Smooth Muscle Cells Produce an Inhibitor of Endothelial Cell Growth

Barbara L. Schumacher, Debra Grant, and Reuben Eisenstein

A potent inhibitor of endothelial cell growth and neovascularization has previously been isolated from both bovine and human aorta. In an attempt to determine if the major cellular component of that tissue, smooth muscle cells, synthesized molecules with this activity, we studied the effects of serum-free conditioned medium from bovine aortic smooth muscle on the growth of cultured endothelial cells from bovine aorta. The smooth muscle conditioned medium was found to contain both growth inhibitory and stimulating activities that could be distinguished by their heat stability. The inhibitor is precipitable by ammonium sulfate, heat stable (80° C), and inactivated by dithiothreitol, trypsin, and 2-mercaptoethanol. It has an estimated molecular weight of approximately 35,000 to 40,000 daltons (by column chromatography). It therefore appears that smooth muscle cells produce an inhibitor of endothelial cell growth that belongs to a class of natural endothelial cell growth inhibitors derived from avascular tissues we tentatively term “avasculins.” Proof of its identity with the inhibitor isolated from intact aorta (or other tissues) must await purification.

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Proliferation of vascular cells, both endothelial (EC) and smooth muscle cells (SMC), are significant events in embryology, developmental and reparative processes, and a number of pathologic processes including atherosclerosis, tumor growth, and proliferative retinopathies. A number of humoral and local factors (including classical hormones, platelet-derived growth factor, fibroblast growth factor, heparan sulfate fragments, and others) are known to affect the growth of one or both of these cell types. Inhibitors of EC growth have been identified in a number of avascular tissues, including cartilage, aorta, and ocular tissues. One potent factor found in aortic extracts, which is as yet only partially purified, passes through filter membranes with a nominal cut-off of 10,000 daltons and is effective in inhibiting neovascular growth in vivo and EC proliferation in culture. In an attempt to determine whether this activity was synthesized by SMC, the major cell type found in the aortic wall, we designed tissue culture experiments to determine the effects of smooth muscle conditioned medium (SMCM) on the growth of EC in culture. The data indicate that cultured SMC produced both inhibitory and stimulatory effects on EC growth. The inhibitory factor has properties in common with the EC growth inhibitor extracted from intact aorta.

Methods

We cultured second to tenth passage EC and SMC from bovine aorta as previously described with slight modifications. Briefly, EC were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with either 10% heat-inactivated calf serum (CS) or 10% heat-inactivated plasma-derived serum (PDS, Hyclone). A standard amount of antibiotics was also added to the growth medium.

SMC were cultured in RPMI-1640 supplemented with 10% heat-inactivated CS and with the same antibiotics used for EC. Balb/c 3T3 cells were cultured in DMEM and B16 F10 mouse melanoma cells (from Isaiah Fidler) in Waymouth's medium, both with 10% heat-inactivated CS. All cells were cultured in a humidified atmosphere of 5% CO₂ air at 37° C.

Preliminary growth inhibition assays were done in Linbro 96 well trays (Flow Laboratories, Inc., McLean, Virginia). Stock cultures of EC were briefly trypsinized and seeded at 20,000 cells/ml, 0.25 ml/well. After 24 hours, the medium was removed and replaced with medium containing test materials or control fresh medium, in both cases after filter sterilization through a Millex 0.45 μm filter (Millipore Corpo-
ration, Bedford, Massachusetts). After 72 hours the cells were counted in a Coulter counter (Model Z1, Coulter Electronics, Inc., Hialeah, Florida).

Subsequent assays were done in either Corning 24-well plates or Nunc or Falcon 35-mm Petri dishes with the use of 0.5-cc-wells or 2 cc-Petri dishes, respectively. Results were similar in all types of culture vessels. When 3T3 cells were compared to EC, both cell types were cultured under identical conditions by using DMEM and 10% heat-inactivated CS. For growth curve experiments with 50 µg/ml of a 90% (NH₄)₂SO₄ precipitate of SMCM, cells were harvested and counted after 24, 48, and 72 hours. To test for reversibility of effect, a dose of 100 µg/ml was used. After 4 and 24 hours, the medium was aspirated from some dishes. Cells were then washed three times with complete medium, and refed with fresh medium. Control dishes, unexposed to this smooth muscle cell product, were treated identically. These cultures were compared with cells cultured continuously in medium containing the test material. Cell counts were done after 24, 48, and 72 hours (Figure 1).

To prepare conditioned medium, second-to-fifth passage SMC were grown to hyperconfluence (2 to 4 cell layers thick) in Falcon 75 cm² flasks. Medium was aspirated, and the cell layer was rinsed three times with Hanks Balanced Salt Solution. Serum-free and antibiotic-free RPMI-1640 was then added, and the cultures were incubated at 37°C for 48 hours. SMCM was then withdrawn and frozen at -40°C. The SMC appeared to be healthy after 48 hours in serum-free medium and were readily passaged.

SMCM from many such cultures was thawed, pooled, centrifuged, sterilized by membrane filtration, and divided into aliquots. Some aliquots were dialyzed against water in a Spectrapor No. 1 bag (Spectrum Medical Industries, Inc., Los Angeles, California) at 4°C and lyophilized (SM-RPMI). 100 ml of SMCM yielded approximately 15 mg of this material. We took other aliquots that were 90% saturated with ammonium sulfate (pH 7.4). The precipitate was centrifuged, dissolved in phosphate-buffered saline (PBS), dialyzed against water in a Spectrapor No. 1 bag at 4°C, and lyophilized (SM-ASP). 100 ml of SMCM yielded approximately 8.4 mg of SM-ASP.

For comparative purposes, an EC-growth inhibitory fraction, termed A10, was prepared as previously described by filtration through PM-10 membranes. Another fraction with such activity was prepared from the filtrate of a 50,000 molecular weight cut-off membrane (Amicon hollow fiber H1X50) and then treated in a manner identical to SM-ASP. This preparation is termed A50-ASP.

A 1.5 x 90 cm column was packed with BioGel P-60 equilibrated with 3 M Guanidinium HCl, 0.005 M 2[N-Morpholino] ethane sulfonic acid (pH 6.0). All samples were dissolved in and eluted from the column in the same solution. Standard proteins, A50-ASP and SM-ASP, were chromatographed separately under identical conditions. We collected 2 ml fractions of the SM-ASP and A50-ASP runs. The absorbance patterns of the standards and the test fractions were compared for a molecular weight estimation. Each fraction of the SM-ASP and the A50-ASP was dialyzed against water at 4°C, lyophilized, and tested for effects on EC growth in culture.

Aliquots of SM-RPMI or SM-ASP were dissolved in a small amount of PBS and then divided into two equal portions. One was heated at 80°C for 20 minutes, and the other was not heated. Each was added separately to complete medium, and the effects on cell growth were determined as described above. Identically treated PBS was used as a control.

Other aliquots of SMCM were dialyzed against 4 mM dithiothreitol and then against water exhaustively before the effects on EC growth were tested; to some aliquots, 2-mercaptoethanol (2ME) was added to a concentration of 5 x 10⁻⁵ M. For these experiments, 2ME or dithiothreitol was added to fresh medium as a control.

All cell experiments were done at least in triplicate and repeated at least twice, always with similar results.

SDS slab gel electrophoresis was performed in 8 M urea and sodium dodecyl sulfate by the method of Anderson et al. and then stained with formaldehyde-Coomassie blue to diminish diffusion in the gel as described by Steck et al.
Results

Early experiments (data not shown) demonstrated that when second to tenth passage cells were used, similar data were generated, regardless of passage. The same was true of EC obtained from different aortas at different times. Because the cells were usually confluent 96 hours after refeeding (120 hours after seeding), experiments were not continued longer than 72 hours after refeeding. Since the geometry of the different culture dishes varied, cell density per unit area also differed with the culture vessels. However, when 20,000 cells/ml were dispensed, data were similar in each type of dish.

In preliminary experiments with nonlyophilized material, SMCM had a biphasic effect on EC growth. After 24 hours, growth was moderately inhibited, but when diluted 1:2 or 1:4, modest stimulation was observed. At 48 and 72 hours only, a stimulatory effect was found that increased with progressive dilution. This suggested that there was more than one material that affected growth. To obtain evidence for this, we heated SMCM as described above. SMCM treated in this manner had an inhibitory effect on EC growth, which diminished upon dilution (Figure 2).

In later experiments with SM-ASP in lyophilized form, modest inhibition of EC growth was observed and there was greater inhibition when the SM-ASP was heated. A typical experiment is shown in Table 1 and Figure 3. At a dose of 50 μg/ml, SM-ASP inhibited EC growth after 24, 48, and 72 hours, but cell numbers increased on each day of culture (Figure 3). With 100 μg/ml, cell numbers diminished between 48 and 72 hours, but in cultures refed with normal medium, the growth curves returned toward normal (Figure 1).

SM-ASP chromatographed on a BioGel P-60 column at a high-salt concentration showed the greatest growth inhibitory activity in a fraction estimated to be approximately 35,000 to 40,000 daltons when compared to protein standards of known molecular weight.

Table 1. Effect of SM-ASP on Endothelial Cells: Percentage of Inhibition of Growth

<table>
<thead>
<tr>
<th>SM-ASP dose (μg/ml)</th>
<th>Cells cultured in DMEM/10% CS</th>
<th>Cells cultured in DMEM/10% PDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-ASP</td>
<td>Unheated SM-ASP</td>
<td>Heated SM-ASP</td>
</tr>
<tr>
<td>50.0</td>
<td>37 ± 3.8</td>
<td>55 ± 2.4</td>
</tr>
<tr>
<td>25.0</td>
<td>13 ± 4.1</td>
<td>23 ± 12.3</td>
</tr>
<tr>
<td>12.5</td>
<td>8 ± 3.5</td>
<td>14 ± 2.2</td>
</tr>
</tbody>
</table>

Smooth muscle conditioned medium was saturated with ammonium sulfate, centrifuged, dissolved in phosphate-buffered saline (PBS), and divided into two equal aliquots (designated SM-ASP), one of which was heated at 80°C for 20 minutes. Both were added to complete medium and fed to cells seeded 24 hours before. Cell counts were done 72 hours later, and are recorded ± standard error. DMEM = Dulbecco's modified Eagle medium; CS = calf serum; PDS = plasma-derived serum.
Figure 4. Gel filtration of SM-ASP. Absorbance pattern is of standard proteins (blue dextran, $2 \times 10^6$ daltons; ovalbumin, 43,000 daltons; soybean trypsin inhibitor, 21,500 daltons; lysozyme, 14,300 daltons; and aprotinin, 6,500 daltons). Vertical bars represent growth-inhibitory activity of SM-ASP chromatographed on the same column under identical conditions. Greatest growth inhibitory activity is in fractions of estimated molecular weight of 35,000 to 40,000.

Table 2. Percentage of Inhibition of Growth by SM-ASP

<table>
<thead>
<tr>
<th>Dose (µg/ml)</th>
<th>EC % Change</th>
<th>3T3 % Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.0</td>
<td>-38</td>
<td>+8</td>
</tr>
<tr>
<td>25.0</td>
<td>-24</td>
<td>+10</td>
</tr>
<tr>
<td>12.5</td>
<td>-10</td>
<td>+5</td>
</tr>
</tbody>
</table>

Endothelial cells (EC) or 3T3 cells were cultured under identical conditions. Nonheated SM-ASP was used. SM-ASP inhibited EC growth in a dose responsive manner. Change in 3T3 cells numbers are considered to be within experimental error. - = decrease; + = increase.
growth in culture. It has been observed\textsuperscript{7-11} that aorta and other avascular tissues contain inhibitors of EC or SMC growth in culture.

It therefore seems likely that there are locally synthesized substances that influence vascular cell growth. Castellot et al.\textsuperscript{7} described a SMC growth-inhibitor produced by EC in culture that was apparently a heparan sulfate, and Gadjoisek et al.\textsuperscript{16} have identified a smooth muscle mitogen produced by the same cells. At least four substances extracted from intact aorta are known to inhibit EC growth in culture, the most potent of which is an only partially purified material that passes through 10,000 MW cutoff filter membranes.\textsuperscript{10} Analogous activity has been extracted from cartilage, vitreous, and lens.\textsuperscript{9, 11, 19, 20} The experiments described here were primarily designed to test whether cultured SMC from bovine aorta synthesized factors with such activity and to partly characterize these factors.

The data indicate that in cell culture SMC secretes at least two factors that affect EC growth in different ways. Initial experiments showed that SMCM stimulates EC growth and that this effect was enhanced on dilution, suggesting that inhibitory materials were also present. This impression was confirmed by the observation that heating SM-ASP at 80° C left only an inhibitory effect, which diminished upon dilution. The absence of visible precipitation after this manipulation indicated that this was not due to loss of active material by the process.

From the design of the experiments, it is not possible to determine whether the effects of SM-ASP on cell growth are due to a direct interaction of cell products with growing cells or are mediated through effect on the serum in the medium. It is clear, however, that these effects are not due to direct alteration of serum by cells, since conditioned medium was prepared serum-free. This effect also does not seem to involve platelet-derived factors, since it is effective in plasma-derived serum.

The data from experiments with SMCM indicate that protein is the major factor responsible for inhibition of EC growth by SMCM, as evidenced by the trypsin, dithiothreitol, and 2-mercaptoethanol experiments, in which all three destroyed growth inhibitory activity.

The chromatographic data indicate that the major EC growth-inhibitor has an estimated MA of 35,000 to 40,000 daltons.

The EC growth curves during exposure to heated SM-ASP at a dose of 50 μg/ml suggest that this effect is largely inhibitory rather than cytotoxic since no dead floating cells were observed and cell number increased in both the experimental group and the control group over the entire 72-hour study period. At a larger dose (100 μg/ml), the growth curve suggested some cytotoxic effect, but the recovery experiment indicated that this was reversible, since the curves returned to normal when the EC were refed with control medium. The activity had at least some degree of cell-specificity, since it did not affect 3T3 or B16-F10 melanoma cell growth. We have previous-

\textsuperscript{10} shown that the growth-inhibitory fraction extracted from bovine aorta is a potent inhibitor of EC and lymphocyte growth in culture, but is much less effective against SMC, neuroblastoma cells, and some tumor cell lines.

Some of the questions posed by these experiments are: whether the inhibitor from cultured SMC is the same as the EC growth-inhibitor extracted from intact aorta; what is the nature of the effector molecule; and what is its mode of action. The EC growth-inhibitor produced by SMC in culture and the aortic growth-inhibitor share many properties. A50-ASP from intact aorta, SM-ASP from intact aorta, and SM-ASP from SM-conditioned medium show major growth inhibitory activity with an estimated MW of 35,000 to 40,000 (with lesser activity of lower MW). They are also heat-stable and are precipitated by 90% ammonium sulfate. Proof of identity awaits more complete purification. One possible explanation for the presence of minor growth-inhibitory activities in lower molecular weight fractions is the formation of proteolytic fragments of a larger parent molecule. Other explanations are possible. The role of this factor in regulating either neovascularization or repair of deendothelized areas in large blood vessels is not clear.

If, as we believe, the growth inhibitor isolated from intact aorta is the same as, or related to, the one from SMC, cultured cells may be a potential source for its eventual purification. Thus, a product of SMC, apparently a polypeptide of estimated MW about 35,000-40,000 can be added to the list of natural EC growth-inhibitors derived from tissues that are normally avascular and which we tentatively term avasculins.

\textbf{Acknowledgment}

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\textbf{References}

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