

# Cultured Endothelial Cells Produce a Heparinlike Inhibitor of Smooth Muscle Cell Growth

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**ABSTRACT** Using cultured cells from bovine and rat aortas, we have examined the possibility that endothelial cells might regulate the growth of vascular smooth muscle cells. Conditioned medium from confluent bovine aortic endothelial cells inhibited the proliferation of growth-arrested smooth muscle cells. Conditioned medium from exponential endothelial cells, and from exponential or confluent smooth muscle cells and fibroblasts, did not inhibit smooth muscle cell growth. Conditioned medium from confluent endothelial cells did not inhibit the growth of endothelial cells or fibroblasts. In addition to the apparent specificity of both the producer and target cell, the inhibitory activity was heat stable and not affected by proteases. It was sensitive to *Flavobacterium* heparinase but not to hyaluronidase or chondroitin sulfate ABC lyase. It thus appears to be a heparinlike substance. Two other lines of evidence support this conclusion. First, a crude isolate of glycosaminoglycans (TCA-soluble, ethanol-precipitable material) from endothelial cell-conditioned medium reconstituted in 20% serum inhibited smooth muscle cell growth; glycosaminoglycans isolated from unconditioned medium (i.e., 0.4% serum) had no effect on smooth muscle cell growth. No inhibition was seen if the glycosaminoglycan preparation was treated with heparinase. Second, exogenous heparin, heparan sulfate, chondroitin sulfate B (dermatan sulfate), chondroitin sulfate ABC, and hyaluronic acid were added to 20% serum and tested for their ability to inhibit smooth muscle cell growth. Heparin inhibited growth at concentrations as low as 10 ng/ml. Other glycosaminoglycans had no effect at doses up to 10  $\mu$ g/ml. Anticoagulant and non-anticoagulant heparin were equally effective at inhibiting smooth muscle cell growth, as they were in vivo following endothelial injury (Clowes and Karnovsk. *Nature (Lond.)*. 265:625–626, 1977; Guyton et al. *Circ. Res.* 46:625–634, 1980), and in vitro following exposure of smooth muscle cells to platelet extract (Hoover et al. *Circ. Res.* 47:578–583, 1980). We suggest that vascular endothelial cells may secrete a heparinlike substance in vivo which may regulate the growth of underlying smooth muscle cells.

A characteristic feature of the normal, healthy arterial wall is that the intimal endothelial cells form a continuous quiescent monolayer, and the underlying medial smooth muscle cells also remain in a quiescent growth state. If the endothelium is damaged, smooth muscle cell proliferation occurs until the endothelium regenerates (9, 29). The regulation of cell growth in the vascular wall is poorly understood. Ross (15, 28) and others (10, 25) have shown that platelet-derived growth factors are required for smooth muscle cell growth in vivo and in vitro. Recently, conditioned medium from macrophages has been found to stimulate smooth muscle cells (23). Gajdusek et al.

(11) have shown that endothelial cells produce a factor which stimulates the growth of smooth muscle cells. Eisenstein et al. (8) have found that extracts from the inner arterial wall can be fractionated to produce both stimulators and inhibitors of smooth muscle cell growth.

We present evidence demonstrating that cultured endothelial cells produce both positive and negative effectors of smooth muscle cell growth. The inhibitory activity appears to be heparin or a heparinlike molecule. These results suggest a possible role for heparin in the regulation of vascular smooth muscle cell growth in vivo.

## MATERIALS AND METHODS

### Purification of Heparinase

*Flavobacterium heparinum* were either grown as described by Linker and Hovingh (22) or were provided by Dr. R. Langer (Massachusetts Institute of Technology, Boston, Mass.). The cells were sonicated at a cell protein concentration of ~8 mg/ml for 12 min with a Branson Sonifier set at 125 W. The sonicate was centrifuged at 20,000 g at 4°C for 30 min. The resulting supernate was treated with protamine sulfate at a level of 15 mg/ml and then exhaustively dialyzed against distilled water.

The crude preparation was subsequently chromatographed on hydroxylapatite and cellulose-phosphate as outlined by Linker and Hovingh (22). We purified the enzyme further by concentrating with ultrafiltration, and then by filtration on Sephacryl S-200 equilibrated in 0.5 M NaCl in 0.01 M Tris-HCl, pH 7.5. The elution profile exhibited a major protein peak at a molecular weight of ~60,000 daltons, as well as several minor protein peaks of lower molecular size. Direct analysis of the first peak with respect to heparin-cleaving potency (22) revealed a constant specific activity of ~3,000 U/mg across the initial two-thirds of the profile. The remaining two components of lower molecular size possessed essentially no glycosaminoglycan (GAG)-cleaving ability. The final enzyme preparation exhibited no capacity to degrade chondroitin sulfate, dermatan sulfate, or hyaluronic acid when employed at concentrations as high as ~200 µg/ml (24). There was no detectable protease activity in this preparation as measured by the radiolabeled casein assay (17).

### Isolation of Bovine Aortic Heparan Sulfate

Glycosaminoglycans were obtained from blood vessels by extensive proteolytic digestion of the intima and media of calf aortas (26). Heparan sulfate was isolated free of other GAGs by chromatography on DEAE-Sephadex A-25 and Sepharose 4B (30). The resulting product was identified by the two-dimensional electrophoretic system of Hata and Nagai (16) in conjunction with appropriate standards. The nature of the heparan sulfate was confirmed by its sensitivity to digestion with bacterial heparinase and its resistance to the action of chondroitinase ABC and testicular hyaluronidase (24).

### Cell Culture

Bovine aortic endothelial cells (BAEC) were isolated from freshly slaughtered calves, as previously described (2, 13, 19). The cells obtained from one aorta are grown in a single 75-cm<sup>2</sup> tissue culture flask in RPMI-1640 medium containing 20% fetal calf serum (FCS), 4 mM glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin (0.25 µg/ml), and gentamicin (50 µg/ml) at 37°C in a humidified, 10% CO<sub>2</sub> atmosphere. At confluence, >95% of the cells are endothelial, as determined by their distinctive morphology and the presence of factor VIII antigen on the cell surface.

Rat aorta smooth muscle cells (rat SMC) from Sprague-Dawley (Charles River, Wellesley, Mass.; CD strain) rats were isolated (18) as previously described. Cells were grown in the same medium and incubation conditions described for BAEC.

Calf aorta smooth muscle cells (calf SMC) were isolated as previously described (27). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (CS), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin (0.25 µg/ml), and gentamicin (50 µg/ml) at 37°C in a humidified, 10% CO<sub>2</sub> atmosphere.

Baby hamster kidney-21/clone 13 cells (BHK) were obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown in DMEM containing 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), and gentamicin (50 µg/ml) at 37°C in a humidified, 10% CO<sub>2</sub> atmosphere. This fibroblast line was used because many laboratories have shown that these cells can be readily growth-arrested, and that the growth of the cells is dependent on serum factors. Thus its growth characteristics are similar to those of smooth muscle cells.

### Conditioned Medium

A week or more after reaching confluence, BAEC were washed and 10 ml (~1 ml/10<sup>6</sup> cells) of RPMI containing 0.4% FCS was added. 48 h later, this medium was removed and immediately filtered through a 0.22 µm filter. Conditioned medium (CM) from exponential BAEC and from exponential and confluent cultures of other cell types was collected in the same manner. Before adding it to cells, CM was mixed 1:1 with medium containing twice the usual serum concentration (e.g., RPMI + 40% FCS, or DMEM + 20% CS), thus yielding a final serum concentration of 20% and 10%, respectively.

### Growth Arrest of SMC

Sparsely plated rat or calf SMC were washed and placed in RPMI + 0.4% FCS or DMEM + 0.2% CS for 72 h. Flow microfluorimetry (32) and [<sup>3</sup>H]-thymidine uptake into DNA were used to determine that the cells were arrested in G<sub>0</sub> (G<sub>1</sub>).

### Inhibition Assay

To assay for inhibitory activity, 6 × 10<sup>4</sup> cells were plated into 16-mm multiwell plates and growth-arrested as described above. Control cultures were released from the G<sub>0</sub> block by placing them in RPMI + 20% FCS. Other cultures were placed in the 1:1 mixture of CM and RPMI + FCS (final concentration FCS 20%). Cell numbers were measured in duplicate samples at daily intervals by washing the cells once in a trypsin-EDTA solution (Grand Island Biological Co., Grand Island, N. Y.), then incubating the cells for several minutes in trypsin-EDTA solution to dislodge the cells. The dislodged cells were collected and counted in a Coulter counter. Trypsinized cultures were routinely checked by direct microscopic examination to ascertain that the trypsinization procedure had not lysed the cells (as determined by the presence of cell debris) and to ensure that all cells were removed from the multiwell. The cells were not fed during the experiment.

### Mitogenesis Assay

To assay for mitogenic activity, exponential BAEC-CM was fractionated and concentrated 50-fold using Amicon XM100 and UM10 filters (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). A concentrated fraction was mixed 1:1 with RPMI + FCS (final concentration FCS 1%) and added to growth-arrested rat SMC. In some control experiments, cells were exposed to a 1:1 mixture of 50-fold concentrated RPMI + 0.4% FCS and RPMI + FCS (final concentration FCS 1%). In other control experiments, cells received RPMI + 20% FCS. Cell numbers were measured in duplicate samples at daily intervals using a Coulter counter as described above.

### Chemicals

Chondroitin sulfate ABC lyase and *Streptomyces* hyaluronidase were purchased from Miles Laboratories Inc., Miles Research Products (Elkhart, Ind.). Unless otherwise stated, heparin was obtained from Upjohn Co., Agricultural Prods. MKT (Kalamazoo, Mich.) or Elkins-Sinn, Inc. (Cherry Hill, N. J.); other GAGs were kindly supplied by Drs. M. Mathews and J. Cifonelli (University of Chicago). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

## RESULTS

### Growth Arrest of SMC

SMC were plated and exposed to low serum as described in Materials and Methods. We used flow microfluorimetry and [<sup>3</sup>H]thymidine uptake into DNA to determine the cell cycle distribution of exponentially growing and serum-deprived SMC (32). The results will be published in detail elsewhere.<sup>1</sup> In brief, >90% of the growth-arrested rat SMC cells have the G<sub>1</sub> DNA content. These cells are most likely arrested at the same point in the cell cycle (G<sub>0</sub>), because growth-arrested cells placed in fresh complete medium undergo a synchronous round of DNA synthesis, with the earliest cells beginning S phase 16–18 h after the addition of serum. The peak of DNA synthesis occurs ~28 h after release. Flow microfluorimetry confirms the time course of this release from G<sub>0</sub>. Calf SMC behaved similarly, except that the onset of S phase began 12–14 h after release, and peaked at ~20 h.

### Effect of Confluent Primary BAEC-CM on SMC

Rat and calf SMC were growth-arrested as described in Materials and Methods. Control cultures were released from the G<sub>0</sub> block by replacing the low serum medium with RPMI

<sup>1</sup> Castellot, J., and M. J. Karnovsky. Manuscript in preparation.

+ 20% FCS. Other cultures were exposed to the 1:1 mixture of confluent primary BAEC-CM and RPMI + FCS (final concentration FCS 20%). Cell growth after exposure to CM is presented in Fig. 1.

Rat SMC exposed to CM go through one to one and a half doublings at the same rate as control cells. After this initial growth, rat SMC stopped proliferating. Control cultures reach confluence at day 4 or 5 under these conditions. If the CM was replaced with normal growth medium at day 4 or 5, the cells proliferated to confluence.

Calf SMC exposed to CM also go through one or two rounds of division at about the same rate as control cells. At this point (usually day 3), the cells in CM often began to round up and slowly detach. Very few cells remained by day 5. Once the cells began to round up, replacing the CM with normal growth medium did not reverse this effect. In approximately one-third of the experiments, the calf SMC did not round up, but their growth plateaued, as shown in Fig. 1. The inhibitory effect was reversible, as the cells in these experiments proliferated to confluence if the CM was replaced with normal growth medium.

Direct observation of cells undergoing mitosis in CM-exposed rat and calf SMC cultures revealed that both daughter cells remained attached and spread normally after cytokinesis. Neither visual inspection nor Coulter counting of the medium indicated the presence of detached cells in CM-treated rat SMC and calf SMC cultures in which the cells did not round up. Flow microfluorimetry data indicate that both CM-treated and control SMC were arrested mainly in  $G_0$  (G1) at the endpoint of the experiment (data not shown). CM-treated SMC

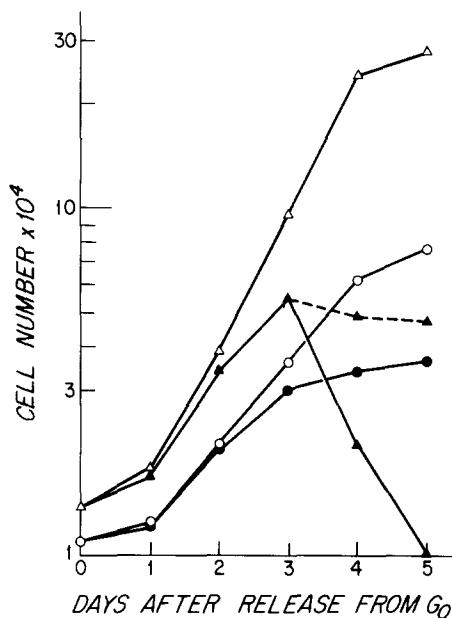


FIGURE 1 Effect of CM from confluent primary BAEC on growth-arrested SMC. Rat and calf SMC were arrested in  $G_0$  by serum-deprivation as described in Materials and Methods. The effect of confluent primary BAEC-CM on SMC growth was assayed as described in Materials and Methods. In approximately two thirds of the experiments, calf SMC exposed to CM began to round up and detach at day 3 ( $\blacktriangle$ — $\blacktriangle$ ). The other third of the time, the calf SMC did not round up ( $\blacktriangle$ — $\blacktriangle$ ).  $\Delta$ — $\Delta$ , calf SMC in RPMI + 20% FCS;  $\circ$ — $\circ$ , rat SMC in RPMI + 20% FCS;  $\bullet$ — $\bullet$ , rat SMC exposed to CM. Data for each cell type are the average of ten or more experiments.

did not stain with trypan blue at any time during the course of the experiment. Finally, the possibility that the detachment of calf SMC is related to something present in the serum is suggested by experiments in which CM was mixed 1:1 with lower concentrations of FCS (final FCS concentrations, 10% and 5%). In these experiments, growth of control calf SMC (i.e., no CM) is reduced to three to four doublings in 10% FCS and two to three doublings in 5% FCS. CM-treated calf SMC did not go through even one doubling, and they did not round up and detach. Because of the potential difficulties in quantifying results of experiments in which calf SMC detached, subsequent data on calf SMC in this paper are based on experiments in which calf SMC did not round up and detach.

To test the possibility that simple nutrient depletion was causing growth inhibition, some experiments were done using a 1:1 mixture of CM and RPMI containing twice the normal nutrient concentration + FCS (final concentration FCS 20%). The results were the same as those in Fig. 1.

### Specificity of the CM Effect

CM was collected from exponential and confluent cultures of BAEC, rat and calf SMC, and BHK cells as described in Materials and Methods. The volume of medium used for conditioning was 1 ml/ $10^6$  cells. All cell types tested for their responsiveness to CM were plated as described in Materials and Methods, except that BHK cells were arrested using 0.2% CS for 60 h. Rat SMC used in these experiments were second or third passage; calf SMC were first or second passage. BAEC were first through fifth passage, except that the confluent BAEC-CM was always collected from primary cultures. Cell numbers in duplicate cultures were measured at daily intervals after exposing cells to CM. The data are presented in Table I as the maximum percent inhibition. We obtain this figure by first subtracting the starting cell number (i.e., the cell number at the time the cells are released from  $G_0$ ) from the cell number at day 5, when growth has essentially ceased in both control and CM-exposed cells, and any growth inhibition was therefore maximal. The net growth in control cells is set at 100%, and the net growth in CM-treated cells is expressed as a percentage of the control. The difference is the maximum percent inhibition. We used this method of expressing our data to (a) compare more readily growth inhibition in experiments using different cell types or different types of CM, (b) to compare more easily the effect of various physical and biochemical treatments on the inhibitory activity, and (c) to calculate an  $ED_{50}$  for heparin and other substances with inhibitory activity.

Confluent, primary BAEC-CM preferentially inhibited rat and calf SMC; BHK and BAEC were much less affected (Table I). Other CM did not substantially inhibit SMC growth. It should be pointed out that when exponential cells are put in 0.4% serum they begin the transition from proliferation to quiescence. We cannot rule out the possibility that a growth effector(s) secreted by truly exponential cells is lost or masked during this transition. With this reservation in mind, the data demonstrate an apparent specificity of the inhibitory activity for both the producer and the target cell.

### Serum Requirements for Release and Effectiveness of the Inhibitory Activity

When we allowed confluent primary BAEC to condition serum-free medium for 48 h, the inhibitory activity decreased to 10–20% of the activity found in CM containing 0.4% serum.

TABLE I  
Specificity of the BAEC-CM Effect

| Type of CM                  | Maximum percent inhibition |      |         |          |
|-----------------------------|----------------------------|------|---------|----------|
|                             | BHK                        | BAEC | Rat SMC | Calf SMC |
| None (normal growth medium) | 0                          | 0    | 0       | 0        |
| Exponential BHK             | 2                          | 11   | 8       | 4        |
| Confluent BHK               | 13                         | 3    | 16      | 10       |
| Exponential rat SMC         | 9                          | 11   | 16      | 7        |
| Confluent rat SMC           | 8                          | 15   | 1       | 12       |
| Exponential calf SMC        | 0                          | 6    | 7       | 9        |
| Confluent calf SMC          | 0                          | 8    | 13      | 18       |
| Exponential BAEC            | 7                          | 0    | 0       | 1        |
| Confluent BAEC              | 6                          | 2    | 63      | >80      |

Cells were tested for their response to CM as described in Materials and Methods and Results. The data are presented as maximum percent inhibition. 0 means only that there was no inhibitory effect of CM. Exponential BAEC-CM (described in text) stimulated cell growth when compared to normal growth medium. Experiments involving BHK cells or BHK-CM were done twice. All other combinations were done four or more times. In all cases, cells in normal growth medium doubled four or more times before reaching confluence.

TABLE II  
Serum Requirement for BAEC-CM Effect

|     | Maximum percent inhibition |        |         |         |                  |
|-----|----------------------------|--------|---------|---------|------------------|
|     | 10% CS                     | 20% CS | 10% FCS | 20% FCS | 10% CS + 10% FCS |
| -CM | 0                          | 0      | 42      | 20      | 12               |
| +CM | 34                         | 30     | 91      | 87      | 80               |

Calf SMC were plated and growth-arrested as described in Materials and Methods. The cells were exposed to the indicated final concentrations of sera with or without BAEC-CM. The data are from a representative experiment in which the calf SMC exposed to CM did not round up and detach.

The overall metabolic rate of confluent BAEC in serum-free medium as measured by the rate of protein synthesis was decreased by only 30% (data not shown). This suggests that production of the inhibitory activity by BAEC depends to a large extent on the presence of serum. FCS and CS were equally effective in releasing the inhibitory activity.

If growth-arrested calf SMC were exposed to a 1:1 mixture of BAEC-CM and RPMI + FCS (final FCS concentration, 20%), the growth inhibition shown in Fig. 1 occurred. However, if growth-arrested calf SMC were exposed to a 1:1 mixture of BAEC-CM and DMEM + CS (final CS concentration, 10%), much less inhibition was observed (Table II). Calf SMC are usually grown in DMEM + 10% CS; when grown in RPMI + 20% FCS, they grow at a slightly slower rate and reach 80–90% of the final cell density of calf SMC grown in DMEM + 10% CS. Mixing CS and FCS does not block the inhibitory activity (Table II). Growth-arrested calf SMC released into DMEM + 20% CS grow at the same rate as they do in DMEM + 10% CS; the inhibitory effect of BAEC-CM when the final CS concentration is 20% is similar to the effect when the final CS concentration is 10% (Table II). When  $G_0$  calf SMC are exposed to RPMI + 10% FCS, they reach ~60% of the final cell density of calf SMC grown in DMEM + 10% CS. The inhibitory effect of BAEC-CM when the final FCS concentration is 10% is slightly greater than in 20% FCS because the cells do not go through even one doubling. These data suggest

that BAEC-CM interacts with something present in greater concentrations in FCS than in CS to generate the inhibitory activity. However, we cannot rule out the possibility that substances present in CS compete with the SMC for the inhibitor. Similar experiments cannot be done with rat SMC because these cells do not grow well in CS. In summary, the release of inhibitory activity by BAEC requires the presence of a small amount of either FCS or CS; however, the inhibitory effect is greater in the presence of FCS than in CS.

### Effect of Passage Number

CM from confluent BAEC at different passages was collected as described in Materials and Methods. It was tested for inhibitory activity on second or third passage rat SMC as described in Materials and Methods. The results are presented in Fig. 2A as the maximum percent inhibition. The data demonstrate that the inhibitory activity in confluent BAEC-CM decreases rapidly as the passage number of the BAEC increases.

Fig. 2B shows the results of experiments in which confluent primary BAEC-CM was tested on rat SMC at different passages. These data show that the inhibition by BAEC-CM decreases as the passage number of rat SMC increases. Calf SMC were always used between passages one and three because they tended to become pleiomorphic at later passages. We have generally noted that third passage calf SMC were slightly less responsive to BAEC-CM (not shown).

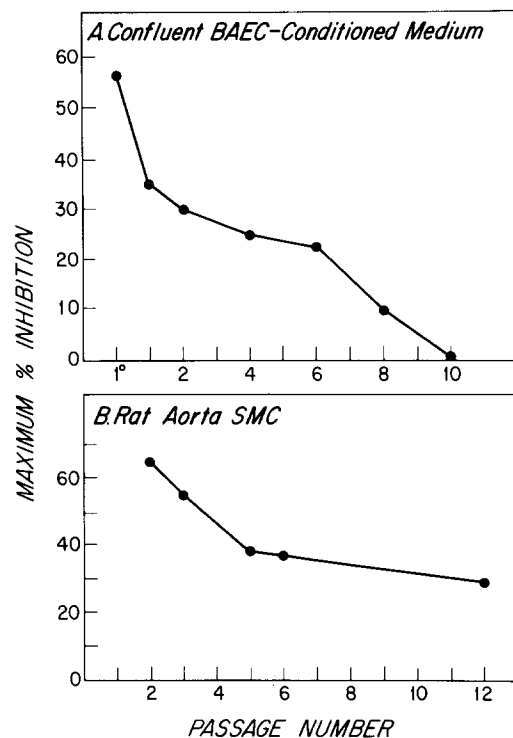


FIGURE 2 Effect of passage number on BAEC-CM effect. (A) The effect of confluent BAEC-CM from the indicated passage numbers on the growth of second or third passage rat SMC was assayed as described in Materials and Methods. (B) The effect of confluent primary BAEC-CM on the growth of rat SMC at the indicated passage numbers was assayed as described in Materials and Methods. All passage number data are the average of three or more experiments, except for BAEC-CM at passages 6, 8 and 10, which were done twice.

## Biochemical Characterization of the Inhibitory Activity

We subjected the CM obtained in 0.4% serum to a variety of treatments to characterize it. To test the heat sensitivity of the inhibitory activity, aliquots of CM were heated at 60°C for 30 min, or at 90°C for 5 min. CM treated this way lost <20% of its activity (Table III).

To determine whether the inhibitory activity was a protein(s), we treated it with 30 µg/ml trypsin for 2 h at 37°C. A tenfold excess of soybean trypsin inhibitor in a small volume was added 30 min before mixing the CM with RPMI + FCS (final concentration FCS 20%). Separate aliquots of CM were treated with 30 µg/ml chymotrypsin for 2 h at 37°C, or with 30 µg/ml subtilisin (Carlsberg) for 60 min at 37°C. A tenfold weight excess of chick egg white ovinhibitor was added to the chymotrypsin-treated CM at least 30 min before mixing the CM with RPMI + FCS (final concentration FCS 20%). Subtilisin-treated CM was heated at 90°C for 5 min to kill the enzyme activity. Less than 10% of the inhibitory activity was lost after these protease treatments (Table III). To control for nonspecific protease or inhibitor effects, the same concentration of proteases and inhibitors (or boiled enzyme in the case of subtilisin) were mixed together for 30 min, then added to RPMI + 20% FCS or the 1:1 CM-RPMI + FCS (final concentration FCS 20%) mixture. The inhibitory activity was unaltered by these treatments.

BAEC-CM was incubated with 1 U/ml of chondroitin ABC lyase for 90 min at 37°C. Separate aliquots of BAEC-CM were treated with 15 U/ml hyaluronidase for 90 min at 37°C. The samples were then boiled for 5 min, a treatment which kills the enzyme activity but does not affect the inhibitory activity. These enzyme treatments resulted in little or no loss of activity (Table III). Boiled hyaluronidase or chondroitin ABC lyase added to RPMI + 20% FCS did not affect SMC growth.

When BAEC-CM was incubated with 10 U/ml purified *Flavobacterium* heparinase for 90 min at 37°C, 80% of the inhibitory activity was lost (Table III). The heparinase preparation has no detectable protease activity, even when tested in a sensitive assay system (17). In addition, the inhibitory activity in BAEC-CM was insensitive to digestion by a broad range of proteases, as described above. The heparinase is specific for a heparin or heparinlike substrate, in that it requires a 1→4 linkage between sugar residues and also requires sulfated iduronic acid or N-sulfated glucosamine as a substrate. Thus, it will not degrade chondroitin sulfates, dermatan sulfate, or hyaluronic acid. The inhibitory activity in BAEC-CM therefore appears to be heparin or a heparinlike molecule.

Two other lines of evidence support this conclusion. First, a crude isolate of glycosaminoglycans from BAEC-CM was prepared by the method of Dietrich and de Oca (7). The TCA-soluble, ethanol-precipitable material obtained was reconstituted in the starting volume of RPMI + 20% FCS. When rat or calf SMC were exposed to this medium, their growth was inhibited with the same kinetics displayed in Fig. 1, although the calf SMC did not round up and detach in these experiments. Treating the crude isolate of GAGs with heparinase destroyed the inhibitory activity. GAGs isolated in the same manner from unconditioned medium (i.e., RPMI + 0.4% FCS) had no effect on SMC growth.

The second approach was to examine the effect of exogenous GAGs on SMC growth. When chondroitin sulfate ABC, dermatan sulfate, hyaluronic acid, or two types of heparan sulfate

TABLE III  
Biochemical Properties of the BAEC-CM Inhibitory Activity

| Treatment   | Maximum percent inhibition |         |
|---|----------------------------|---------|
|   | Calf SMC                   | Rat SMC |
| None  | 84 ± 8                     | 58 ± 5  |
| 60°C, 30 min  | 76 ± 9                     | 53 ± 10 |
| 90°C, 5 min   | 93 ± 4                     | 62 ± 5  |
| Trypsin<br>(30 µg/ml, 2 h, 37°C)                                | 82 ± 5                     | 60 ± 10 |
| Chymotrypsin<br>(30 µg/ml, 2 h, 37°C)                           | 85                         | 54      |
| Subtilisin<br>(30 µg/ml, 60 min, 37°C)                          | —                          | 51      |
| Hyaluronidase<br>(15 U/ml, 90 min, 37°C)                        | 94 ± 4                     | 50      |
| Chondroitin SO <sub>4</sub> ABC lyase<br>(1 U/ml, 90 min, 37°C) | 88 ± 7                     | 58 ± 2  |
| Heparinase<br>(10 U/ml, 90 min, 37°C)                           | 19                         | 9 ± 6   |

CM from BAEC was subjected to the indicated treatments and then assayed for its ability to inhibit growth-arrested calf and rat SMC when mixed 1:1 with RPMI + FCS (final concentration 20% FCS). SD are given in those instances when the treatments were performed three or more times. In other cases, the treatments were performed twice.

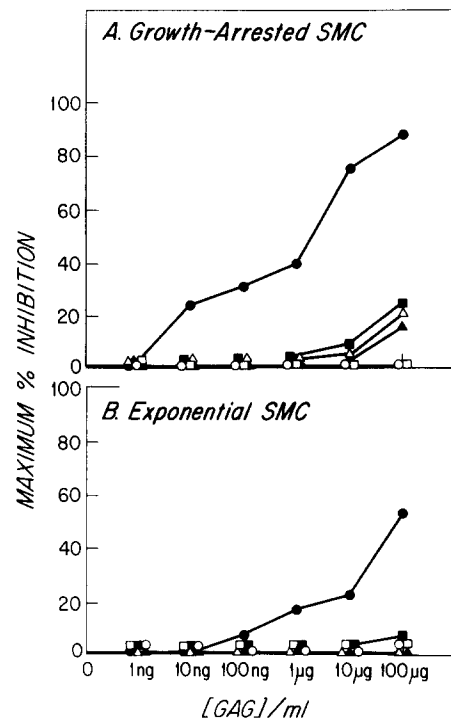


FIGURE 3 Effect of GAGs on rat SMC growth. (A) Rat SMC were plated and growth-arrested as described in Materials and Methods. The cells were then exposed to RPMI + 20% FCS containing the indicated concentrations of GAGs. Cell number was measured at daily intervals. Data are expressed as maximum percent inhibition. (B) Rat SMC were plated at  $6 \times 10^3$ /16-mm well. After 24 h, the medium was aspirated and replaced with fresh RPMI + 20% FCS with or without the indicated concentrations of GAGs. Data are expressed as maximum percent inhibition. ●—●, heparin; □—□, bovine aortic heparan sulfate; ■—■, beef lung heparan sulfate; △—△, dermatan sulfate; ▲—▲, chondroitin ABC sulfate; ○—○, hyaluronic acid. Data for the heparan sulfates are the average of three experiments; data for other GAGs are the average of five or more experiments.

were added to either growth-arrested or exponential cultures of rat SMC, no inhibition of growth occurred at concentrations up to 10  $\mu\text{g/ml}$  (Fig. 3). At 100  $\mu\text{g/ml}$ , slight inhibition (up to 20%) of growth-arrested cells was observed with these GAGs (Fig. 3). In contrast, heparin inhibited SMC growth by 50% at 1–2  $\mu\text{g/ml}$  in growth-arrested SMC, and at 90–100  $\mu\text{g/ml}$  in exponential SMC (Fig. 3). Anticoagulant and non-anticoagulant heparin (21) were equally effective in inhibiting SMC proliferation.

Note that growth-arrested SMC are nearly 100 times more sensitive to heparin than exponential SMC (Figs. 3 and 4). Although the  $\text{ED}_{50}$  for heparin is rather variable from experiment to experiment, the 100-fold difference in sensitivity to heparin was very consistent.

We also examined the effect of heparin on BAEC growth. Exponential and growth-arrested BAEC were not inhibited by heparin at doses <100  $\mu\text{g/ml}$  (Fig. 4). There was actually a slight stimulation of BAEC, which ranged from 10–30% in different experiments. This is in sharp contrast to the effect of heparin on SMC. Heparin did not affect the growth of either exponential or growth-arrested BHK cells at doses <100  $\mu\text{g/ml}$  (data not shown).

### Mitogenic Activity in BAEC-CM

When we attempted to fractionate BAEC-CM on Amicon filters, nearly all the inhibitory activity was lost. However, we noticed a small but consistent increase in cell number when the 10,000- to 100,000-dalton and >100,000-dalton fractions were added to RPMI + 20% FCS. In light of the recent observations by Gajdusek et al. (11) that BAEC secrete a SMC mitogen, we fractionated CM from exponential BAEC at passages five through seven. The mitogenic potential of these Amicon fractions was assayed on rat SMC as described in Materials and Methods. The >100,000-dalton fraction supported two rounds of SMC division, whereas the 1% FCS (final concentration) control supported less than one doubling (Fig. 5). The 10,000- to 100,000-dalton fraction had even greater mitogenic activity, but the background from this fraction of

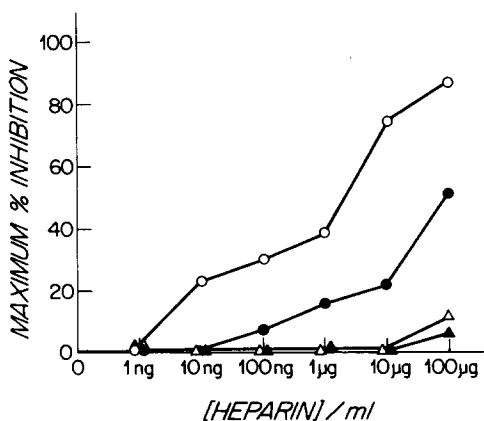


FIGURE 4 Effect of heparin on BAEC and rat SMC growth. Exponential cultures of BAEC, and exponential and growth-arrested cultures of rat SMC were obtained as described in Materials and Methods. Growth-arrested BAEC were obtained by placing  $6 \times 10^3$  BAEC growing in 16-mm wells in RPMI + 0.4% FCS for 72 h. These cells were exposed to the indicated concentrations of heparin as described in Fig. 3.  $\circ$ — $\circ$ , growth-arrested rat SMC;  $\bullet$ — $\bullet$ , exponential rat SMC;  $\Delta$ — $\Delta$ , growth-arrested BAEC;  $\blacktriangle$ — $\blacktriangle$ , exponential BAEC. Data are the average of five or more experiments.

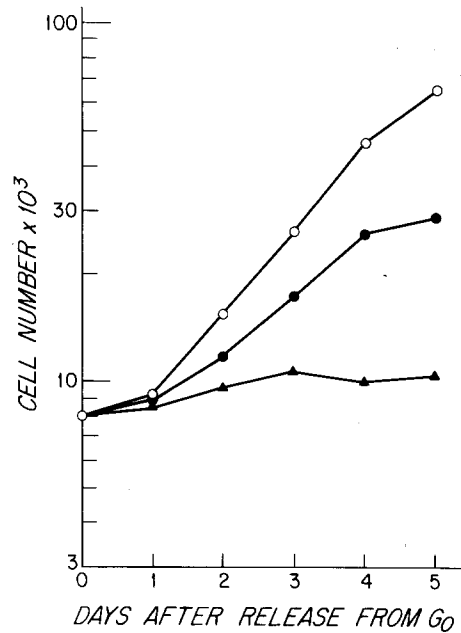


FIGURE 5 Mitogenic activity in fractionated BAEC-CM. CM from exponential BAEC (passages 5–7) was fractionated using Amicon filters. The ability of a concentrated >100,000-dalton fraction ( $\bullet$ — $\bullet$ ) to stimulate the growth of  $G_0$ -arrested rat SMC was assayed as described in Materials and Methods, and compared to growth in 20% FCS ( $\circ$ — $\circ$ ) and 1% FCS (final concentration) + concentrated 0.4% FCS ( $\blacktriangle$ — $\blacktriangle$ ; see Materials and Methods). Data are from a representative experiment.

concentrated RPMI + 0.4% FCS was also higher and more variable. This is probably the result of concentrating the low molecular weight growth factors, e.g., platelet factors, known to be present in serum. The results from this fraction are thus more difficult to interpret. These results confirm the observations of Gajdusek et al. (11) on the production of an SMC mitogen by BAEC.

### DISCUSSION

We have shown that cultured BAEC are capable of secreting both positive and negative effectors of SMC growth. The mitogen is probably similar to the one described by Gajdusek et al. (11). The inhibitor appears to be heparin or a heparinlike molecule. Using CM from confluent cultures of primary BAEC, we observed a slow-onset inhibition of proliferation of growth-arrested SMC (Fig. 1). The difference between our observations of an inhibitory activity in BAEC-CM and those of Gajdusek et al., in which a mitogenic activity dominates, are due likely to one or more of the following differences in the systems used: (a) we collected CM only from primary BAEC that have been confluent for at least a week, whereas Gajdusek et al. used later passage BAEC to collect CM; (b) we collected our CM in the presence of serum, whereas Gajdusek et al. used plasma-derived serum; and (c) we used growth-arrested SMC to assay for inhibitory activity, whereas Gajdusek et al. used exponential SMC to assay for mitogenic activity.

The issue of cytotoxicity is difficult to address directly. Several observations suggest that the inhibitory effect of BAEC-CM is not strictly cytotoxic: (a) the rat SMC never detach, and the calf SMC detach only two-thirds of the time in experiments using 20% FCS; (b) experiments in which the final concentration of FCS was varied suggest that the detachment of calf SMC may be an artefact of serum concentrations; (c)

the medium does not contain detached cells (except in those calf SMC experiments in 20% FCS in which cells detach); (d) daughter cells are able to spread normally after cytokinesis; (e) flow microfluorimetry shows that both control and CM-exposed SMC have the same cell cycle distribution at the end of the experiment; (f) the CM-treated SMC never take up trypan blue; and (g) the BAEC-CM effect is reversible. Even if the growth inhibition is due to cytotoxicity, however, it is still important, because the inhibitory activity is specific for both producer and target cell (Table I). This type of specificity could be important in: (a) the normal vessel, in which this mechanism of SMC growth regulation may be involved in maintaining the SMC in a quiescent growth state; (b) the injured vessel, where the cessation of myointimal proliferation occurs after re-endothelialization of the damaged area (9); and (c) regulation of blood vessel morphogenesis during development and remodeling of the vasculature. The lack of inhibition of BAEC growth by BAEC-CM is corroborated by the observation that commercially-prepared heparin, while inhibiting SMC proliferation, did not inhibit BAEC (Fig. 4) or BHK cells.

Serum is required for production of the heparinlike inhibitor by BAEC. The nature of the serum requirement is not known, but at least two general possibilities exist: (a) serum provides a necessary biosynthetic precursor or cofactor for heparin synthesis; and (b) serum provides a substance that releases heparin from the endothelial cell surface. Because heparan sulfate has been shown to cause release of more heparan sulfate from the cell surface (4, 5), it is possible that the serum provides either heparan sulfate or an enzyme which liberates heparin. These possibilities remain to be tested.

The secretion of a heparinlike inhibitor of SMC by BAEC fits in well with observations made in this and other laboratories. Buonassisi (3), Gamse et al. (12), and Busch et al. (5) have shown that cultured BAEC secrete heparan sulfates (of which heparin may be considered a subclass). Indeed, about half of all the GAGs synthesized by BAEC are heparan sulfates secreted into the medium. In contrast, SMC and fibroblasts secrete only ~10% of their GAGs as heparan sulfates (12, 20, 31). In addition, Gamse et al. (12) found that the heparan secreted by BAEC was more heparinlike (i.e., more highly sulfated) than the heparan made by bovine aortic SMC.

The BAEC must be confluent before the inhibitory activity can be detected. Indeed, exponentially growing BAEC produce a SMC mitogen (Fig. 5). We suggest that the cessation of BAEC growth may be an important step in the regulation of vascular smooth muscle cell growth in vivo. This idea is supported by recent observations that in a nonregenerating (i.e., quiescent) vascular bed, heparan sulfates are the predominant cell-associated GAGs, whereas in an actively regenerating vascular bed, the amount of heparan sulfate is greatly diminished (1).

When purified GAGs were added to growth-arrested SMC cultures, heparin was by far the most potent inhibitor of growth (Fig. 3). Preliminary experiments (not shown) suggest that the degree of sulfation plays an important role in the antiproliferative effect of heparin. This idea is supported by the fact that the inhibitory activity of GAGs correlates with their degree of sulfation; heparin has 2.5–3 sulfate groups per hexosamine; lung heparan sulfate, dermatan sulfate, and chondroitin ABC sulfate have about one sulfate per hexosamine; aortic heparan sulfate and hyaluronic acid contain 0.3 and zero sulfates per hexosamine, respectively.

The effect of heparin on SMC proliferation in vivo has been

studied in this laboratory for several years. Clowes and Karnovsky (6), using an air-dry model for vessel injury (9), showed that heparin markedly suppressed myointimal proliferation when infused in rats 24 h after the injury. More recently, Guyton et al. (14), also using the air-dry model for vessel injury, found that heparin prevented myointimal proliferation, and that anticoagulant and non-anticoagulant heparin were equally effective. In a series of parallel studies, Hoover et al. (18) demonstrated that heparin blocked the stimulation of cultured rat SMC growth by platelet extract, and showed that the inhibition was probably not mediated via a direct interaction with platelet-derived growth factor. Again, anticoagulant and non-anticoagulant heparin were equally effective. The experiments using exogenous heparin and other GAGs reported here confirm and extend these previous studies.

Based on the in vivo and in vitro demonstrations that non-anticoagulant heparin is as potent as anticoagulant heparin in inhibiting SMC growth, it is possible that non-anticoagulant heparin might be useful in those clinical situations (balloon embolectomy, endarterectomy, vein grafting, etc.) in which suppression of myointimal proliferation is important to the patient, because the problem of increased bleeding that accompanies the use of anticoagulant heparin would be avoided. Synthetic, non-anticoagulant analogs of heparin which retain their SMC antiproliferative capacity might also be useful. The growth-arrested SMC assay would provide a good screening system for developing such compounds.

Experiments are now underway to examine the nature of the serum requirement for production and release of heparin from BAEC, the chemical structure of antiproliferative heparin, and the mechanism of heparin-induced inhibition of SMC proliferation. This system should prove useful in elucidating the molecular mechanisms involved in the regulation of vascular cell growth.

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