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Isolation and Pure Culture of Microvascular Endothelial Cells from the Fetal Skin

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Microvascular endothelial cells were purely isolated from human fetal skin using magnetic particles. The principle of this technique is based on the selective binding of the lectin Ulex europaeus I (UEA I) to the endothelial cell surface via fucose residues. Initially UEA I was covalently bound to tosyl-activated magnetic polydisperse polymer particles (Dynabeads) and then the UEA I-coated beads were collected using a magnetic particle concentrator (MPC). Endothelial cells were isolated by extracting microvascular segments from trypsin-treated fetal skin tissue and were purified by sieving with nylon mesh and by 35% Percoll gradient centrifugation. For further purification, the obtained cells were incubated with UEA I-coated Dynabeads. The endothelial cells bound to the Dynabeads were collected using MPC. This is a simple and reproducible technique for isolating a pure population of microvascular endothelium from the fetal skin.

Key Words: Fetal skin, microvascular endothelial cells, isolation, Ulex europaeus I, magnetic beads

Endothelial cells are critical in diverse biologic process, including leukocyte trafficking and homing, inflammation, wound healing, tumor metastasis, and angiogenesis. Investigations of these processes have, until recently, used endothelial cells isolated from the large blood vessels such as bovine aorta or human umbilical vein. However, it is now clear that there are many fundamental differences between large vessel endothelial cells and microvascular endothelial cells (Zetter, 1981; Gumkowski *et al.* 1987). The active involvement of the microvascular endothelium in the physiology and pathology of the skin is becoming increasingly apparent. Thus to understand the development of human skin disease an important first step is the isolation and culture of human dermal microvascular endothelium.

In vitro culture of microvascular endothelium remains a difficult procedure because it is not easy to isolate the microvascular endothelial cell population. The major problem in the isolation and culture of microvascular endothelial cells is the presence and rapid overgrowth of fibroblasts. Many investigators have tried to eliminate these contaminating cells. The manipulations of culture media encourage the selective growth of endothelial cells and inhibit fibroblast proliferation. The preferential uptake of acetylated low-density lipoprotein has been utilized to obtain pure human umbilical vein endothelial cells by flow cytometry (Voyta *et al.* 1984). Also, contaminating cells have been physically destroyed from endothelial cell cultures by surgical diathermy under phase microscopy (Marks and Penny,

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1986). However, these methods have given limited success, are time consuming and do not give a reproducible yield of early passage endothelial cells.

Human endothelial cells possess a specific property to bind selectively to the lectin Ulex europaeus I (UEA I) (Holthofer *et al.* 1982). This lectin specifically binds to a glycoprotein having an α-L-fucosyl group on endothelial cells (Hormia *et al.* 1983).

Recent advances in cell culture techniques have made it possible to isolate microvascular endothelial cells from synovium by covalently coupling UEA I to magnetic polydisperse polymer particles (Dynabeads) (Abbot *et al.* 1992).

There are some differences in fetus and adult skin. Especially, the basic difference in fetal wound healing and adult wound healing is that typical acute inflammatory reaction is absent in fetal wound healing. Although fetal skin has been used as a model of wound healing, there has not yet been a study on isolation and culture of microvascular endothelial cells from fetal skin. Since fetal dermis contains many dispersed premature pleuripotential cells with vasculatures, contamination and rapid growth of fibroblasts have made the previous isolation method of microvascular endothelial cells impossible.

In this study, we described the method to isolate and cultivate fetal dermal microvascular endothelial cells (FMEC) using UEA I-coated magnetic particles. The identification and growth pattern of cultured cells were described.

MATERIALS AND METHODS

Preparation of ulex europaeus I-coated dynabeads

Tosyl-activated Dynabeads (Dynal; Dynatech, Alexandria, VA, USA) were washed in distilled water. UEA I lectin (Sigma Chemical Co., St. Louis, MO, USA) was covalently bound to the beads by vortex suspension in 0.5 M borate solution, pH 9.5, containing 10 μg/ml UEA I. Equal volumes of washed Dynabeads and UEA I solution were mixed for 24 hours at 22℃ by slow rotation. The coated beads were collected using a magnetic particle concentrator (MPC). The beads were washed in 0.01 M phosphate buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin for 10 minutes, then for a further 30 minutes, followed by an overnight wash at 4℃.

FMEC isolation

Fetal skin specimen was cut into 3-mm squares and placed in PBS containing 0.3% trypsin (Sigma Chemical Co.) and 1% EDTA (Sigma Chemical Co.) at 37℃ for 10 minutes. The skin segments were washed with Hank's balanced salt solution (HBSS) several times and placed in a petri dish in HBSS with the keratinized surface down. They were then individually compressed with the side of a scalpel blade to express microvascular fragments from the cut surfaces of the skin. The microvascular fragments were passed through a 105 μm nylon mesh (Small Parts Inc., Miami, FL, USA) and collected. The microvascular fragments in 1 ml of HBSS were layered onto a 35% solution of Percoll (Pharmacia AB, Uppsala, Sweden) in HBSS and spun at 400 × g for 15 minutes at room temperature. The fraction with a density less than 1.048 g/ml, which was rich in microvascular fragments, was removed. Those portions of the gradient containing the microvascular segments were spun at 400 × g for 15 minutes again.

Cell purification

UEA-I coated Dynabeads were added to the cell suspension at an approximate concentration of 3 per endothelial cell. Cells and beads were gently mixed at 4℃ for 10 minutes, after which endothelial cells bound to the Dynabeads were isolated using the MPC. Cell-bead complexes were then resuspended in HBSS, and the procedure was repeated twice.

Cell culture

Isolated endothelial cells were cultured in endothelial basal media (Clonetics Corp., San Diego, CA, USA) with 5 ng/ml epidermal growth factor (Clonetics Corp.), 1 μg/ml hydrocortisone acetate (Sigma Chemical Co.), 5 ×

10^{-5}M dibutyryl cyclic AMP (Sigma Chemical Co.), 100 μg/ml streptomycin, 250 μg/ml amphotericin B (Sigma Chemical Co.), and 30% human serum (Irvine Scientific, Santa Ana, CA, USA). Cells were maintained at 37℃ in a humidified atmosphere of 95% air: 5% CO_2. The resulting cell cultures were consistently 100% pure, as assessed by morphologic and immunochemical criteria. Experiments were conducted with endothelial cells at passages 2-8.

Characterization of FMEC

Four criteria were used to establish the endothelial origin of cells isolated: ① the characteristic cellular morphology and growth pattern by phase-contrast microscopy; ② the presence of Factor VIII related antigen by indirect immunofluorescence; ③ the uptake of acetylated low-density lipoprotein (LDL); and ④ the regulation pattern of the expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) after treatment with interleukin 1α (IL-1 α) and tumor necrosis factor a (TNF-α).

Staining for factor VIII antigen

Cells were grown and stained on 12-mm gelatin-coated glass coverslips. The coverslips were fixed in 100% methanol for 10 minutes at −20℃, washed in HBSS supplemented with Ca^{2+} and Mg^{2+} ions, and incubated for 30 minutes at room temperature with a 1:50 dilution of mouse Factor VIII antigen (Immunotech, Marseilles, France). The cultures were washed three times for 15 minutes each in HBSS and incubated for an additional 30 minutes with a 1:20 dilution of fluorescein-conjugated anti-mouse IgG. After additional wash in HBSS, the coverslips were inverted over a drop of glycerol prior to viewing.

Uptake of acetylated low-density lipoprotein

Endothelial cells possessing a "scavenger pathway" selectively ingest modified LDL (Voyta *et al.* 1984). FMEC on glass coverslips were incubated with 10 μg/ml of 1,1'-dioctadecyl-1-(3,3,3',3'-tetramethyl)-indocarboxyamine perchlorate-labeled acetylated LDL (DiI-Ac-LDL; Biogenesis, Bournemouth, UK) at 37℃ for 4 hours. The cultures were washed three times in HBSS and examined for fluorescence at 480 nm with standard filter sets for rhodamine fluorescence.

ELISA for the study of expression of ICAM-1 and VCAM-1 in response to IL-1α or TNF-α

FMEC were plated into 96-well flat-bottomed microtiter plates at a concentration of 4×10^4 cells per well and were preincubated with either cell culture media alone or with 100 unit/ml IL-1α or 100 unit/ml TNF-α at 37℃ in an atmosphere of 5% CO_2 for 4 hours. Subsequently, cells were fixed and stained for ICAM-1 and VCAM-1 using an ELISA as previously described (Lee *et al.* 1992).

RESULTS

Morphological characteristics of FMEC

FMEC was isolated by Ulex-coated beads, and its morphology was observed by phase-contrast microscope. Primary cultures of FMEC, 24 hours after isolation, showed typical endothelial morphology. Ulex-coated beads bound to FMEC at a ratio of 3 to 5 beads per cell (Fig. 1). The number of beads bound per cell decreased as the cells replicated due to a diluting effect. By the second passage, no attached beads can be seen and the endothelial cells form a typical cobblestone-like confluent monolayer-i.e., a hexagonal or cuboidal shape with a large round nucleus and very little overlapping of adjacent cells (Fig. 2). Overgrowth of contaminating cells could not be observed.

Expression of factor VIII antigen on FMEC isolated

Endothelial cell expresses Factor VIII antigen on the cell surface. To identify the isolated cells originated from endothelium, the cultured cells were stained for Factor VIII-related antigens. Virtually almost all of the cells were stained positively for Factor VIII-related antigens. They demonstrated a pattern of dot staining localized in cytoplasmic granules, es-

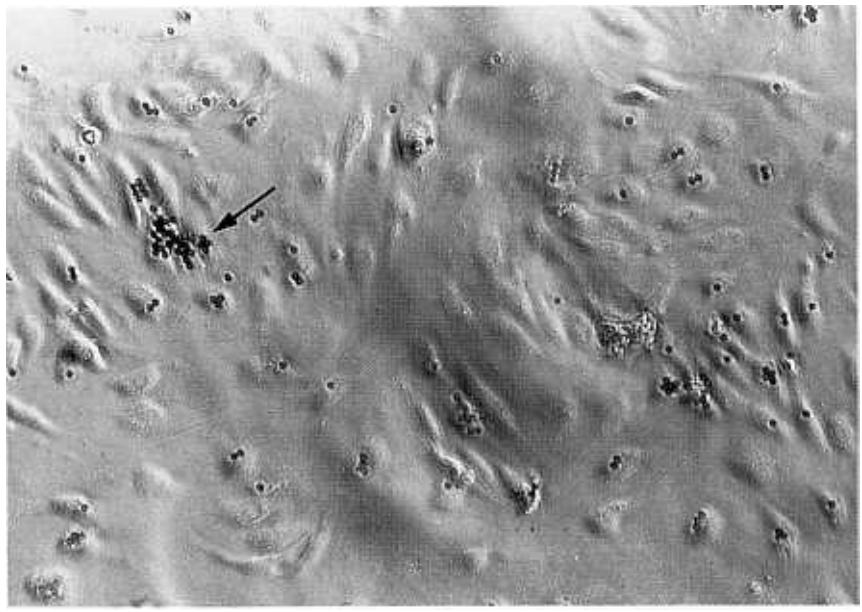

Fig. 1. *Morphology of fetal dermal microvascular endothelial cells, 24 hours after isolation with Ulex europaeus I-coated beads (×100). Some of the cells show cytoplasmic extension typical to endothelial morphology. Ulex europaeus I-coated beads (arrow) were bound to endothelial cells at a ratio of 3 to 5 beads per cell.*

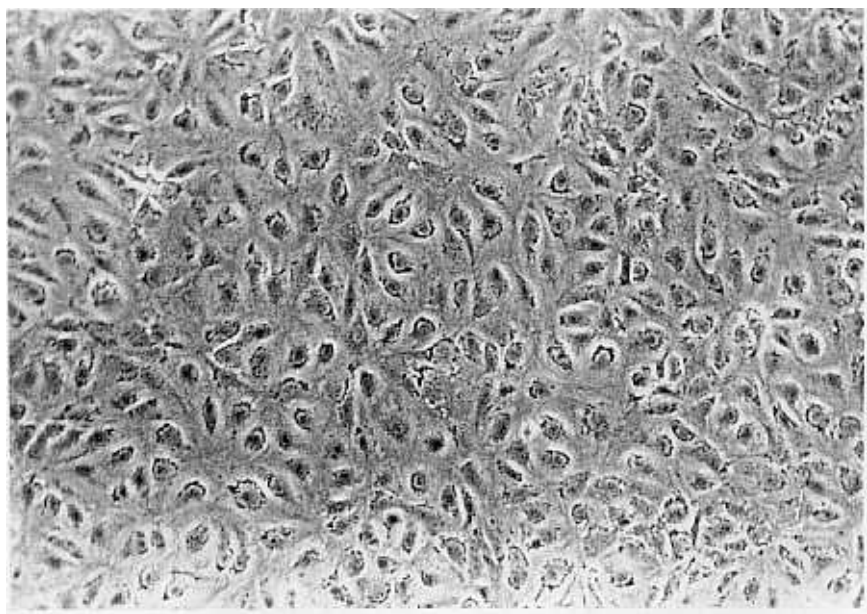

Fig. 2. *Morphology of a monolayer of fetal dermal microvascular endothelial cells, 2 weeks after isolation (×100). Cells show contact inhibition and typical cobblestone-like appearance.*

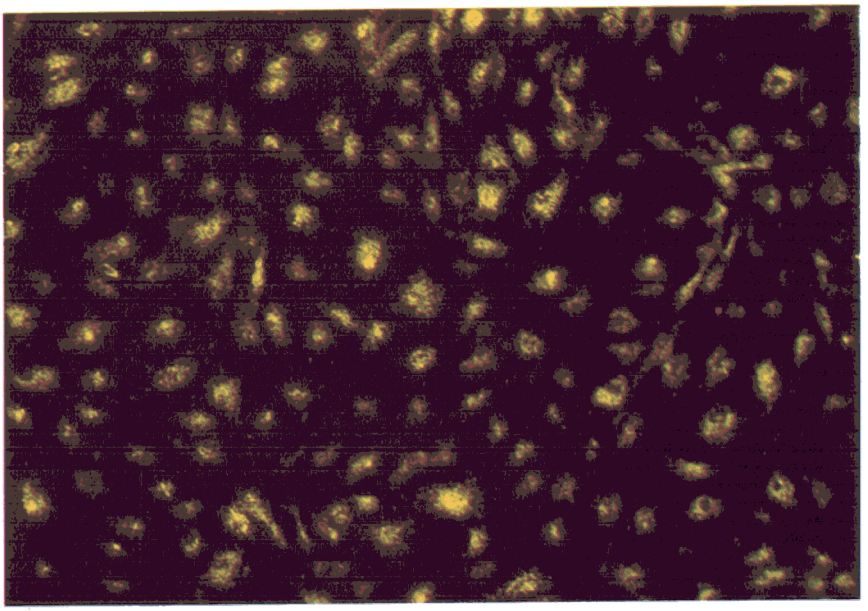

Fig. 3. *Immunohistochemical staining of the FMEC for Factor VIII-related antigen. They showed discrete cytoplasmic granules (×200).*

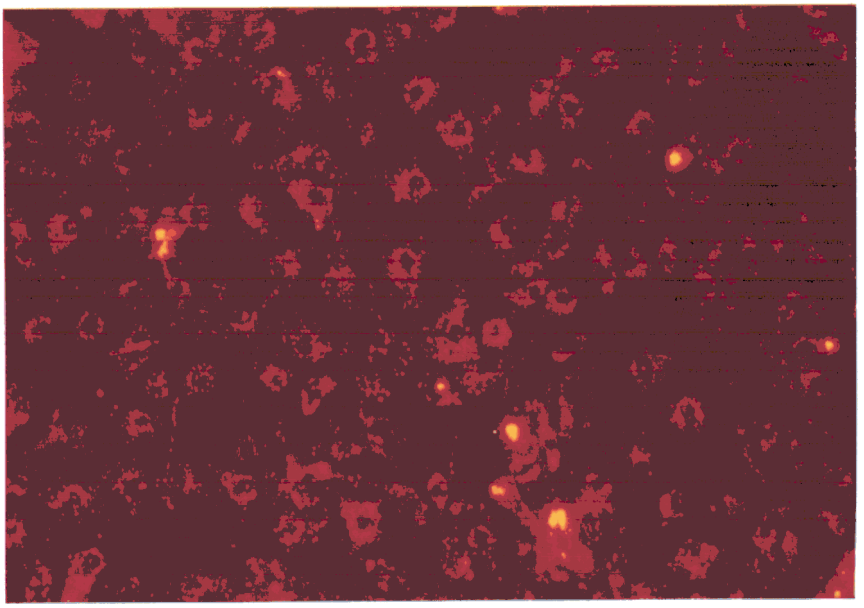

Fig. 4. *Fluorescence of ingested 1,1'-dioctadecyl-1-(3,3,3',3'-tetramethyl)-indocarboxy-amine perchlorate-labeled acetylated low-density lipoprotein (DiI-Ac-LDL) metabolized by endothelial cells. They fluoresced brightly (×200).*

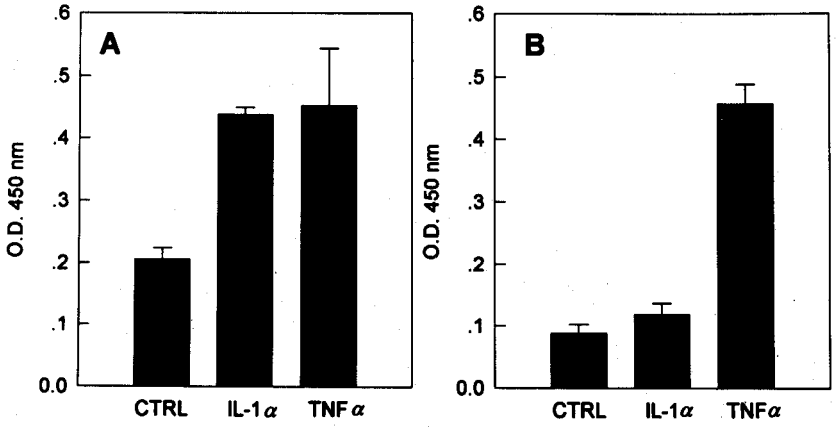

Fig. 5. Expression of ICAM-1 (A) and VCAM-1 (B) on FMEC in response to IL-1a or TNF-α. Expression of ICAM-1 on FMEC was significantly increased after treatment with IL-1a or TNF-α. VCAM-1 was significantly expressed on FMEC in response to TNF-a only.
O D: optical density, CTRL: control.

pecially at the perinuclear region, that revealed the presence of the antigen in primary cultures of FMEC (Fig. 3).

Uptake of acetylated low-density lipoprotein

Endothelial cells selectively ingest modified LDL. Following incubation with DiI-Ac-LDL, cultured FMEC fluoresced brightly. The fluorescence was predominantly punctate with a perinuclear distribution (Fig. 4).

Expression of ICAM-1 and VCAM-1 in response to cytokines

To estimate immunologic activity of cultured FMEC, expression of ICAM-1 and VCAM-1 in response to 100 unit/ml IL-1α or 100 unit/ml TNF-α was examined using ELISA. Expression of ICAM-1 on FMEC was significantly increased after treatment with IL-1α or TNF-α (Fig. 5A), as has been previously reported (Lee *et al.* 1992). VCAM-1 was also expressed on stimulated FMEC, but only TNF-α induced expression of VCAM-1 (Fig. 5B).

DISCUSSION

Microvascular endothelium differs from large vessel endothelium in several ways (Zetter, 1981; Gumkowki *et al.* 1987). Microvascular endothelial cells are involved in angiogenesis (Form *et al.* 1986), inflammation (Harlan, 1985), hematogenous metastasis (Auerbach *et al.* 1987) and many pathologic states. They also act as a permeability barrier to plasma, allowing the selective, active transfer and metabolism of many substances (Scholefl, 1963). Therefore, it is inappropriate to use endothelial cells cultured from major blood vessels in these kinds of studies.

The study of vascular endothelial cell biology, in general, has increased since the routine culture and identification of these cells isolated from umbilical veins were first demonstrated (Jaffe *et al.* 1973). However, the cultivation of microvascular endothelial cells is more difficult than that of large vessel endothelial cells. Early attempts to culture microvascular endothelial cells were unsuccessful because of an inability to grow them in homogeneous populations. The main problems are difficulties in isolation of microvascular endothelial cells and fastidious culture conditions. To inhibit contaminating cells such as fibroblasts and permit growth of endothelial cells selectively, Eagle's Minimal Essential Medium (MEM), Dulbeco's Modified Eagle's Essential

Medium (DMEM), Medium 199, or Endothelial Basal Medium (EBM) has been used as culture media (Folkman *et al.* 1979; Davison *et al.* 1980). The culture media was also manipulated by substituting D-valine for L-valine (Gilbert and Migeon, 1975), adding cyclic AMP (Marks *et al.* 1985), or using high serum concentrations (Marks *et al.* 1985).

The principal method for identifying endothelium has been the use of specific antisera against an endothelial cell product, Factor VIII-related antigen. However, all vascular endothelia do not appear to produce Factor VIII (Mukai *et al.* 1980). UEA I is a good general marker for vascular endothelium in human tissue and reacts preferentially with vascular endothelium irrespective of the blood group type via an endothelial cell surface glycoprotein that contains α-L-fucosyl group (Hormia *et al.* 1983). It is a more sensitive marker for identifying human endothelium than others. Therefore we used it as a reagent for isolating fetal dermal microvascular endothelium.

Dynabeads are super-magnetic polystyrene beads with a ferrous core that can be activated for chemical binding of peptides, proteins or glycoproteins to their surfaces. They have been used to select both negatively and positively for cells. Lea *et al.* (1985) previously demonstrated the usefulness of immunomagnetic cell fractionation for the depletion of cell populations expressing a specific surface marker. However, the cells positively selected under those conditions had strongly reduced functional capacity. Significant inhibition of cell adhesion to plastic was observed when the bead-to-cell ratio exceeded 10:1. This was not due to toxic substances being eluted from the particles, but was probably a result of physical stress on the cells created by the large excess of magnetic beads employed. Jackson *et al.* (1990) reported this problem was avoided by removing attached beads from isolated cells by competitive binding of free 0.1M fucose to the beads at 4℃. This technique, however, did not prove to be reproducible. In early experiments, the magnetic susceptibility of the particles allowed for removal of cells binding as few as 1-2 beads (Lea *et al.* 1985). Accordingly, we tried to reduce the amount of particles during isolation. With a 3:1 ratio between particles and cells expressing the selecting marker, we could obtain a pure endothelial cell population. Positive selection under such conditions gave cell suspensions performing normally in a functional assay system. We found that a ratio of 3:1 gave a good harvest of endothelial cells without interfering with adhesion to plastic and subsequent proliferation. The cultured endothelial cells were viable, morphologically intact and grew to confluence as a cobblestone-like monolayer. They continued to replicate at passages 2-8 and express Factor VIII-related antigen. The cultured FMEC expressed significantly higher levels of ICAM-1 and VCAM-1 in response to IL-1α or TNF-α.

Isolating endothelial cells by UEA I-coated Dynabeads has distinct advantages over other isolation techniques. This technique is fast compared with established procedures. After a specimen was taken, we could obtain pure microvascular endothelial cell population within 3 hours. In contrast, earlier methods required 3-4 months to obtain pure cultures (Folkman *et al.* 1979). Another advantage is that the method is reproducible and most endothelial cells are selected.

In summary, we have demonstrated that purifying endothelial cells with UEA I-coated Dynabeads is an improved method for specifically isolating endothelial cells from the fetal skin without affecting cell viability. Utilizing this technique, we have increased the speed and efficiency of techniques used for the isolation of pure cultures of microvascular endothelial cells. In addition, cultured FMEC will be useful as models for investigation of microvascular endothelial cell biology of fetal skin.

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