INTERMEDIATE-SIZED FILAMENTS OF HUMAN ENDOTHELIAL CELLS

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ABSTRACT

Human endothelial cells prepared from umbilical cords are characterized in parallel by electron microscopy and indirect immunofluorescence microscopy using specific antibodies against different classes of intermediate-sized filaments. The strongly developed, loose bundles of intermediate-sized filaments typically found in these cells are not decorated by antibodies against prekeratin or antibodies against smooth muscle desmin. They are, however, strongly decorated by antibodies directed against murine "vimentin," i.e., the 57,000 mol wt polypeptide which is the major protein of the intermediate-sized filaments predominant in various cells of mesenchymal origin. Cytoskeletal preparations greatly enriched in intermediate-sized filaments show the enrichment of a polypeptide band comigrating with murine vimentin. This shows that the intermediate-sized filaments that are abundant in human endothelial cells are predominantly of the vimentin type and can be demonstrated by their cross-reaction with the vimentin of rodents. These data also strengthen the evidence for several subclasses of intermediate-sized filaments, which can be distinguished by immunological procedures.

KEY WORDS   intermediate filaments · 10-nm filaments · cytoskeleton · vimentin · endothelial cells

The occurrence of unbranched, 7- to 11-nm thick filaments, different from microfilaments, has been described in a wide variety of vertebrate cells ("intermediate-sized filaments," "100 Å filaments"; see references 1, 3–9, 11–15, 19, 22–24, 27–29, 32, 33, 35–40). Examples include the tonofilaments and the filaments of keratin, the 10-nm filaments present in various muscle cells and in special muscle structures, the neurofilaments, and the arrays of intermediate-sized filaments

observed in many culture cells. This filament class also includes the aggregates of intermediate-sized filaments which are formed, frequently in conspicuous perinuclear bundles and coils, spontaneously or upon prolonged treatment of cells with certain antimitotic drugs (1, 9, 22, 24, 37). Immunological similarities (4) and similarities in the chemical composition (37, 38) of the intermediate-sized filaments observed in the diverse cell types and intracellular arrangements have been emphasized. On the other hand, we (11–15) and others (1, 6, 8, 19, 28, 40) have recently found immunological and protein chemical differences among the var-

ious subclasses of this morphologically defined category of filaments.

In studies of intermediate-sized filaments, the filament bundles occurring in cultured endothelial cells have been of special interest because they occur spontaneously and are often found in extensive aggregates (e.g., references 2-4, 17, 18, 20, 21, 25). We have therefore attempted to characterize the type of intermediate-sized filaments present in these cells by immunofluorescence microscopy, using antibodies against the major constitutive proteins of the following three different classes of 7- to 11-nm filaments: (a) prekeratin, i.e., a group of polypeptides (mol wt 48,000-68,000) characterisitc of tonofilaments and the predominant type of intermediate-sized filaments occurring in various epithelial and epithelia-derived cells ("cytokeratins"; cf. references 7, 11-16, 32, 39, 40); (b) a protein of ~57,000 mol wt which is the major constituent of the intermediate-sized filaments of various mesenchymal and mesenchyme-derived cells (references 12, 15; cf. 1, 23, 40) and for which the name "vimentin" has been proposed (12)—this protein is also present in the filament aggregates induced by treatment of cells with Colcemid (reference 12; cf. 37); (c) desmin (or "skeletin"; cf. references 5, 6, 8, 28, 29), i.e., the major protein of the 10-nm filaments characteristic of smooth muscle (mol wt 50,000-55,000). This protein is found also in cultured smooth and cardiac muscle cells (1, 8, 28, 29), in differentiated myoblasts and in myotubes of skeletal muscle (1, 8). Here, we show that the bundles of intermediate-sized filaments abundant in cultured human endothelial cells are specifically decorated by antibodies to murine vimentin and thus fall into the category of the filaments that are usually predominant in cells of mesenchymal origin.

MATERIALS AND METHODS

Cells

Pieces of human umbilical cords obtained under sterile conditions after Caesarean section were placed in Dulbecco's minimal essential medium (DMEM), and venous endothelial cells were prepared essentially according to the procedure described by Blose and Chacko (2) for abdominal veins of guinea pigs. The umbilical cord veins were perfused with DMEM. The veins were then everted by longitudinal cuts and were mounted as flattened sheets with fine needles on silicon plates. The whole was incubated for 10 min at 37°C in Mg^{2+}- and

Ca^{2+}-free, phosphate-buffered saline (PBS) containing 0.4% collagenase and 0.125% trypsin. After gentle rinsing, the detached cells were collected by centrifugation at 150 g for 5 min. The cells were resuspended in DMEM supplemented with 20-30% fetal calf serum and plated in 7-cm^2 Petri dishes. For maintenance and growth of secondary cultures, DMEM containing 20% fetal calf serum or 20-30% human serum was used. Use of the human serum resulted in a somewhat higher growth rate. Some cell cultures were treated with 10^{-6} M Colcemid for 24 h.

Antibodies

The guinea pig antibodies to purified bovine hoof prekeratin have been described (12-16). We used immune sera as well as purified IgGs which were made monospecific by affinity chromatography. In addition, we used guinea pig antisera elicited against individual, isolated, hoof prekeratin polypeptides (16).

Guinea pig antibodies against murine vimentin electrophoretically purified from high salt buffer-extracted cytoskeletons of mouse 3T3 cells (reference 12; for conditions of preparative gel electrophoresis, elution and precipitation with acetone, see Fig. 1) were prepared as described (12). The specificity of the antibodies used here (preparation GP_{2M}) was demonstrated in Ouchterlony immunodiffusion tests performed as described (13) and by immunoreplica techniques (see below). The typical separation of vimentin on gel electrophoresis as well as the specificity of the guinea pig antibodies obtained is presented in Fig. 1. The typical appearance of arrays of vimentin-containing fibrils in cultured rodent cells of mesenchymal origin seen by indirect immunofluorescence microscopy (see below) is shown in Fig. 2. In addition, we used antibodies against vimentin that had been made monospecific by affinity chromatography on vimentin covalently coupled to Sepharose 4B essentially as described previously for rabbit antibodies to tubulin (42) and guinea pig antibodies to prekeratin (16).

Rabbit and guinea pig antisera against desmin from chicken gizzard, essentially prepared according to Lazarides and Hubbard (29), and from smooth muscle of porcine uterus were used. Our antisera to avian desmin showed significant cross-reaction with mammalian desmin as judged by the decoration, in frozen sections, of smooth muscle and intercalated disk-associated filaments of rat heart. The guinea pig antisera were used preferentially in the present study since they did not contain the "autoantibodies" decorating various forms of intermediate-sized filaments that are often observed in various "normal" sera of rabbits (reference 32; cf. 1, 19).

Purified monospecific rabbit antibodies to actin and tubulin have been described previously (see references 32, 42). Rabbit antisera against antihemophilic factor VIII (clotimmun-AHG-associated protein) were obtained from Behringwerke AG (Marburg, Federal Republic of Germany). Monospecific antibodies to bovine procollagen, which cross-reacted with human procolla-
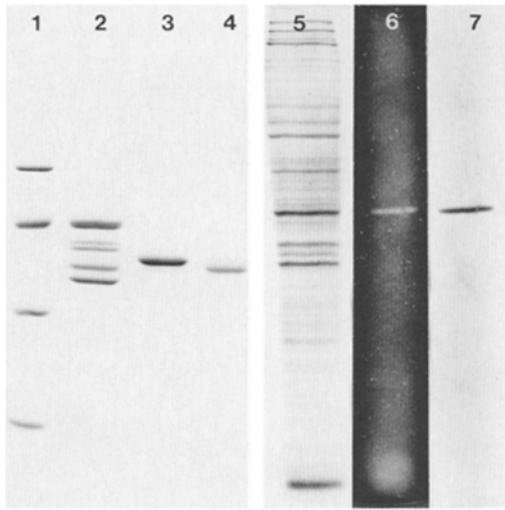
gen and form I collagen, were a generous gift from Dr. R. Timpel (Max-Planck-Institute for Biochemistry, Martinsried, Federal Republic of Germany). Preimmunsera were routinely used as controls.

Indirect Immunofluorescence Microscopy

Cells grown on cover slips were processed for indirect immunofluorescence microscopy as described (e.g., references 12, 30, 32, 41). Staining for factor VIII was performed on ethanol-methanol fixed cells as described (20).

Electron Microscopy

Cells grown on cover slips were fixed and processed for electron microscope examination of ultrathin sections as described (10, 11).

Preparations of Cytoskeletons and Gel Electrophoresis

Cytoskeletal preparations were made essentially as described (13). Briefly, the cell layers were washed twice with PBS and incubated for 5 min in TN buffer (10 mM Tris-HCl, 140 mM NaCl, pH 7.6) containing 1% Triton X-100 and then for 30 min in TN buffer containing 1.5 M KCl and 0.5% Triton X-100. Cell material was scraped off and the combined and suspended residual material was collected by 15-min centrifugation at 3,500 g. The pellets obtained were washed twice in 10 mM Tris-HCl (pH 7.6) and centrifuged. The final pellets were processed for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described (13).

Immunoreplica Tests

Immunoreplicas were made using a modification of the procedure described by Showe et al. (34). The gel was layered (~100 $\mu l/cm^2$) with agarose-serum solution (1.2% agarose solution containing 1.8% NaCl and 0.2% SDS was prepared at the temperature of a boiling water bath, then cooled at 50°C, and mixed with an equal volume of the specific serum). The polyacrylamide gel - agarose gel sandwich was incubated for 6 h at 37°C in a moist chamber. The agarose gel overlay was then floated off, at room temperature, in PBS and extensively washed in the same buffer (usually 3-4 d). Precipitin bands in the agarose gel were made visible by scattered light and were photographed, after 1, 2, and 4 d of washing, using lateral slit illumination. In addition, precipitin bands were demonstrated, after 4 d of washing, by 5-min incubation of the agarose gel in 0.025% Coomassie blue in aqueous solution containing 20% methanol and 7% acetic acid, followed by destaining of the gel in 7.5% acetic acid solution containing 7.5% methanol.

FIGURE 1 Characterization of murine vimentin and the guinea pig antisera raised against it by gel electrophoresis and immunoreplica techniques. The typical mobility of vimentin on SDS-polyacrylamide gel electrophoresis is seen, in comparison with the constitutive polypeptides of other intermediate-sized filaments, in the gel containing slots 1–4. Slot 1 presents, for comparison, molecular weight reference proteins (from top to bottom: phosphorylase a, bovine serum albumin, rabbit skeletal muscle actin, chymotrypsinogen); slot 2 contains purified prekeratin from desmosome-attached tonofilaments of calf muzzle (for preparation and apparent molecular weights of polypeptides, see references 7 and 13); slot 3 contains electrophoretically purified vimentin from mouse 3T3 cells (cf. reference 12); slot 4 contains electrophoretically purified desmin from chicken gizzard (mammalian desmin such as isolated from porcine uterus smooth muscle comigrates with the avian desmin under these conditions; cf. reference 12) The purified vimentin shown in slot 3 has been used for immunization in guinea pigs. When the antisera obtained (shown here is the specific antisera preparation GP$_{2M}$) are used in the agarose immunoreplica technique, it can be demonstrated that an immunoprecipitate is formed only with vimentin: slot 5 shows the separation of total proteins of mouse 3T3 cells on SDS-polyacrylamide gel electrophoresis (very low molecular weight polypeptides are not shown in the particular gel); slots 6 and 7 present the agarose immunoreplica of the same gel shown in slot 5 as seen in scattered light (slot 6) after relatively brief washing (24 h) and after Coomassie blue staining of the agarose gel (slot 7) performed after 4 d of washing. Similar specific immunoprecipitate bands have been obtained with gels of total proteins from various rodent cell cultures but also, though less strongly, with gels of proteins from cultured human cells.
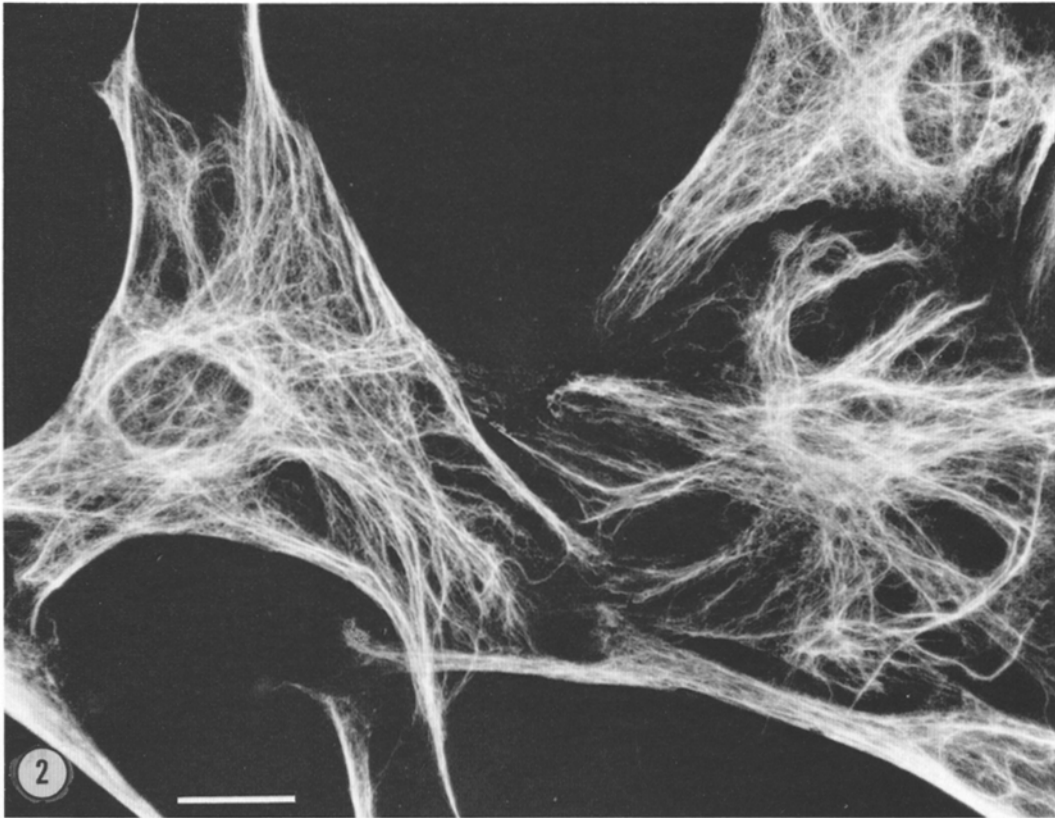
FIGURE 2 Immunofluorescence micrograph showing the typical arrays of fibrils decorated by antibodies to murine vimentin in a rodent cell culture derived from abdominal vein wall of rat (RVF cells, for details of cell preparation and culture see reference 15). These cells, which show a myofibroblastoidal morphology, are particularly rich in intermediate-sized filaments of the vimentin-type. Bar, 20 μm. × 800.

RESULTS

Human endothelial cells prepared and grown as described could be identified by the typical polygonal, epithelioid morphology displayed at confluence (cf. references 17, 18, 20, 21, 25, 26, 31, 43), the abundance of intermediate-sized filaments often arranged in relatively loose bundles (Fig. 3a and b), the typical formation of plasma membrane caveolae and endocytotic vesicles in the cell periphery (Fig. 3b and c), the occurrence of characteristic "Weibel-Palade bodies" (Fig. 3d), and the strong and specific fluorescence after decoration with antibodies to factor VIII protein (Fig. 3e). These criteria are generally accepted as a positive demonstration of the endothelial character in blood vessel-derived cell cultures (2–4, 17, 18, 20, 21, 25, 26, 43). The cells described

here did not react with antibodies against bovine procollagen I, in contrast to human fibroblasts and vascular smooth muscle-derived cells prepared from human umbilical cord. A variable percentage of the cells showed spontaneously formed bundles of aggregated intermediate-sized filaments, visible by phase contrast optics in the living cell (cf. reference 3).

In indirect immunofluorescence microscopy, the human endothelial cells showed normal displays of fibers decorated by antibodies to actin (Fig. 4a and b), often in the form of "cables" (cf. reference 30), corresponding to the bundles composed of microfilaments seen in the electron microscope (cf. Fig. 3a). Antibodies to tubulin decorated an extensive microtubular system (e.g., Fig. 4c; as to documentation of differences in the
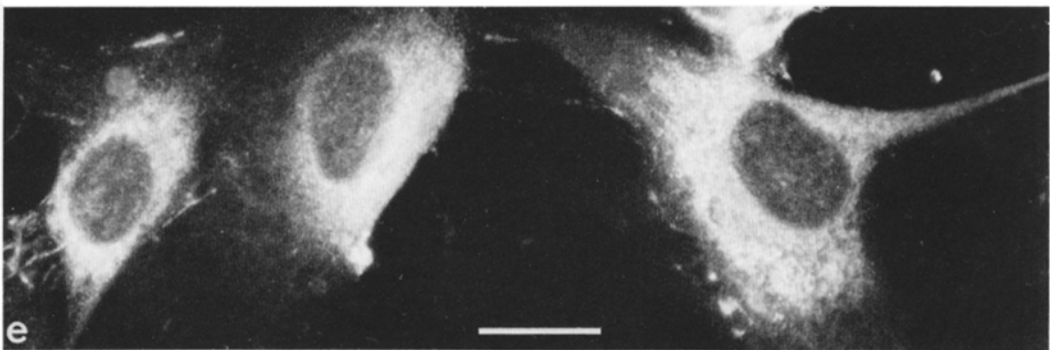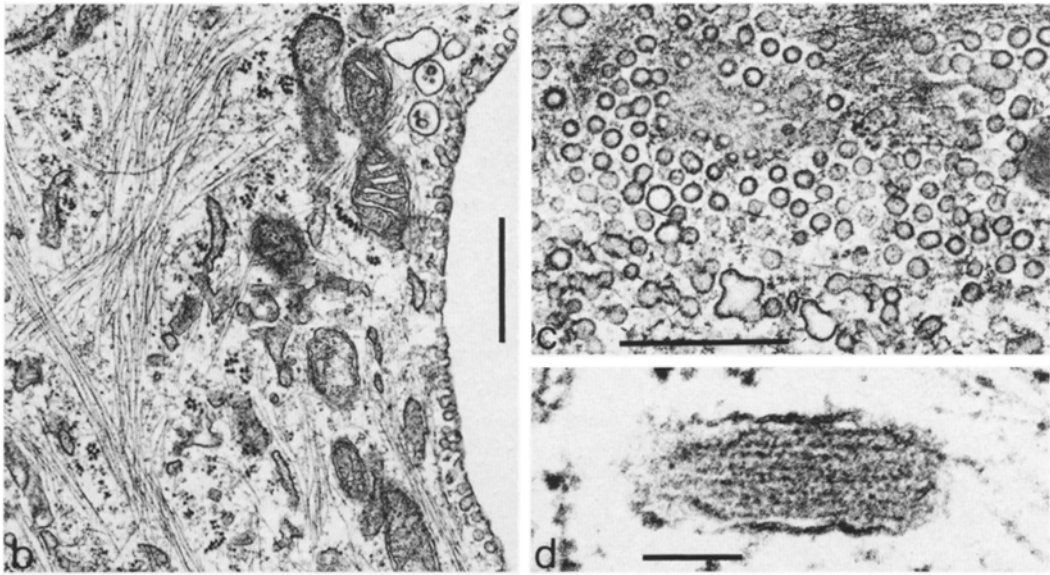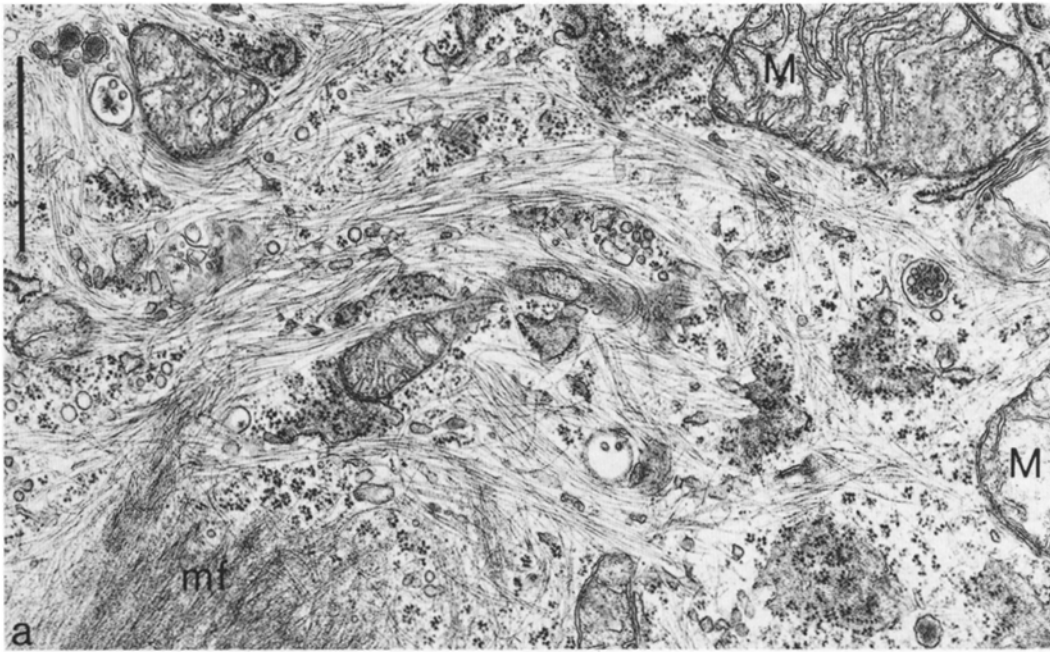
appearance of actin cables, microtubules and intermediate-sized filaments in other cell types, see, e.g., references 1, 12, 23, 27, 32). The cells did not contain fibrous elements which could be significantly decorated with the various preparations of antibodies to bovine prekeratin (see Materials and Methods), although the antiprekeratin sera used cross-reacted over a wide range of vertebrate species, ranging from amphibia to man (Fig. 4d; cf. references 12-16). Cytoplasmic fiber decoration was also not observed with antibodies directed against desmin from chicken gizzard (Fig. 4e) and porcine uterus. However, strong and specific fibril decoration was observed with the antibodies to murine vimentin (Fig. 5a-d). The specificity of these antibodies to the vimentin is illustrated for rodent cells in Fig. 1. The fibrillar arrays decorated by these antibodies corresponded to the bundles of intermediate-sized filaments shown by electron microscopy as well as to the wavy fibril contours of such filaments observed with the same antisera in other cells (12). Various forms of fibril arrangements were seen. Arrays characterized by the occurrence of radially oriented fibrils, resembling those often seen in large, flat mesenchymal cells (cf. Fig. 2) were seen in most of the cells (Fig. 5a and b). An especially frequent display was that presented in Fig. 5a, showing densely aggregated perinuclear "streams" of fibrils from which finer wavy fibrils seemed to emerge and extend toward the cell periphery. Other arrangements of fibrils decorated by antibodies to murine vimentin are shown in Figs. 5b-d and include the heavy decoration of the massive filament bundles that partly or completely encircle the nuclei of some cells (Fig. 5d).

When the cells were treated with Colcemid for prolonged times, the fibrillar material decoratable with antibodies to murine vimentin appeared to be aggregated into perinuclear whorls, coils or rings (Fig. 5e-g). Electron microscopy of such drug-treated cells fully confirmed the observations made by Blose and Chako (3) and showed the

often highly bizarre-shaped aggregates of intermediate-sized filaments. Sometimes, finer fibrils of vimentin-containing material were seen to extend from these perinuclear coils into the cytoplasm (e.g., Fig. 5g), but usually the more peripheral cytoplasm did not show the fibril arrays seen in the non-treated cells.

During mitosis of the endothelial cells the fibril system decoratable with antibodies to vimentin was rearranged in a dense "basket" surrounding the mitotic apparatus, similar to that observed during mitosis in other cells (cf. reference 12, 19, 23). The arrangements of the decorated fibrils in such mitotic cells could be traced by differential focusing and seemed to correspond to the arrays of bundled intermediate-sized filaments described in mitosis of cultured guinea pig endothelial cells (3).

Cytoskeletal material extracted in high salt buffer, which consisted primarily of loose tangles of intermediate-sized filaments as judged by electron microscopy of ultrathin-sectioned and negatively stained preparations, was examined by gel electrophoresis (Fig. 6). Such preparations invariably showed the enrichment of a polypeptide component of apparent mol wt 57,000 which comigrated with murine vimentin, together with some residual actin and a high molecular weight protein tentatively identified as fibronectin (Fig. 6; for the demonstration of fibronectin in cultured bovine endothelial cells, see reference 43; for reference on the occurrence of similar high molecular protein in other cytoskeleton preparations, see 13). None of the polypeptide bands enriched in the endothelial cytoskeletons showed an electrophoretic mobility similar to that of those of the prekeratin polypeptides (Fig. 6) or avian and porcine desmin (cf. Fig. 1a). In immunoreplica tests which showed a strong reaction of the vimentin band in various cells of rat and mouse (cf. reference 15; Fig. 1), a less intense, though significant, precipitin band was seen in the position of vimentin in human endothelial cells.

FIGURE 3 (a-e) Cultured venous endothelial cells from human umbilical cord as recognized by electron microscopy of flat ultrathin sections (a-d) and indirect immunofluorescence microscopy using rabbit antibodies against factor VIII protein (e). Note the abundance of intermediate-sized filaments (a and b) and of peripheral caveolae and endocytotic vesicles (b, cross-section; c, grazing section), and the occurrence of typical "Weibel-Palade bodies" with contents arranged in a paracrystalline array (d). M, mitochondria; mf, microfilament bundle. Bars, (a) 1 μm, (b and c) 0.5 μm, and (d) 0.1 μm; bar in the light micrograph (e), 20μm. a, × 26,000; b, × 33,000; c, × 45,000; d, × 130,000; e, × 850.
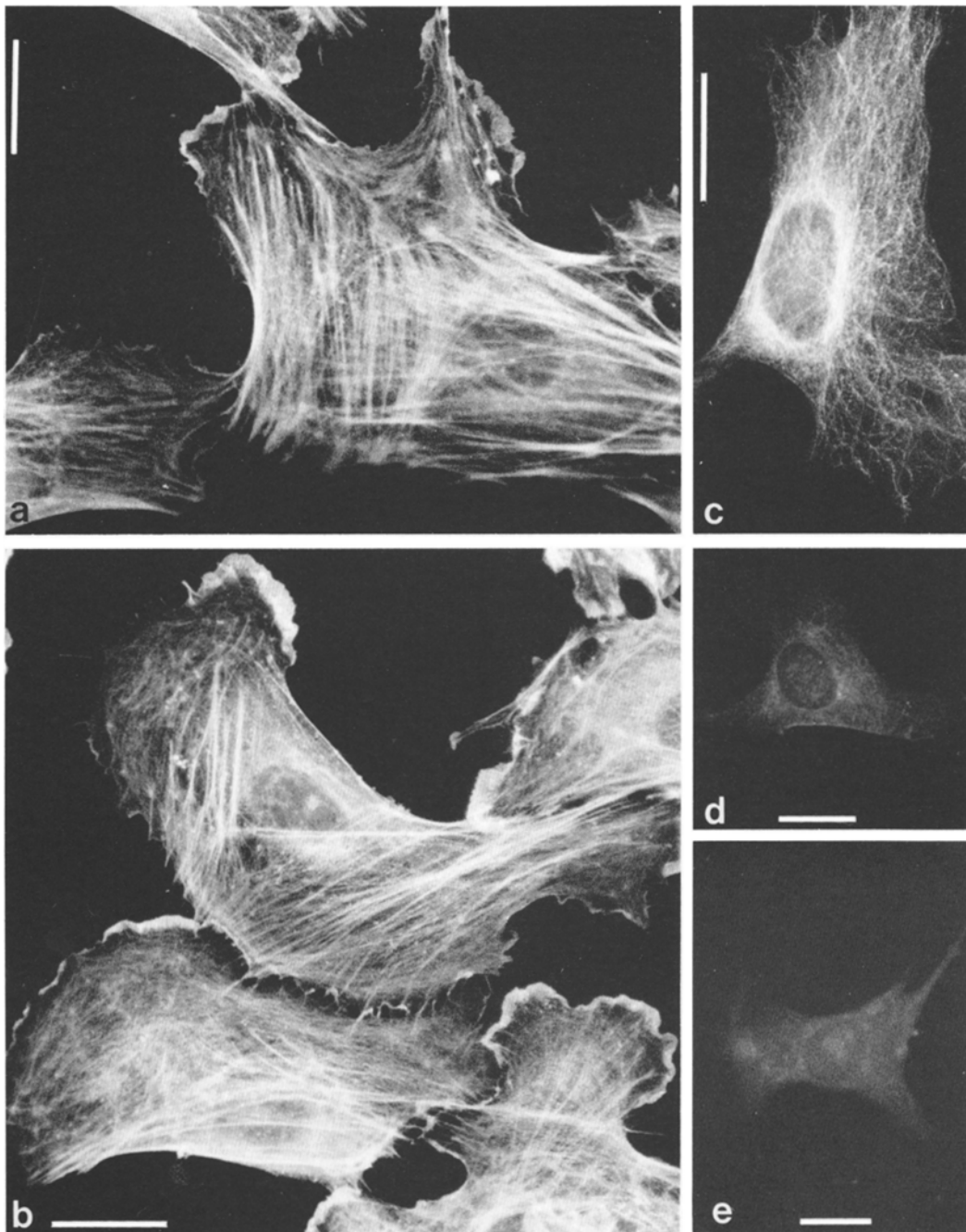
FIGURE 4 (a–d) Immunofluorescence micrographs of human endothelial cells after decoration with antibodies to actin (a and b), tubulin (c), bovine prekeratin (d), and with an antiserum against chicken gizzard desmin (e) which is known to show cross-reaction with mammalian muscle structures containing 10-nm filaments of the muscle type. Note various forms of arrangement of cable-like bundles of microfilaments and peripheral "ruffles" decorated by antibodies to actin (a and b) and microtubular arrays decorated by anti-tubulin (c), as opposed to absence of significant fibril staining with antibodies to prekeratin (d) and desmin (e). Note also points of attachment of microfilament bundles containing actin to the adherent plaque-junctions of adjacent endothelial cells (b; for electron microscopy of such junctions and of microfilament cables see also reference 18). Bars, 20 μm. a, × 750; b, × 800; c, × 900; d and e, × 550.
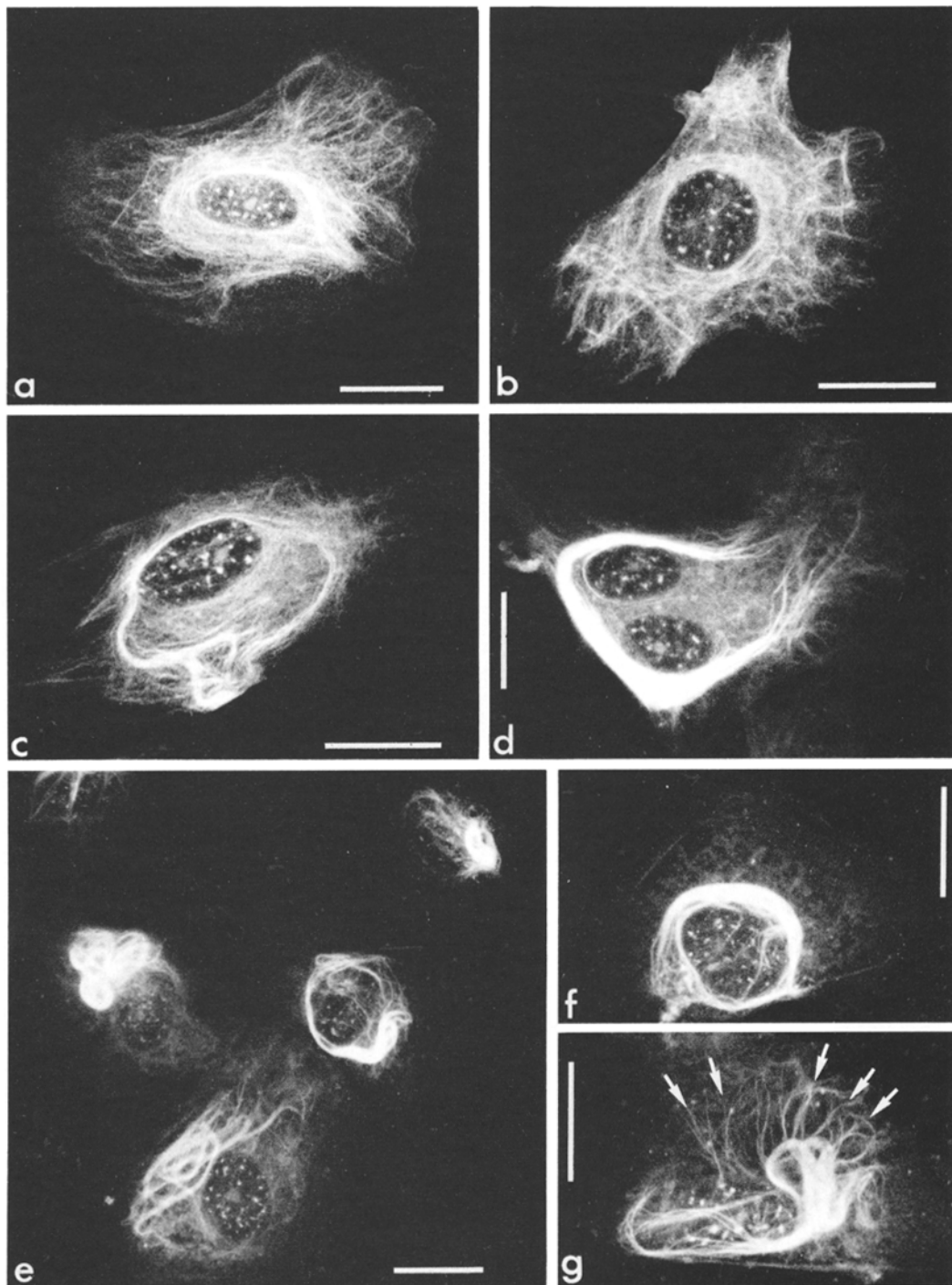
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FIGURE 5 (a-g) Immunofluorescence micrographs of human endothelial cells after decoration with antibodies to murine vimentin. The fibril arrays of intermediate-sized filaments shown in a-d are in untreated cells; perinuclear coils of stained fibrils shown in e-g are typical of cells treated with Colcemid (the arrows in g denote fine fibrillar extensions from the coil aggregate). Bars, 20 μm. a, × 750; b and c, × 850; d, × 700; e, × 650; f, × 800; g, × 850.
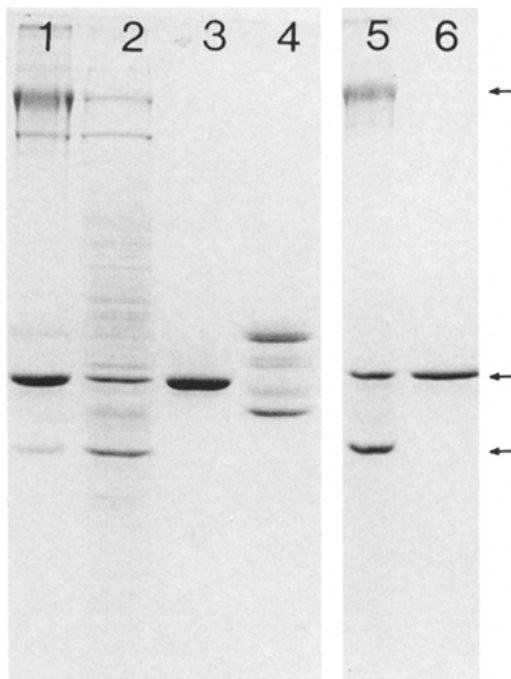
FIGURE 6 SDS-polyacrylamide (7.5%) gel electrophoretic separation of polypeptides of total proteins (slot 2) and cytoskeletal preparations (slots 1 and 5; the material shown in slot 1 has been extracted twice with the high salt buffer, cf. Material and Methods) of cultured human endothelial cells prepared from umbilical cord veins (low molecular weight polypeptides not shown in the gels presented here). The polypeptides of human endothelial cell preparations are compared with murine vimentin (slots 3 and 6; for preparation see references 12 and 13) and prekeratin prepared from desmosome-tonofilament fractions of *stratum spinosum* of bovine muzzle (slot 4; cf. references 7, 13). Note the predominance of actin and a polypeptide component comigrating with murine vimentin in whole human endothelial cells (slot 2) and the enrichment of the latter polypeptide in cytoskeletal preparations (slots 1 and 5), together with a high molecular weight polypeptide (apparent mol wt ~200,000, probably identical with fibronectin) and some non-extracted actin. The arrows in the right denote the position of this high molecular weight component, of vimentin, and actin (from top to bottom). None of the polypeptides enriched in the cytoskeletal preparations of human endothelial cells comigrates with one of the prekeratin polypeptides (bovine and human prekeratin polypeptide patterns are similar; for references see also 11, 39, 40) and with desmin (cf. Fig. 1).

DISCUSSION

Our observations show that the intermediate-sized filaments abundantly present in endothelial cells

in culture are of the vimentin-type which is the typical, predominant subclass of 7- to 11-nm filaments found in mesenchymal cells (12). Prekeratin-containing filaments, which are characteristic of various epithelial cells (11-16, 40), and desmin-containing filaments, which so far have only been observed in cells of muscle or myogenic origin (1, 5, 6, 8, 28, 29, 35), appear to be absent in endothelial cells. The characterization of the intermediate-sized filaments of endothelial cells as belonging to the vimentin-containing subclass is also in agreement with the strong reaction observed after decoration with antibodies to vimentin in the walls of blood vessels, capillaries included, found in frozen sections through various tissues including muscle and brain (12). Our data, however, seem to be at variance with those reported by Blose et al. (4) who have shown that the aggregates of intermediate-sized filaments of guinea pig endothelial cells could be decorated by rabbit antisera against a preparation of filaments from bovine brain. This observation has been interpreted as indicating immunological similarity between the intermediate-sized filaments present in endothelial cells and the neurofilaments of neuronal cells. It seems possible, however, that the results described by these authors might have been influenced by spontaneous rabbit "autoantibodies" that decorate filaments of the vimentin-type (cf. references 19, 32) or by contaminants of vimentin in their "brain filament" preparation (cells of brain capillaries and meninges are rich in filaments of the vimentin type; cf. reference 12). Although our present data leave open the interpretation of Blose et al. (4) that the intermediate-sized filaments of endothelial cells and the neurofilaments are antigenically related, it is worth emphasizing that our guinea pig antibodies to murine vimentin have not decorated any neuronal cell structures as seen in frozen sections of brain (cf. reference 12). Thus, the intermediate-sized filaments of endothelial and neuronal cells cannot be identical although they might still be related.

As we have discussed in detail elsewhere (12), murine vimentin is related to, if not identical with, the major protein of the intermediate-sized filaments described in normal and transformed hamster fibroblasts of the NIL 8 line (23). It is also related to the component of the intermediate-sized filaments decorated by a rabbit autoantibody in various mammalian cells (19). Similarly, it seems related to at least one component of the fibrillar aggregates induced with colchicine or Col-

cemid as suggested by a comparison with the decoration of such structures using autoantibodies of rabbits (e.g., 19, 32) as well as of human cancer patients (27) and by the detailed analyses by Goldman and his colleagues (37, 38). Holtzer and coworkers (8, 22) have recently described by immunofluorescence microscopy in various chicken cells the location of a similar protein (mol wt ~58,000) in the intermediate-sized filaments of the "fibroblast-type", including the Colcemid-induced aggregates, in chick cells.

Antibodies to vimentin-like proteins so far described by us (12) and others (1, 8, 23) seem to have a limited species cross-reactivity, in contrast, for example, to the antibodies to prekeratin (12-14, 16, 40). While we have observed strong decoration with the antibodies to mouse vimentin in mesenchyme-type cells of various rodents, we have found markedly stained fiber arrays in cells of other vertebrates regularly only after aggregates of intermediate-sized filaments have been formed by treatment of the cells with antimitotic drugs (12, 14). Thus, it is of special significance that the naturally occurring bundles of intermediate-sized filaments present in human endothelial cells demonstrate some antigenic relatedness of murine and human vimentin, and antibodies of the type described here may be of value in studies of human cells. Future immunological and biochemical work will be required to clarify the similarities and differences among the various forms of intermediate-sized filaments in general and among the filaments of the vimentin-type in different species in particular.

Note added in proof: We have recently obtained guinea pig antibodies to murine and human vimentin which show perfect cross-reaction in diverse vertebrate species, including mammals, birds, and amphibia. With such broadly cross-reacting antibodies the vimentin nature of the intermediate-sized filaments prominent in human endothelial cells has been fully confirmed.

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