Effects of Porcine Aortic Smooth Muscle Cell Conditioned Medium on Endothelial Cell Replication

Gayle A. Hoover, Suzanne McCormick, and Norman Kalant

Previous studies suggested that arterial smooth muscle cells (SMC) may be involved in regulating the growth of capillaries into atherosclerotic plaques. In the present study, we determined the effect of SMC products on porcine aortic endothelial cell (EC) replication in vitro. Quiescent or slowly growing EC in medium without endothelial cell growth factor (ECGF) were stimulated to proliferate in the presence of porcine aortic SMC conditioned medium, while the same conditioned medium inhibited the growth of rapidly dividing EC in high serum concentrations or with ECGF. The magnitude of both activities depended on SMC conditioned medium concentration. The dose-dependent increase in EC number stimulated by ECGF was completely inhibited by SMC conditioned medium. This effect was not due to a direct interaction of conditioned medium with ECGF because SMC conditioned medium inhibited the growth of EC that were rapidly proliferating in 10% serum without ECGF. The inhibitory activity was retained by an ultraviolet membrane with an exclusion limit of 1000 daltons; the stimulatory activity was recovered in the ultraviolet and remained stable after boiling, treatment with acid or base and trypsin, and repeated freezing and thawing, but was removed by activated charcoal. The growth-promoting activity could not be accounted for by release of cell contents from lysed cells or of thymidine into the medium. Conditioned medium from SMC incubated in the presence of serum contained less EC growth-stimulatory activity but more growth-inhibitory activity than that from SMC in serum-free medium.

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There is increasing attention to the presence and mechanisms of cell-cell interactions observed in tissue culture and their potential role in vivo. In the vascular wall, the two main cell types are the endothelial cells (EC) and the smooth muscle cells (SMC); their interactions, direct and indirect, may be significant in the maintenance of the normal equilibrium of the cell populations and in the changes associated with atherogenesis. Several effects of EC on SMC have been documented: EC in culture release into the medium a polypeptide that is an SMC growth factor;1 they oxidize low density lipoproteins (LDL), rendering them less susceptible to uptake by the LDL receptor pathway of SMC but more susceptible to uptake by macrophages;2,3 and they produce a factor that stimulates production of glycosaminoglycans by SMC.4 There is less information on the effect of SMC on EC. Eisenstein et al 5 found that normal aorta contains an EC growth inhibitor, and they subsequently demonstrated6 that such an inhibitor is produced by SMC in culture. They noted that SMC conditioned medium also had EC growth-stimulatory activity but did not characterize it beyond establishing its heat lability. The purpose of the present work was to obtain more information about this EC growth stimulator.

Methods

Cell Culture

EC were obtained from descending thoracic aortas of young pigs by collagenase digestion techniques adapted from the procedure described by Gimbrone et al.7 They were grown in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, Mississauga, Ontario) supplemented with 1 mM nonessential amino acids (GIBCO Canada, Burlington, Ontario), sodium bicarbonate (2.25 g/l), and 10% (vol/vol) defined calf bovine serum (supplemented) (Hyclone Laboratories, Logan, UT). After the first 24 hours in primary culture, the cells received endothelial cell growth factor (ECGF) in the form of a crude extract of bovine brain8 at 75 to 150 µg protein/ml medium, depending on the maximum activity in a given batch of ECGF.

SMC were derived from explants9 of the middle media layers of descending thoracic aortas of young pigs. They were grown in DMEM supplemented as for EC except that 10% (vol/vol) fetal bovine serum (FBS) replaced the supplemented calf serum (SCS). Both sera were heat-inactivated for 30 minutes at 56°C before use in cell culture.

Stock cultures of both cell types were grown in 100-mm tissue culture dishes precoated with human plasma fibro-

From the lady Davis Institute for Medical Research of the Sir Mortimer B. Davis-Jewish General Hospital, Montreal, Quebec, Canada.

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Address for reprints: Norman Kalant, M.D., Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital, 3755 Cote Saint-Catherine Road, Montreal, Quebec, Canada H3T 1E2.

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nectin® and maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. They were passaged at confluence, once weekly, by treatment with 0.05% trypsin-0.02% disodium EDTA in Earle's balanced salt solution (GIBCO), followed by a wash in serum-containing medium. They were redistributed at a 1:20 (EC) or 1:10 (SMC) split ratio and given fresh medium every 2 days.

**Preparation of Conditioned Medium**

Confluent stock cultures of SMC were washed three times with Dulbecco's phosphate-buffered saline (DPBS, GIBCO) and given 6 ml of serum-free DMEM. After a 24-hour incubation, the conditioned medium was collected under sterile conditions and was filtered through a 0.45-μm membrane filter to remove cell debris before being assayed or subjected to further treatment as described below. The SMC were released with trypsin:EDTA and counted in a hemocytometer to determine their density for each batch of conditioned medium. The average cell number/confluent 100-mm culture dish was 2.6 x 10⁴ (range 1.2 to 4.4 x 10⁴). Within this density range, there was no significant correlation between SMC density and activity in conditioned medium. Serum-free DMEM incubated in parallel cell-free dishes and treated in an identical way to SMC conditioned medium served as control, cell-free (GF) conditioned medium in every assay.

**Endothelial Cell Growth Assay**

EC, which were released with trypsin:EDTA from confluent stock cultures, were seeded at 2 x 10⁴ cells/35-mm tissue culture dish precoated with human plasma fibronectin (5 μg/cm²) to increase plating efficiency. The effects of conditioned medium were the same whether or not fibronectin was used. The cells were seeded in DMEM containing SCS at a concentration necessary to maintain the cells in a quiescent state for the duration of the assay. For most EC strains, this was 2.5% (vol/vol) SCS, but for some, 1% was sufficient, and in one case, levels of up to 10% had no significant effect on cell number. After 24 hours (day 1), cell counts were determined in three dishes, and the remainder were refed 2 ml fresh medium with appropriate supplements of SCS, ECGF, and conditioned medium. Generally, the conditioned medium was assayed at a level of 50% (vol/vol) of the total medium. The cells were given fresh medium every second day until the end of the experiment. At the specified times, cells were released with trypsin:EDTA and counted in a hemocytometer. Assays were done in triplicate and each experiment was repeated two or more times; the data shown are representative of these.

**Characterization of Conditioned Medium**

Large pools of filtered SMC and CF conditioned media were each divided into equal aliquots for various treatments. Conditioned medium was dialyzed in Spectra/por membrane tubing (nominal molecular weight cut-off, 3500; Fisher Scientific, Montreal, Quebec) for 24 hours against either an equal volume or a 35 x volume of fresh DMEM. After determining that the EC growth-promoting substance in SMC conditioned medium was dialyzable and the inhibitory factor was retained by the dialysis tubing, we subsequently separated this activity by filtering conditioned medium through a Dialo YM2 ultrafiltration membrane (nominal molecular weight cut-off, 1000; Amicon Canada Limited, Oakville, Ontario) in an Amicon model 52 ultrafiltration cell with stirring at 4°C, under an N₂ pressure of 55 psi. Filtration was stopped when the retentate volume reached 2 to 5 ml. The retentate was brought up to the original volume of conditioned medium with serum-free DMEM.

Samples of ultrafiltrate were heated to 56°C for 30 minutes or 100°C for 4 minutes, were cooled rapidly on ice, and were centrifuged 10 minutes at 2000 g to remove precipitates. Stability of changes in pH was assessed by treating conditioned medium ultrafiltrate with HCl to bring it to pH 3 or with NaOH to bring it to pH 11. After 5 hours at 4°C, the samples were neutralized and centrifuged as above. To determine trypsin sensitivity, samples of ultrafiltrate were incubated for 3 hours at 37°C with 50 μg/ml trypsin (1:250, GIBCO)/ml of ultrafiltrate. Soybean trypsin inhibitor (200 μg/ml, Boehringer-Mannheim Canada, Dorval, Quebec) was added. Controls containing soybean trypsin inhibitor (200 μg/ml) incubated with ultrafiltrate for 3 hours followed by the addition of trypsin (50 μg/ml), or soybean trypsin inhibitor added alone were not different than untreated ultrafiltrate samples in the growth assay. Other samples of conditioned medium ultrafiltrate were mixed with 20 mg washed activated charcoal (Norit-A, neutral, Fisher Scientific)/ml filtrate at room temperature for 1 hour on a magnetic stirrer. The charcoal was removed by centrifugation. Stability to freezing was assessed after samples of ultrafiltrate were frozen at −20°C and thawed three times, followed by centrifugation to remove precipitates. All treated samples and untreated controls of conditioned medium ultrafiltrate were passed through a 0.2-μm membrane filter for testing in the EC growth assay.

To determine if the growth stimulator was being released into medium during lysis of dead SMC, we compared the effects of an SMC cytosol with that of conditioned medium ultrafiltrate. SMC cultured in DMEM were washed free of medium, then lysed by sonication in the cold; the lysate was centrifuged at 10,000 g for 30 minutes and filtered through a 0.2-μm membrane filter. Aliquots of SMC conditioned medium ultrafiltrate and of the cytosol derived from equal numbers of cells were then tested in the EC growth assay.

To assess the thymidine content of SMC conditioned medium, normal human foreskin fibroblasts were used in a competitive ³H-thymidine uptake assay. Fibroblasts from stock cultures grown in DMEM with 10% SCS were plated in 35-mm dishes at a density of 50,000 cells/dish and incubated for 24 hours in 10% SCS in DMEM. The medium was removed, and the cells were washed four times with DPBS (37°C). To each dish was added 1.0 ml of serum-free DMEM containing 5 μCi of ³H-thymidine (specific activity 67 μCi/mmol) and various additions of either unlabeled thymidine or conditioned medium ultrafiltrate. After incubation for 1 hour at 37°C, the medium was removed; the cells were washed four times with cold DPBS, and precipitated with 10% trichloroacetic acid.
EC seeded at a density of $2 \times 10^4$ cells/35-mm culture dish (2.5 $\times 10^3$ cells/cm$^2$) in 2.5% SCS (level of serum depending on cell strain) without ECGF remained quiescent or grew slowly over the next 7 to 10 days. Porcine aortic SMC conditioned medium at a concentration of 50% (vol/vol) in this medium markedly stimulated their replication (Figure 1). In contrast, when ECGF was included, SMC conditioned medium inhibited EC growth compared to cell-free conditioned medium (Figure 1).

EC number on day 7 of the assay showed a dose-dependent response to both of these effects (Figure 2). In 2.5% SCS without ECGF, increasing concentrations of SMC conditioned medium up to 50% of the total medium caused a linear increase in cell number; at levels of 75% or more, the stimulatory effect fell (Figure 2A). In 10% SCS with ECGF, which was optimum growth medium for these EC, increasing concentrations of SMC conditioned medium progressively decreased EC number at day 7 (Figure 2B).

With CF conditioned medium, EC number showed a minimal dose response to serum. In the presence of SMC conditioned medium, EC number increased in an approximately linear fashion with increasing SCS concentration (Figure 3).

Figure 4 shows the ECGF dose response of EC number with 50% SMC or cell-free (CF) conditioned medium (TCA) for 1 hour in the cold. After removal of the TCA, the precipitate was washed repeatedly with cold 10% TCA, then dissolved in 1 N NaOH, aliquots were used for assays of protein and of radioactivity.

**Results**

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medium in 2.5% SCS. Again, without ECGF, SMC conditioned medium increased EC number. At all levels of ECGF, SMC conditioned medium inhibited the stimulating effect of ECGF on EC growth. Higher levels of ECGF were ineffective in overcoming this growth-inhibitory effect.

To determine whether the EC growth-inhibitory effect of SMC conditioned medium in the presence of ECGF was due to an interaction with ECGF per se or was related to the growth rate of the EC, we determined the effect of conditioned medium on a strain of EC that grew well in 10% SCS without ECGF. Figure 5 illustrates that in CF conditioned medium and 10% SCS alone, this strain initially grew at a somewhat lower rate than with ECGF, but after 7 days, cell numbers in the two groups were the same. SMC conditioned medium abolished the increase in the rate of cell growth produced by ECGF and lowered the final cell count whether or not ECGF was present. Thus the inhibition of growth was not dependent on the presence of ECGF.

We initially determined that the EC growth-promoting substance in SMC conditioned medium was absent in the retentate after dialysis and was recovered in the dialysate. Subsequently, we filtered the conditioned medium through an ultrafiltration membrane with an exclusion limit of 1000 daltons. The ultrafiltrate contained potent EC growth-promoting activity that was evident in the absence of ECGF. No additional activity was provided by the conditioned medium ultrafiltrate when cells were already growing maximally in ECGF and 10% SCS (Figure 6A). Inhibitory activity for EC growth was retained by the ultrafiltration membrane as indicated in Figure 6B.

To determine if the activities were produced by SMC in the presence of serum, we prepared conditioned medium using 1% FBS in DMEM instead of the usual serum-free DMEM, and we filtered it through the YM2 membrane. Figure 7A shows that filtered SMC conditioned medium made with 1% FBS contained much less growth-stimulatory activity than conditioned medium made without serum. In other experiments, we found no EC growth-promoting activity in conditioned medium made with 10% FBS (data not shown). Figure 7B illustrates that the YM2 membrane retentate of SMC conditioned medium made with 1% FBS contained EC growth inhibitor, apparently more than conditioned medium made without serum.

Growth assays of the SMC conditioned medium ultrafiltrate indicated that the stimulatory activity was stable to trypsin, heat, changes in pH in the ranges 3 to 11, and repeated freezing and thawing (Figure 8). All of the activity was removed by treatment with charcoal.
Figure 5. Inhibition of endothelial cell (EC) proliferation by porcine aortic smooth muscle cell (SMC) conditioned medium. EC were seeded on day 0 at approximately 2.0 × 10⁴ cells/35-mm culture dish in 2 ml Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) supplemented cell serum (SCS). After 24 hours, three dishes of cells were counted and the remainder were given 10% SCS in DMEM containing 50% (vol/vol) porcine aortic SMC (C) or cell-free (■) conditioned medium with (—) or without (—) endothelial cell growth factor at 100 μg/ml. Medium and supplements were refreshed every other day. Points represent the mean of triplicate dishes counted on the days indicated. Coefficients of variation averaged 10%.

Although SMC death was not notable during the conditioning period in serum-free DMEM (cell counts were similar before and after the 24-hour period), the possibility remained that some lysis of dead cells may have been responsible for activity in the conditioned medium. Therefore, we compared the activity of conditioned medium with a preparation of SMC cytosol. Table 1 shows that the addition of cytosol had a marked stimulatory effect on EC growth in the presence of ECGF and an inhibitory effect in its absence. Dilutions of cytosol in fresh medium (1:25 and 1:50) had no effect on EC growth in the presence or absence of ECGF.

It has been shown that thymidine is a growth factor for EC; to determine if the SMC conditioned medium contained enough thymidine released from the SMC to account for the EC growth stimulation, we determined the thymidine content of the medium by a competitive ³H-thymidine uptake assay in human foreskin fibroblasts. We found, as anticipated, that unlabeled thymidine at concentrations that stimulate EC growth suppressed uptake of labeled thymidine; however, ultrafiltrate prepared from conditioned medium did not affect thymidine uptake (Table 2).

**Discussion**

In these experiments, medium from cultured vascular SMC had both stimulatory and inhibitory effects on in vitro EC proliferation. The effect that predominated depended on the growth rate of the EC. In a dose-dependent manner, SMC conditioned medium stimulated replication of low density EC maintained in a quiescent state or growing slowly, but inhibited the growth rate and the final cell density of EC that were rapidly proliferating in the presence of a high serum concentration or of ECGF; both effects were apparently dependent on the rate of cell growth per se and not on the level of serum or the presence of ECGF in the assay. The conditioned medium effects were the result of two distinct activities that could be separated on the basis of molecular weight. Furthermore, the production of the stimulatory activity by SMC was low or absent in the presence of serum factors, whereas the inhibitory activi-
ity in a given amount of conditioned medium was greater with as little as 1% serum during the conditioning period.

Our data demonstrating, in SMC conditioned medium, a nonfilterable inhibitory activity for EC proliferation are consistent with that of Schumacher et al. who described a 35 to 40 K dalton peptide in bovine aortic SMC conditioned medium that inhibits growth of proliferating EC. Other investigators have noted briefly that media of cultured SMC from calf pulmonary artery and bovine aorta stimulate bovine aortic EC or bovine capillary EC growth in vitro, but the nature of these activities has not been described further. Schumacher et al. described an EC growth stimulator in whole conditioned medium of bovine aortic SMC as heat labile, but we found that our EC growth-promoting substance, separated from the inhibitor by ultrafiltration, was retained after boiling with visible loss of other materials as precipitates. This EC growth stimulator had a molecular weight less than 1000 daltons, was stable to extreme pH, was not degraded by trypsin, and was completely stable to repeated freezing and thawing. These attributes suggest that it is not a peptide. Its removal from conditioned medium by activated charcoal demonstrates a hydrophobic nature. This preliminary characterization indicates that this activity is different from in vitro ECGFs found in conditioned media of other cell types including the nondialyzable, trypsin-sensitive factor secreted by human embryo fibroblasts, the heat- and protease-stable activity with a molecular weight greater than 10 000 in conditioned medium from differentiated 3T3 adipocytes, and the polypeptides secreted by human hepatoma cells or neural tumor lines. Its relationship to the low molecular weight mitogen for porcine aortic EC from platelets and to the low molecular weight angiogenic factors from tumors and synovial fluid remains to be determined by further characterization of all of these activities.

During the 24-hour incubation used for preparing conditioned medium, a small number of cells died and some of these may have been lysed, with release of cell contents into the medium. Clearly, SMC cytosol did have effects on EC growth similar to those of conditioned medium (Table 1). Because conditioned medium was serum-free, it was assumed that its protein content would be a reasonable index of the amount of cytosol released by cell lysis; comparison of conditioned medium with cytosol diluted to an equivalent protein concentration should then indicate whether the effects of the conditioned medium might be due to its content of cytosol. We found, however, that the protein concentration of the medium was immeasurable by standard procedures; consequently we used arbitrary dilutions of cytosol (1:25 and 1:50) for the comparison. Because these dilutions, which contained a measurable amount of protein, had no effect on EC growth, we conclude that the effects of conditioned medium are probably due to sequestration of the active agent(s) by living cells, rather than to release of cell contents on lysis of dead cells.
Figure 7. A. Effect of serum on smooth muscle cell (SMC) conditioned medium content of endothelial cell (EC) growth-stimulatory activity. EC were seeded on day 0 at approximately 2.0×10⁶ cells/35-mm culture dish in 2 ml Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) supplemented calf serum (SCS). Cells in three dishes were counted on day 1, and the remainder received fresh DMEM, 10% SCS, and the ultrafiltrate (50%, vol/vol) of porcine aortic SMC (C) or cell-free (CF) conditioned medium made in serum-free DMEM (----) or in DMEM containing 1% (vol/vol) fetal bovine serum (FBS) (- - -). Medium was refreshed on days 3 and 5. Points represent the mean of triplicate cell counts on days 3, 5, and 7. Coefficients of variation averaged 10%. B. Effect of serum on SMC conditioned medium content of EC growth-inhibitory activity. EC were seeded and counted on day 1 as in A. On days 1, 3, and 5, cultures received 10% SCS, endothelial cell growth factor (EGF) (75 μg/ml), and the ultrafiltrate membrane retentate (50%, vol/vol) of porcine aortic SMC (C) or CF (■) conditioned medium made in serum-free DMEM (-----) or in DMEM containing 1% FBS (- - -). As a quiescent EC control, a further group of dishes received 10% SCS without EGF and with the retentate of CF conditioned medium made in 1% FBS (x - x). (Similar results [not shown] were seen with the equivalent SMC conditioned medium.) Points represent the mean of triplicate cell counts on days 3, 5, and 7. Coefficients of variation averaged 12%.

Figure 8. Effect of various treatments on endothelial cell (EC) growth-stimulatory activity in ultrafiltrate of smooth muscle cell (SMC) conditioned medium. EC were seeded in Dulbecco's modified Eagle's medium (DMEM) with 10% supplemented calf serum (SCS). On days 1, 3, and 6, cells received fresh DMEM, 10% SCS, and the ultrafiltrate (50%, vol/vol) of either cell-free (CF) or porcine aortic SMC conditioned medium that had been treated as described in the Methods section. A. Untreated conditioned medium ultrafiltrate. B. Digested with trypsin for 3 hours. C. Heated to 56°C for 30 minutes. D. Heated to 100°C for 4 minutes. E. Adjusted pH 3. F. Adjusted to pH 11. G. Frozen and thawed. H. Treated with activated charcoal. Bar height represents the day 7 cell number in SMC conditioned medium samples treated in the same manner. Data are the mean±SE of results from two experiments.

Although thymidine is a low molecular weight growth factor for EC,11 our results indicate that the growth factor in conditioned medium did not compete with 3H-thymidine at tracer concentration for uptake by growing cells; thus, if thymidine is present in the conditioned medium, its concentration is too low to account for the stimulation of EC growth.

The growth of EC may be important in two aspects of vascular disease: in repairing damage to the endothelial lining of the vascular tree, and in the development of new capillaries which grow into the intima during atherogenesis.24,25 The present data raise important questions as to what factors control the expression by SMC of EC growth inhibitor and stimulator. We have seen that serum factors decrease the secretion of stimulator and increase the amount of inhibitor in SMC conditioned medium. It will be important to determine the nature of the serum factors responsible for these effects. Also of particular interest will be the effects on the expression of the SMC activities of various factors known or suspected to be associated with atherogenesis, including lipoproteins, growth factors from platelets,14 EC,1,25 and macrophages,26 and extracellular matrix components known to affect the growth27 and proteoglycan metabolism28 of cultured aortic SMC. Furthermore, our data indicate the importance of determining how the growth phase of the EC influences its responsiveness to SMC-derived stimulants.
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