Isolation and Culture of Microvascular Endothelium from Human Adipose Tissue

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ABSTRACT The study of human endothelial cells in tissue culture has been previously limited to umbilical vein, a large vessel source, and microvascular endothelium from human foreskin, spleen, and adrenal. Microvascular endothelium cultured from these sources have required matrix-coated culture flasks, tumor-conditioned medium, or 50% human serum for growth and subcultivation. To obtain cultures of microvascular endothelium with less stringent growth requirements, human adipose tissue was digested with collagenase and endothelial cells were separated from other stromal elements by sequential filtration and layering cells onto 5% albumin. Using standard medium containing 10% fetal calf serum, these cells grew readily to confluence and survived serial passages. When the cultures were subconfluent, cytoplasmic extensions and a capillary-like morphology were observed. Confluent cultures displayed the "cobblestone" appearance characteristic of other endothelial preparations. Electron microscopy demonstrated the presence of characteristic tight junctions and pinocytotic vesicles. Immunofluorescent staining for Factor VIII was positive, and cultures contained angiotensin-converting enzyme activity. Thus, cultures of human microvascular endothelium were readily obtained from adipose tissue and required only standard medium with 10% serum for growth and subcultivation. This system can be used to study human endothelial cell biology and may prove useful in the study of pathologic states such as diabetic microvasculopathy and tumor angiogenesis.

INTRODUCTION

The vascular endothelium is important in numerous structural and metabolic functions. Endothelial cells act as a permeability barrier to plasma, providing a nonthrombogenic surface and allowing the selective, active transfer and metabolism of many substances (1–5). In addition, the endothelium is involved in the pathogenesis of many disease states, including atherosclerosis (6), diabetic microangiopathy (7, 8), and tumor angiogenesis (9). The propagation of endothelium in tissue culture can be very useful in the study of these metabolic processes, since the cellular environment can be controlled.

The cell biology of large vessel endothelium, cultured from bovine or porcine aorta and human umbilical vein, has been extensively studied (1, 10). In contrast, microvascular endothelium has been more difficult to cultivate. Human capillary endothelial cells from foreskin microvessels have required fibronectin-coated culture flasks and 50% human serum to grow to confluence and survive subcultivation (11, 12). Fibroblast contamination has been a problem in this system (12), and other preparations of microvascular endothelial cells have required treatment with thimerosal, a mercurial cell toxin, to remove stromal cells (13, 14). Other sources of microvascular endothelium have also required matrix-coated flasks (11, 15–17), high concentrations of serum (11), or tumor-conditioned medium (16). Thus, because an optimal medium for cell growth has not yet been defined, the study of regulatory processes in these cells is complicated.

In this report, we describe the isolation, characterization, and culture of microvascular endothelium from human adipose tissue. This system offers a readily available source of tissue from which pure cultures of microvascular endothelial cells are easily obtained. In addition, these cells do not require matrix-coated

flasks, tumor-conditioned medium, or 50% serum for growth and subcultivation. We feel this represents a model in vitro system for studying the regulatory processes of human microvascular endothelium and the pathophysiology of numerous disease states.

METHODS

Histology. Two random samples of omental fat were fixed in Bouin's fixative, embedded in paraffin, sectioned at 6 μm, and stained with hematoxalin and eosin.

Isolation of human microvascular endothelial cells. After informed consent was obtained, $\sim$10 g of omental and/or subcutaneous adipose tissue was removed during elective surgery for nonmalignant conditions from patients who were in overall good health. The tissue was immediately placed into sterile, iced phosphate-buffered saline (PBS) with 1.0 mM Ca^{+2} and processed within 2 h. Using a modification of the technique of Rodbell (18), the tissue was divided into 2–3-g pieces that were minced and transferred into a plastic flask containing 7 ml of 2 mg/ml collagenase (Worthington Biochemicals, Freehold, NJ) in Kreb's-Ringer bicarbonate buffer with 3 mM glucose and 4% bovine serum albumin (BSA). Digestion proceeded at 37°C in a gyratory water bath shaker for 15 min (19). The suspension was then passed through 250-μm pore nylon mesh (Nitex, Tetko, Inc., Elmsford, NY) to remove the undigested pieces. To inactivate the collagenase, Medium 199 (Gibco Laboratories, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Reheis Chemical Co., New York), 100 U/ml penicillin, and 100 μg/ml streptomycin (both from Gibco Laboratories) was added. In the filtrate, the adipocytes floated to the surface and the medium below the adipocytes, containing endothelium and stromal cells, was removed and centrifuged at 300 g for 10 min. The cell pellet was resuspended in 5 ml of serum-containing medium, and this in turn was passed through 30-μm nylon mesh (Nitex). Clumps of endothelium, containing 4–15 cells, were retained by the mesh, while single cells, including most of the nonendothelial stromal cells, passed through. The cell aggregates were then washed into a plastic centrifuge tube, recentrifuged, and the pellet was resuspended in 5 ml of serum-containing medium. This suspension was then gently layered on 40 ml of PBS containing 1.0 mM Ca^{+2} and 5% BSA, to allow separation of the larger endothelial cell aggregates from the few remaining single cells. After 10 min at room temperature and at unit gravity, the top 10 ml of the PBS-albumin was removed and the remaining 30 ml was centrifuged. The pellet containing $\sim$1,000 cells, was resuspended in 4 ml of Medium 199 containing 10% serum, and plated in a 25-cm² flask (Corning Glass Corp., Corning, NY). Cultures were incubated at 37°C in a humid 5% CO_2, 95% air environment. The culture medium was changed every 2–3 d. Serial passage was accomplished by a brief exposure to 0.25% trypsin with 0.01% EDTA, with the flasks split three for one.

Characterization, Factor VIII antigen staining. Primary cultures were grown to confluence on 25-mm round coverslips (Scientific Products Div., American Hospital Supply Corp., McGaw Park, IL), fixed in a 1:1 solution of glacial acetic acid and methanol at 0°C for 10 min, and washed with PBS. After incubation for 60 min at 37°C in a 1:80 dilution of rabbit antiserum to human Factor VIII (Calbiochem-Behring Corp., La Jolla, CA), the coverslips were washed with PBS three times for 15 min each. The cells were then incubated for 60 min at 37°C in a 1:20 dilution of fluorescein-conjugated anti-rabbit IgG (Calbiochem-Behr-

ing). After three additional 15-min washes, coverslips were mounted on glass slides using a 1:1 solution of glycerol and PBS, and observed under a Zeiss photomicroscope equipped with epifluorescent illumination (Carl Zeiss, Inc., New York). Negative controls for each experiment included endothelial cells exposed to fluorescein-conjugated anti-rabbit IgG without prior exposure to anti-human Factor VIII antiserum.

Factor VIII antigen staining was also carried out by a direct immunofluorescent technique using a partially purified fluorescein-conjugated anti-human Factor VIII. This antiserum has been demonstrated to lose its ability to stain endothelial cells with prior absorption with human Factor VIII (20); and to retain its staining characteristics when absorbed with serum from patients with von Willebrand's disease (Clark, R. A. F. Unpublished observation).

Electron microscopy. Samples of omental adipose tissue were promptly fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3, dehydrated in graded alcohols, and embedded in EM bed 812 (Epon). Samples were then stained with uranyl acetate and lead citrate and examined in a Zeiss EM 95. Primary cultures of microvascular endothelial cells were prepared similarly. Cells were fixed both while attached to the culture dish and after being scraped directly into glutaraldehyde-cacodylate buffer.

Angiotensin-converting enzyme activity. The activity of this enzyme was assayed in monolayers of confluent endothelial cultures using [^{3}H]benzoyl-phenyl-alanine-proline (Ventrex Corp., Portland, ME) as a substrate (21). SQ14,225 (captopril, a gift of Mr. S. J. Lucania, Squibb Institute of Medical Research, Princeton, NJ) in a final concentration of 5×10^{-6} M was used as a specific inhibitor of the enzyme (22) and served as a negative control. Activity was expressed as units per confluent 25-cm² flask ($2.05\pm0.2 \times 10^6$ cells/flasks, mean$\pm$SEM, $n = 5$), one unit being defined as the amount of angiotensin-converting enzyme required to hydrolyze substrate at an initial rate of 1%/min at 37°C.

RESULTS

Microvascular endothelium in adipose tissue. Omental fat from two subjects was fixed and stained with hematoxalin and eosin to estimate blood vessel size. Numerous microvessels were embedded in a stroma composed predominantly of adipocytes (Fig. 1). The luminal diameter of vessels cut in cross section was 19.3$\pm$3.2 μm (mean$\pm$SEM, $n = 27$).

Electron microscopy of omental adipose tissue demonstrated numerous capillaries and microvessels separated from adipocytes by a distinct basal lamina (Fig. 2a). These endothelial cells had the characteristic fine structural appearance of endothelial cells from other sources. Pinocytotic vesicles were a prominent feature of these cells, and adjacent cells were connected by a characteristic junctional complex (Fig. 2b). Parallel arrays of microfilaments were also abundant. Although uncommon in the preparations examined, Weibel-Palade bodies, organelles specific to endothelial cells, were present (Fig. 2b).

Morphology of microvascular endothelium in tissue culture. At the completion of the primary isolation, aggregates of 4–15 endothelial cells could be seen floating in the medium. Virtually all the cells from the initial inoculum attached to the culture flask within
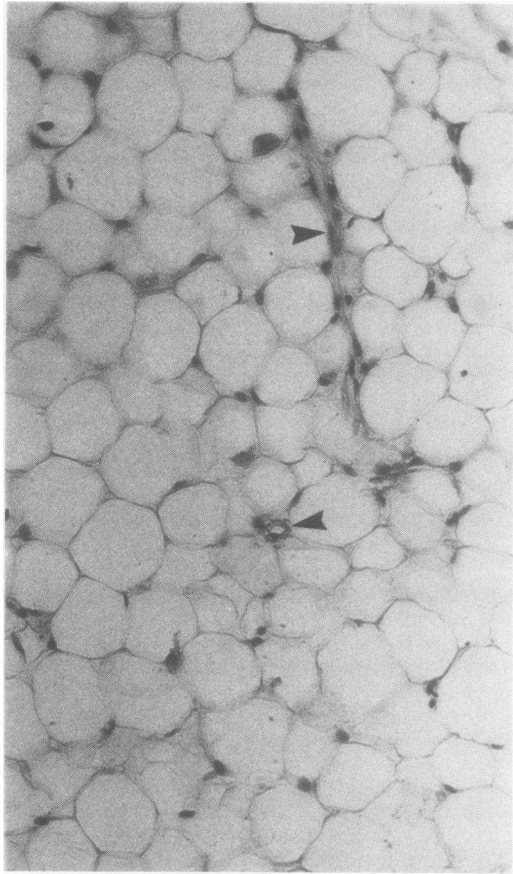
FIGURE 1 Light microscopy of a section through omental adipose tissue (hematoxalin and eosin). Two microvessels (arrows) can be seen embedded in a stroma composed mostly of adipocytes (×120).

24 h. Within 72 h, the cells began to assume the morphology characteristic of endothelium. The cells proceeded to spread out, becoming flat and polygonal with round or oval nuclei containing several nucleoli (Fig. 3A). Many cytoplasmic extensions reached out toward adjacent cells with some cells displaying a crescent or ring configuration (Fig. 3A–C). The growth of each endothelial colony occurred rapidly and was directed radially away from its center. At various stages of colony growth, many cells displayed a dense perinuclear phase-contrast lucent band (Fig. 3A). As the cells approached confluence, the numer of cytoplasmic extensions decreased and fewer cells exhibited a capillary-like appearance. After 1–3 wk, depending on the initial inoculum, a tightly packed monolayer with the characteristic cobblestone morphology was evident (Fig. 3D). Primary isolations have been repeatedly performed with no fibroblast or smooth muscle cell contamination noted by phase-contrast microscopy.

After several months at confluence, cells began to develop a granular cytoplasm and lift off the flask. This appearance of cellular senescence, however, usually disappeared with passage into subculture. To date, these human microvascular endothelial cells have been maintained for up to five passages in subculture. By the fifth passage there was increasing plemorphism of cells, and some delay in reaching confluence.

Electron microscopy of cultured cells. Endothelial cells in culture displayed most of the features noted in situ. Cells contained a moderate number of pinocytotic vesicles and numerous cytoplasmic parallel arrays of intermediate filaments (Fig. 4a). Characteristic endothelial junctional complexes were abundant in areas of cell-cell contact (Fig. 4a,b). Weibel-Palade bodies could not be reliably identified in these cells.

Anti-Factor VIII staining. Indirect and direct immunofluorescent staining with antiserum to human Factor VIII demonstrated the presence of the antigen in eight primary cultures and one first-passage culture of microvascular endothelium. Cytoplasmic illumination was produced (Fig. 5), with some granules of stained material concentrated in the perinuclear space. Cultures of human preadipocytes and mouse 3T3-L1 cells failed to stain with the Factor VIII antiserum used in these characterizations, while cultures of bovine aortic and first passage human microvascular endothelium produced staining of a similar intensity to the primary cultures of human microvascular endothelium.

Angiotensin-converting enzyme activity. All five cultures of microvascular endothelium assayed contained angiotensin-converting enzyme activity. The mean enzyme activity in primary cultures was 12.3 ± 1.5 U/min per confluent 25-cm^2 flask (mean$\pm$SEM, $n = 5$). Negative controls included flasks of endothelial cells incubated with angiotensin-converting enzyme substrate in the presence of SQ14,225, a specific kininase inhibitor, and flasks of several other cell types (T47D human breast cancer cells, rat hypothalamus, rat chondrocytes, and buffalo rat hepatocytes) cultured in the same serum-containing medium.

DISCUSSION

The isolation and culture of human microvascular endothelium has been reported previously from human foreskin, adrenal cortex, and spleen (11, 12, 16). In each system, the cells would grow to confluence only when supplied with a growth matrix on the culture flask and either tumor conditioned medium (16) or medium containing 50% human serum (11). One report of microvascular endothelium from human foreskin described growth in 5% serum (12). These cultures, however, were contaminated with up to 20% fibroblasts and would not grow in subculture. Rela-
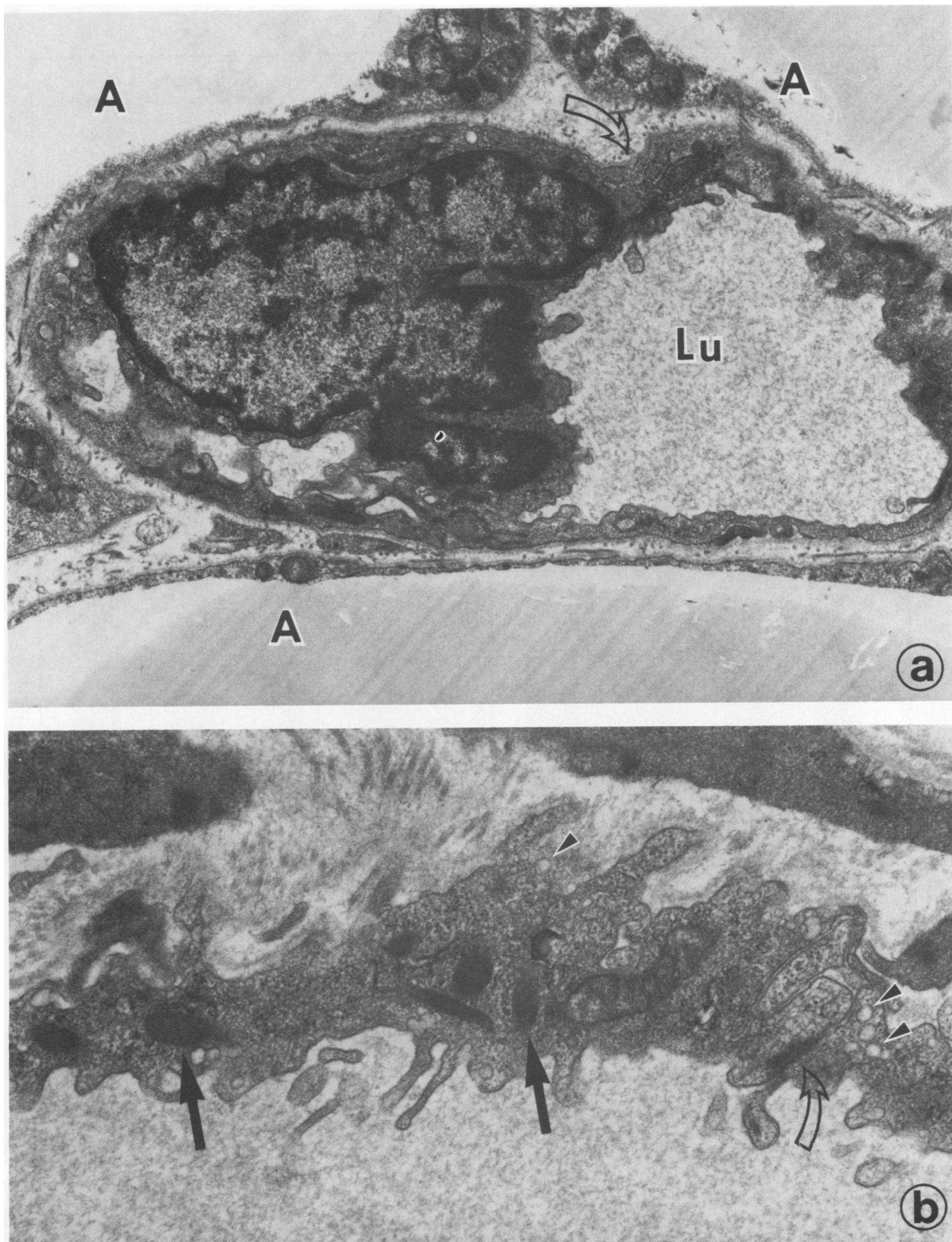
FIGURE 2 Electron microscopy of omental adipose tissue. (a) Low power view showing a capillary endothelial cell forming a lumen (Lu) and surrounded by a basal lamina (arrow). The adjacent cells are adipocytes (A) ×13,800. (b) Higher power view of endothelial cell processes. Cells are connected by a junctional complex (arrow) and have numerous pinocytotic vesicles (arrowheads). Several Weibel-Palade bodies are evident (arrows) ×29,300.
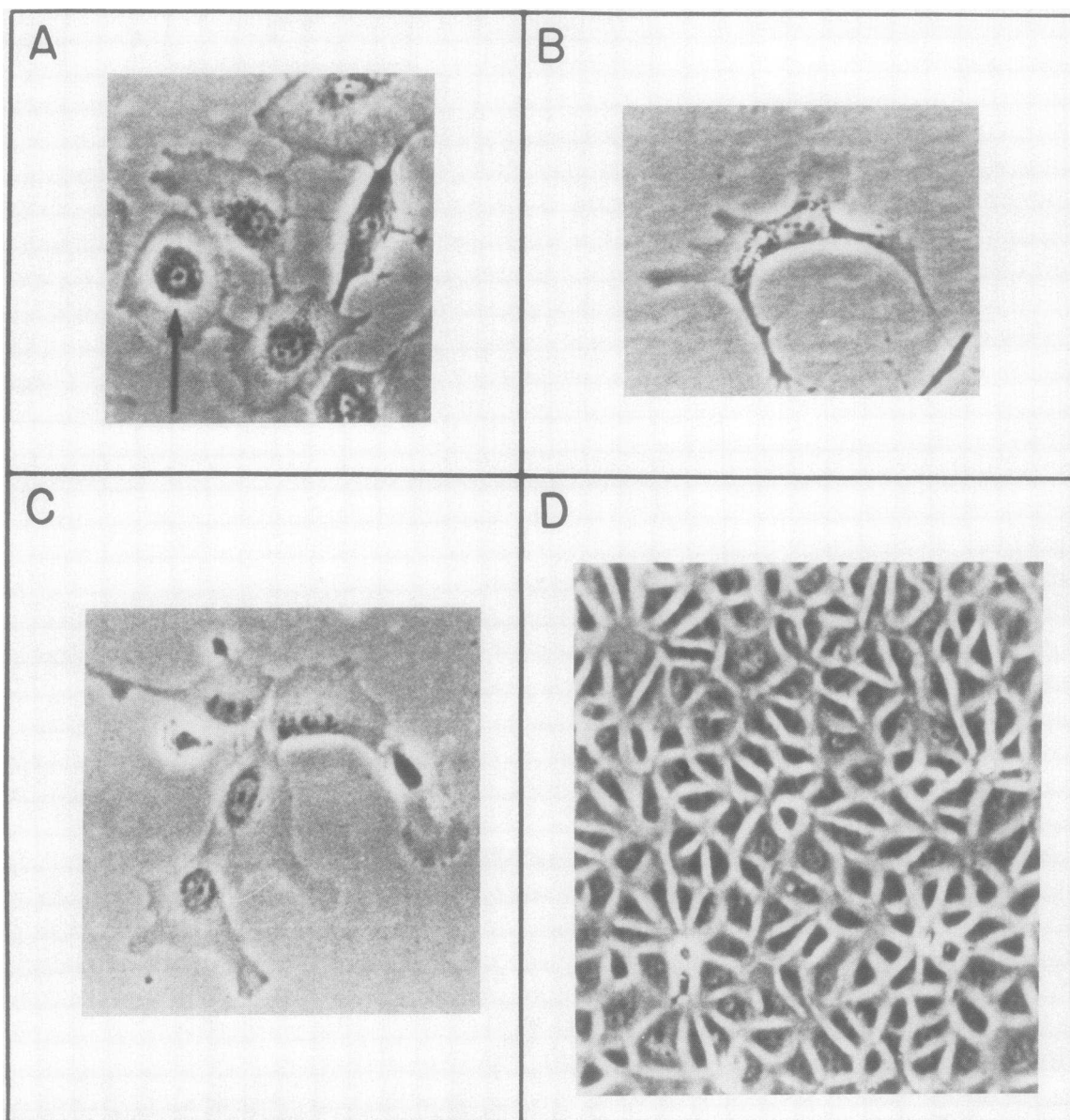
FIGURE 3 Phase-contrast microscopy of human microvascular endothelial cells in culture demonstrating: (A) numerous cytoplasmic extensions and a phase-lucent perinuclear band (arrow) in cells 3 d after primary isolation (×320); (B) a ring-like morphology displayed by an endothelial cell at day 3 (×320); (C) endothelial cells at day 5 arranged in a capillary-like network (×320); and (D) microvascular endothelial cells at confluence (×160).

tively pure cultures of endothelium were reported previously from rat epididymal fat pad, which grew to confluence in 20% serum (13). These cultures, however, required prolonged treatment with thimerosal to eliminate contaminating cells.

Adipose tissue represents an excellent source for the isolation of microvascular endothelium. With routine histologic sectioning and staining, and on electron microscopy, adipose tissue displays numerous capillaries and microvessels. Because most of the cells in fat are adipocytes, they can be separated from endothelial cells simply by flotation. After a 15-min digestion, endothelial cells are in cell aggregates that can be separated from other cell types by filtration through a 30-μm mesh. The few remaining fibroblasts that cling to the filter are removed by sedimentation on 5% al-
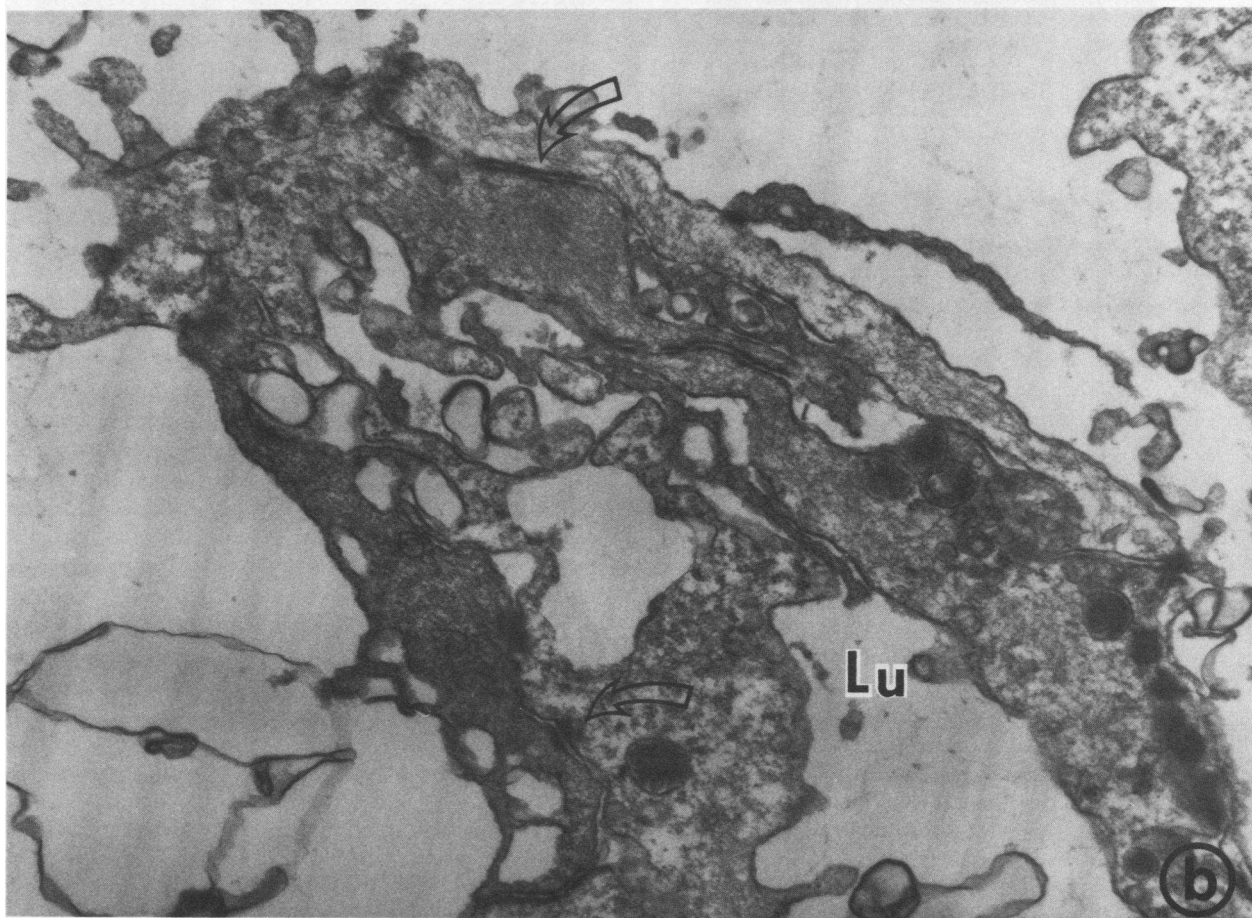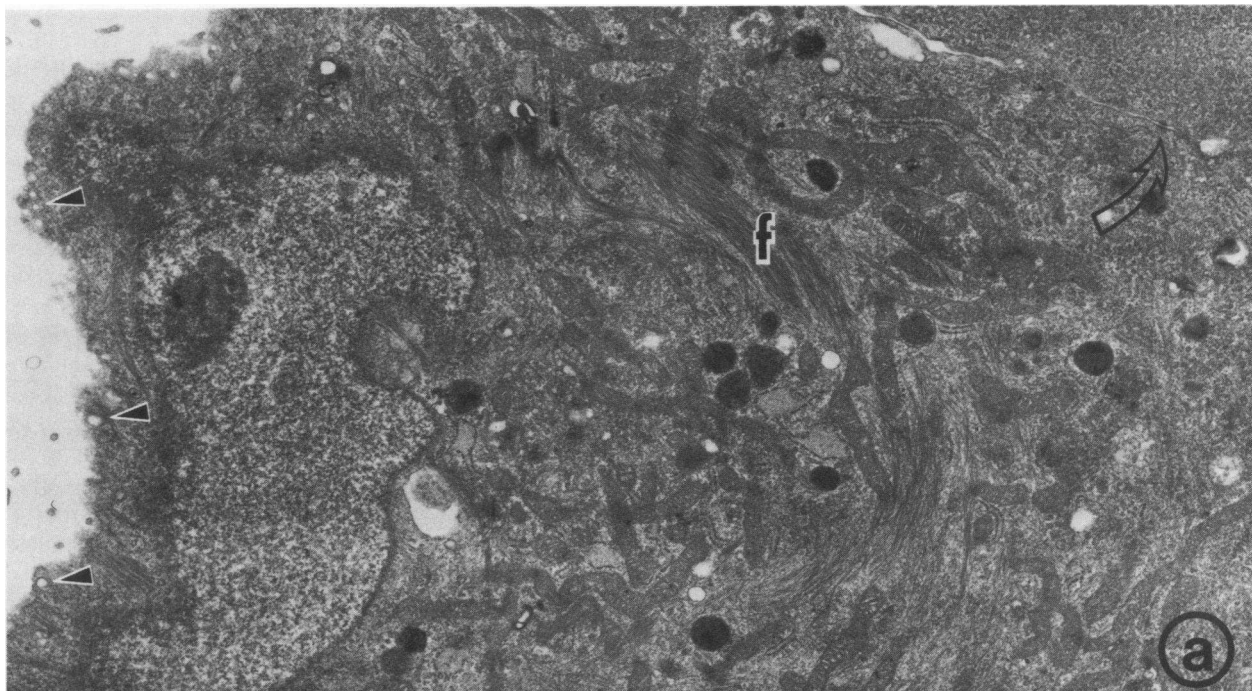
FIGURE 4 Electron micrographs of cultured microvascular endothelial cells. (A) *En face* section through the nucleus and cytoplasm showing bundles of filaments (f), a junctional complex (arrow), and pinocytotic vesicles (arrowheads; ×10,200). (B) Endothelial cell processes forming a lumen (Lu). Numerous junctional complexes (arrows) are apparent (×26,400).
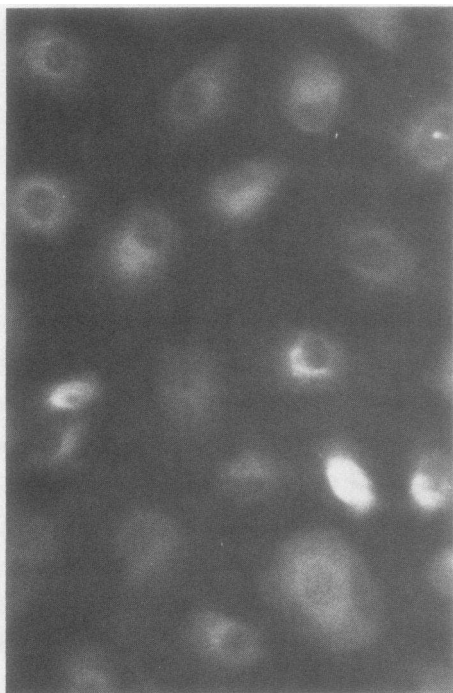
FIGURE 5 Indirect immunofluorescent staining for Factor VIII antigen. Cytoplasmic illumination is evident with some granules concentrated in the perinuclear space. Primary culture is at confluence (×370).

bumin. Once in culture, the endothelial cells divide rapidly and survive serial passage when cultured in Medium 199 with 10% serum. No basement membrane component was needed to serve as a matrix for these cells, and the addition of growth factors, other than those present in 10% whole serum, was not required. The reason for these less fastidious growth requirements is not clear. Adipose tissue is very active metabolically, and clearly requires a rich network of capillaries to facilitate the lipoprotein lipase-mediated hydrolysis of triglyceride-rich lipoproteins at the endothelial surface (23). Therefore, adipose cells may be instrumental in stimulating capillary endothelial growth, an effect that may be carried over into tissue culture.

The phase-contrast microscopic morphology of these cells at confluence is characteristic of endothelium as reported in other systems (11–16, 24). Numerous cytoplasmic extensions, often forming a capillary-like structure, are present as the cells spread out during the initial growth phase. The presence of a phase-lucent perinuclear ring on cells in culture has been shown previously to represent circumferential bands of perinuclear intermediate filaments (25–27). This pattern of intermediate filaments is believed to be characteristic of endothelial cells (25).

Electron micrographic features of these cells that are characteristic of endothelial cells in other systems include pinocytotic vesicles and the junctional complexes at cell-cell interfaces. Weibel-Palade bodies are endothelium-specific cytoplasmic organelles of unknown function that have been found in abundance in large vessel endothelium (1, 28). These structures, however, have been either absent (13–16) or present in a lesser frequency (1, 11, 12, 28, 29) in other preparations of microvascular endothelial cells. In the microvascular endothelium from this preparation, Weibel-Palade bodies were found in low frequency in cells in situ and were not present in cells in culture. Thus, the relative paucity of Weibel-Palade bodies in this system is consistent with other preparations of microvascular endothelium, and may represent an important morphologic feature that distinguishes large vessel from microvascular endothelial cells.

Factor VIII antigen is present only in endothelium, megakaryocytes, and platelets (30) and staining for this antigen was positive in all cultures from this preparation that were examined. Angiotensin-converting enzyme has been shown to be a sensitive marker for endothelium, although brush border epithelium from renal proximal tubule (31) and human dermal fibroblasts (32) also contain the enzyme. All cultures of adipose tissue microvascular endothelium assayed contained angiotensin-converting enzyme activity in quantities >10-fold higher than reported previously by the same assay technique in bovine aortic endothelial cells (21, 26).

Microvascular endothelium differs from large vessel endothelium in several ways. Each cell type displays a different morphology in situ (33). In addition, microvascular endothelial cells have demonstrated the ability to migrate in response to tumor-conditioned medium (34) and to form three-dimentional vascular networks in tissue culture (17). Disease states such as diabetic microangiopathy (8) and tumor angiogenesis (16) occur at the level of the microcirculation, and not in large arteries or veins. It remains to be determined whether these differences between large and small vessel endothelium represent a divergence in cellular differentiation, or differences in cell properties imposed by their different environments. The ability to propagate microvascular endothelium in culture will permit investigation of this basic question. In addition, since a subcutaneous fat biopsy can be performed without significant risk, endothelial cell growth and metabolism in individual patients can be studied. Thus, human microvascular endothelium from adipose tissue represents an easily obtainable tissue culture system that will facilitate the study of human endothelial cell biology and pathologic states involving the microvasculature.

ACKNOWLEDGMENTS

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