Leukocyte Interleukins Induce Cultured Endothelial Cells to Produce a Highly Organized, Glycosaminoglycan-rich Pericellular Matrix

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ABSTRACT We report here that interleukins have a dramatic effect on extracellular matrix production by cultured endothelial cells. Human umbilical vein endothelial cells incubated with growth media conditioned by lectin-activated human peripheral blood mononuclear leukocytes undergo marked changes in cell shape and elaborate a highly organized extracellular material that is not detectable in untreated cultures. This material has the following characteristics: (*a*) it is not recognizable by electron microscopy unless the cationic dye, Alcian blue, is added to the fixative; (*b*) it is visualized as a network of branching and anastomosing fibrils of various thickness that can be resolved into bundles of fine filaments; (*c*) it is associated with the cell surface, extends between contiguous cells, and coats the culture substrate; (*d*) it is removed by digestion with glycosaminoglycan-degrading enzymes, such as crude heparinase and chondroitinase ABC. These results demonstrate that soluble factors released by activated peripheral blood mononuclear leukocytes (interleukins) stimulate cultured human umbilical vein endothelial cells to produce a highly structured pericellular matrix containing glycosaminoglycans (probably chondroitin sulfate and/or hyaluronic acid) as a major constituent. We speculate that this phenomenon corresponds to an early step of angiogenesis as observed in vivo as a consequence of interleukin release.

Because of its unique and strategic position, vascular endothelium participates in a wide range of normal and pathological processes, including inflammation and lymphocyte traffic through specialized postcapillary venules in lymphoid tissue (14, 34). The role played by endothelial cells in these events would be better defined if the factors that modulate their physiological properties were identified.

There is increasing evidence that lymphoid cell products may profoundly affect vascular endothelium. Antigen- or mitogen-activated mononuclear leukocytes synthesize and release an array of soluble factors (collectively called lymphokines or interleukins) that have a wide spectrum of regulatory activities on both lymphoid (38, 44) and nonlymphoid cells (33, 44, 54), and several observations suggest that endothelial cells can also be a target of interleukin activity (2, 9, 12, 13, 39, 41, 49). This prompted us to investigate the effect of interleukins on endothelial cells in an in vitro system. For this purpose, monolayer cultures of human umbilical vein endothelial (HUVE)[1] cells were incubated with cell-free supernatants from cultures of lectin-activated mononuclear leukocytes and examined by electron microscopy. The results of this study show that interleukins induce cultured HUVE cells to elaborate a highly organized pericellular matrix containing cytochemically recognized glycosaminoglycans (GAG) as a major constituent.

MATERIALS AND METHODS

Cell Culture: Primary cultures of HUVE cells were prepared by an adaptation of previously described methods (17, 28). Briefly, the umbilical vein was perfused with 50 ml of Hanks' balanced salt solution to wash out the blood, and filled with Medium 199 (Gibco Laboratories, Grand Island, NY) containing 1 mg/ml collagenase (CLS, Type I, Worthington Biochemical Corp., Freehold, NJ). After a 20-min incubation at 37°C, the contents of the vein were

[1] *Abbreviations used in this paper:* GAG, glycosaminoglycans; HUVE (cell), human umbilical vein endothelial (cell); PBL, peripheral blood mononuclear leukocyte; PHA, phytohemagglutinin.

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flushed out by perfusion with 10 ml of Medium 199. In our experimental conditions, this collagenase effluent usually contained only a limited number of endothelial cells (10^4–10^5), the majority of which did not exclude Trypan blue, and was therefore discarded. The vessel was refilled with Medium 199 without collagenase and clamped at its extremities, and the cord was gently kneaded between the fingers for ~2 min to loosen the residual endothelial cells. The umbilical vein was then perfused with 40 ml of Medium 199, and the cells were collected by centrifugation at 150 *g* for 7 min, washed once, and resuspended in Medium 199 (we are grateful to Dr. C. Ody for suggesting this procedure, which routinely yielded 0.5–1.5 × 10^6 viable cells). The endothelial cells were grown in 16-mm Nunclon plastic culture wells (Nunc, Kampstrup, Roskilde, Denmark) with Medium 199 containing 20% fetal calf serum (Gibco Laboratories), 400 U/ml penicillin, 200 μg/ml streptomycin, and 4 mM L-glutamine. The culture medium was renewed at 48-h intervals.

Primary cultures of pig aortic endothelial cells were kindly provided by Dr. A. Junod (Pneumology Division, University Hospital, Geneva, Switzerland) and cultured on gelatin-coated dishes with medium RPMI-1640 containing 20% fetal calf serum and 4 mM L-glutamine. Fetal bovine heart endothelial cells (CRL 1345) were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (Gibco Laboratories) supplemented with 10% newborn calf serum and 100 μg/ml fibroblast growth factor (Collaborative Research, Waltham, MA) (19). Cloned bovine capillary endothelial cells, a kind gift of Dr. B. Zetter (Children's Hospital, Boston, MA), were cultured on gelatin-coated dishes with a 1:1 mixture of Dulbecco's modified Eagle's medium and sarcoma-conditioned medium supplemented with 10% calf serum as described (15).

A variety of nonendothelial cells were also used in this study: HeLa cells were obtained from Dr. J. Roth, and normal rat liver cells from Dr. G. Vergani; human Chang liver cells (CCL 13), human intestine epithelial cells (Henle 407, CCL 6), and baby hamster kidney fibroblasts (BHK-21, CCL 10) were purchased from the American Type Culture Collection. These cells were grown in Medium F11 (Gibco Laboratories) supplemented with 10% fetal calf serum.

Preparation of Activated Peripheral Blood Mononuclear Leukocyte Supernatants: Peripheral blood mononuclear leukocytes (PBL) were isolated from heparinized blood of healthy human volunteers by centrifugation on a Ficoll-Hypaque gradient, according to a modification (25) of the method of Böyum (5). The cells were suspended in medium RPMI-1640 and incubated at 37°C in 100-mm plastic culture dishes (Falcon Plastics, Oxnard, CA). After 60 min, nonadherent cells were recovered and cultured at a concentration of 2 × 10^6 cells/ml in medium RPMI-1640 supplemented with 3.4 mM L-glutamine and 5% decomplemented human serum, with or without 2 μg/ml phytohemagglutinin (PHA) (Wellcome, Beckenham, England) for 48 h at 37°C. At the end of the incubation period, the cells were sedimented by centrifugation and the culture supernatants (which are referred to as activated PBL supernatants) were filtered through 0.22-μm Millipore filters (Millipore Corp., Medford, MA) and stored at −20°C until use. The biological activity of the supernatants was tested for interleukin 2 as described (20) using an interleukin 2-dependent mouse cell line. All supernatants had high IL_2 activity. Controls consisted of either medium RPMI-1640 containing 2 μg/ml PHA, or supernatants from unstimulated cultures to which PHA was added immediately before collection. For some experiments, the supernatant was concentrated 10-fold by ultrafiltration through an Amicon PM-10 membrane (Amicon Corp., Danvers, MA) with a molecular weight cutoff of 10,000, and the retentate was diluted to the original volume by the addition of medium without serum. Supernatants from pig blood and bovine spleen leukocytes were prepared in a similar way.

Incubation of Cells with Activated PBL Supernatants: Endothelial and nonendothelial cells plated on 16-mm plastic culture wells were grown to confluency (usually 3–5 d in the case of HUVE cells) in standard medium before starting the experiments. Supernatants from activated PBL were then added to the culture media to obtain a final concentration of 300 μl/ml, and renewed at 48-h intervals. Control cultures were exposed to the same dilution of RPMI-1640 medium containing 2 μg/ml PHA or, in some experiments, to the supernatant of unstimulated PBL. After 4–6 d of incubation, the cells were fixed by replacing the culture medium with either 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, or the same fixative containing 0.5% Alcian blue 8GX (Lot 13794, Gurr Ltd., London, England) (4).

Treatment of Endothelial Cells with GAG-degrading Enzymes: To investigate the nature of the material staining with Alcian blue, live endothelial cell cultures (previously exposed to activated PBL supernatants as described above) were incubated with each of the following enzymes: (*a*) crude heparinase from *Flavobacterium heparinum*, which degrades all known GAG except keratan sulfate (16, 29, 30); (*b*) chondroitinase ABC, which degrades chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, and hyaluronic acid, but not heparin and heparan sulfate (10, 56, 57); and (*c*) human platelet heparitinase, which is specific for heparan sulfate and heparin (35, 37, 55). Crude heparinase (a generous gift of Dr. Alfred Linker, Veterans Administration Hospital, Salt Lake City, UT) was dissolved in either phosphate-buffered saline (PBS) or serum-free Medium 199 (pH 7.0) at a concentration of 0.5–1 mg/ml. The endothelial cells were incubated with the enzyme for 1–3 h at 33°C. Chondroitinase ABC (Miles Laboratories Inc., Elkhart, IN) was dissolved in Hanks' balanced salt solution (pH 7.4) at a concentration of 2 U/ml (53). The cells were incubated for 3 h at 37°C. Partially purified human platelet heparitinase (35, 55) (a generous gift of Dr. Åke Wasteson, Biomedical Center, Uppsala, Sweden) was dialyzed against PBS (pH 6.5) and used without further dilution. Incubations were done for 1–3 h at 37°C.

Control cultures were incubated under the same conditions of pH and temperature as experimental cultures, but in enzyme-free buffer solutions. At the end of the incubation period, the cells were rinsed in buffer, fixed in glutaraldehyde-Alcian blue as described above, and further processed for electron microscopy.

The possible presence of proteolytic activity in the crude heparinase and chondroitinase ABC was assayed by adding 100 μl of enzyme solution to 50 μl of a 2% (wt/vol) suspension of casein (Carnation Instant non-fat dry milk heated for 20 min in a boiling water bath and spun at 300 *g* for 10 min to remove aggregates), and measuring the decrease in absorbance at 410 nm with a Dynatech AM 120 Microelisa-auto-reader spectrophotometer (Dynatech Corp., Alexandria, VA). Casein lytic activity was compared with that of dilutions (0.01–10 μg/ml) of trypsin (Trypsin 1:250, Difco Laboratories, Detroit, MI).

Processing for Electron Microscopy: After primary fixation in glutaraldehyde or glutaraldehyde-Alcian blue (see above), the cultures were rinsed in 0.1 M cacodylate buffer and postfixed for 20 min in 1% osmium tetroxide in Veronal acetate buffer. They were then treated quickly (~1 min) with 0.1% tannic acid (Mallinckrodt Inc., St. Louis, MO) in 50 mM cacodylate buffer, pH 7.0, washed, stained en bloc with 0.5% uranyl acetate in Veronal acetate buffer for 20 min, dehydrated in graded ethanols, and embedded in Epon.

Sectioning: After polymerization, the Epon disks covering the cell monolayers, together with the plastic base of the culture wells, were cut into small "matchsticks" with a cross-sectional area of ~1 mm^2, using a fine circular saw. The small square of culture well plastic that remained attached to the Epon matchstick was removed with forceps, thus leaving the embedded monolayer directly exposed for sectioning. To achieve an adequate visualization of the alcianophilic material coating the culture substrate, care was taken to collect the very first sections obtained from each Epon matchstick, the sectioning plane being almost parallel to the base of the exposed monolayer. In this way, at least one edge of each section grazed the substrate-attached material, providing useful en face views of its organization. To cut sections perpendicular to the plane of the culture, the matchsticks were reembedded in flat moulds to obtain the desired orientation. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 300 (Philips Electronic Instruments, Inc., Mahwah, NJ) or a Zeiss EM 10 electron microscope (Carl Zeiss, Inc., New York).

Immunofluorescence Microscopy: Cells grown on glass coverslips were fixed in 3.5% formaldehyde in PBS for 10 min, and permeabilized with 0.5% Triton X-100 in PBS for 10 min. They were then incubated for 2 h at room temperature in a moist chamber with rabbit antihuman Factor VIII antiserum (Nordic Immunology, Tilburg, The Netherlands) at 1:20 dilution, washed in PBS, and further incubated for 1 h with a 1:100 dilution of sheep anti-rabbit IgG antiserum labeled with fluorescein isothiocyanate (Institut Pasteur, Paris, France). For the visualization of fibronectin matrices, control and heparinase-treated cultures were incubated with a 1:20 dilution of rabbit anti-mouse fibronectin antiserum (a kind gift of Dr. B. Dumont, Dept. of Pathology, University of Geneva) followed by washing and indirect staining for rabbit IgG as described above. After additional washing in PBS, the coverslips were mounted on microscope slides with glycerol/PBS and photographs were taken with a Zeiss photomicroscope equipped with epifluorescence optics.

RESULTS

General Observations

The endothelial nature of the cells recovered from the umbilical vein was ascertained by the following criteria: (*a*) presence of Factor VIII antigen in all the cells as demonstrated by indirect immunofluorescence (26); (*b*) presence of Weibel–Palade bodies (17, 28) in thin sections; (*c*) occurrence of endothelial-type tight junctions (50) in freeze-fracture replicas (results not shown); and (*d*) typical "cobblestone" appearance of confluent cultures (17, 28) (cf. Fig. 1 *a*). Contamination by

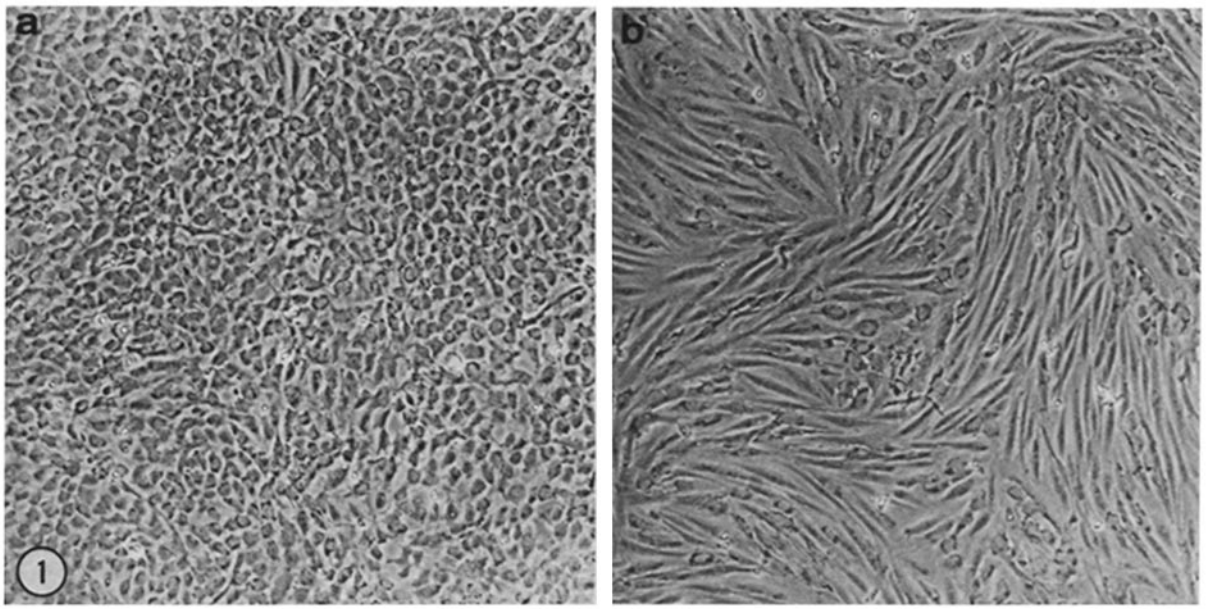

FIGURE 1 Morphological changes induced by activated PBL supernatants in confluent cultures of HUVE cells (phase contrast). (*a*) Endothelial cells grown for 4 d in the presence of 2 μg/ml PHA have a characteristic epithelioid "cobblestone" appearance. (*b*) Endothelial cells incubated for 4 d with supernatants from PHA-activated PBL display an elongated fibroblastoid morphology. × 70.

smooth muscle cells or fibroblasts was never observed.

Addition to the culture medium of supernatants from PHA-activated PBL resulted in marked morphological changes. Whereas confluent endothelial cells grown in the presence of either PHA (2 μg/ml) or supernatants from unstimulated PBL maintained a characteristic polygonal, epithelial morphology (Fig. 1 *a*), cells incubated with activated PBL supernatants assumed, within 2–5 d, a spindle-shaped, fibroblastic appearance (Fig. 1 *b*). This effect was reversible upon interruption of the treatment and was much more evident in low density cultures, where the cells took on an extremely elongated and/or dendritic morphology. A detailed account of these changes and of the accompanying reorganization of the endothelial cell cytoskeleton will be given in a separate communication.

Except for these modifications, major ultrastructural differences between control and interleukin-treated endothelial cells were not noticed in thin sections of glutaraldehyde-fixed cultures. However, a striking difference between the two experimental conditions was observed when the cationic dye Alcian blue was added to the fixative solution. This difference consisted of the presence, in interleukin-treated endothelial cells, of a highly organized alcianophilic pericellular material. This material, which is described below, was found in 16 different HUVE cell cultures, and using five distinct preparations of activated PBL supernatants. In contrast, it was never observed in cultures exposed to PHA alone, or to supernatants of unstimulated PBL.

Alcianophilic Pericellular Material

When Alcian blue was included in the fixative (4), sections parallel to the culture plane revealed a cell coat consisting of a fine feltwork of moderately electron-dense material (Fig. 2). The cell coat of interleukin-treated (but not of control) endothelial cells contained an additional component appearing as highly electron-dense rodlike structures (Fig. 2 *b*). However, sections tangential to the cell surface showed that the Alcian blue-stained material was in fact organized as a complex network of branching and anastomosing fibrils (Fig. 3). In addition to forming a cage around the cell periphery (Fig. 3 *a*), the fibrillar network extended from the cell surface into the extracellular space and joined contiguous endothelial cells (Fig. 3 *b*). In suitable sections, the Alcian blue-stained material appeared to form a highly structured extracellular matrix composed of interconnecting fibrils of various thickness (Fig. 4 *a*). At high magnification, the coarser and medium-sized fibrils were seen to consist of bundles of fine filaments (perhaps corresponding to the thinnest 8–12-nm threads stretched between the fibrils) (Fig. 4 *b*). A further characteristic localization of the alcianophilic material was on the culture substrate (Fig. 5). Sections grazing the substrate (see Materials and Methods) usually offered the best and easiest visualization of the alcianophilic material, probably because they were nearly parallel to the plane of the matrix over a large area (a rare situation in sections cut at different levels, even when passing tangential to the cell surface).

In sections perpendicular to the culture plane, the upper surface of both control and interleukin-treated endothelial cell monolayers was covered with a thick feltwork of moderately electron-dense material. In interleukin-treated cells, this material contained, in addition, irregularly-spaced globular or rod-shaped dense structures (Fig. 6), most probably representing transverse or oblique sections of the fibrils described earlier. Similar structures also bridged the gap between contiguous cells (Fig. 6 *a*) and were deposited on cell-free areas of the culture substrate (Fig. 6, *c* and *d*). Perpendicular sections thus showed the continuity between the material associated with the endothelial cell surface and that present in the extracellular space or coating the culture substrate (Fig. 6).

Alcianophilic material with a slightly altered appearance could still be seen 7 d after interruption of the interleukin treatment (not shown).

When Alcian blue was omitted from the fixative, no structures were seen which corresponded to those described above,

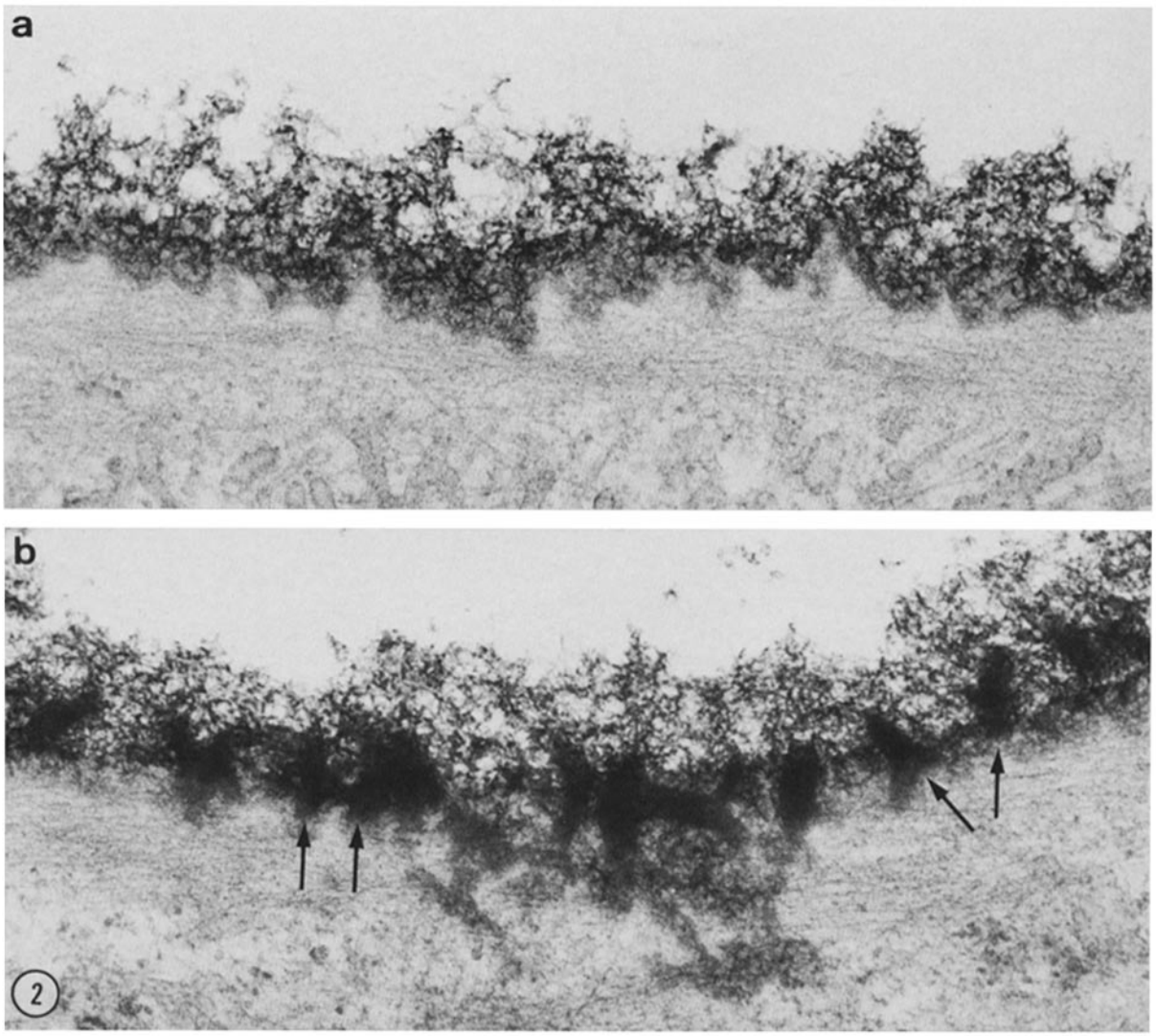

FIGURE 2 Thin sections slightly oblique to the surface of HUVE cells fixed in glutaraldehyde-Alcian blue. (*a*) Endothelial cell exposed for 6 d to PHA alone (control). The plasma membrane is covered with a fine feltwork of moderately electron-dense material. (*b*) Endothelial cell exposed for 6 d to supernatants from PHA-activated PBL. In addition to the feltlike material, the cell surface presents highly electron-dense rodlike structures (arrows). (*a*) × 56,000. (*b*) × 55,000.

and the surface of both control and interleukin-treated cells had a similar appearance. In these preparations, fibrillar material morphologically indistinguishable from fibronectin matrices (8, 51) was frequently observed in the extracellular space and/or associated with the cell surface. However, its organization and topographical distribution was different from that of the Alcian blue-stainable network.

To establish whether the elaboration of an Alcian blue-stainable pericellular matrix was a phenomenon specific to HUVE cells or common to other cell types exposed to the same culture conditions, we also incubated the following cells with human PBL supernatants and fixed in glutaraldehyde-Alcian blue: endothelial cells from pig aorta (primary culture), fetal bovine heart (passages 12–17), and bovine adrenal capillaries; human intestine epithelial cells (Henle 407) and HeLa cells; rat liver cells and BHK-21 hamster fibroblasts. In addition, because some interleukin activities have a narrow species specificity, pig aortic endothelial cells were also exposed to pig leukocyte supernatants, and bovine capillary endothelial cells were exposed to bovine leukocyte supernatants. In no instance did we observe a pericellular matrix similar to that seen with HUVE cells treated with activated human PBL supernatants.

Enzyme Digestion Studies

The chemical composition of the alcianophilic matrix surrounding interleukin-treated endothelial cells was studied by incubating intact cultures with various degradative enzymes prior to Alcian blue staining. In selecting test enzymes, we were guided by the known affinity of Alcian blue for GAG and by its ability to preserve and visualize extracellular matrix proteoglycans in preparative procedures for electron microscopy (4, 52). Alcianophilic pericellular material was not demonstrable after incubation of the cultures with crude *Flavobacterium heparinum* heparinase, a GAG-degrading enzyme preparation of relatively broad specificity (16, 29, 30), or with chondroitinase ABC, which acts on chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, and hyaluronic acid (but not on heparin and heparan sulfate) (10, 56, 57).

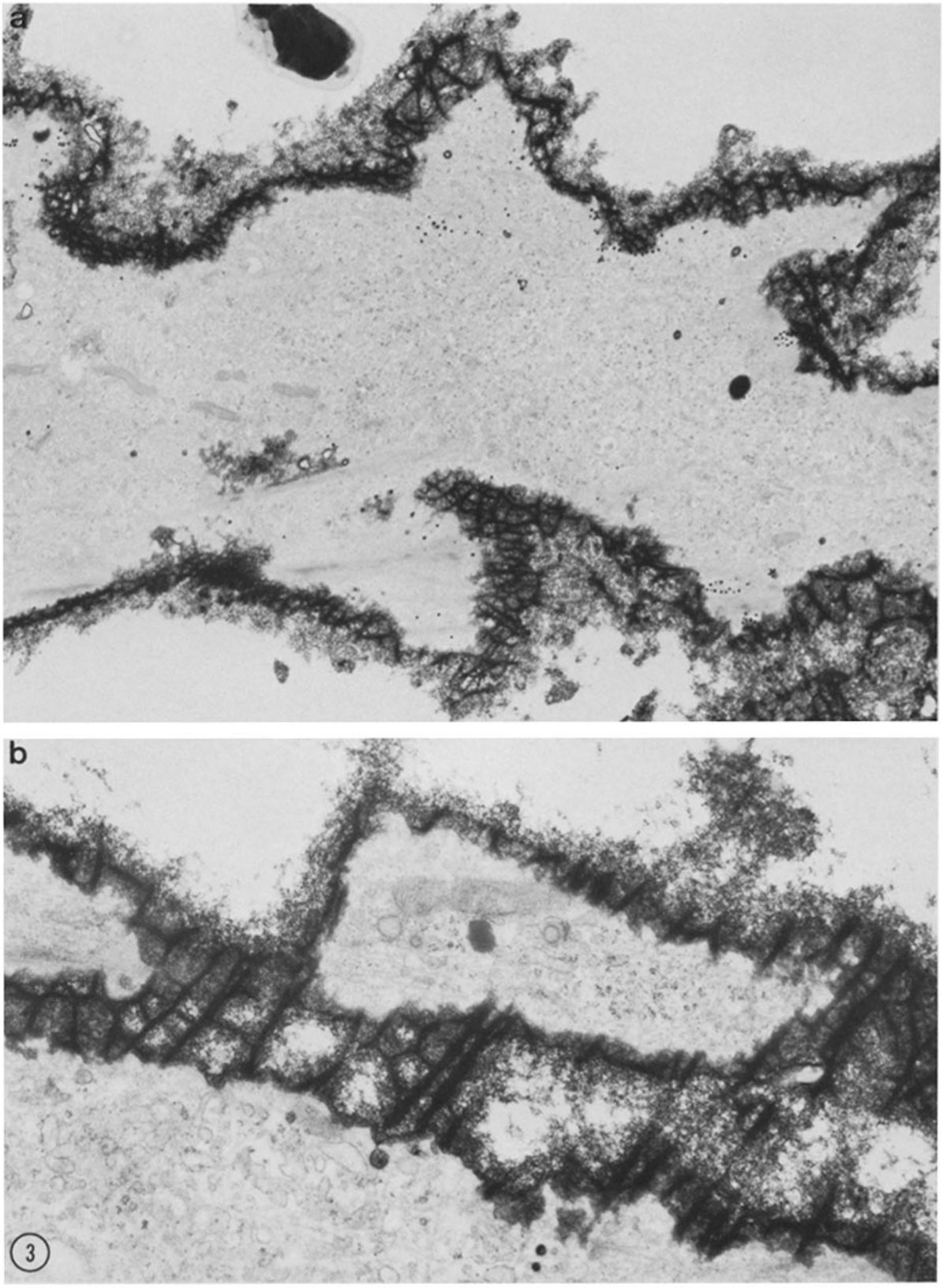

FIGURE 3 Interleukin-treated endothelial cells. In sections tangential to the cell surface, the electron-dense material appears organized as a network of anastomosing fibrils that form a cage around the cell periphery (*a*), and extend between contiguous cells (*b*). Glutaraldehyde–Alcian blue fixation. (*a*) × 7,500. (*b*) × 18,500.

In contrast, the alcianophilic material resisted digestion with partially purified human platelet heparitinase, which selectively attacks heparin and heparan sulfate (35, 37, 55) (Fig. 7). These results are summarized in Table I. Incubation with enzyme-free buffer solutions under the same conditions of pH and temperature as experimental cultures did not affect

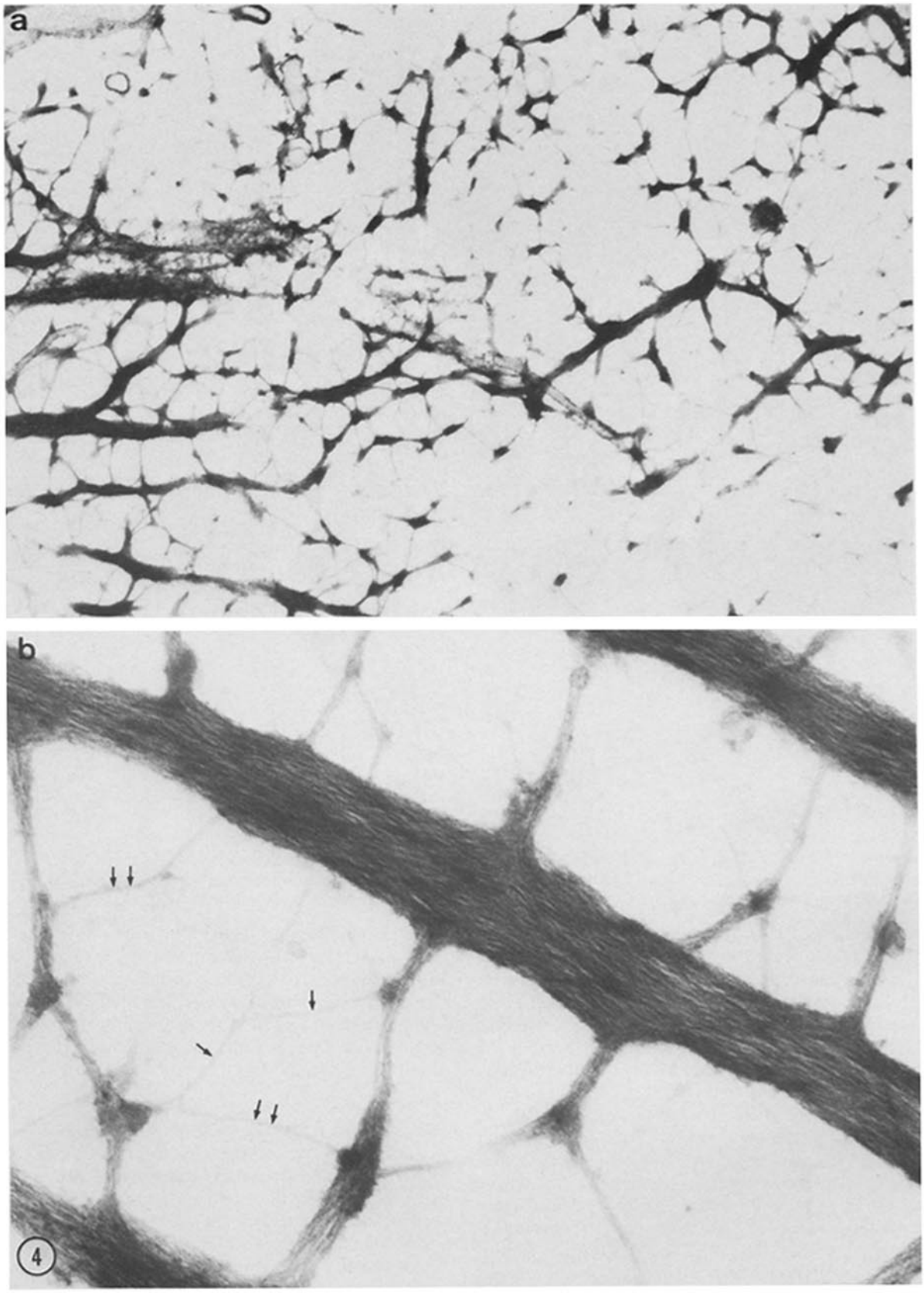

FIGURE 4 Thin section parallel to the plane of the culture. (*a*) Alcianophilic material forming a highly organized fibrillar matrix in the extracellular space. The matrix consists of interconnecting fibrils of various thickness; (*b*) higher magnification showing the filamentous substructure of the electron-dense fibrils, and the thinnest 8–12 nm threads (arrows) extending between them. (*a*) × 14,000. (*b*) × 70,000.

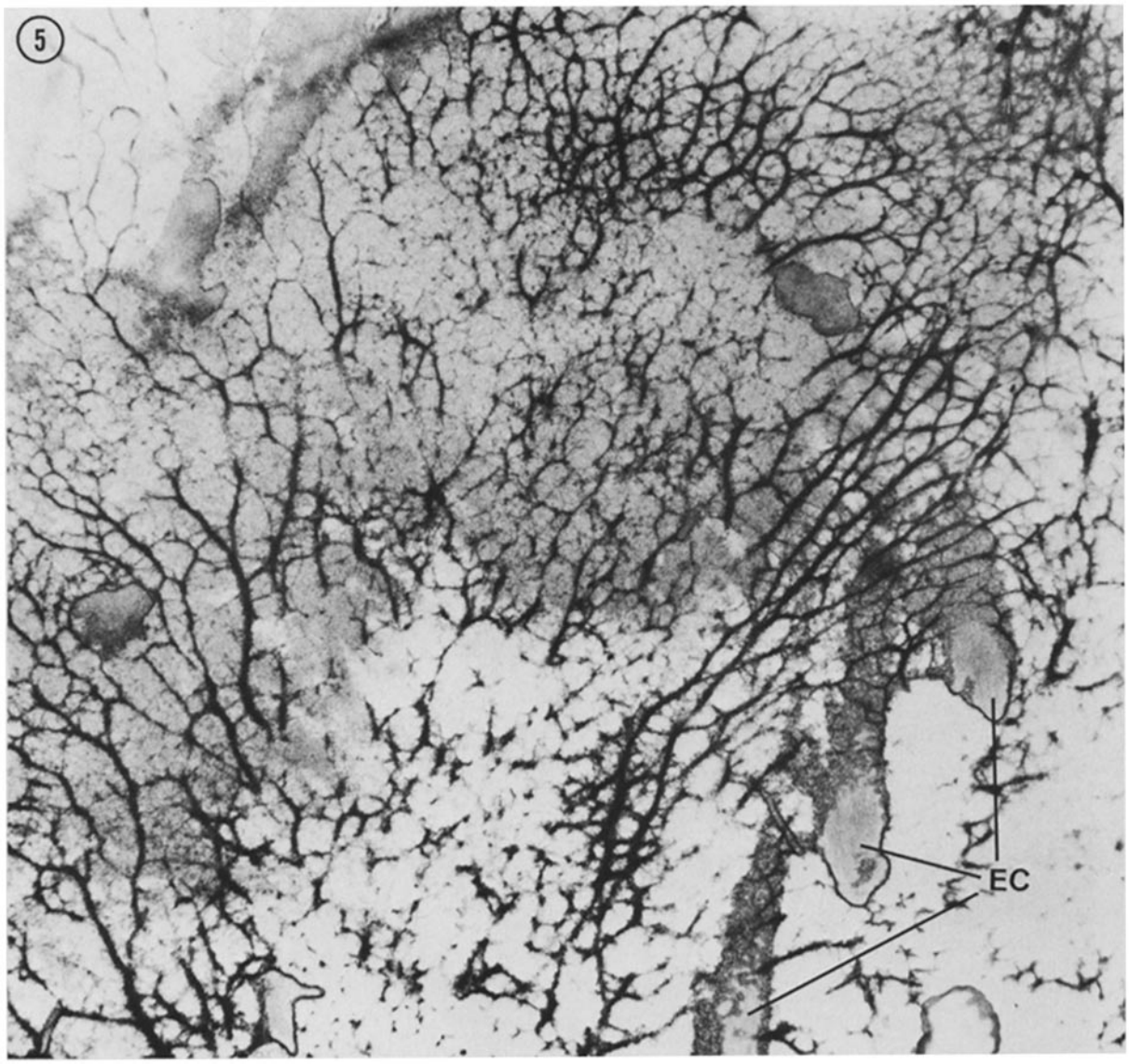

FIGURE 5 Thin section grazing the culture substrate (see Materials and Methods). The alcianophilic material associated with the surface of endothelial cell processes (*EC*) appears in continuity with that present in the extracellular space (lower right of the micrograph) and that laid down on the culture substrate (grayish upper and left part of the micrograph). × 11,000.

the alcianophilic material.[2] In addition, the possibility that this material was removed by proteases contaminating the GAG-degrading enzymes was ruled out by the following controls: (*a*) proteolytic activity was undetectable in either heparinase or chondroitinase ABC by a spectrophotometric casein lysis assay under conditions capable of revealing 0.1 μg/ml of trypsin (see Materials and Methods); (*b*) incubation of the cultures with 1 μg/ml trypsin, a concentration 10 times higher than the detection threshold of the assay cited above, did not affect the alcianophilic network (high concentrations of proteolytic enzymes could not be tested, because they caused complete cell detachment); (*c*) in experiments similar to those of Gill et al. (16), crude heparinase released more than 2.5 times as much of ^{35}S-labeled material from endothelial monolayers as that released by the PBS control; in contrast, release of [^{3}H]leucine-labeled material by heparinase was not significantly different from release by incubation with PBS alone (Table II); (*d*) the fibronectin matrix, which is extremely sensitive to most proteinases, was not affected by heparinase digestion: as seen by immunofluorescence microscopy (see Materials and Methods), heparinase-treated cultures contained a dense network of fibronectin, indistinguishable from the control (cf. also reference 24).

DISCUSSION

Since the recent development of techniques for maintaining vascular endothelial cells in vitro, there has been a continuous interest in establishing what extracellular matrix components are synthesized by these cells and which factors regulate their production (for a review, see reference 31). It has been demonstrated that cultured endothelial cells produce a variety of extracellular components, including several collagen types (46), elastin (32), fibronectin (27), laminin (18), and GAG (6, 36). In this study, we have shown that supernatants from cultures of PHA-activated PBL induce HUVE cells to produce a highly structured extracellular material which can be preserved and/or visualized by including the cationic dye, Alcian

[2] The presence of an alcianophilic matrix in these cultures (which were incubated for 1–3 h in buffer and further washed before fixation) precludes the possibility that it represents material precipitated from the culture medium during fixation.

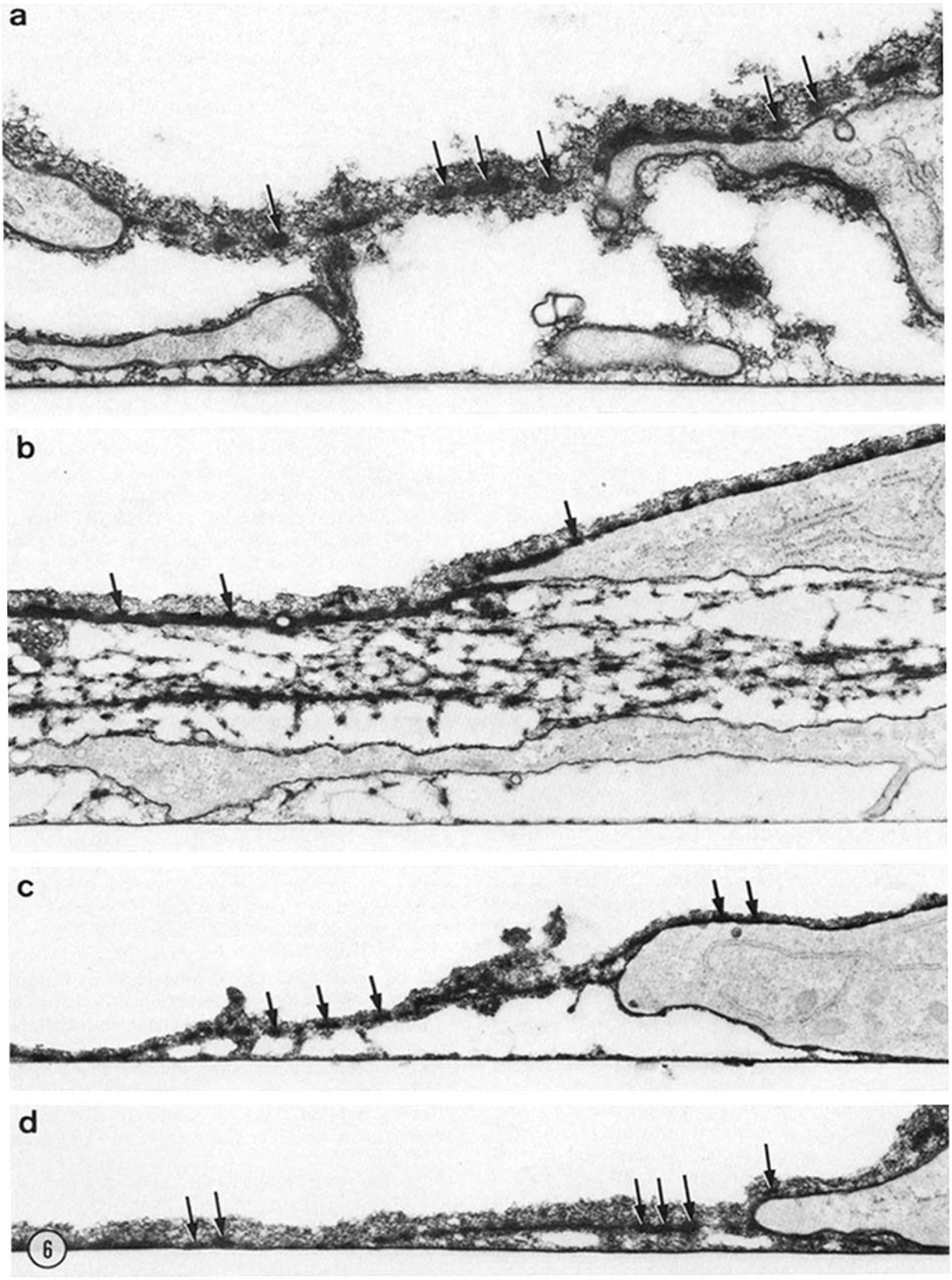

FIGURE 6 Thin sections perpendicular to the culture plane. A thick feltlike material containing globular or rod-shaped dense structures (arrows) covers the upper surface of endothelial cells (*a–d*) and extends as a bridge between contiguous cells (*a* and *b*) or cells and culture substrate (*c* and *d*). In *b*, a delicate network of granular and fibrillar densities also fills the intercellular space. The ventral cell surface (*a* and *c*), the dorsal surface of cells undergrowing other endothelial cells (*a* and *b*), as well as the regions of the substrate located beneath the cells (*a–c*) are mostly devoid of associated dense material. Gaps between endothelial cells, such as those seen in these micrographs, are especially frequent in interleukin-treated cultures. (*a*) × 34,000. (*b*) × 18,500. (*c*) × 10,500. (*d*) × 25,000.

TABLE I

Susceptibility of the Alcianophilic Matrix to Digestion with GAG-degrading Enzymes

Enzymes	Substrate specificity	Effect on Alcianophilic matrix
Crude heparinase	All known glycosaminoglycans except keratan sulfate	Removed
Chondroitinase ABC	Chondroitin-4- and 6-sulfate Dermatan sulfate Hyaluronic acid	Removed
Heparitinase	Heparan sulfate Heparin	Not affected

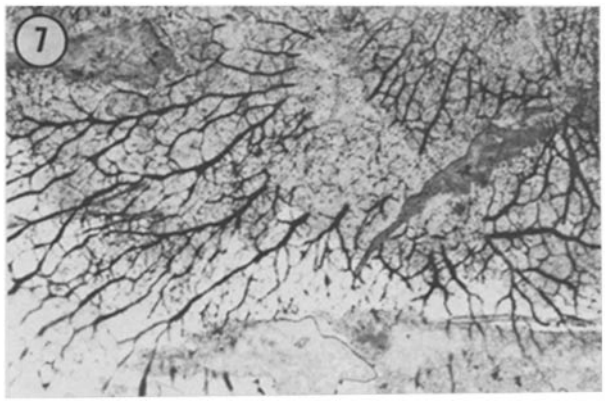

FIGURE 7 Interleukin-treated endothelial cell culture incubated with human platelet heparitinase before Alcian blue staining. The network of electron-dense material coating the culture substrate (cf. Fig. 5) has not been removed by the enzyme treatment. × 4,500.

blue, in the fixative solution.

The alcianophilic material is organized as a network of branching and anastomosing fibrils of various thickness which, at an appropriate magnification, can be resolved into bundles of fine filaments. Individual 8–12-nm threads are also seen to interconnect the coarse fibrils. Although clearly associated with the endothelial cell surface, this material also extends between contiguous cells throughout the extracellular space, and is deposited onto the culture substrate forming, therefore, an extensive pericellular matrix.

Enzyme digestion studies were necessary to ascertain the chemical nature of this alcianophilic material, in that at the pH used during fixation for electron microscopy, Alcian blue staining is not specific for GAG as protein carboxyl groups and other negative charges may participate in dye binding (4). Sensitivity to crude heparinase, a GAG-degrading enzyme preparation of broad specificity (16, 29, 30), resistance to human platelet heparitinase (specific for heparin and heparan sulfate; see references 35, 37, and 55), and sensitivity to chondroitinase ABC (10, 56, 57) indicate that the alcianophilic material contains chondroitin sulfate and/or hyaluronic acid. Although crude heparinase and chondroitinase ABC have been reported previously not to contain proteolytic activity (16), controls were done which verified that the removal of the alcianophilic matrix was not caused by contaminating proteases (see Results).

Interaction with cationic dyes and subsequent tissue processing probably alter the native conformation of GAG causing collapse of the once extended macromolecules (21). Although proteoglycans usually appear in the electron microscope as dense "granules" (22, 23), in some instances they have been seen as elongated rod-shaped particles or branching fibrils (7, 42, 43, 45, 47, 48), which are thought to result from less extensive condensation of the native macromolecules and to more closely reflect the original organization of the proteoglycan matrix (7, 42, 43, 45). Thus, the pericellular fibrillar network observed in endothelial cultures stimulated by interleukin-rich supernatants probably represents GAG that have been only partially collapsed during fixation.

Cultured endothelial cells have also been shown to synthesize GAG (6, 36) in the absence of interleukin stimulation. However, in the present study, pericellular networks were observed only in endothelial cell cultures previously exposed to interleukin-containing supernatants and not in control cultures. The most likely explanation is that, in our culture conditions, interleukins strongly enhance GAG secretion. Soluble products from lectin-activated PBL have been shown to stimulate GAG synthesis by cultured synovial fibroblastic cells (1) and, more recently, to enhance the formation of hyaluronic acid-rich cell coats (as visualized in light microscopy by red blood cell exclusion) by cultured glioma cells (11). Although it is possible that interleukins play a role in the modulation of GAG secretion by different cell types, among the different cells examined in our study, only HUVE cells appeared to produce alcianophilic pericellular matrices in response to incubation with activated PBL cell supernatants. However, caution should be exercised in interpreting the lack of reactivity of endothelial cells from pig aorta, as well as of the fetal bovine heart and bovine capillary endothelial cell lines, because this may result from the absence of the active factor in pig and bovine supernatants or, in the case of the bovine cell lines, from the loss of differentiated properties due to repeated subculture.

An interesting phenomenon accompanying the elaboration of alcianophilic matrix in interleukin-treated endothelial cultures is the change of cell shape. The relationship between these two consistently associated effects of PBL supernatants is presently unclear, but normal endothelial cells plated on the extracellular matrix produced by other, interleukin-treated, endothelial cells (following removal of the treated cells with Triton X-100) maintain the usual polygonal morphology (unpublished results). Thus, there is no evidence that shape changes result from an altered matrix environment.

The effect of interleukin-containing supernatants on the elaboration of GAG-rich extracellular matrix by cultured HUVE cells raises the question of the functional significance of this phenomenon and its possible relevance to biological processes occurring in vivo. Soluble products released by either lymphocytes (2) or macrophages (40) have been shown

TABLE II

Release of Radioactively Labelled Substrates by Treatment of Endothelial Cell Monolayers with Crude Heparinase

	^{35}S	[^{3}H]Leucine
	cpm	
Crude heparinase	1702	11,964
PBS	639	11,483

Interleukin-treated endothelial cell cultures were incubated for 48 h with either 80 μCi/ml ^{35}S or 100 μCi/ml [^{3}H]leucine, extensively washed, and treated with crude heparinase or PBS as described in the text. Radioactivity released in the supernatant was measured in a liquid-scintillation-counting system, with Aquassur (New England Nuclear, Boston, MA) as the scintillation cocktail.

to induce the formation of new blood vessels (angiogenesis) in various test systems. Angiogenesis is a poorly understood phenomenon, and has not been studied ultrastructurally with methods allowing the detection of proteoglycans until the recent work of Ausprunk et al. (3) on tumor-induced angiogenesis in rabbit cornea. The salient finding of the latter study was the demonstration that the growing tips of capillary sprouts are surrounded by a network of proteoglycan granules differing in arrangement and composition from those associated with mature microvessels. Stimulation of proteoglycan synthesis by endothelial cells may thus be one of the first steps in the formation of new blood vessels. An attractive hypothesis, presently under investigation, is that the endothelial cell changes we have observed after interleukin stimulation (i.e., the elaboration of a highly organized proteoglycan matrix) represent the in vitro equivalent of one of the first stages of angiogenesis.

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Note Added in Proof: In recent experiments, we tested the effect of highly purified or recombinant interleukins on HUVE cell cultures. While recombinant interleukin 2 had no effect, an alcianophilic pericellular matrix was induced by combined treatment with recombinant γ-interferon and interleukin 1 (which also reproduced the shape changes elicited by PBL supernatants) and, occasionally, with γ-interferon alone. However, it could not be induced by interleukin 1 alone. This suggests a synergistic action of γ-interferon and interleukin 1 on endothelial cells.

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