

The Role of Membrane–Membrane Interactions in the Regulation of Endothelial Cell Growth

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ABSTRACT A cell surface preparation from confluent endothelial cells can inhibit DNA synthesis of actively growing endothelial cells. The decrease in the rate of [³H]thymidine incorporation is concentration dependent and levels off at 47% of the control. The preparation has no effect on the growth of vascular smooth muscle cells. A similar preparation from smooth muscle cells does not show inhibitory activity with either endothelial or smooth muscle cells. The inhibition of growth can also be demonstrated by a decrease in thymidine index and growth rate. The inhibition is transient and after 48 h, the growth rate is similar to that of the control. In a wound edge assay, both migration and proliferation are inhibited. The inhibitory activity is partially labile to trypsin and abolished by pepsin, heating at 100°C, or reduction. Cell surface iodination and analysis of the proteins removed by urea treatment by SDS polyacrylamide gel electrophoresis show at least 11 bands with apparent molecular weights from 250,000 to 18,000. These radiolabeled proteins, as well as the active component of the cell surface preparation, are sedimentable at 100,000 g for 1 h. They are both solubilized in 30 mM octyl glucoside but not by treatment with 0.1 M sodium carbonate, pH 11.5. These results suggest that the activity is due to a cell-surface membrane fraction and may provide a basis for studying the mechanism of density-dependent inhibition of growth in a normal cell of defined origin.

Growth of endothelial cells is characterized by the formation of a highly ordered monolayer. There is considerable evidence that cell density and cell–cell contact play prominent roles in the control of endothelial cell growth (1). Whereas growth of endothelial cells from a sparse density may be dependent on growth factors, the final cell density is independent of serum concentration (2, 3). Once a contiguous monolayer is formed, stimulation of endothelial cell growth has only been shown for agents that disrupt the continuity of this structure (4–6). Growth cannot be reinitiated by an increase in the serum concentration or by the addition of growth factors.

Further evidence that cell–cell contact is a central factor in controlling endothelial cell growth is shown by cells, *in vivo* or *in vitro*, regenerating at a wound edge (7–11). Endothelial cells respond to a wound by migrating as a highly coordinated cell sheet. Approximately 24 h after wounding, endothelial cell movement is followed by cell replication (1). When this movement is inhibited by cytochalasin B, the cells fail to enter the cell cycle, which suggests some critical event associated with rearrangement of cell–cell relationships in the advancing sheet (12).

Whereas the contact inhibition of movement (13, 14) is a

well-established phenomenon, inhibition of growth by cell–cell contact is controversial. A number of studies have centered on the density-dependent inhibition of growth observed in 3T3 cells. This established cell line was originally selected by repeated passage at low density (15). The saturation density of 3T3 cells is markedly dependent on the concentration of serum, and that concentration effect appears to be attributable to the availability of specific growth-promoting substances in the medium and release of cellular growth inhibitors (16–20). Growth of 3T3 cells can be reinitiated by the addition of mitogens (21). Growth at wound edges is not inhibited by cytochalasin B (22). This has led to the careful distinction of density-dependence as being a general phenomenon as opposed to more specific mechanisms requiring cell–cell contact.

More direct support for density-dependent inhibition of growth by cell–cell contact has come from reports of cell surface membrane fractions that can regulate the replication of cultured 3T3 cells (23–25). Lieberman and Glaser (26) have suggested that such surface components may be similar to factors that are involved in cell–cell adhesion. Whereas none of these factors have been purified, they seem to share certain properties: they specifically inhibit the cell of origin;

they do not inhibit transformed cells; inhibition of replication does not appear to be total; and the inhibition is reversible.

There has been no direct evidence for a similar membrane component involved in growth control of the endothelium. There is, however, evidence that the composition of the endothelial cell surface is dependent on cell-cell contact (27). Striking differences were observed in the cell surface proteins available to lactoperoxidase-catalyzed iodination when confluent endothelial cells were compared with growing cells. Fibronectin and a 60-kD cell surface protein, CSP-60, were described as being uniquely present at saturation density. Disruption of the monolayer by mild urea treatment showed a loss of both CSP-60 and fibronectin. Reformation of the monolayer appeared to correlate with the reappearance of CSP-60. We have confirmed these results and found that CSP-60 is markedly decreased on sparsely plated cells but reappears rapidly when cells are plated at confluent density. Enzymatic iodination of smooth muscle cells shows a different pattern that lacks a cell surface protein corresponding to CSP-60.

We report here that a urea extract from confluent endothelial cells contains a number of cell surface proteins, including CSP-60. A protein in this extract appears to be capable of inhibiting endothelial cell replication in a way similar to the effect reported by others for 3T3 cell membrane fractions (23).

MATERIALS AND METHODS

Cell Cultures: Endothelial cells isolated from bovine aorta were grown in culture as previously described (28). When confluent, the cells (passage 6–12) were fed weekly with Waymouth's complete medium supplemented with 10% fetal bovine serum (KC Biological, Lenexa, KS). Smooth muscle cells were isolated from explants of bovine aorta and were used between passage two and four (29). Cultures were periodically screened for mycoplasma by DNA fluorescent staining.

Radiolabeling of Cultured Cells: Cultures that were nearly confluent were metabolically labeled for 12 h using 20 $\mu\text{Ci/ml}$ L-[^{35}S]methionine (970 Ci/mmol, New England Nuclear, Boston, MA) in serum-free Waymouth's methionine-free medium. Cell surface labeling was carried out using a modification (manuscript submitted for publication) of the method of Hubbard and Cohn (30). In brief, confluent endothelial monolayers were rinsed twice with Dulbecco's phosphate-buffered saline (PBS)¹ and then with cold PBS containing 10 mM glucose. Lactoperoxidase (Calbiochem-Behring Corp., La Jolla, CA) covalently attached to cyanogen bromide-activated Sepharose beads (10 mU/ml, Pharmacia Inc., Piscataway, NJ), glucose oxidase (5 mU/ml), and 150 $\mu\text{Ci/ml}$ Na- ^{125}I (carrier-free, New England Nuclear) were added. After 20 min at 4°C, the reaction medium was removed, and the cell layer was washed four times with cold PBS containing 1 mM KI.

Isolation of Cell Surface Fraction: For the isolation of surface proteins, cells were cultured in 100-mm dishes. Confluent cultures were washed in PBS and then incubated in serum-free media for at least 30 min. The cells were washed with PBS and then incubated with 7.5 ml of freshly prepared 1 M urea (ultra pure, Schwarz/Mann, Cambridge, MA) in PBS, pH 7.3, for 10 min at 37°C. The extract was removed, the cell layer was washed with 5 ml PBS, and fresh medium was added back. The combined wash and extract were centrifuged at 2,000 g for 10 min, the supernatant was chilled, and phenylmethylsulfonyl fluoride (0.1 mM) (Pierce Chemical Co., Rockford, IL) was added. The supernatant was then dialyzed overnight at 4°C against 10 mM acetic acid and was lyophilized. Cell loss from the extraction was <0.01% of the total. Dialysis tubing (M_r 12,000 cutoff) was prepared by boiling in sodium bicarbonate, EDTA, and distilled water.

Alternatively, the urea extract was centrifuged in a Type 60 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) at 100,000 g for 1 h at 4°C in a Beckman L2-65B ultracentrifuge. The pelleted samples were suspended with either 0.1 M sodium carbonate, pH 11.5 (31), 5 mM EDTA in calcium- and magnesium-free phosphate-buffered saline, or with 30 mM octyl glucoside (Calbiochem-Behring Corp.) in 10 mM HEPES, pH 7.4 (32), and centrifuged

at 100,000 g for 1 h in a Type 50.3 Ti rotor (Beckman Instruments, Inc.). The 100,000 g urea extract (0.2 mg protein) was incubated in 2 ml of 30 mM octyl glucoside in 10 mM HEPES, pH 7.4, and 0.01 mM dithiothreitol for 30 min at 0°C. The octyl glucoside-solubilized sample was exhaustively dialyzed against 10 mM HEPES, pH 7.4, at 4°C and then against PBS.

Inhibition Assay: To measure DNA synthesis, cells (1×10^4) were plated in 2-cm² wells in 0.5 ml medium containing 10% fetal bovine serum. After 48 h, 0.2 ml of PBS containing the suspended extract was added. The lyophilized extract was previously suspended in PBS and dialyzed overnight at 4°C against PBS, then sterilized under ultraviolet light before being added to cells. Samples were added in triplicate, then 24 h later pulsed for 2 h with 1 $\mu\text{Ci/ml}$ [^3H]thymidine ([^3H]TdR) (6.7 Ci/mmol, New England Nuclear). Cells were washed twice with cold PBS and incubated with 2 ml 10% trichloroacetic acid at 4°C for 30 min. Cells were then washed once with cold 10% trichloroacetic acid and solubilized with 0.3 ml of 0.5 M NaOH. This was transferred to a scintillation vial with 0.3 ml water and 5 ml Aquasol (New England Nuclear). Cell number was determined in triplicate using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) after the cells were detached from the culture dishes with 0.05% trypsin in 0.54 mM EDTA in calcium- and magnesium-free phosphate-buffered saline. Trypsinized cultures were checked by microscopic examination to ensure that all cells were removed.

Autoradiography was performed by labeling the cells (2×10^4) in 35-mm culture dishes as described above with [^3H]TdR for 2 h. Cells were rinsed with PBS at 4°C and fixed in 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for at least 1 h. Cells were then rinsed with PBS containing 50 mM glycine, were dehydrated, and layered with NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, NY). After 2 wk of exposure at 0°C, the emulsion was developed in D19, fixed, and finally stained with hematoxylin. The percentage of labeled nuclei was quantitated by light microscopy.

Wounding Procedure: Cells were first grown to stationary density in 35-mm culture dishes. Wounds were made with a 12-mm-wide fragment of a stainless steel razor blade drawn laterally across the dish surface (33). Wells were rinsed once with PBS to remove nonadherent cells, and fresh medium with the sample to be tested was added. For autoradiography, [^3H]TdR was added to cultures to a final concentration of 1 $\mu\text{Ci/ml}$ for 2 h before termination of the experiment.

Characterization of Cell Surface Proteins: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed on 6–15% gradient slab gels (34). Lyophilized samples or radiolabeled cultures were suspended in sample buffer with or without 4% 2-mercaptoethanol and placed immediately in a boiling water bath for 2 min. After electrophoresis, the gel was stained with Coomassie Blue G250 and either dried for autoradiography and exposed with an enhancing screen (lightning plus, DuPont Co., Wilmington, DE) or prepared for fluorography (35). The following proteins were used to calculate apparent molecular weights: chicken muscle myosin heavy chain (M_r 200,000), β -galactosidase (M_r 116,000), phosphorylase B (M_r 94,000), bovine serum albumin (BSA) (M_r 67,000), ovalbumin (M_r 43,000), and carbonic anhydrase (M_r 30,000). Protein was measured according to the method of Lowry (36), using BSA as a standard. The following enzymes were assayed as indicated: alkaline phosphodiesterase I (EC 3.1.4.1) (37), 5'-nucleotidase (EC 3.1.3.5) (38), glucose-6-phosphatase (EC 3.1.3.9) (39), and acid phosphatase (EC 3.1.3.2) (23). The specific activities of the enzymes are reported as $\mu\text{mol product} \times \text{h}^{-1} \times \text{mg protein}^{-1}$.

Chemicals: Trypsin (bovine pancreas, TPCK-treated 241 U/mg), soybean trypsin inhibitor, and pepsin (2,600 U/mg) were from Worthington Biochemical Corp. (Freehold, NJ). Pepstatin A was from Sigma Chemical Co. (St. Louis, MO), and guanidine hydrochloride from Heico Inc. (Mendota, IL). All other materials were reagent grade or better.

RESULTS

Cell Surface Proteins

Under mild conditions, iodination with lactoperoxidase attached to beads selectively modifies proteins on the upper cell surface of confluent endothelial cells. In autoradiograms of thick sections, >85% of the grains are present in the upper half of the cell (manuscript submitted for publication). Analysis of solubilized preparations by SDS PAGE (Fig. 1, gels 5 and 6) shows a simple pattern of radiolabeled bands ranging in molecular weight from 241,000–13,000 with five major heavily labeled bands. The matrix component, fibronectin, represents <1% of the radiolabeled cell surface proteins. This

¹ Abbreviations used in this paper: PBS, Dulbecco's phosphate-buffered saline; TdR, thymidine.

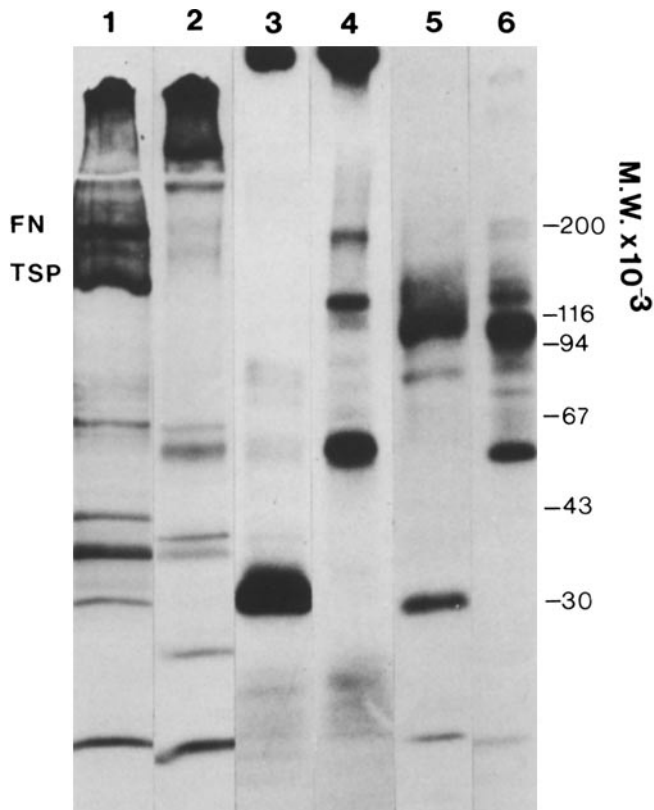


FIGURE 1 SDS PAGE of urea released endothelial cell surface proteins. Cultures that were nearly confluent were metabolically labeled for 12 h with L-[³⁵S]methionine in serum-free Waymouth's methionine-free medium. To radiolabel the cell surface, lactoperoxidase covalently attached to Sepharose beads, glucose oxidase, and Na-¹²⁵I were added to confluent endothelial cultures in PBS containing 10 mM glucose. Cell surface proteins were prepared by treating a confluent monolayer with 1 M urea in PBS for 10 min at 37°C. The extract was removed, the cell layer was washed, and the combined fractions were dialyzed and lyophilized. Solubilized samples were analyzed on 6.5–15% polyacrylamide slab gels. After staining, the gels were dried for autoradiography or prepared for fluorography. Gel 1, urea extract prelabeled with [³⁵S]methionine, reduced. Gel 2, urea extract prelabeled with [³⁵S]methionine, non-reduced. Gel 3, urea extract prelabeled ¹²⁵I, reduced. Gel 4, urea extract prelabeled with ¹²⁵I, non-reduced. Gel 5, total cell surface ¹²⁵I-labeled, reduced. Gel 6, total cell surface ¹²⁵I-labeled, non-reduced. FN, fibronectin; TSP, thrombospondin.

is consistent with the previous demonstration of fibronectin that is on the basal surface only of confluent cultures of endothelial cells (40). Intracellular proteins, such as actin or vimentin, were not radiolabeled. We have used this approach to specifically label the cell surface.

Analysis of Extracted Proteins

Treatment of cells with low concentrations of urea has previously been used to isolate cell surface proteins (24, 41). In the presence of 1 M urea for 10 min, confluent endothelial cells retract and become rounded, thus disrupting the continuity of the monolayer. Numerous blebs are seen protruding from the cell surface while the cells remain attached to the dish by cytoplasmic processes. After washing and re-feeding the cells, we show by time-lapse video microscopy that the cells regain their normal flattened morphology within 30 min; by 2 h, <1% of the cells exhibit an abnormal cell surface. Less

than 4% of the cells are trypan blue positive. Viability is also demonstrated by the ability of urea-treated cells to proliferate when they are re-plated. Extraction after prelabeling with L-[³⁵S]methionine and analysis of the released proteins by SDS PAGE (Fig. 1, gels 1 and 2) show that numerous proteins are present in the extract. One of the cell surface proteins extracted from fibroblasts by urea treatment was fibronectin (41). Our results are similar. Two of the high molecular weight components released by urea were identified as fibronectin and thrombospondin by immunoblotting (42).

Extraction of the confluent cell layer with urea also removes ~16% of the iodinated cell surface proteins. Autoradiography of the extract after SDS PAGE (Fig. 1, gels 3 and 4) shows that several cell surface labeled proteins are removed by urea treatment. After reduction, seven radiolabeled protein bands are seen with apparent molecular weights of 98,000, 92,000, 84,000, 70,000, 62,000, 30,000, and 20,000. Longer exposure of the autoradiogram shows additional radiolabeled bands at molecular weights of 220,000, 140,000, 116,000, and 54,000. One of the major cell surface iodinated proteins, CSP-60, has an apparent molecular weight before reduction of 60,000, which after reduction appears at 30,000. It is the major iodinated protein in the urea extract and is enriched fourfold. Our previous experiments have confirmed the studies by Vlodaysky and co-workers (27) on the appearance of CSP-60 after trypsinization and re-plating. CSP-60 is evident only when cells become confluent. It is only weakly labeled on subconfluent growing endothelial cells.

Growth Regulation

To examine whether the cell surface extract from confluent endothelial cells might have any influence on growth, the urea extract was concentrated by lyophilization after dialysis and was added to sparsely growing endothelial cells for 24 h (Fig 2A). DNA synthesis, measured as the rate of [³H]TdR incorporation into acid insoluble counts, decreased with addition of a suspension of the lyophilized extract. Addition of the urea extract decreased the rate of [³H]TdR incorporation by endothelial cells in a concentration-dependent manner, leveling off at 47% of the control rate at 1.5 μg of protein added. Endothelial cultures receiving equivalent amounts of a PBS extract from confluent endothelial cells were not affected. At a concentration of urea extract that maximally inhibited endothelial cells, sparse smooth muscle cells showed no change in the rate of DNA synthesis (Fig. 2B). At concentrations of endothelial urea extract that were fully inhibitory, extracts from confluent smooth muscle cells produced either no inhibition or a slight inhibition of [³H]TdR incorporation rate of endothelial cells (Fig. 2A). Addition of the smooth muscle extract to sparse smooth muscle cells, which grow in a multilayered pattern, showed no effect compared with the control. Equivalent amounts of urea extract from confluent 3T3 cells also showed slight or no inhibition of endothelial cell growth but did inhibit the growth of 3T3 cells (results not shown). Lieberman and co-workers found similar results with 3T3 membranes that were not as effective in arresting growth of the human fibroblast cell line IMR 91 as were IMR 91 membranes (43).

Autoradiography of sparse endothelial cells labeled with [³H]TdR (Fig. 3) shows that the fraction of labeled nuclei decreases with increasing concentrations of cell surface extract. The extract from confluent endothelial cells did not decrease the fraction of labeled smooth muscle cell nuclei.

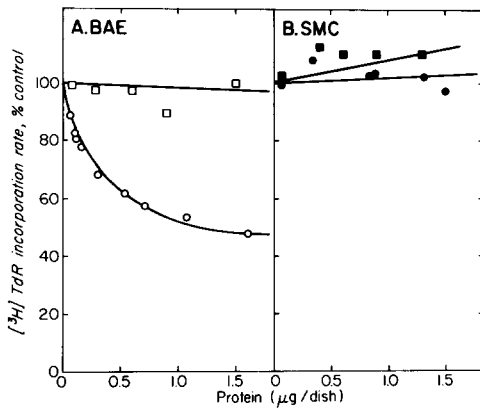


FIGURE 2 $[^3\text{H}]\text{TdR}$ incorporation rate by endothelial and smooth muscle cells as a function of cell surface extract from confluent endothelial or smooth muscle cells. To examine the effect of the urea extract on growth, the extract is concentrated by lyophilization after dialysis and is added to sparse cells growing in media containing 10% fetal bovine serum. 48 h after plating, 0.2 ml of PBS alone or samples in triplicate containing the suspended extract were added. $[^3\text{H}]\text{TdR}$ incorporation into DNA is determined 24 h later. The range of tritium counts incorporated into DNA in controls varied between 17,500 and 20,000 cpm. (A) \circ , urea extract from endothelial cells assayed with endothelial cells (BAE); \square , urea extract from smooth muscle cells assayed with endothelial cells (BAE). (B) \bullet , urea extract from endothelial cells assayed with smooth muscle cells (SMC); \blacksquare , urea extract from smooth muscle cells assayed with smooth muscle cells (SMC). All points are the mean from three independent determinations of total counts per well.

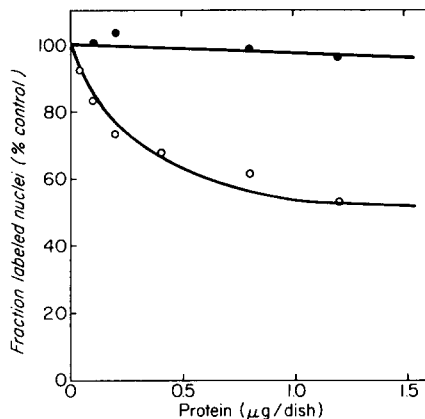


FIGURE 3 Fraction of labeled nuclei in sparse endothelial and smooth muscle cells. The cells were plated and treated as described in Materials and Methods. Cells were pulsed with $[^3\text{H}]\text{TdR}$, then washed with PBS and prepared for autoradiography. The percentage of cells with labeled nuclei for control cultures was 52 and 44 for endothelial and smooth muscle cells, respectively. \circ , urea extract from endothelial cells added to endothelial cells. \bullet , urea extract from endothelial cells added to smooth muscle cells.

The inhibition of $[^3\text{H}]\text{TdR}$ incorporation in growing endothelial cells treated with urea extract reflects a reduction in the percentage of cells synthesizing DNA. This was confirmed by an analysis of cell number as shown in Fig. 4. At a concentration of urea extract in which the effect was nearly maximal in $[^3\text{H}]\text{TdR}$ incorporation, a decrease in growth rate was also observed. Unless the cell surface material was re-added, the effect was transient and after 48 h, the growth rate returned to that of the control and proliferated to confluence. This indicates that the inhibitory effect is reversible. The cells in

the control have a doubling time of 28 h in the culture conditions. Repeated daily additions of extract maintained at 61% reduction in growth rate compared with the control rate. There was no striking difference in the morphology of the treated cells as compared with the control cells.

Effect on Migration and Replication

The effect of the urea-extracted cell surface components on the response of a monolayer to wounding is shown in Fig. 5. The number of cells migrating into the wound is inhibited by the addition of a centrifuged cell surface fraction (Fig. 5A). Migration across the scratch, defined by the number of nuclei crossing the wound, is reduced by 66%. The distance of migration of the leading cell is 56% that of the control (Fig. 5B). The morphology of the treated cells appears to be normal, and the inhibition does not appear to be the result of a toxic effect. In addition, the percentage of cells undergoing DNA synthesis is reduced as demonstrated by autoradiography. This is consistent with other studies that show that migration and proliferation are apparently linked in endothelial cells (5, 12).

Biochemical Characterization

The ability of the urea extract to inhibit DNA synthesis was significantly decreased by treatment with trypsin (Table I). Pepsin addition, heating at 100°C , or reduction with dithiothreitol under denaturing conditions abolished the inhibitory activity (44). To terminate the proteolytic digestions, a 10-fold molar excess of soybean trypsin inhibitor or pepstatin A was added to the appropriate reaction. The protease inhibitors had no effect on the inhibitory activity of control samples.

When the urea extract was centrifuged at $100,000\text{ g}$ for 1 h, all of the inhibitory material was pelleted. This indicates it is associated with a large molecular aggregate. Treatment of membranes with 0.1 M sodium carbonate buffer, pH 11.5, which converts closed vesicles into open membrane sheets, releases content proteins and extrinsic membrane proteins

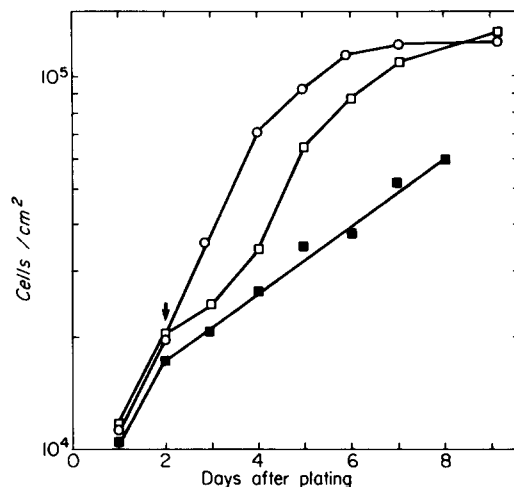


FIGURE 4 Growth rate of endothelial cells after addition of the cell surface extract from confluent endothelial cells. Endothelial cells (1×10^4) were plated in 0.5 ml of media containing 10% fetal bovine serum, and 48 h later the urea extract ($0.5\ \mu\text{g}$) was added as a single dose (\square) or a repeated addition (\blacksquare) every 24 h, changing the media at the time of each addition. A single addition (0.2 ml) of PBS (\circ) was added to the control. Arrow indicates initial addition. Cell numbers were determined in triplicate.

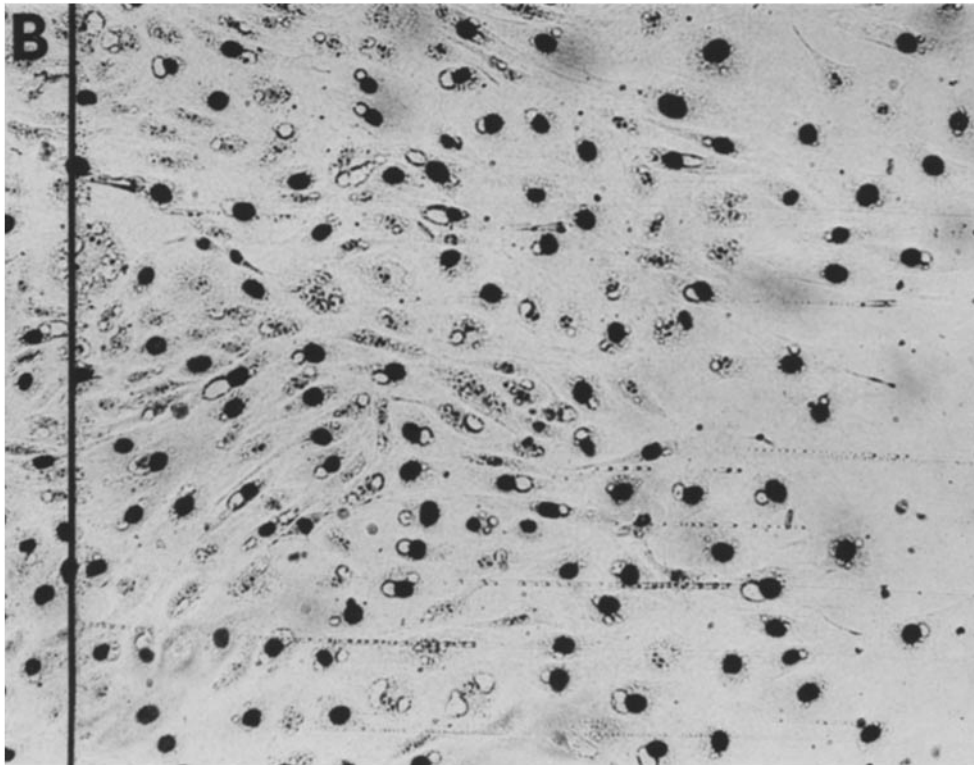
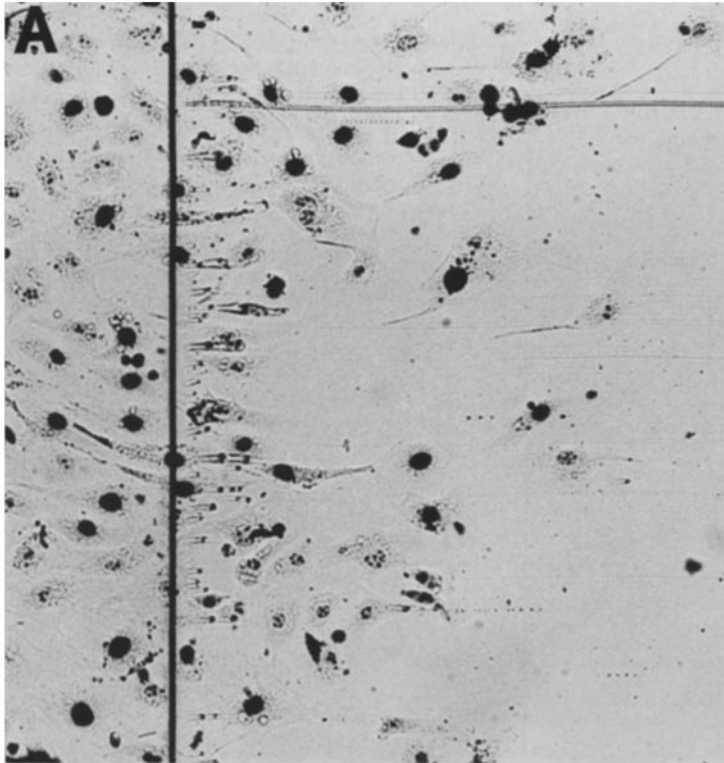


FIGURE 5 Autoradiographs of a wound, whose site is indicated by the vertical lines in *A* and *B*, in confluent endothelial cultures after the addition of the cell surface extract. Samples were added at the time of wounding. The cultures were exposed to [³H]-TdR for 2 h before termination at 48 h and autoradiography. (*A*) 100,000 g pellet of the cell surface extract (1.4 μg) was added at the time of wounding and again with new media at 24 h. (*B*) Control. × 100.

(31). This treatment followed by centrifugation solubilized 20% of the protein, but all of the inhibitory activity remained in the pellet fraction. Treatment of the pelleted urea extract with the nonionic detergent octyl glucoside followed by centrifugation resulted in the extraction of the inhibitory components. The comparison between the pelleted urea extract and the octyl glucoside extract of the inhibition of DNA synthesis is shown in Fig. 6. After solubilization, the inhibitory

activity is still nondialyzable. Maximum inhibition of the 100,000 g urea extract is seen at 0.3 μg and for the octyl glucoside solubilized extract, 0.2 μg.

Analysis of the 100,000 g urea extract for biochemical markers of subcellular organelles shows the presence of 5'-nucleotidase, alkaline phosphodiesterase I, glucose-6-phosphatase, and a trace of acid phosphatase. The specific activities for alkaline phosphodiesterase I and glucose-6-phosphatase

TABLE I
Biochemical Properties of the Endothelial Cell Surface Urea
Extract

Treatment	[³ H]TdR incorporation % control ± SD
None	47 ± 2
100°C, 3 min	101 ± 4
Dithiothreitol*	104 ± 7
Trypsin (1:10 wt/wt), 37°C, 1 h†	84 ± 2
Pepsin (1:10 wt/wt), 37°C, 1 h†	99 ± 1
100,000 g, 1 h—pellet	43 ± 2
—supernatant	98 ± 3
5 mM EDTA, pH 7.4—pellet	53 ± 7
—supernatant	99 ± 4
0.1 M Carbonate, pH 11.5—pellet	51 ± 2
—supernatant	94 ± 6
30 mM Octyl glucoside—pellet	105 ± 8
—supernatant	32 ± 3

The urea extract from confluent endothelial cells was subjected to the indicated treatments and then assayed for its ability to inhibit [³H]TdR incorporation of sparsely plated endothelial cells.

* Reduction with dithiothreitol was carried out in 6 M guanidine-HCl as described by Crestfield et al. (44) and compared with a control treated with guanidine alone. The sample was then exhaustively dialyzed against PBS at 4°C before it was added to cells.

† Pepsin digestion was in phosphate-buffered saline, pH 5. After treatment with proteases, the reaction was terminated with a 10-fold molar excess of soybean trypsin inhibition (for trypsin) or pepstatin A (for pepsin) and the samples were added to Waymouth's media containing 10% fetal bovine serum. Pepsin-treated samples were dialyzed against PBS before addition to the assay. Controls without urea extract showed no inhibition of [³H]-TdR incorporation. Values are means of triplicate wells for maximum inhibition.

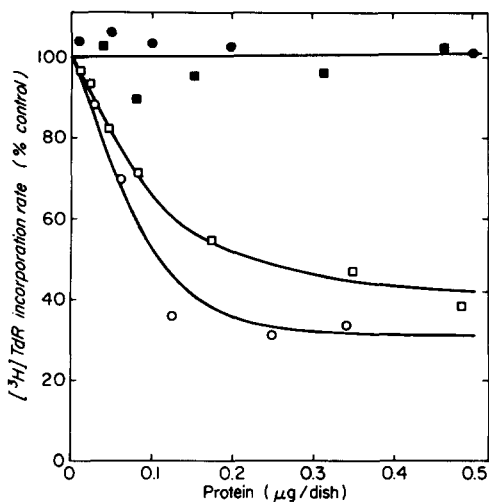


FIGURE 6 Comparison of the effect on [³H]TdR incorporation rate of the supernatant and pellet fractions after treatment of the cell surface extract. The urea extract was centrifuged at 100,000 g for 1 h at 4°C, and the supernatant and pellet fractions were concentrated by lyophilization after dialysis. The pelleted material was then treated with 30 mM octyl glucoside in 10 mM HEPES, pH 7.4, for 30 min at 4°C and recentrifuged. ■, 100,000 g supernatant from urea extract. □, 100,000 g pellet from urea extract. ○, 100,000 g supernatant from octyl glucoside-treated urea extract. ●, 100,000 g pellet from octyl glucoside-treated urea extract.

are 24 and 10 U, respectively. This represents a ten- and sixfold purification over the cell homogenate. The distribution of alkaline phosphodiesterase I as a plasma membrane marker has previously been shown in endothelial cells (37). Both the radioiodinated cell surface proteins shown in Fig. 1 and phosphodiesterase I parallel the growth inhibitory activity

through the fractionations shown in Table I. CSP-60 is still the major iodinated cell surface protein, but radioiodinated proteins with molecular weights of 116,000, 98,000, 84,000, and 70,000 are enriched in the octyl glucoside supernatant fraction (data not shown). These results suggest that the effect of urea is to cause release from the cell of membrane fragments that contain cell surface labeled proteins. We have confirmed the inhibition by using a preparation of plasma membranes prepared from confluent endothelial cells as described previously (23), but we found only a slight effect on growth of smooth muscle cells (data not shown).

DISCUSSION

A suspension of cell surface components prepared by urea treatment of confluent endothelial cells inhibits DNA synthesis, thymidine index, and growth rate when added to subconfluent growing endothelial cells. This inhibition of DNA synthesis is reversible, which suggests that the active factor is turned over, and that the effect is not the result of cell injury or cell death. The growth inhibitory material appears to be present on cells that show density-dependent inhibition but is not demonstrable with a similar extract from smooth muscle cells. This is in contrast to the heparin-like inhibitor of smooth muscle cell growth described by Castellot and co-workers (45).

Several pieces of evidence imply that the inhibitory activity is associated with a membrane fragment as an integral protein. The activity is sedimentable at 100,000 g for 1 h but is solubilized by octyl glucoside. In contrast, sodium carbonate treatment, which removes extrinsic membrane proteins (31), does not solubilize the activity. Our results are comparable to previous studies (23, 32) using a plasma membrane preparation from 3T3 cells. Their inhibitory activity is a protein that can antagonize the growth stimulatory effects of mitogens (25). Maximum inhibition of growth of endothelial cells is achieved with 0.3 µg of the centrifuged urea extract derived from confluent endothelial cells. Whittenberger et al. (32) in a similar assay required 2 µg of isolated plasma membranes from 3T3 cells. 3T3 plasma membrane fractions provide about a 50% inhibition of 3T3 cell replication. The effect on 3T3 cell growth is counteracted by the addition of excess serum. As with serum deprivation, addition of 3T3 membrane fractions increases the proportion of cells in G₁, implying a physiologic G₀ block or prolonged G₁ interval. The 3T3 inhibitory activity appears to be a membrane protein and is solubilized with the detergent octyl glucoside. A similar activity has been described by Natraj and Datta (24). The material in their studies appears to be a glycoprotein and is released from cells by treatment with a low concentration of urea.

It is interesting to speculate on the possible location and identity of proteins with specific growth inhibitory properties. Margolis et al. (46) have suggested that the ability of cells to be nonthrombogenic is related to the ability to restrict attachment of its neighbors to this surface with inhibition of growth. Upon transformation of endothelial cells, there is an alteration in morphology and thrombogenicity due to an abnormal deposition of fibronectin on the upper surface of the endothelial cell (47). This suggests that contact inhibition and non-thrombogenicity might depend on the restriction of specific sets of proteins to the upper and lower cell membranes. Vlodaysky et al. (27) have speculated about the role of an endothelial cell surface protein, CSP-60, in this process. CSP-60 is present in the urea extract, and we have shown that it is

one of the major proteins on the upper cell surface that can be iodinated (manuscript submitted for publication). Whether this protein is the active agent will have to await purification or identification of specific antibodies directed at an active component.

It is also conceivable that the growth inhibitory protein might be specifically localized to the cell junction. Both cultured endothelial and 3T3 cells have simple junctional complexes. Tight junctions are absent and gap junctions are small and sparsely distributed (48). There are, however, extensive areas of apposition of cell membranes of adjacent cells without any specific structure. A recent report of monoclonal antibodies to 3T3 cell surfaces is of interest (49). One antibody could localize an M_r 100,000 protein to continuous structures in the cell junctions. A specific protein located at this site or at the cell surface would be an obvious candidate for the agent responsible for the effect reported here.

The issue of how endothelial cells control their growth is central to current concepts of vascular response to injury in atherosclerosis. These data suggest that the control may be dependent on some component of the endothelial cell surface. The obvious implication is that the results reported here might have great value in the understanding of how normal endothelial cells maintain a continuous nonthrombogenic layer but do not overgrow in an uncontrolled fashion.

We thank Serena L. Wang for her expert technical assistance. Antibodies to fibronectin and thrombospondin were generous gifts from Dr. Paul Bornstein.

This work was supported by National Institutes of Health Grant HL-18645 and by a Grant-in-Aid from the Heart Association of Washington.

Received for publication 26 July 1984, and in revised form 8 February 1985.

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