Human adipose tissue endothelial cells promote preadipocyte proliferation

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Human adipose tissue endothelial cells promote preadipocyte proliferation. Am J Physiol Endocrinol Metab 281: E1037–E1044, 2001.—Adipogenesis is preceded by development of a microvascular network, and optimal functioning of adipose tissue as an energy store and endocrine organ is dependent on extensive vascularization. We have examined the role of endothelial cell-derived factors that influence the proliferation of human preadipocytes. Microvascular endothelial cells and preadipocytes were isolated from human omental adipose tissue by use of a developed procedure of collagenase digest, immunoselection, and differential trypsinization. Conditioned medium from human umbilical vein, is that only MVEC are involved in regulation of adipose tissue growth such as occurs in the development of obesity. Furthermore, adipose tissue functions as both the major site of energy storage in the body and as an endocrine organ synthesizing and secreting a number of important molecules involved in regulation of energy balance (3, 6). For optimum functioning, therefore, adipose tissue requires extensive vascularization.

There is evidence that endothelial cells secrete factors that are involved in regulation of growth and differentiation of a variety of mesenchymal cells, including adipose precursors (7, 8, 31). Furthermore, endothelial cells are as diverse in structural and biochemical characteristics as the organs and tissues in which they are found (12, 23). It has also been demonstrated that endothelial cells derived from the microvascular structures of specific tissues differ significantly from large-vessel endothelial cells (10, 17, 20). One very important difference between microvascular endothelial cells (MVEC) and large-vessel endothelial cells, such as those derived from human umbilical vein, is that only MVEC are involved in angiogenesis (16). Therefore, it is important, when studying the paracrine effects of these cells, that the cells are isolated from vessels of the appropriate size and organ (5).

Recently, techniques for immunoselection and subsequent culture of microvascular endothelium from various tissues have been described. Isolation techniques utilizing magnetic beads coated with a monoclonal antibody to platelet-endothelial cell adhesion molecule 1 (PECAM-1) have been reported by a number of groups (9, 13, 15, 17, 22, 29). However, the presence of initially small numbers of contaminating
cells, such as fibroblasts and smooth muscle cells, which quickly outgrow the endothelial cells, has remained a major problem.

We have developed a simple technique that utilizes anti-PECAM-1-coated magnetic beads and subsequent differential trypsinization steps, which allows isolation and separate culture of both MVEC and preadipocytes derived from subcutaneous and omental adipose tissue depots. This isolation of both target cell types from one digest procedure allows optimization of the limited tissue available from any one individual. It also ensures that the MVEC are from the same tissue depot as the preadipocytes.

Because endothelial cells have common as well as specialized roles in different tissues and organs, we hypothesized that endothelial cells from human adipose tissue secrete a factor(s) that influences preadipocyte proliferation and differentiation. We also hypothesized that depot specificity in these effects might exist. In these experiments, we aimed to assess the effect of MVEC conditioned media on proliferation of human preadipocytes and to compare and contrast these effects in preadipocytes and endothelial cells from omental (intra-abdominal) and subcutaneous sites.

MATERIALS AND METHODS

All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

Production of Anti-PECAM-1 Antibody-Coated Magnetic Beads

Dynabeads M-450 with covalently bound sheep anti-mouse IgG1 (Dynal) were coated with purified mouse anti-human monoclonal antibody to PECAM-1 (CD31) (Pharmingen) according to the manufacturer's instructions. Dynabeads coated with anti-PECAM-1 antibody were resuspended and stored sterile at 4°C in deionized phosphate-buffered saline (DPBS) + 0.1% BSA at a concentration of 30 mg/ml. Prepared beads remained active for ≥4 mo.

Subjects

Paired omental (O) and abdominal subcutaneous (S) adipose tissue biopsies were obtained from 4 male [average age 69 yr, range 66–70 yr; average body mass index (BMI) 27, range 26–29] and 5 female (average age 55 yr, range 39–67 yr; average BMI 27, range 20–32) patients undergoing elective open-abdominal surgical procedures (either gynecological or vascular surgery). None of the patients had diabetes or severe systemic illness, and none were taking medications known to affect adipose tissue mass or metabolism. The protocol was approved by the Research Ethics Committees of the Princess Alexandra Hospital and the Queensland University of Technology. All patients gave their written informed consent.

Isolation of Stromovascular Cells

Biopsies were transported to the laboratory in Ringer solution (transport time 15 min). Preadipocytes and MVEC were isolated from the same biopsies. Arabic numerals refer to Fig. 1. 1) After removal of visible nerves, blood vessels, and fibrous tissue, the fat was finely minced and incubated for 1 h

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Fig. 1. Method for isolation and separate culture of microvascular endothelial cells (MVEC) and preadipocytes (PA) from human adipose tissue. DPBS, deionized PBS; RT, room temperature; HBSS, Hank's balanced salt solution; FCS, fetal calf serum; EC, endothelial cells; PECAM-1, platelet-endothelial cell adhesion molecule 1.
at 37°C in digest solution (in mM: 25 HEPES, 5 glucose, 129 sodium chloride, 50 potassium chloride, and 1 calcium chloride) containing 3 mg/ml type II collagenase and 1.5% BSA. The ratio of digest solution to adipose tissue was 4:1. The resultant digest material was filtered through a 250-μm mesh (Sigma), and adipocytes and free oil were separated from the stromovascular components by centrifugation at 250 g for 5 min at 4°C. The stromovascular pellet was suspended, washed, and centrifuged in DPBS + 10% BSA (600 g, 5 min, 4°C). This was repeated and followed by a final wash in DPBS alone. The resulting pellet was incubated in 0.25% trypsin containing 1 mM EDTA [trypsin-EDTA (T-V); Commonwealth Serum Laboratories, Brisbane, Qld, Australia] for 15 min at room temperature with occasional agitation. Trypsin was neutralized by addition of Hanks’ balanced salt solution (HBSS) containing 5% fetal bovine serum (FBS; ICN Biomedical Australasia). Large fragments of connective tissue were removed by filtration through 100-μm mesh (Sigma). The filtrate was centrifuged (600 g, 5 min, 4°C), and the pellet was resuspended and plated into 1% gelatin-coated 25-cm² culture flasks (Corning) in endothelial cell growth medium (M-199; ICN Biomedical Australasia) containing 10% FBS, 100 IU penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all ICN Biomedical Australasia), 90 μg/μl heparin, 30 ng/ml β-EC growth factor (β-ECGF), 0.014 M HEPES, and 0.15% NaHCO₃. This mixed cell population was cultured for 3–5 days at 37°C in 5% CO₂.

Selection of MVEC with anti-PECAM-1 Dynabeads. After a short culture period (∼3 days), the cells were incubated with T-V for 4–5 min, followed by neutralization of trypsin with HBSS + 5% FBS and centrifugation. The pelleted cells were resuspended in 1 ml HBSS + 5% FBS and incubated with 50 μl of anti-PECAM-1 coated Dynabeads (15 min, 4°C). The cell/bead suspension was brought to a total volume of 10 ml with HBSS + 5% FBS, and EC were selected using a magnetic particle concentrator for 3 min at room temperature. With the tube still in the magnet, nonselected cells were resuspended in 1 ml HBSS containing 5% FBS and reselected using the magnetic particle concentrator (3 min). This wash/selection procedure was repeated five times. Selected cells (endothelial cells) were plated onto 1% gelatin-coated culture flask in endothelial cell growth medium (as above). Nonselected cells (preadipocytes) in the wash were transferred to a new tube. Endothelial cells were then washed with a further 10 ml HBSS + 5% FBS and reselected using the magnetic particle concentrator (3 min). This wash-selection procedure was repeated five times. 9a) Selected cells (endothelial cells) were plated onto 1% gelatin-coated culture flask in endothelial cell growth medium (as above). 9b) Nonselected cells (preadipocytes) were centrifuged and resuspended in DMEM-lial cell growth medium (as above). This wash/selection procedure was repeated five times.

Purification of endothelial cell cultures. Separation of EC from contaminating fibroblastic cells was achieved by treating the cultures with T-V for 30–40 s, neutralizing the T-V with HBSS + 5% FCS, and transferring the nonadherent EC to a 1% gelatin-coated flask with endothelial cell growth medium. This trypsinization and transfer procedure was repeated one or two times over the first 2 wk of culture until homogeneous EC cultures were obtained.

Cell Culture

Cells were maintained at 37°C in an atmosphere of 5% CO₂. The medium was changed every 2–3 days, and cells were routinely passaged with T-V. Endothelial cells were maintained in gelatin-coated flasks in endothelial cell growth medium while preadipocytes were in uncoated culture flasks in preadipocyte growth medium. As endothelial cell numbers increased, the concentration of β-ECGF in the endothelial cell growth medium was decreased from 30 to 10 ng/ml. Both endothelial cells and preadipocytes were used in experimental work between passages 2 and 4.

Culture of Other Cell Types.

The human dermal MVEC line CADMEC (Cell Applications, San Diego, CA; cultured under the same conditions as adipose-derived primary endothelial cells) and human skin fibroblasts (HSF; obtained by punch biopsy and cultured under identical conditions as the human PA) were used as positive and negative controls, respectively, for EC studies.

Characterization of Endothelial Cells

MVEC obtained from adipose tissue biopsies were characterized in a number of ways.

Morphology. Cultures were examined by inverted phase-contrast microscopy for the characteristic cobblestone morphology of endothelial cells (Fig. 2A).

Immunofluorescence. Cells were evaluated by immunofluorescence with the use of specific monoclonal antibodies for expression of von Willebrand’s Factor (vWF) (clone F8/86, DAKO; Fig. 2B) and PECAM-1 (CD31) (clone JC/70A, DAKO; Fig. 2C). Cells were grown to confluence in individual wells of 24-well plates (1% gelatin coated). Control cells (CADMEC), primary cultures of human PA, and HSF were processed in parallel. After removal of medium, cells were fixed in 2% paraformaldehyde (BDH Laboratory Supplies, Dorset UK) for 2 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 (Ajax Chemicals, Auburn NSW, Australia) for 30 s at room temperature. Fixed and permeabilized cells were washed and blocked with 1% BSA in PBS (3×) before incubation for 4 h at 4°C with primary antibodies applied after dilution in PBS + 1% BSA (all antibodies used at 1:100 dilution). To preclude false positives produced by nonspecific binding of secondary antibodies, all cell types were also treated in a similar manner with either buffer substituting for primary antibody or with nonimmune antibody (isotype control). The cells were washed with PBS (3×) and then incubated at room temperature for 30 min with fluorescein isothiocyanate (FITC)-labeled secondary antibody (rabbit anti-mouse IgG FITC; DAKO) at 1:50 dilution in PBS + 1% BSA. Cells were washed (2×) with PBS, and then nuclei were counterstained with propidium iodide (stock: 5 mg propidium iodide in 100 ml 0.1 M trisodium citrate; working solution: 1 part stock to 3 parts 0.1 M PBS) for 5 min at 4°C. Cells were washed a further two times with PBS before being examined and photographed with a Nikon Eclipse TE300 inverted microscope with a Nikon TE-FM Epi-Fluorescence attachment and a Nikon F70 camera with Kodak MAX 400 ASA film. The expression of E-selectin (CD62E) was also investigated by use of a monoclonal antibody (clone BBIG-E4, R&D Systems) using fluorescence-activated cell sorting (FACS) analysis. The expression of E-selectin in preadipocytes was not detected.

Gene expression. MVEC and CADMEC were examined for expression of endothelial nitric oxide synthase (eNOS) by the NOS3 gene (Fig. 4). Total RNA was extracted from the cells using TRI-reagent (Sigma) according to the manufacturer’s instructions. Two micrograms of RNA were converted into cDNA by use of Expand Reverse Transcriptase (Roche) with standard methodologies. PCR was performed in a total reaction volume of 25 μl containing 1× PCR buffer, 1 μl of cDNA, 12.5 pmol of each primer, 1.5 mM MgCl₂, and 0.625 U of Taq DNA polymerase. Primer sequences and thermal cycling

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conditions were as previously described (28). PCR products were separated on 1.2% agarose gels containing 1 mg/ml of ethidium bromide in 1/3 Tris-borate-EDTA buffer and viewed and photographed under ultraviolet light. FX174 markers were used.

Characterization of PA

As previously described (14), PA were characterized on the basis of morphology (phase contrast microscopy and cell counts) and differentiation capacity. The latter was assessed by glyceraldehyde-3-phosphate dehydrogenase (G3PDH) enzyme activity and triacylglycerol accumulation.

G3PDH activity. Activity was assessed as previously described (1, 18) (Fig. 5A).

Triacylglycerol accumulation. Cell counts and Nile red assay were used to assess lipid accumulation.

Cell counts. After 14 days of treatment in differentiation medium, the number of lipid-containing cells in each treatment was estimated under phase contrast microscopy using a Neubauer hemacytometer (Sigma-Aldrich, Castle Hill, Australia) at ×100 magnification. For each treatment, 10 different areas were examined, and both total number of cells and percentage of lipid-containing cells were evaluated (data not shown).

Nile red assay. As previously described (18), PA cultured in 6-well plates were washed 3 times in PBS (pH 7.4), and 150 μl of T-V were added to each well. Cells were incubated at 37°C for 10 min until cells detached from the culture plate. PBS containing Nile red, at a final concentration of 1 mg/ml, was added to each well, and cells were further incubated at room temperature for 5–7 min. Fluorescence was measured at room temperature in a spectrofluorometer (Aminco Bowman Series 2 Luminescence Spectrometer) at 488 nm excitation/540 nm emission. Results were normalized to surface area (Fig. 5B). Each treatment was carried out in triplicate.

Preparation of Conditioned Medium

Separate cultures of MVEC, CADMEC, and HSF, all at confluence on 1% gelatin-coated cultureware, were each exposed to endothelial cell growth medium (see Isolation of Stromovascular Cells) containing 10 ng/ml β-ECGF for 48 h at 37°C in 5% CO2. This medium was then collected, filtered using a 0.22-μm low protein-binding filter, and stored at 4°C for subsequent use.

Fig. 2. Characterization of adipose tissue-derived MVEC. A: phase-contrast photomicrograph of MVEC isolated from human adipose tissue. Note the typical cobblestone morphology and the prominent, centrally located nuclei. B: immunocytochemical staining for von Willebrand’s factor (vWF) shows prominent perinuclear cytoplasmic staining. C: immunocytochemical staining for PECAM-1 shows junctional staining consistent with plasma membrane expression. In B and C, nuclei counterstained with propidium iodide. (Bar = 10 μm; original magnification ×200).

Fig. 3. E-selectin expression by adipose-derived MVEC. A: after 4-h stimulation with TNF-α (10 ng/ml) immunostaining for E-selectin was diffusely cytoplasmic. B: control for A was adipose-derived MVEC that were maintained in normal medium without TNF-α for 4 h (Bar = 10 μm; approximate magnification ×200).

Fig. 4. Endothelial nitric oxide synthase (eNOS) gene expression in adipose-derived MVEC and in a dermal endothelial cell line (CADMEC).

Fig. 5. E-selectin expression by adipose-derived MVEC. A: after 4-h stimulation with TNF-α (10 ng/ml) immunostaining for E-selectin was diffusely cytoplasmic. B: control for A was adipose-derived MVEC that were maintained in normal medium without TNF-α for 4 h (Bar = 10 μm; approximate magnification ×200).
Preadipocyte Proliferation Assays

Subcutaneous and omental PA and HSF were plated separately at $1\times 10^5$ cells/well (subconfluent) in 96-well plates in DMEM-Ham’s F-12 (1:1) + 10% FCS (PA growth medium) and allowed to adhere at 37°C in 5% CO₂ for 16–20 h. The medium was then changed to EC growth medium that had been conditioned (see Preparation of Conditioned Medium) by exposure to confluent subcutaneous or omental MVEC, HSF, or wells containing no cells (blank control). Just before use, each medium was thawed, and a further 5% FCS was added to each. Cell numbers were assessed using a formazan colorimetric assay (Promega). The water-soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (SRB) was added to each well at a concentration of 200 μg/ml. After incubation at 37°C for 4 h, absorbance at 490 nm was measured with a Bio-Rad 3550 microplate reader. The validity of this assay was tested in two ways: 1) preadipocytes were plated at 250, 500, 1,000, 2,000, and 4,000 cells per well (in quadruplicate), and absorbance was measured at 490 nm; and 2) after measurement at 490 nm, the cells were subsequently stained with propidium iodide, and direct cell counts were carried out using fluorescence microscopy. A total of four fields per well was counted, and these results were compared with those obtained with formazan absorbance at 490 nm.

Statistics

The correlation between cell number and optical density was estimated by means of Pearson’s correlation coefficient. Proliferation data were evaluated by one-way analysis of variance for repeated measures. Post hoc comparison for the within-condition effect was handled with paired t-tests at $\alpha = 0.005$.

RESULTS

Isolation of Adipose-Derived MVEC

After selection for MVEC with the use of anti-PECAM-1-coated Dynabeads, individual cells coated with numerous beads could be seen by means of phase contrast microscopy. These cells grew quite rapidly in distinctive cobblestone patches, and the number of beads on each cell decreased with mitosis. The presence of beads on the cells was not found to have any adverse effects. Contaminating fibroblastic cells, characterized by morphology and absence of beads, were also found in the MVEC cultures after initial selection with Dynabeads. We have determined that, after T-V treatment, rapidly growing MVEC lift off cultureware within 1–2 min, whereas contaminating fibroblastic cells remain adherent for a much greater time. This allowed separation of the MVEC from other cell types present in the culture, and homogeneous cultures of adipose-derived MVEC were achieved. These MVEC displayed typical cobblestone morphology and formed contact-inhibited monolayers (Fig. 2A). These cells were successfully grown to passage 6 with no changes in morphology. Cells between passages 2 and 4 were used in experimental work.

Isolation of PA

PA negatively selected during Dynabead selection for MVEC displayed typical fibroblastic morphology, which contrasted markedly with the cobblestone morphology of the MVEC. These cells were also used between passages 2 and 4.

Characterization of MVEC

Table 1 summarizes the results of characterization studies. Confluent monolayers of adipose-derived MVEC were examined by phase-contrast microscopy and showed a homogeneous monolayer of cells with morphology typical of endothelial cells (Fig. 2A). The endothelial nature of these cells was confirmed by immunofluorescence studies, which demonstrated expression of vWF, PECAM-1 (CD31), and E-selectin, all of which are endothelial cell-specific markers (16, 17, 24). Immunofluorescence microscopy showed that vWF is expressed throughout the cytoplasm of MVEC, particularly in the perinuclear region (Fig. 2B), whereas PECAM-1 is expressed on the cell membrane, particularly at points of cell-cell contact (Fig. 2C). The CAD-MEC line showed the same pattern of expression of vWF and PECAM-1 as adipose-derived MVEC (not shown). Incubation with 10 ng/ml TNF-α for 4 h induced E-selectin expression in the adipose-derived MVEC (Fig. 3A), with immunostaining being charac-
teristically perinuclear cytoplasmic. This staining was absent in unstimulated controls (Fig. 3B). HSF and PA did not express any of the markers. In all cases, there was no nonspecific binding either with secondary antibody or with the isotype control (not shown). RT-PCR studies demonstrated that both MVEC and CADMEC strongly express the NOS3 gene (Fig. 4), which codes for eNOS, a marker of functional endothelium (30).

MVEC isolated using the described method were shown to express vWF, PECAM-1, and E-selectin from passage 1 to passage 6, and eNOS expression was present even after recovery from cryopreservation.

Characterization of PA

When exposed to differentiation-permissive medium, PA started to accumulate intracytoplasmic lipid after 5–6 days. By 21 days in this medium, cell counts demonstrated that between 25 and 40% of cells contained lipid (results not shown). G3PDH activity and triacylglycerol accumulation were also measured at 21 days, and both were found to be markedly increased over the negligible levels in PA maintained during this period in nondifferentiating PA growth medium (Fig. 5). HSF cultured under the same conditions as the preadipocytes did not accumulate lipid, and no G3PDH activity was demonstrated (data not shown). MVEC obtained from adipose tissue did not survive in the aforementioned serum-free differentiation medium. PA isolated using this procedure were morphologically and biochemically indistinguishable from those isolated using standard techniques (14). This was demonstrated on the basis of G3PDH activity and triacylglycerol accumulation (Fig. 5).

Effect of MVEC Conditioned Media on PA Proliferation

To determine whether any soluble factors affecting PA proliferation were secreted by MVEC, the human PA were exposed to 48-h treatment with MVEC conditioned medium. Results demonstrated a significant increase in the rate of proliferation of PA (both subcutaneous and omental) compared with controls (Fig. 6; \( P < 0.001 \)). This result was similar for PA treated with MVEC conditioned media from both S and O adipose tissue sites; however, PA treated with S MVEC showed a slightly higher trend in proliferation rate than those treated with O MVEC (Fig. 6). Figure 6 demonstrates that the mitogenic effect of factors produced by adipose-derived MVEC on PA shows some specificity, as proliferation induced by CADMEC was not as great as that induced by adipose-derived MVEC (\( P = 0.001 \)). Conditioned medium from HSF had no increased proliferative effect on PA over the blank control. The proliferation assay used in these studies was validated using known numbers of PA, and results demonstrated a linear relationship between cell number and absorbance at 490 nm (\( r^2 = 0.9 \)). In a limited number of experiments, direct cell count was also used to validate the results and showed a positive correlation (Pearson correlation coefficient = 0.97) with formazan absorbance at 490 nm in both test and experimental assays.

DISCUSSION

In this study, we have described a method for isolation and culture of MVEC and preadipocytes from the human adipose tissue. The isolation of these cells using a serum-free method is important for the study of their function in vitro and in vivo. The use of MVEC conditioned medium in the culture of PA allowed for the demonstration of a significant increase in proliferation rate. This result suggests that MVEC produce soluble factors that promote PA proliferation. The specificity of this effect is supported by the observation that CADMEC, which are derived from the dermal layer of human skin, do not produce the same mitogenic effect as adipose-derived MVEC.
same human adipose tissue sections. We have also demonstrated a paracrine effect of the MVEC from both subcutaneous and omental sites on the proliferation of preadipocytes.

Isolation and culture of microvascular endothelial cells has been difficult due mainly to small initial yields and overgrowth of contaminating cells (17). However, recently described techniques utilizing magnetic beads (Dynabeads) coated with either Ulex europaeus agglutinin 1 or a monoclonal antibody to PECAM-1 have alleviated some of the earlier problems associated with the isolation of MVEC from a number of different tissues (4, 15, 17, 19).

We have developed a modification of this method and chosen to coat the magnetic beads with anti-PECAM-1, because it has been reported to be very specific for MVEC in adipose tissue (15). This is particularly important in adipose tissue from omental sites, which also contain mesothelial cells (16, 24). This cell type shares several morphological and functional features with endothelial cells but does not express PECAM-1 (17). PECAM-1 was also chosen as the selection antigen because it is one of the few endothelial cell surface molecules that shows trypsin resistance and persists in culture over several passages (13).

In our hands, the use of Dynabeads coated with anti-PECAM-1 proved successful in the initial selection of MVEC from adipose tissue, but nonendothelial cells contaminating the culture were still a problem. We had previously observed that MVEC, when treated with trypsin-EDTA, lifted off the cultureware surface much faster than the nonendothelial cells. Using this differential sensitivity to trypsin-EDTA, we developed a simple technique for separating rapidly growing MVEC from contaminating cells and were thus able to obtain homogenous cultures of MVEC from adipose tissue biopsies (see Fig. 2A). These cells were characterized as endothelial by their distinctive cobblestone morphology and the expression of vWF (Fig. 2B), PECAM-1 (Fig. 2C), and E-selectin (after TNF-α stimulation; Fig. 3A). These markers are specific for MVEC from adipose tissue, as vWF is not found in any other cell type present in adipose tissue (24), and apart from endothelial cells, PECAM-1 is found only in myeloid cells and platelets, none of which are viable in the culture conditions used (29). Contamination by mesothelial cells of endothelial cell cultures obtained from omental sites can occur; however, we have ruled out this possibility, because mesothelial cells do not express vWF, PECAM-1, or E-selectin (17). The use of PECAM-1 in both the selection and characterization of adipose-derived MVEC demonstrates that expression of this surface protein is present at the time of isolation and persists over several passages. MVEC were also shown to strongly express eNOS, which has been shown to be a marker of both endothelial cell identity (21, 27) and endothelial cell function (30). All markers of endothelial cell identity were present both in CADMEC and in MVEC derived from adipose tissue (passages 1–6). This would indicate that these markers are not affected by the MVEC isolation procedure and persisted over several subcultures. The presence of eNOS and PECAM-1 gene expression gives strong indication that these cells are functional MVEC (30), and expression of E-selectin after cytokine stimulation is further evidence that these cells are MVEC in which the integrity of functional properties has been preserved. MVEC were used in experimental procedures between passages 2 and 4.

Preadipocytes negatively selected using this isolation technique had the same morphological appearance as those isolated using standard procedures. These cells developed the same degree of G3PDH activity after 21 days in differentiation medium containing thiazolidinedione as did those obtained by standard procedure (P > 0.5; see Fig. 5). Overall, we did not detect any cell loss, morphological changes, changes in growth characteristics, or degree of differentiation between preadipocytes obtained using standard isolation procedures and those obtained using the present technique.

We have used the MVEC isolated as discussed to study their paracrine interactions with human preadipocytes. The MVEC and preadipocytes were isolated from the same adipose tissue biopsies and were cultured separately. Results demonstrated a significant mitogenic effect of the MVEC conditioned medium (Fig. 6). Overall, there was no significant site-specific difference in proliferation of human preadipocytes treated with conditioned medium from MVEC derived from either subcutaneous or omental sites, but subcutaneous preadipocytes treated with subcutaneous MVEC conditioned media showed a slightly higher trend. The greater proliferative response of preadipocytes to conditioned medium from adipose-derived MVEC over the response to CADMEC indicates some specificity of effect. This is consistent with the organ-specific characteristics of endothelial cells (11). Human skin fibroblasts also responded to the mitogenic effect of adipose-derived MVEC to the same degree as did the preadipocytes. This is not altogether surprising, because microvascular endothelial cells have been shown to secrete a number of mitogens, including IGF-1 (26) and members of the heparin-binding fibroblast growth factor family (6), all of which would have a proliferative effect on a number of cell types.

These findings demonstrate paracrine interactions between microvascular endothelial cells and preadipocytes present within each adipose tissue depot. Because the hyperplastic capacity of adipose tissue resides in the fibroblast-like preadipocyte pool, these results show that factors produced by microvascular endothelial cells may play an important role in the development of human obesity (25). The factor(s) produced by MVEC responsible for the demonstrated increase of human preadipocyte proliferation remains to be determined. MVEC are known to produce several growth factors, and we are currently investigating a number of candidate molecules to determine whether these may be responsible for the observed effects.

Growth of adipose tissue also depends on the adipose conversion of the preadipocytes contained within fat.
tissue. Future studies will investigate the role that MVEC may play in development of adipose tissue stores, concentrating specifically on the differentiation of human preadipocytes.

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