medium containing appropriate test substances and continued for 30 hr. After fixation and drying, cells were coated with emulsion, exposed, and then developed. The percentage of nuclei labeled with [^{3}H]thymidine was determined and expressed as the mean of a minimum of six determinations ± 1 SEM. Control cells received no added factors. PDGF and EGF were given at 5 ng/ml. The concentration of TS was 20 μg/ml. Note that PDGF alone substantially increased DNA synthesis in SMC, whereas EGF or TS alone had small but detectable effects. TS and EGF added together acted synergistically to markedly stimulate SMC mitogenesis (see text). The experimentally observed value for the concerted effects of TS and EGF (exp.) was significantly greater ($P < 0.001$ by one-way analysis of variance) than the calculated additive effect of TS and EGF (add.). ▨, Observed data ± SD; □, arithmetically derived data; - - -, percentage of labeled nuclei in control SMC (Con).

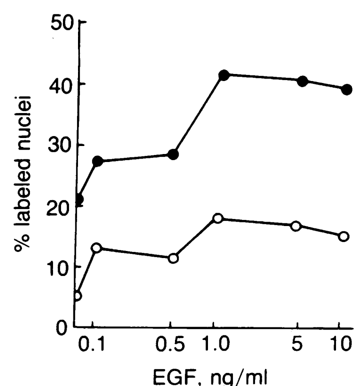

FIG. 2. Response of vascular SMC to increasing amounts of EGF with or without added TS. The mitogenic response to EGF (0.1–10 ng/ml) was determined in the presence (●) or absence (○) of TS at 20 μg/ml as described in the legend to Fig. 1. Note that SMC respond only weakly to increasing amounts of EGF alone unless TS is present.

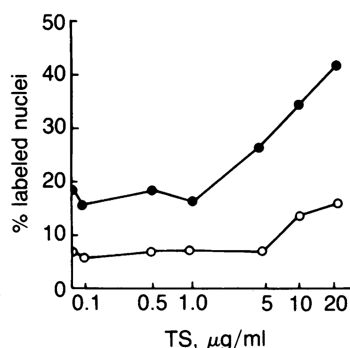

FIG. 3. Response of vascular SMC to increasing amounts of TS with or without added EGF. The mitogenic response to TS (0.1–20 μg/ml) was determined in the presence (●) or absence (○) of EGF at 5 ng/ml as described in the legend to Fig. 1. Note that TS alone has weak mitogenic activity at concentrations > 10 μg/ml. Mitogenesis in response to EGF is enhanced by concentrations of TS > 5 μg/ml.

may reflect its short half-life in the matrix (28, 30), the possible indirect nature of the effect, and possible differences between cell-derived and platelet TS.

Growth-Promoting Effects of TS Are Specific for EGF. The facilitative effect of TS on SMC growth was limited to EGF (Fig. 4). Other progression factors such as IGF-1 (5 ng/ml) or insulin (1 μg/ml) were not effective as SMC mitogens when given alone (Fig. 4 *Upper*) or in concert with TS (Fig. 4 *Lower*). One-way analysis of variance was used to compare the observed values with the values expected for additive effects. Only EGF and TS in combination stimulated mitogenesis to a level significantly greater than the calculated additive effect ($P < 0.005$) (Fig. 4 *Lower*). Therefore, TS was not able to confer "competence" on SMC as PDGF or fibroblast growth factor would be expected to do (26). In additional experiments not shown, TS alone did not stimulate SMC to enter G_1 as did PDGF. Thus, TS-treated SMC appear to respond to EGF by initiating cell cycle progression from a G_0 state.

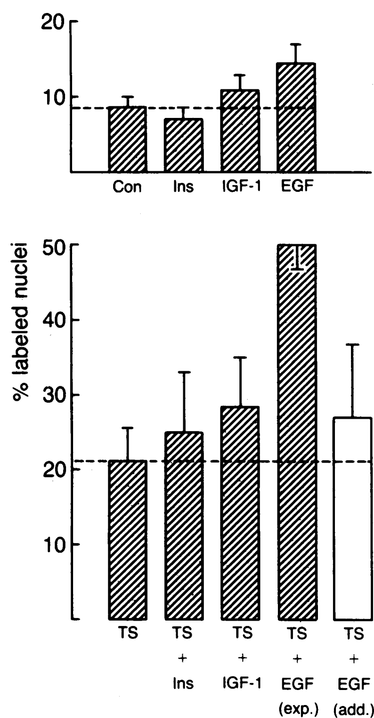

FIG. 4. Specificity of the synergistic effect of TS and EGF on SMC mitogenesis. The mitogenic response of SMC to insulin (Ins) at 1 μg/ml, IGF-1 at 5 ng/ml, and EGF at 5 ng/ml was determined in the presence (*Lower*) or absence (*Upper*) of TS at 20 μg/ml as described in the legend to Fig. 1. Note that SMC responded only weakly to IGF-1, insulin, or EGF when added alone (*Upper*). TS potentiated the mitogenic effect of EGF but not that of insulin or IGF-1 (*Lower*). The observed experimental value for the effect of TS and EGF together (exp.) is significantly greater ($P < 0.005$ by one-way analysis of variance) than all other values shown, including the calculated additive effect of TS and EGF (add.). ▨, Observed data ± SD; □, arithmetically derived data; ---, percentage of labeled nuclei in control cells (Con) (*Upper*) or in cells treated with TS alone (*Lower*).

Heparin Inhibits TS-Mediated Mitogenesis. Heparin (100 μg/ml), which inhibits the binding of TS to fibroblasts (30), to the SMC extracellular matrix (12), and to glycolipids in cell membranes (31), blocked the facilitative effects of TS while having a small but variable effect on PDGF-mediated mitogenesis and no effect on EGF-mediated DNA synthesis (Fig. 5). The slight mitogenic activity of TS alone (see Fig. 1) was essentially nullified by heparin in every experiment; the ability of heparin to inhibit the stimulation by TS and EGF together was consistent but showed more variability. We have described the effects of other glycosaminoglycans on TS–SMC interactions in a previous report (12).

Growth-Facilitative Activity Resides in the TS Molecule. A variety of experiments (listed below but not always shown) were performed to prove that the growth-promoting activity in our TS preparations was due to TS and not to small amounts of a platelet contaminant. Only EGF was effective in stimulating DNA synthesis in TS-treated SMC (Fig. 4); other progression factors were not active, helping to rule out the possibility of a contaminating, competence-inducing growth factor (e.g., PDGF or fibroblast growth factor). Heparin inhibited TS-mediated mitogenesis, while the mitogenic effects of other growth factors were unaffected (Fig. 5). The sensitivity of the growth-facilitative activity to heparin is consistent with known properties of the TS molecule—i.e., the presence of a heparin-binding domain in TS (23, 32, 33) and the ability of heparin to block the binding of TS to cell surfaces (30), to the SMC extracellular matrix (12), and to sulfated membrane glycolipids (31). Inhibition of growth factor binding by heparin has not been observed, to our knowledge, for any mitogen studied to date.

Antibodies against human PDGF, in quantities shown to inhibit SMC mitogenesis induced by PDGF at 5 ng/ml, did not affect the activity in our TS preparations. On the other hand, affinity-purified antibodies to human platelet TS, which showed no activity against PDGF in ELISA assays,

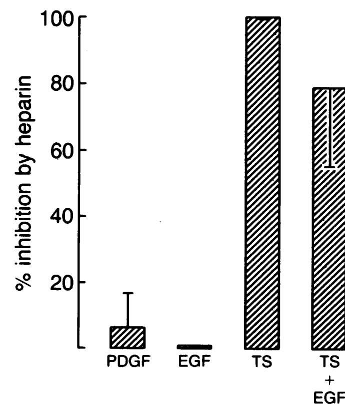

FIG. 5. Heparin-sensitivity of the SMC response to mitogens and TS. The mitogenic response of vascular SMC to PDGF (5 ng/ml), EGF (5 ng/ml), TS (20 μg/ml), or EGF and TS together was determined in the absence and presence of heparin at 100 μg/ml as described in the legend to Fig. 1. Note that PDGF- or EGF-mediated mitogenesis is not significantly inhibited by heparin, while mitogenic pathways involving TS show nearly complete inhibition by heparin.

reduced SMC mitogenesis (17, 34). Unlike PDGF-mediated mitogenesis, stimulation by EGF and TS together did not elicit morphological alterations in SMC, even at similar levels of DNA synthesis. Addition of TS to quiescent SMC, followed by metabolic labeling with [^{35}S]methionine, did not reveal any phenotypic alterations such as those seen when PDGF is added (12). Cross-incubation studies of labeled SMC proteins failed to detect the presence of protease activity in our TS preparations. Finally, the mitogenic activity was coeluted with TS from a Sepharose CL-4B column, and the growth-facilitative activity was not removed by exhaustive dialysis.

The growth-facilitative activity is unlikely to be due to contamination of our preparations with a known competence factor, as discussed above, or with transforming growth factor α or EGF, as evidenced by our dose–response data showing a lack of mitogenic response to EGF up to 100 ng/ml. Transforming growth factor β, another platelet mitogen, is growth-inhibitory to SMC in monolayer culture (58). Thus, the available data support our contention that the activity is present in the TS molecule itself. The data also support our supposition that TS must bind to the SMC extracellular matrix or cell membrane to express its growth-facilitative activity.

DISCUSSION

TS: A Mitogen-Inducible Matrix Glycoprotein with Autocrine Growth-Regulatory Properties. TS is an apparently homotrimeric glycoprotein of M_r 450,000 found in platelet α granules (35), synthesized by a variety of cells in culture (13, 36–40) and present in the extracellular matrix of cultured endothelial cells (13), fibroblasts (13, 39), and SMC (12, 13). TS binds to cell surfaces (30), presumably through an interaction with sulfated membrane glycolipids (31) and possibly glycoproteins, and interacts with components of the extracellular matrix such as fibronectin (41), heparin (33, 42), and collagen (43, 44). While a role for platelet TS in the later stages of platelet aggregation has been clearly established (45), the functional role of cell-derived matrix TS remains unclear. Current data suggest that TS is a modular glycoprotein capable of a variety of molecular interactions and, consequently, may be involved in a variety of seemingly unrelated functions (see ref. 17 for a recent review).

In previous work (12), we have shown that synthesis of TS by vascular SMC is induced in a rapid and transient fashion in response to PDGF. Increased synthesis of TS occurred within 1 hr after addition of PDGF, peaked at 2 hr, and returned to baseline levels by 5 hr. The induction was inhibited by actinomycin D, suggesting regulation at a transcriptional level. The production of TS was induced by PDGF, but not by other growth factors, in a dose-dependent fashion and at concentrations of PDGF suboptimal for mitogenesis. After synthesis, TS was rapidly secreted and incorporated into the SMC extracellular matrix. Thus, the elaboration of a TS-rich extracellular matrix may occur concomitantly with the entry of SMC into the cell cycle, supporting a previous suggestion (46, 47) that one function of mitogens may be to direct the production of a growth-supportive extracellular matrix, specific for a particular cell type. We hypothesized that a TS-rich extracellular matrix may be facilitative to, or required for, SMC growth (12), a concept supported by our antibody data showing inhibition of SMC migration and proliferation in the presence of monospecific anti-TS (17, 34).

In this paper we show that TS increases the sensitivity of SMC to EGF, another platelet mitogen (48). EGF is a potent mitogen for epidermal cells (49) and has been described as a progression factor for mesenchymal cells (26, 50). In our system, rat SMC respond only slightly to EGF alone. However, in the presence of TS, SMC respond vigorously to EGF as a primary mitogen. We hypothesize (Fig. 6) that the formation of a TS-rich extracellular matrix in response to PDGF may facilitate SMC growth in an autocrine fashion by (*i*) allowing or optimizing the SMC response to EGF as a progression factor or (*ii*) allowing neighboring, TS-sensitized cells to respond to EGF alone. Thus, according to this model, a single PDGF-mediated mitogenic signal may be amplified or prolonged in the absence of additional PDGF. Alternately, platelet-derived TS and EGF may combine to stimulate DNA synthesis in SMC without mitogenic involvement of PDGF. A TS–EGF mitogenic pathway may be especially relevant at low concentrations of PDGF, since induction of TS production by SMC occurs at concentrations of PDGF lower by a factor of 4 than that required for a maximal mitogenic response (12). The physiological relevance of these mitogenic pathways cannot be assessed at present because of the paucity of knowledge of the relative concentrations and functional roles played by various platelet components *in vivo*.

Role of TS in Heparin-Mediated Inhibition of SMC Growth. Heparin-like glycosaminoglycans are synthesized and secreted in the form of heparan sulfate proteoglycans by cultured vascular endothelial (9, 51, 52) and smooth muscle (10) cells. These molecules are believed to be normal components of the intact arterial wall (53) and, when provided exogenously to SMC *in vivo* or *in vitro*, can inhibit SMC replication (9, 14, 15, 54) and migration (16). The mechanism(s) underlying these inhibitory effects is not known but is likely to be extracellular, given the demonstrated affinity of heparin for a wide

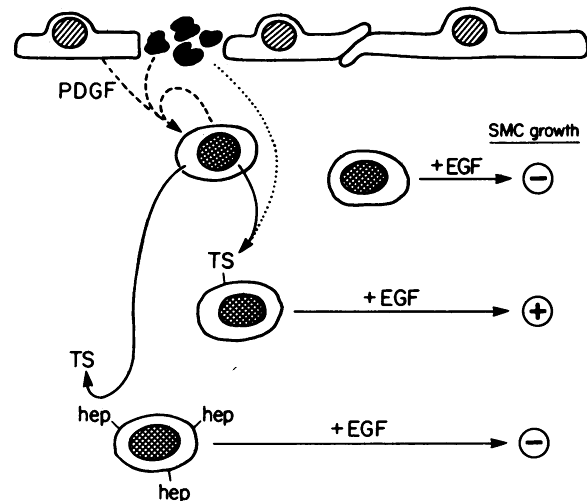

FIG. 6. A model for the role of TS in SMC mitogenesis *in vivo*. PDGF (dashed line) derived from platelets (solid), endothelium (hatched nuclei), or SMC (crosshatched nuclei) induces SMC to synthesize and secrete TS, a protein that is also released from platelet α granules (dotted line). The induction of TS by PDGF can occur at levels of PDGF that are less by a factor of 4 than that required for a full mitogenic response. TS can bind to neighboring SMC and "sensitize" them to the growth-promoting effect of EGF (middle arrow), a platelet factor that normally is a weak mitogen for SMC (top arrow). In the presence of endogenous heparin-like growth inhibitors (hep), TS may not interact appropriately with the SMC extracellular matrix or cell surface, and facilitation of growth will not occur (bottom arrow). Thus, significant SMC growth may occur without involvement of PDGF (utilizing platelet-derived TS and EGF) or at submitogenic levels of PDGF (utilizing SMC-derived TS). In addition, TS may allow or enhance the SMC response to EGF as a progression factor and may expand or prolong an initial PDGF-mediated event. Therefore, TS may act as an extracellular integrator of growth-stimulatory and -inhibitory signals. The relative strengths of these signals would determine, for any given vascular injury, the extent of the SMC proliferative response.

variety of matrix components and the small fraction of added heparin actually internalized by endocytosis during binding studies on cultured SMC (55).

We have shown (12) that heparin and related glycosaminoglycans block the incorporation of newly synthesized TS into the SMC extracellular matrix. This effect was observed at concentrations of heparin as low as 1.0 μg/ml. Heparin did not block the induction of synthesis of TS by PDGF but rather acted extracellularly to prevent the mitogen-induced expression of a TS-rich matrix. If such a matrix is facilitative to or required for cell growth, as suggested by our data, then such a mechanism (see Fig. 6) may account for the ability of heparin to block SMC proliferation. Wright *et al.* (56) have shown that EGF, but not PDGF or IGFs, was able to reverse the heparin-mediated inhibition of cervical epithelial cell growth. Thus, heparin may inhibit cellular proliferation through a mechanism involving EGF, consistent with the data presented in this study.

We propose that SMC growth is controlled, at least in part, by influences from the extracellular matrix. Heparin-like growth inhibitors, which themselves are matrix components, direct SMC to synthesize and secrete a pair of noncollagenous proteins with M_r values of 37,000 and 39,000 (57) as well as a unique short-chain (M_r 60,000) collagen (20). PDGF-treated SMC elaborate a TS-rich extracellular matrix whose production is inhibited extracellularly by heparin-like glycosaminoglycans (12). In this study we have shown that TS does, in fact, facilitate SMC growth along at least one unique, heparin-sensitive, mitogenic pathway. Thus, we have identified an autocrine growth-promoting mechanism whereby mitogen-stimulated SMC secrete TS, a matrix glycoprotein that, in turn, facilitates the cell's response to EGF or sensitizes neighboring cells to respond to EGF. A similar autocrine inhibitory mechanism may also be operative in SMC, involving cell-derived inhibitory heparan sulfate glycosaminoglycans (10) and heparan-mediated inhibition of a TS-rich matrix.

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