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Density-Dependent Endothelial Cell Production of an Inhibitor of Smooth Muscle Cell Growth

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Abstract Embryonic data and ultrastructural analyses suggest that the primitive endothelium signals undifferentiated mesenchymal cells to migrate to the forming blood vessel and subsequently regulates mural cell growth and behavior. Upon maturation of the blood vessel, chemotactic and mitogenic signals are apparently diminished and differentiated smooth muscle cells normally remain quiescent. This homeostasis is seemingly upset in conditions which lead to pathologies characterized by smooth muscle cell hyperplasia such as atherosclerosis. By culturing endothelial cells at different densities, we attempted to re-create the various stages of vascular development. Whereas media conditioned by sparse endothelial cells stimulate smooth muscle cells, media conditioned by dense endothelial cell cultures are inhibitory. Culture of sparse smooth muscle cells in media conditioned for 3 days by postconfluent endothelial cell cultures leads to dose-dependent and reversible smooth muscle cell inhibition. Furthermore, in the presence of the endothelial cell-derived inhibitor, smooth muscle cells are rendered refractory to mitogens such as fibroblast growth factor and platelet-derived growth factor. The inhibitory activity is not attributable to the well-characterized inhibitors of smooth muscle cell growth, transforming growth factor type-β, prostaglandin I_2, or heparan sulfate proteoglycan. Partial characterization of the inhibitory conditioned media suggests that the active molecule is smaller than 1,000 da, and stable to boiling as well as proteinase K and heparinase digestion. These findings support the concept that there is intercellular communication between endothelial cells and smooth muscle cells and provide evidence for a novel endothelial cell-derived smooth muscle cell growth inhibitor.

Key words: intercellular communication, vascular growth control, blood vessel development

Although a number of studies have described the cellular process leading to blood vessel formation, little is known about the molecular basis of these events. In spite of the fact that useful information has been obtained from in vivo models, the complexity of these systems often makes data interpretation difficult. Thus, we have used tissue culture cells in an attempt to reproduce various aspects of blood vessel growth in vitro. Since mature vessels consist primarily of two cell types, endothelial cells (EC) and mural cells, pericytes in the microvasculature and smooth muscle cells (SMC) in the macrovasculature, we have focused on understanding the role of heterotypic interactions (both physical and chemical) in vascular growth. We previously showed that pericytes and SMC suppress EC proliferation in a contact-dependent manner via the activation of transforming growth factor type-beta (TGF-β) [Antonelli-Orlidge et al., 1989]. We suspect that this interaction is reciprocal and that EC influence the growth of mural cells as well.

A role for interactions between EC and SMC in vascular growth control has been suggested by developmental studies. During vasculogenesis SMC are derived locally from undifferentiated mesenchymal cells [Ekblom et al., 1982] and it is hypothesized that the immature EC in growing vessels recruit SMC and influence their growth and behavior [Schwartz et al., 1990]. Morphometric analyses of mature blood vessels reveal that there is a reproducible and apparently well-controlled relationship between the number of EC and SMC in the vascular tree (personal communication, Dr. J. Folkman). Further evidence that EC are involved in the regulation of SMC growth comes from studies which utilize animal and organ culture models of neointima formation. In synthetic grafts placed into the aortoiliac circulation of baboons, the migra-

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tion and proliferation of SMC in the intima is temporally correlated with endothelialization [Clowes et al., 1986]. Since in this model the neointima forms in the absence of platelets, EC are the likely source of the SMC mitogens [Clowes et al., 1986].

Although it is well documented that EC produce a variety of diffusible factors known to influence SMC behavior [Castellot et al., 1981; Eldor et al., 1983; Furchgott and Zawadzki, 1980; Dodge et al., 1991; Bowen-Pope et al., 1989; Vlodavsky et al., 1987; D'Amore and Smith, *1993*], the signals that regulate the expression of these factors in EC and the precise role that EC-mural cell interactions play in vascular growth control remain elusive.

The proliferative state of vascular cells varies throughout the lifespan of a blood vessel. During vessel development, both EC and mural cells grow and proliferate, but once the blood vessel matures, these cells rarely divide [Engerman et al., 1967]. Since we hypothesize that EC are involved in the control of mural cell proliferation we have systematically investigated the effect of EC and their growth state on SMC proliferation. In this report we provide evidence for the density-dependent production of a novel SMC growth inhibitor by EC.

MATERIALS AND METHODS

Cell Culture

Endothelial cells. Bovine aortic endothelial cells (BAE) were isolated as previously described by scraping the intima of a bovine aorta with a scalpel blade and digesting the cell layer with 1% collagenase (171 U/mg) (Worthington Biochemical Co., Freehold, NJ)/1% BSA-Fraction V (Integren Co., Purchase, NY) for 15 min [Gimbrone, 1976]. After removing the collagenase, the cells were grown in Dulbecco's modified Eagle's media (DMEM) (JRH Biosciences, Lenexa, KS), 10% CS (JRH Biosciences), L-glutamine (29.2 mg/ml) pen-strep (10,000 U/ml) (Irvine Scientific, Santa Ana, CA) (DMEM/10% CS). The cells were identified as endothelial by staining with antisera to von Willebrand factor (Boehringer Mannheim, Indianapolis, IN) and by their uptake of acetylated low-density lipoprotein (Biomedical Technologies, Stoughton, MA) [Voyta et al., 1984]. To establish homogeneous populations of EC the cells were passaged by serial dilution. BAE were routinely used between passage 7 and 18.

Smooth muscle cells. SMC were explanted according to standard methods [Ross, 1971] and grown in DMEM/10% CS. The cells were identified as smooth muscle by staining with antisera against smooth muscle actin (provided by Dr. Ira Herman, Tufts University, Boston, MA) and by their lack of uptake of acetylated low density lipoprotein. SMC were used at passages 1–4. All cells were routinely demonstrated to be free of mycoplasma by staining with 4-6-diamidine-2-phenyl-dihydrochloride (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the protocol provided by the manufacturers.

Proliferation assays. SMC were plated into 24 well plates at 5,000 cells/cm^2 in 0.5 ml DMEM/10% CS and allowed to attach overnight. The next day the number of cells plated (T_0) was determined by counting the cells from triplicate wells. The growth media were replaced with CM at indicated concentrations or DMEM/10% CS, in the presence or absence of various neutralizing antibodies, including anti-TGF-β (R+D Systems, Minneapolis, MN) or test agents, including TGF-β (R+D Systems), platelet-derived growth factor-BB (PDGF-BB) (Creative Biomolecules, Hopkinton, MA) and basic fibroblast growth factor (bFGF) (Takeda Chemical Industries, LTD, Osaka, Japan). Three days later (unless otherwise stated), the cells of quadruplicate wells were trypsinized and counted electronically. We have previously determined that SMC in DMEM/10% CS undergo 3–4 population doublings in 3 days.

Preparation of BAE conditioned media (CM). To collect media conditioned by BAE that were either actively growing (subconfluent) or quiescent (postconfluent), cells were plated sparsely at 10,000 cells/cm^2/0.5 ml DMEM/10% CS. At 3-day intervals, the time required for BAE in DMEM/10% CS to undergo one population doubling, CM were collected, clarified by centrifugation, and stored at 4°C; the cells were refed with fresh growth media. Each time CM were collected the number of BAE on a parallel plate was determined electronically using a Coulter counter. Subconfluent densities of 4–8 × 10^4 cells/cm^2 were achieved between days 3 and 6, whereas confluent densities of 1–1.5 × 10^5/cm^2 were obtained after 9–12 days in culture. The number of BAE remained constant during the generation of postconfluent CM, which were collected at 3-day intervals between 12 and 28 days after the cells were plated. Unless other-

wise stated the CM were assayed at 50%, diluted with fresh DMEM/10% CS.

Characterization of the inhibitory activity. To elucidate the nature of the inhibitory activity and to determine if the inhibitor was any of the well-known SMC inhibitors such as TGF-β, heparan sulfate, or prostaglandins, CM were subjected to the treatments described below. Following treatment, the CM and appropriate controls were assayed for their effects on SMC proliferation.

Heparinase sensitivity. To determine whether the EC-derived inhibitor was heparin, inhibitory CM, control DMEM/10% CS, and DMEM/10% CS containing 100 μg/ml heparin (152 U/mg) (Industries, Inc., Franklin, OH) were incubated with 4.5 μg heparinase (provided by Dr. Robert Langer, Massachusetts Institute of Technology, Cambridge, MA) for 1.5 hr at 37°C. Heparinase selectively cleaves glycosidic linkages between N-sulfated hexosamine and iduronic acid 2-0-sulfate [Linker and Hovingh, 1984]. Both heparin and heparan sulfate are susceptible to heparinase treatment [Poole, 1986]. In addition, we took advantage of the fact that, due to their sulfation, heparin and heparan sulfate bind to protamine. Postconfluent CM, DMEM/10% CS, and DMEM containing 100 μg/ml heparin were incubated with protamine linked to agarose beads or control agarose beads (Sigma Chemical Co., St. Louis, MO) with shaking overnight at 4°C. Beads were removed by centrifugation and media assayed for their effect on SMC proliferation. Similar experiments were performed with an anion exchanger, DEAE Sephadex (Pharmacia, Piscataway, NJ).

Protease sensitivity. To determine whether the inhibitor was a protein inhibitory CM, control DMEM/10% CS, and DMEM/10% CS containing 5.0 ng/ml PDGF-BB were incubated with proteinase K (1.5–5 U) immobilized to agarose beads (0.1 g) (Sigma Chemical Co., St. Louis, MO) or the same volume of control agarose beads (Sigma Chemical Co.), shaking at 37°C for 1.5 hr. PDGF-BB is a polypeptide growth factor known to stimulate SMC proliferation, and was included in these experiments to ensure the effectiveness of the proteinase K treatment. Following removal of the beads, the media were assayed for their effect on SMC proliferation.

Heparin affinity. To assess heparin affinity of the inhibitors, CM and control DMEM/10% CS were incubated overnight at 4°C with heparin–Sepharose beads (Sigma Chemical Co.) equilibrated with 10 mM Tris M/0.6 NaCl. The nonbinding flowthrough was tested in SMC proliferation assays.

Estimation of molecular weight. During the course of the characterization, dialysis of the inhibitory CM in tubing with a molecular weight cutoff of 10,000 revealed that the inhibitory component of the postconfluent EC-CM was smaller than 10,000 da. To obtain a crude estimate of the inhibitor's molecular weight subsequent experiments were performed in which CM were concentrated using a centrifugal microconcentrator (Filtron Tech. Corp., Northboro, MA) with a molecular weight cutoff of 1,000. Since the filtrate no longer contained calf serum components greater than 1,000 da (which presumably includes most growth stimulators) the filtrate was supplemented with calf serum to a final concentration of 10% prior to assay. The retentate, which contained serum components greater than 1,000 da, was returned to the original volume with serum free-DMEM.

Prostaglandin extraction. A number of prostaglandins are known to act as inhibitors of SMC proliferation. Therefore, prostaglandins were extracted from inhibitory CM using Sep-Pak C_{18} cartridges (Waters Associates, Millford, MA) [Nigam, 1987]. Briefly, CM, control DMEM, and DMEM with 20 uM prostaglandin E_2 (Sigma Chemical Co.) were acidified to pH 3.0, ethanol added to a final concentration of 15% and applied to columns. Following washes with 15% aqueous ethanol and petroleum ether, the prostaglandins were eluted with ethyl acetate. The organic solvent was evaporated under nitrogen, the residue reconstituted in ethanol for a final concentration of 0.01% in DMEM/10% CS and assayed for its effect on SMC growth.

RESULTS

Effect of Media Conditioned by BAE on SMC Proliferation

To investigate the density-dependent effects of EC on SMC, media conditioned for 72 hr by subconfluent, confluent, or postconfluent cultures of BAE were assayed for their effects on growing SMC. CM collected from actively growing (subconfluent) BAE stimulated SMC growth by 38% over DMEM/10% CS controls during 3 days (Fig. 1). In contrast, media conditioned by confluent BAE had no significant effect on SMC and CM from postconfluent BAE inhibited the

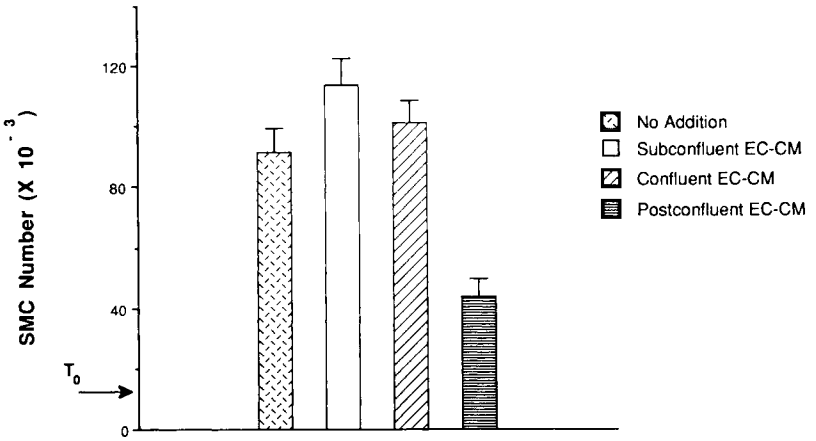

Fig. 1. Effects of EC-CM on SMC proliferation: Dependence on EC growth state. Media were conditioned for 3 days by sparse and dense EC and assayed at 1:1 with DMEM/10% CS for their effects on SMC growth. At day 3 SMC numbers were determined electronically and compared to those numbers of SMC grown in DMEM/10% CS. Results are means of quadruplicate samples ±SD, $P = 0.01$.

growth of SMC by approximately 50% (Fig. 1). Control SMC underwent three population doublings whereas SMC cultured in the presence of postconfluent CM underwent less than one doubling. The effects of confluent CM on SMC growth varied among experiments, from having no significant effect to a slight but significant inhibitory effect. This variability is most likely attributable to the variation in the degree of confluence attained by the cells after 9 days in culture. On the other hand, media conditioned by postconfluent BAE reproducibly inhibited SMC proliferation by 50–60%.

The CM collected from BAE at different densities also had different effects on SMC morphology. SMC grown in media conditioned by sparse BAE were spindle-shaped and formed multilayers (Fig. 2b) like control SMC (Fig. 2a). In contrast, inhibitory CM from postconfluent BAE induced a dramatic change in SMC morphology; the growth-inhibited SMC were retracted, had elongated cell bodies and grew as a single layer (Fig. 2c).

EC synthesize several polypeptide growth factors which likely function in a paracrine manner to regulate SMC behavior, including bFGF and PDGF-BB. Using neutralizing antisera against bFGF or PDGF BB, we blocked a significant portion of stimulatory activity in the sparse CM (data not shown). We are currently investigating the nature of the remaining stimulatory activity.

Postconfluent BAE-CM prepared in the absence of bovine calf serum lacked significant inhibitory activity (Fig. 3). However, media conditioned in the presence of serum as low as 1% contained inhibitory activity that suppressed SMC growth to the same extent as media conditioned in the presence of 10% CS.

To ensure that the growth-inhibitory effect of media conditioned by postconfluent BAE was not due to toxicity, the reversibility of the inhibition was examined. SMC were grown for 3 days in postconfluent inhibitory CM and then refed with DMEM/10% CS, while other cultures were refed with the inhibitory CM. Following the removal of the inhibitory CM, SMC resumed growth at a rate similar to that of untreated control SMC. SMC that remained in the presence of postconfluent CM were completely growth-arrested (Fig. 4). The morphological changes induced by the postconfluent CM were also reversed within 2–3 days following replacement of the inhibitory CM (Fig. 2d).

We suspected that density-dependent shifts between the balance of EC-derived growth stimulators and growth inhibitors might account for the differential effects of the subconfluent and postconfluent CM on SMC growth. To address this possibility, various concentrations of media conditioned by subconfluent and postconfluent BAE were examined for their effects on SMC. Postconfluent CM at 50% significantly inhibited SMC proliferation (Figs. 1 and 5), whereas lower concentrations of the same CM stimulated or had no effect on SMC proliferation (Fig. 5). Media from subconfluent EC was stimulatory when assayed at 50% or at lower concentrations of 12.5 and 6%. Surprisingly, the same media assayed at 25% was inhibitory. This finding was

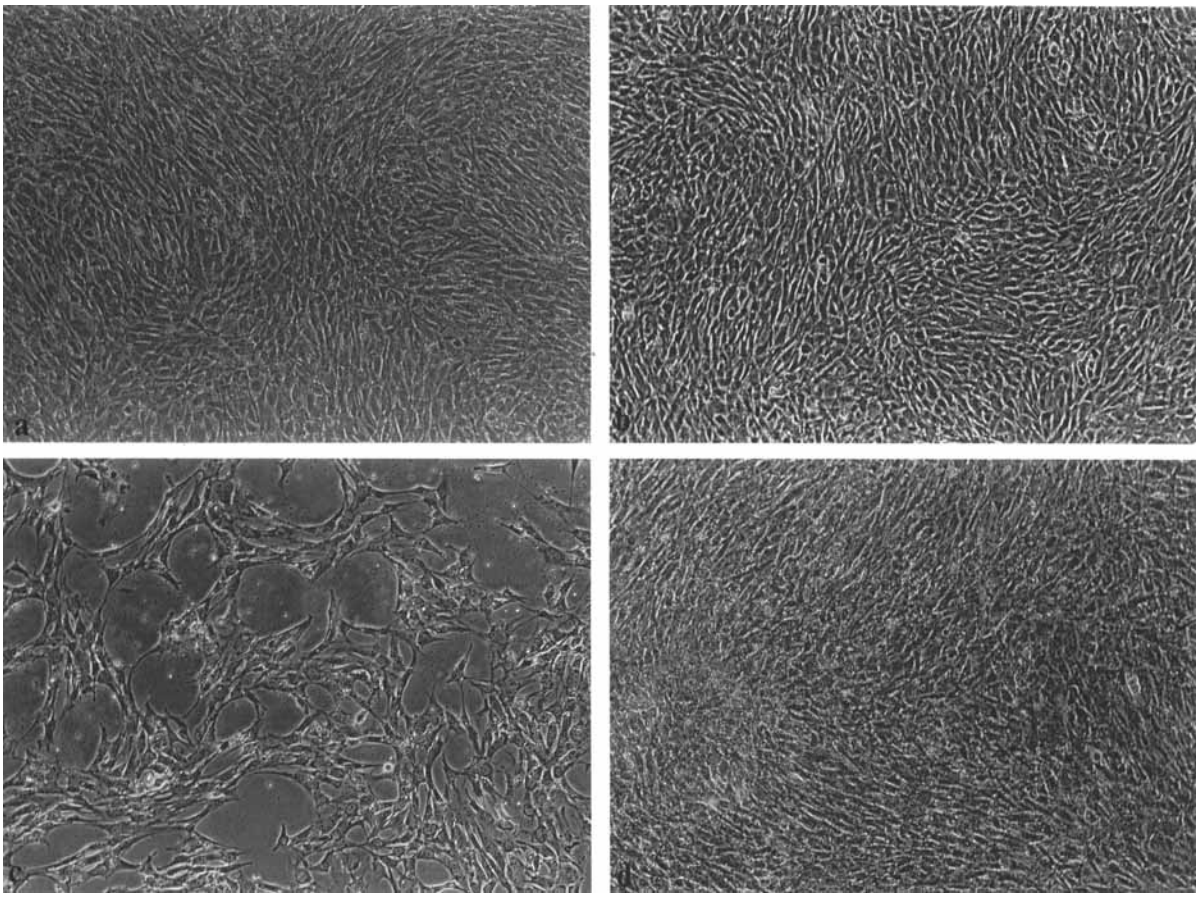

Fig. 2. Effects of EC-CM on SMC morphology: dependence on EC growth state. SMC were cultured for 3 days in **(a)** DMEM/10% CS, **(b)** media conditioned by sparse EC (stimulatory media) at 50%, or **(c)** media conditioned by dense EC (inhibitory media) at 50%. Cell densities under each condition were (a) 89,100/cm^2 ± 7,944, (b) 98,280/cm^2 ± 10,488, (c) 16,565 ± 1063. Three days following the removal of the inhibitory CM the shape change inhibitors were reversible **(d)**.

reproducible though the extent of the inhibition at 25% CM varied.

The inhibitor is not TGF-β. To determine whether the inhibitory activity generated by postconfluent BAE was due to TGF-β1 or TGF-β2, well-known SMC growth inhibitors [Assoian and Sporn, 1986], inhibitory CM were assayed for their effects on SMC in the presence of specific neutralizing antisera. The addition of the neutralizing antibodies had no effect on the CM-induced SMC growth inhibition; the antibodies effectively blocked the inhibitory effects of exogenously added human platelet TGF-β1 (Fig. 6) and TGF-β2, (data not shown). Furthermore, the inhibition of SMC proliferation by exogenously added TGF-β1 and TGF-β2 was accompanied by a change in SMC morphology that was different from that induced by postconfluent CM. Whereas the CM induced SMC elongation and cell body retraction (Fig. 2C), TGF-β1 and TGF-β2 promoted spreading of the SMC.

These results, which indicate that the inhibitor is not TGF-β, were further supported by assaying the postconfluent CM on mink lung epithelial cells (MLE); the postconfluent BAE-CM inhibited DNA synthesis in MLE, but this inhibition was not blocked by addition of neutralizing antisera against TGF-β1 or TGF-β2 (data not shown).

The inhibitor is not a heparin-like molecule. Heparan sulfate, another known inhibitor of SMC proliferation, has been reported to be secreted by EC in a density-dependent manner [Castellot et al., 1981]. Results from experiments in which media conditioned by postconfluent BAE were treated with heparinase suggest that the inhibitory component of the CM is not heparin or heparan sulfate (Fig. 7). Heparinase

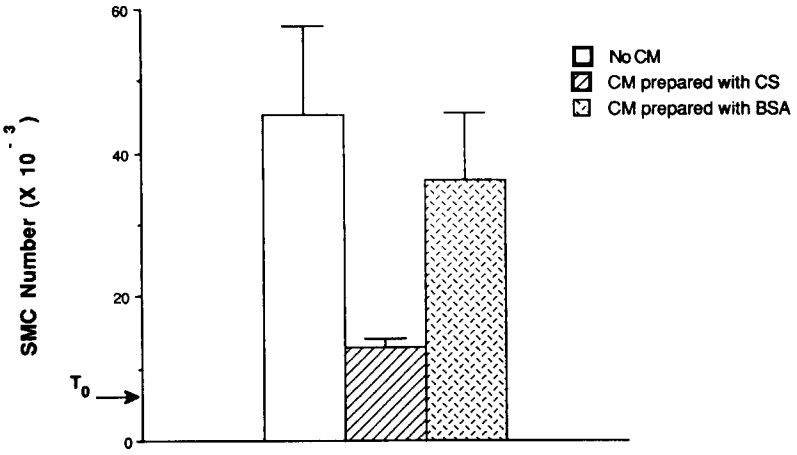

Fig. 3. Serum requirement for EC-derived SMC growth inhibitor. Parallel plates of postconfluent EC were used to prepare CM and serum-free CM. BSA (1%) was added to the serum-free DMEM. Following collection of CM calf serum was added to a final concentration of 10%. Each batch of CM was assayed at 1:1 with DMEM/10% CS for its effects on SMC proliferation. Results are means of quadruplicate samples ±SD.

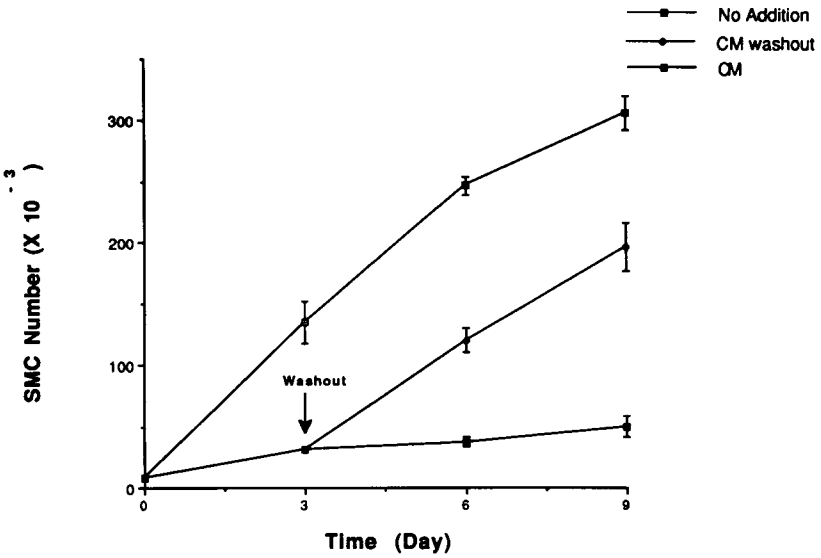

Fig. 4. Reversibility of CM-induced SMC growth inhibition. SMC were grown for 3 days in inhibitory CM and then refed with DMEM/10% CS or again with inhibitory CM. Media were replenished again at day 6. SMC numbers were determined at days 6 and 9. Results are means of quadruplicate samples ±SD.

treatment eliminated the SMC growth inhibition induced by 100 μg/ml of exogenously added heparin but failed to abrogate the inhibitory activity of the postconfluent CM. In fact, CM treated with heparinase was 85% more inhibitory than untreated CM. We suspect that the BAE-CM contains stimulatory factors such as bFGF or heparin binding epidermal growth factor (HB-EGF) which require heparin-like molecules for their activity. As further evidence that the inhibitor is not heparin, the activity was not removed from the CM by incubation with protamine-agarose or with the anion exchanger DEAE-Sepahadex (Table I).

Partial characterization of inhibitor. After observing that the inhibitory activity was lost during dialysis, inhibitory CM were separated using a centrifugal microconcentrator with a molecular weight cut-off of 1,000 and the filtrate and retentate assayed for their effects on growing SMC. Whereas the retentate (MW > 1000) either stimulated or had no effect on SMC proliferation, the filtrate (MW < 1000) reproducibly inhibited SMC growth by at least 50% (Table II).

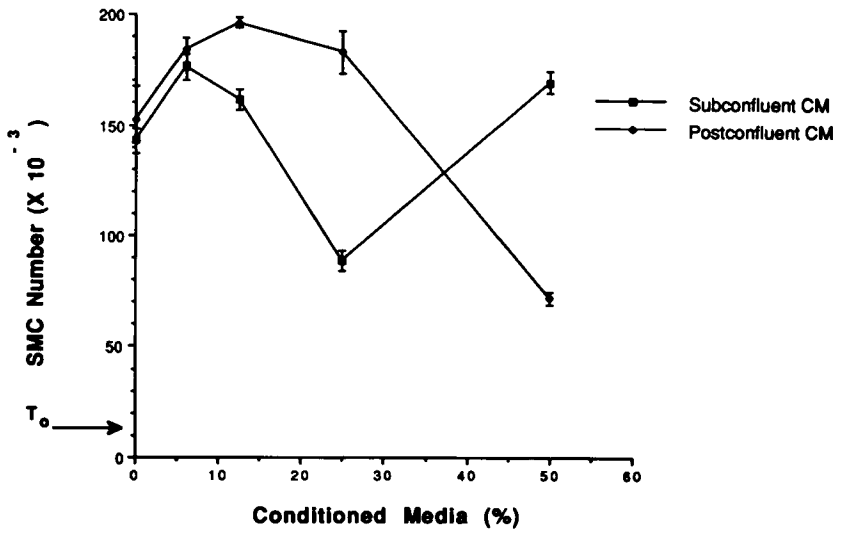

Fig. 5. Dose response of subconfluent and postconfluent EC-CM on SMC proliferation. Media conditioned by sparse and dense EC cultures were assayed at 50, 25, 12.5, and 6% for their effects on the growth of sparse SMC. Results are means of quadruplicate samples ±SD, $P < 0.01$.

The addition of the filtrate to SMC promoted a change in morphology similar to the unfractionated CM. Stimulation of SMC proliferation by the retentate, supports the idea that there is indeed a balance between the levels of growth stimulators and growth inhibitors produced by BAE. Inhibitor(s) predominate in postconfluent BAE-CM and could do so by overcoming the stimulatory activity and/or by rendering the cells refractory to the EC-derived growth stimulators. To examine these possibilities inhibitory CM were assayed in the presence of exogenous bFGF and PDGF. Consistent with the latter speculation, the inhibitor generated by the postconfluent BAE rendered SMC refractory to addition of these well-known SMC mitogens assayed at optimal concentrations (Fig. 8a,b).

Since several prostaglandins are potent inhibitors of SMC proliferation we extracted prostaglandins from the inhibitory CM and assayed the media on growing SMC. Removal of prostaglandins from the inhibitory CM did not eliminate the suppression of SMC growth.

Preliminary biochemical characterization of the postconfluent CM revealed that the inhibitory activity is not sensitive to freeze-thawing, heating at 56°C for 1 hr or boiling for 10 min (Table I). Treatment of the inhibitory CM with proteinase K did not abrogate its inhibitory effects on SMC growth. In addition, the inhibitor has no affinity for heparin and does not bind to cation or anion exchangers (Table I).

DISCUSSION

We have demonstrated that EC in vitro produce both stimulators and inhibitors of SMC proliferation. The effects are dependent on the density and/or growth state of EC and are mediated by diffusible factors. Contact between EC and SMC is not necessary to observe either the stimulatory or inhibitory effects, though a greater degree of stimulation was observed when the cells were cocultured with contact. SMC growth stimulators are predominant in CM obtained from sparse, growing EC, whereas media conditioned by quiescent, contact-inhibited EC suppress SMC proliferation. As EC approach confluence there is a period when stimulators and inhibitors appear to balance one another; CM from cells at this stage have no effect on the growth of SMC. The comparison of the effects of media conditioned by sparse and postconfluent EC provide further support for the idea that these CM contain both stimulators and inhibitors. Media that are strongly inhibitory for SMC at 50% show mild stimulation at lower concentrations. The lack of inhibition at the lower CM concentrations may be due to levels of inhibitor that are insufficient to block the effect of growth stimulators. Similarly, CM stimulatory at 50% was inhibitory when assayed at 25% (but not at lower concentrations), again indicating a fine balance between positive and negative growth regulators.

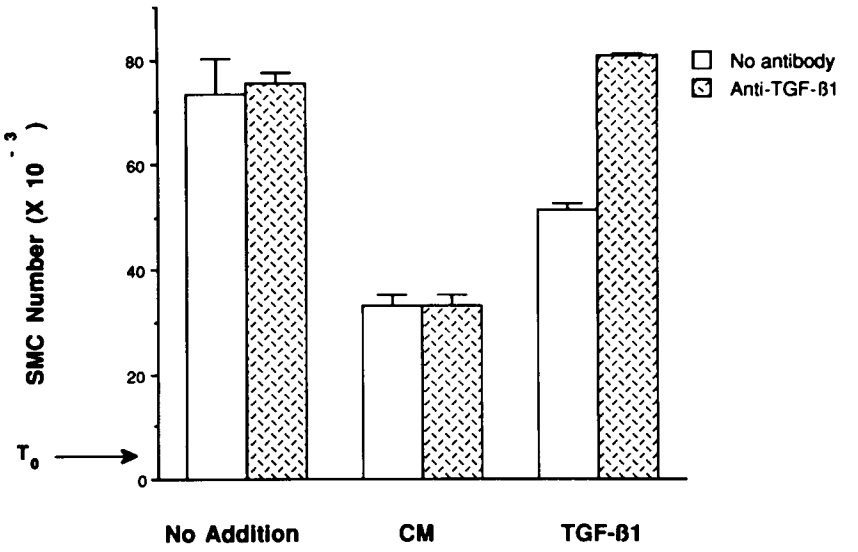

Fig. 6. The effect of anti-TGF-β1 IgG on CM-induced SMC growth inhibition. Inhibitory CM were assayed in the presence of 100 μg/ml neutralizing antisera against TGF-β1, a concentration sufficient to neutralize 1.0 ng/ml TGF-β1. SMC numbers were determined at day 3 and compared to SMC incubated in inhibitory CM. Results are means of quadruplicate samples ±SD.

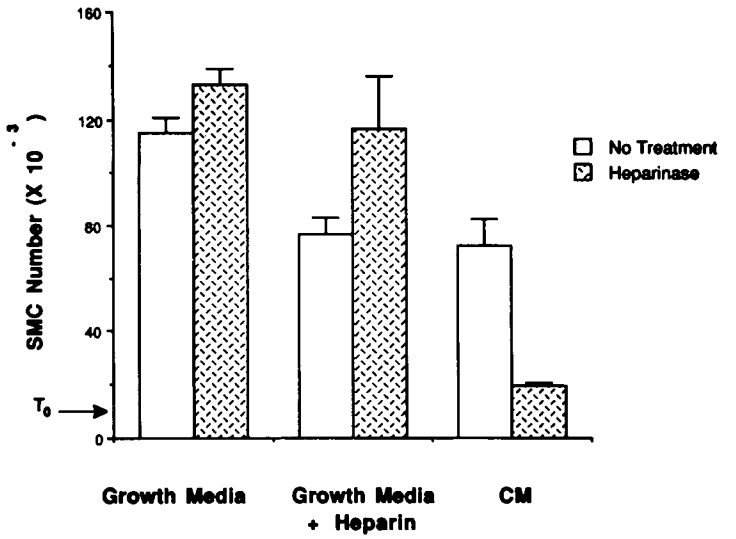

Fig. 7. Effect of heparinase on CM-induced SMC growth inhibition. Inhibitory CM were incubated with heparinase overnight at 4°C, a treatment sufficient to destroy the inhibitory activity of 100 μg/ml heparin. The CM were assayed at 50% for its effects on SMC proliferation. Results are means of quadruplicate samples ±SD.

Our tissue culture observations are consistent with the effect of EC on SMC proliferation in several different in vivo assays where SMC growth appears to be a function of the growth state of the EC. Using a porcine organ culture model of neointimal growth, SMC proliferation was observed to peak at the time when EC turnover was greatest [Koo and Gotlieb, 1991]. In this study the cessation of SMC growth also coincides with EC quiescence. Intravenous administration of acidic FGF, a known vascular mitogen, to rats following carotid artery injury led to rapid re-endothelialization and a dose-dependent reduction in intimal thickening [Bjornsson et al., 1991]. On the other hand, perivascular application of bFGF to the media of uninjured rat carotid arteries does not lead to an increase in SMC proliferation [Edelman et al.,

TABLE I. Characterization of EC-Derived SMC Growth Inhibitor

Molecular weight	<1,000 Daltons
Heat Treatment	
56°C, 60 min	Stable
Boiling, 10 min	Stable
Heparin affinity	None
Protamine affinity	None
Charge[a]	None
Heparinase sensitivity	None
Proteinase K sensitivity	None

[a]The inhibitory activity did not bind DEAE Sephadex or Biorex beads.

TABLE II. Molecular Weight Estimate of EC-Derived SMC Growth Inhibitor

	SMC Number	
Media	Retentate	Filtrate
Nonconditioned	64,655 ± 8,932	62,628 ± 4,655
Postconfluent conditioned	81,493 ± 8,764	19,008 ± 2.102

Media were conditioned for 3 days by postconfluent EC and fractionated using a centrifugal microconcentrator with a molecular weight cutoff of 1,000 da. The filtrate was reconstituted to 10% CS and the filtrate and retentate were assayed as described in Materials and Methods. The results are the means of quadruplicate samples ±SD.

1992]. Identical treatment of carotid arteries in which the EC have been injured via balloon catheter results in SMC proliferation and enhanced neointimal thickening [Edelman et al., 1992]. Our findings, which indicate that quiescent EC release an inhibitor which renders SMC refractory to mitogens, provide a possible explanation for the observed phenomenon.

Previous studies have shown that EC produce other factors which effect SMC behavior and that their production depends on EC culture conditions. In comparison to confluent quiescent EC, subconfluent EC have been reported to have increased expression of c-sis mRNA [Jaye et al., 1985] and to release more bFGF to the media [Healy and Herman, 1992]. Consistent with these observations, we find that a portion of the stimulatory activity present in sparse EC-CM is blocked with neutralizing antisera against PDGF-BB and bFGF. These findings agree well with results of in vivo studies in which nearly half of the mitogenic activity in baboon graft perfusates was neutralized using antibodies against PDGF, with bFGF theorized to be responsible for the remaining activity [Golden et al., 1991]. The fact that we are unable to account for all of the mitogenic activity suggests that other factors are involved. Two likely candidates are connective tissue growth factor (CTGF), a 38-kD cysteine-rich secreted protein that is immunologically related to PDGF [Bradham et al., 1991] and HB-EGF which has 40% homology with EGF, but possesses an N-terminal extension thought to confer heparin affinity [Higashiyama et al., 1991]. Both CTGF and HB-EGF, mitogens and chemoattractants for mesenchymal cells, are known to be made by EC [Bradham et al., 1991, Yoshizumi et al., 1992]. Since neutralizing antibodies against these mitogens are not available, we are unable to assess their contribution to the EC-derived stimulatory activity.

In contrast to the stimulators released by dividing BAE, media conditioned by quiescent BAE were inhibitory. Reversibility studies indicate that the inhibitory activity was not the result of toxicity. Furthermore, since the CM were assayed at 1:1 with DMEM 10% CS, the growth suppression is not due to the depletion of serum products. BAE have previously been shown to synthesize two known SMC inhibitors, PGI_2 and heparan sulfate, in a density-dependent manner [Castellot et al., 1981, Eldor et al., 1983]. Incubation of the EC-CM with heparinase, protamine-agarose beads or DEAE-Sephadex failed to eliminate the CM's inhibitory activity, indicating that the inhibition is not due to heparin-like molecules. Extraction of prostaglandins from EC-CM with a Sep-Pak C_{18} cartridge, reveals that the activity is not attributable to PGI_2 or other inhibitory prostaglandins including PGE_2, PGA, and PGJ [Sasaguri et al., 1992]. Another well-characterized inhibitor of SMC growth is TGF-β [Assoian and Sporn, 1986]. The addition of neutralizing antibodies against TGF-β1 or TGF-β2 to the EC-CM did not block the inhibitory activity. Though we have previously reported that the conversion of latent TGF-β to a biologically active form does occur in EC-SMC cocultures, the activation requires direct heterotypic contacts and the solo cultures release predominantly latent TGF-β [Antonelli-Orlidge et al., 1989]. Therefore, it is not surprising that the inhibitory activity in CM derived from solo cultures of EC is not due to activated TGF-β.

Partial characterization of the inhibitory CM indicates that the active molecule is less than 1,000 da. The EC-derived inhibitor is stable to

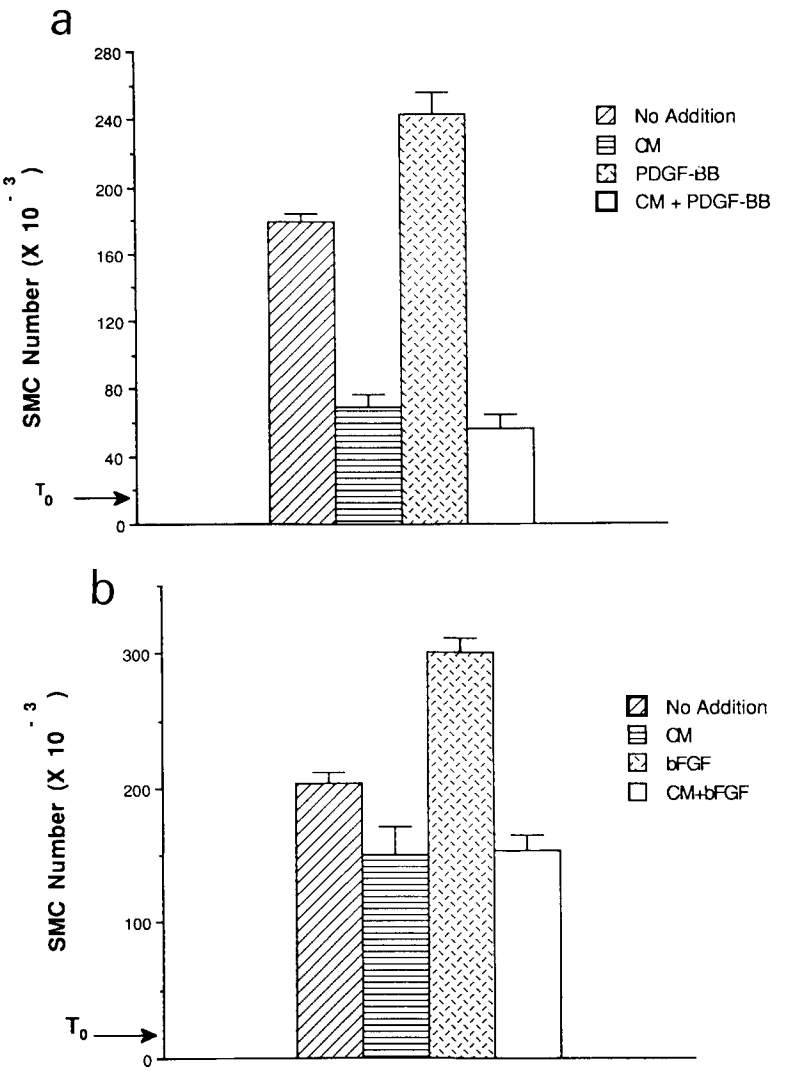

Fig. 8. Effect of CM on SMC growth response to PDGF-BB and bFGF. The mitogens (a) PDGF-BB (10.0 ng/ml) or (b) bFGF (5.0 ng/ml) were added to SMC along with inhibitory CM. SMC numbers were determined 3 days later and compared to the effects of the mitogens in the absence of inhibitory CM. Results are means of quadruplicate samples ±SD.

boiling, freeze-thawing, and proteinase K treatment. The inhibitor does not bind heparin and does not appear to be charged as it has no affinity for anion or cation exchangers. Generation of the inhibitory activity requires serum. Although the serum requirement does not appear to be dose-dependent our assay may not be sensitive enough to detect differences. These findings suggest that the EC-derived SMC growth inhibitor is a novel molecule. Cultured pulmonary artery EC have been reported to release a low molecular weight inhibitor (<10,000 da) that suppresses the growth of pulmonary artery SMC, but has no effect on the growth of aortic SMC [Hassoun et al., 1989]. Aortic SMC growth inhibitory activity derived from media conditioned by cultured BAE has been reported previously [Xu et al., 1991], although few data are available on its characteristics.

In summary we have demonstrated density-dependent effects of EC on SMC proliferation in vitro. We have identified and partially characterized a novel, low molecular weight, EC-derived SMC growth inhibitor. These findings have important implications for understanding interactions between EC and mural cells during development, as well as in the processes leading to vessel pathology.

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