Interleukin-10 Induces E-Selectin on Small and Large Blood Vessel Endothelial Cells

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Summary

In vitro, expression of E-selectin is largely restricted to endothelial cells activated by inflammatory cytokines. Under activated conditions, cytokines such as interleukin (IL) 10, released by keratinocytes in large quantities, may also increase the expression of E-selectin on the dermal microvasculature. The aim of the present study was to investigate the expression of E-selectin on cultured human dermal microvascular endothelial cells (HDMEC) isolated from neonatal foreskins when exposed to IL-10. Expression of E-selectin was determined by immunofluorescence microscopy, FACS® analysis, an HL-60 cell-binding assay, and quantitative polymerase chain reaction (PCR) analysis. For comparison with large blood vessel cells, the expression of E-selectin on human umbilical vein endothelial cells (HUVEC) was also determined in parallel by FACS® and reverse transcriptase–PCR analysis under identical conditions. These studies demonstrate that IL-10 induces the expression of E-selectin on both HDMEC and HUVEC and that the level of expression on HDMEC is comparable with that induced by IL-113 and tumor necrosis factor-α. When HL-60 cells are incubated with HDMEC pretreated with IL-10, a consistent increase in adherence of HL-60 to endothelial cells is observed. This adherence was found to be mediated by E-selectin. PCR analysis and the quantification of E-selectin cDNA by a novel, highly sensitive and specific PCR–immunoassay demonstrate the induction of E-selectin mRNA at the transcriptional level. The induction of the expression of E-selectin by IL-10 on HDMEC may provide additional insights into the pathogenic mechanism of neutrophil accumulation at the site of inflammation in inflammatory skin diseases.

Migration of leukocytes to the site of inflammation is a hallmark of an inflammatory response. Multiple lines of evidence indicate that increased adherence of circulating neutrophils, lymphocytes, and monocytes to the microvascular endothelium is an essential early event in the initiation of acute inflammatory responses, regularly preceding the migration of these cells through vessel walls and accumulation at sites of tissue injury (1, 2). Binding of lymphocytes, monocytes/macrophages, and granulocytes to vascular endothelial cells is mediated by specific cell adhesion molecules, a process crucial to the generation of immune and inflammatory responses (3, 4).

E-selectin is an endothelial-specific leukocyte adhesion molecule expressed on endothelial cells after exposure to inflammatory mediators and is known to be involved in the adhesion of PMN to the endothelium in vitro (5, 6). In vivo, Engelberts et al. (7) found expression of E-selectin on endothelial cells in skin biopsies of patients severely ill with peritonitis at a distance from the primary inflammatory site. Other studies of septic shock using cynomolgus monkeys also demonstrate a role for E-selectin in the pathogenesis of acute inflammation (8). The cutaneous lymphocyte-associated antigen (CLA) is the major T cell ligand for E-selectin and it has been proposed to be involved in the selective targeting of memory T cells reactive with skin-associated Ag to cutaneous inflammatory sites (9). Santamaria et al. (10) reported a functional linkage between CLA expression and disease-associated T cell effector function in patients with atopic dermatitis. E-selectin has also been reported to be a major adhesion molecule for the binding of adult T cell leukemia cells to endothelial cells (11). These studies underscore the critical involvement of E-selectin in inflammation.

Since adhesion of leukocytes to vascular endothelium is the first step in their passage from blood into inflammatory sites, cytokines modulating adhesion molecules may regulate leukocyte accumulation, and hence, the nature and progression of inflammatory responses. A series of studies have demonstrated that human umbilical vein endothelial cells (HUVEC) when incubated with IL-1β, TNF-α, and LPS, express E-selectin and intercellular adhesion molecule...
IL-10 is a recently discovered cytokine with the ability to inhibit cytokine synthesis, T cell proliferation, and nitric oxide formation, suggesting its immunosuppressive potential (14, 15). Wogensen et al. (16) reported recently that expression of IL-10 in the islets of Langerhans of transgenic mice led to a pronounced pancreatic inflammation and vascular changes such as expression of cell adhesion molecules and accumulation of neutrophils and T lymphocytes. The possible involvement of IL-10 in inflammation was further supported by the finding that IL-10 was secreted by keratinocytes activated by LPS in murine (17) and by UVB light in human systems (18). These observations suggest the important regulatory role that IL-10 may have on other cells of the immune system in skin. The direct effects of IL-10, however, on a physiologically relevant site such as the dermal microvasculature, which could explain how monocytes and neutrophils accumulate in skin, have not been determined.

The present study was carried out to better understand the factors responsible for inflammation in skin. In addition to the known effects of IL-10 on monocytes and T lymphocytes, we demonstrate here that IL-10 also has the ability to induce E-selectin on human dermal microvascular endothelial cells (HDMEC). Identification of cytokine networks and their mechanism of action may begin to explain the regulatory role of IL-10 and other cytokines on skin endothelial cells leading to allergic and inflammatory reactions. Since this is a novel and unexpected finding, the similarities and differences in effect of IL-10 in particular (14, 15). Wogensen et al. (16) reported recently that expression of IL-10 is also determined on a cell type widely used (HUVEC) in studies of inflammation.

Materials and Methods

Isolation and Culture of HDMEC. Stable cultures of HDMEC were established as described previously with the following modifications. HDMEC were plated in complete IMDM (Abbot Laboratories, North Chicago, IL) which was supplemented with 200 U/ml penicillin, 100 mg/ml streptomycin, 20 mg/ml gentamycin, 2 mM glutamine, 5 × 10⁻⁴ M dibutylryl 3’5’-cAMP (Sigma Chemical Co., St. Louis, MO), 3.3 × 10⁻⁵ M isobutylmethylxanthine (Sigma Chemical Co.), 8% newborn calf serum (Flow Laboratories, McLean, VA), and 2% human prepartum maternal serum, referred to as complete IMDM. The suspended cells were then plated onto 35-mm plastic tissue culture dishes (Miles Scientific, Naperville, IL) that had been precoated with 1% gelatin. Cultures were expanded when cells reached confluence. Cells were identified as HDMEC by their characteristic morphology and growth pattern as well as by the presence of Factor VIII-associated protein detected by indirect immunofluorescence. Cells in the third passage were used in all assays. Absence of macrophages was monitored by the failure of cells to express CD14. HDMEC cultures that had >1% contamination with other cell types (melanocytes) were not used for functional studies.

Isolation and Culture of HUVEC. Primary cultures of HUVEC were maintained in Endothelial Cell Growth Medium (both purchased from Clonetics Corp., San Diego, CA) supplemented with 2% FCS. Cells in the third passages were used in all experiments and were free of melanocytes and spindle shaped cells.

HL-60 Cells. HL-60 cells (American Type Culture Collection, Bethesda, MD) were cultured in RPMI-1640 medium supplemented with glutamine (50 mg/ml) and 10% FCS. HL-60 cells are a continuous human myeloid cell line derived from peripheral blood leukocytes of a patient with acute promyelocytic leukemia (20, 21). Its characteristics have been associated with differentiated granulocytes. HL-60 cells were maintained by replacing the medium at 3-d intervals. For the adherence assay, HL-60 cells were washed and suspended in IMDM with 1% FCS at a density of 10⁶ cells/ml.

Reagents. Human rIL-10 was purchased from Beckton Dickinson & Co. (Bedford, MA), rTNF-α from R&D Systems, Inc. (Minneapolis, MN), and rIL-1β from Amersham Corp., (Arlington Heights, IL). Both blocking and staining mAb to E-selectin were purchased from Amac Inc. (Westbrook, ME). FITC-labeled goat anti-rat Ab, BSA, sodium azide, polymyxin B, LPS, goat serum, and thimerosal were purchased from Sigma Chemical Co. CD15 mAb was purchased from Beckton Dickinson & Co. (San Jose, CA). The cytokines were diluted in sterile pyrogen-free IMDM. All preparations were reported to be free of endotoxin in bioassays.

Immunohistochemistry. Cytokine-treated HDMEC were fixed with ice-cold methanol and stained with a mAb to E-selectin for 60 min at 37°C. The cells were washed twice with cold PBS supplemented with 1% BSA and incubated with a 1:50 dilution of a FITC-labeled goat anti–rat Ab, BSA, sodium azide, polymyxin B, LPS, goat serum, and thimerosal were purchased from Sigma Chemical Co. CD15 mAb was purchased from Beckton Dickinson & Co. (San Jose, CA). The cytokines were diluted in sterile pyrogen-free IMDM. All preparations were reported to be free of endotoxin in bioassays.

Flow Cytometry. HDMEC were trypsinized and incubated in V-bottomed microtiter plates (Flow Laboratories; 10⁶ cells/well) with 50 μl of primary mAb to E-selectin (Amac Inc.) or mAb to CD15 (Becton Dickinson & Co.) containing 10% goat serum, 0.01% thimerosal and 1% BSA for 60 min at 4°C. After two washes with PBS containing 0.02 mM sodium azide and 1% BSA, the cells were incubated with FITC-conjugated goat anti–rat Ab (Sigma Chemical Co.) for 30 min at 37°C. After two washes with PBS-1% BSA, fluorescence was read on a fluorescence microscope (Nikon, Melville, NY). To confirm that IL-10 was not contaminated with LPS, HDMEC were incubated with 400 μg/ml of polymyxin B (Sigma Chemical Co.) along with IL-10, as indicated.

Flow Cytometry. HDMEC were trypsinized and incubated in V-bottomed microtiter plates (Flow Laboratories; 10⁶ cells/well) with 50 μl of primary mAb to E-selectin (Amac Inc.) or mAb to CD15 (Becton Dickinson & Co.) containing 10% goat serum, 0.01% thimerosal and 1% BSA for 60 min at 4°C. After two washes with PBS containing 0.02 mM sodium azide and 1% BSA, the cells were incubated with FITC-conjugated goat anti–rat Ab (Sigma Chemical Co.) for 30 min at 37°C. After three washes with PBS-1% BSA, the labeled cells were analyzed by flow microfluorometry on a FACScan® (Becton Dickinson & Co.) within 2 d.

HL-60 Cell Adherence Assay. The functional assay for binding of HL-60 cells was performed as described (3, 22) with the following modifications. HDMEC were plated in complete IMDM into 24-well, 8-mm-diameter tissue culture plates (Falcon) at ~2.5 × 10⁶ cells/well and grown to confluence. Before assay, the medium was removed and the HDMEC monolayer washed once with IMDM. The monolayers were incubated with 500 μl of
complete IMDM containing one of the following cytokines for 4 h at 37°C: IL-1β (10 U/ml), TNF-α (10 ng/ml or 200 U/ml), or IL-10 (200 U/ml). Cytokine-treated HDMEC were incubated with the blocking mAb to E-selectin (Amac Inc.) or nonrelevant anti-human IgG at a concentration of 10 μg/ml for 30 min, as indicated. After incubation, the monolayers were washed once with serum-free complete IMDM, and 500 μl of IMDM plus 1% FCS containing 5 × 10⁵ HL-60 cells was added. The monolayers were incubated at 37°C for 30 min in a 5% CO₂ incubator. After the incubation, the nonadherent HL-60 cells were removed by completely filling the well with IMDM plus 1% FCS, covering, inverting, and centrifuging at 100 g for 5 min at 4°C. The medium was removed and 250 μl of 0.5% crystal violet in 50% methanol was added to each well and incubated at room temperature for 15 min. The plates were gently washed in running tap water. For the determination of bound HL-60 cells, five microscopic fields were randomly selected from each well and the number of bound HL-60 cells was determined by counting. Each parameter was tested in quadruplet wells. The results were expressed as the number of HL-60 cells bound per endothelial cell.

**PCR Analysis.** Messenger RNA was isolated from 1.5 × 10⁷ HDMEC by the Oligotex kit (Qiagen Inc., Chatsworth, CA; 23). PCR analysis was carried out using GeneAmp RNA PCR kit (Perkin-Elmer Corp., Norwalk, CT). 250 ng of mRNA was reverse transcribed using random hexamers as primer (Perkin-Elmer Corp.) and M-MLV reverse transcriptase (RT) (Perkin-Elmer Corp.) according to the procedure described (24) in a 20-μl reaction. 10 μl RT (equivalent of 500 ng of total RNA) was used directly for each amplification reaction. Conditions for PCR were as follows: in a 40-μl reaction, 200 nM of each primer, 250 mM each of dGTP, dATP, dCTP, and dTTP (Perkin-Elmer Corp.), 50 mM KCl, 20 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, 100 mg/ml BSA, and 1 U Taq polymerase (Perkin-Elmer Corp.). Primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense primer 5'-GGGGAGCGAGATCCCTCCCAAATCAAGTGGGG-3' (nucleotide [nt] 51-82) and antisense primer 5'-GGGTCAATGATGTTAACCCTTTGCAGCA-TACAAAGTTG-3' (nt 1002-1033) (25); E-selectin sense primer 5'-AGCAAGGGCATGATGTTAACC3' (nt 3260-3191) and antisense primer 5'-GCAATTCCTCTTCTCCAGAGC-3' (nt 2801-2822) (26). For quantitative PCR, the 5' end of antisense primers for E-selectin and GAPDH were biotinylated and the 5' end of sense primer was fluoresceinated. 35 rounds of PCR were carried out to observe the amplified cDNA on 5% polyacrylamide gel. Sequence data are available from EMBL/GenBank/DBJ under accession numbers J04038 for GAPDH and M58017 for E-selectin.

**PCR-Immunooassay.** Since cDNA is amplified in a geometric progression, the sensitivity of PCR-immunoassay (IA) is limited to only a certain number of rounds of amplifications where the amplification is a first order reaction and the total amount of cDNA is linearly related to the concentration of mRNA. The exact number of cycles required for quantitative analysis is initially determined by drawing samples at the end of each cycle. The present procedure included 25 cycles of amplification. Extensive washing followed by filtering with Centricon 100 filters (Amicon, Beverly, MA) was carried out to ensure that unbound primers that might interfere with the assay were not carried over. The assay for quantitative analysis, designated as the amplified DNA assay, has been described by Holmstrom et al. (27), where biotinylated amplified cDNA is captured onto a streptavidin-coated, 96-well microtiter plate surface. The original colorimetric assay was modified into a fluorometric assay. The fluorescence detection was achieved using fluorescein primers and endpoint was measured on a Fluoroskan (Bio-Rad Laboratories, Hercules, CA). Streptavidin-coated 96-well plates were purchased from Pierce Chemical Co. (Rockford, IL).

**Results**

**IL-10 Induces E-selectin on HDMEC and HUVEC.** FACS® analysis of HDMEC and HUVEC treated with

![Figure 1](image-url)
increasing concentrations of IL-10 (10, 100, 200, and 500 U/ml) for 4 h demonstrated that expression of E-selectin was dose dependent (Fig. 1, A and B). Maximum expression of E-selectin on HDMEC and HUVEC occurred at 200 U/ml. Therefore, this concentration was used in all subsequent studies. As shown in Fig. 1, a small population of HUVEC constitutively expresses E-selectin. Moreover, at 200 and 500 U/ml of IL-10, another fraction of HUVEC fails to express E-selectin. To further analyze the kinetics of induction of E-selectin, HDMEC and HUVEC were incubated with IL-10 (200 U/ml) for 4, 24, and 48 h. Fig. 2 shows that the kinetics of maximum expression of E-selectin on HDMEC (A) and HUVEC (B) occurred at 4 h and declined after 24 h. The persistence of E-selectin for 24 h on HDMEC is only slightly higher than that of the HUVEC population.

To determine the cellular location of E-selectin, immunofluorescence microscopy was performed on HDMEC grown on glass coverslips and treated with IL-10 (200 U/ml) for 4, 24, and 48 h followed by fluoresceinlabeled staining for E-selectin (Fig. 3). Untreated HDMEC (Fig. 3 A) did not show any detectable expression of E-selectin, whereas treatment with IL-10 for 4 h resulted in the expression of the molecule in the perinuclear area as well as in the cytoplasm (Fig. 3 B); treatment for 24 h caused distribution of E-selectin throughout the entire cell (Fig. 3 C). Cells treated for 48 h did not show positive staining for E-selectin (Fig. 3 D).

Comparison of the Induction of E-selectin Expression by IL-18, TNF-α, and IL-10. In Fig. 4, the induction of E-selectin by the inflammatory cytokines IL-18, TNF-α, and IL-10 on HDMEC and HUVEC is compared. In HDMEC (A) and HUVEC (B), all cytokines examined induced E-selectin expression. In HDMEC, IL-10 showed maximal induction of E-selectin as compared with IL-18 and TNF-α. In HUVEC, IL-10 was the least effective in inducing expression of E-selectin and a large fraction of HUVEC did not express E-selectin after incubation with IL-10. In Fig. 5, it is demonstrated that incubation of HDMEC with IL-10, together with polymyxin B (400 μg/ml), did not inhibit the ability of IL-10 to induce E-selectin, whereas the ability of LPS to induce E-selectin was inhibited in the presence of polymyxin B, indicating that the effect of IL-10 on HDMEC was not likely due to a possible contamination with LPS.

IL-10 Upregulates Adherence of HL-60 Cells to HDMEC. In Fig. 6, the differences in the adherence of HL-60 cells to untreated (A) and IL-10–treated HDMEC (B) is illustrated. When untreated HDMEC (control) were incubated with HL-60 cells, few HL-60 cells bound to the monolayer as compared with incubation with IL-10–treated cells. In Fig. 7, quantitative determination of the adherence of HL-60 cells to HDMEC treated with IL-18, TNF-α, and IL-10 for 4 h is displayed. Significant increases in binding of HL-60 cells for all cytokines was observed with a maximal increase induced by TNF-α. A neutralizing mAb specific for E-selectin reduced the binding of HL-60 to the monolayer to control values (Fig. 7), showing that the binding of HL-60 cells to cytokine-activated HDMEC was E-selectin specific. Incubation in the presence of a nonrelevant anti–human IgG did not interfere with the binding of HL-60 cells to IL-10–treated HDMEC.

IL-10, IL-18, and TNF-α Induce E-selectin mRNA in HDMEC and HUVEC. Using RT-PCR analysis it was found that untreated HDMEC expressed a baseline level of E-selectin mRNA, whereas HDMEC and HUVEC treated with IL-18, TNF-α, and IL-10 expressed higher and different levels of E-selectin mRNA (data not shown). In Fig. 8 the quantification of the expression of E-selectin mRNA analyzed by PCR–1A is shown. Untreated HDMEC and HUVEC showed a comparable baseline expression of mRNA. In HDMEC treated with IL-10, IL-18, and TNF-α, a significant increase in mRNA was found. In
Discussion

IL-10 is synthesized by several cell types including keratinocytes (17), monocytes (28), and T lymphocytes (29). In skin, IL-10 may play an important role in the cytokine network that determines the magnitude of inflammatory as well as other symptomatic responses such as allergy. The expression of E-selectin on cytokine-treated HUVEC for 4–6 h suggested an orchestrated response of various cell adhesion molecules in the inflammatory process (3, 4). The persistence of E-selectin on IL-10–treated HDMEC for 24 h suggests the possibility that under physiological conditions, IL-10 may play a central role in mediating immune and nonimmune inflammatory processes. The nonidentical levels of the E-selectin expression in response to IL-1β, IL-10, and TNF-α may be another example of the several differences in cellular physiology between cells of the large blood vessels and those from the microvasculature (30–32). Other differences in the expression of E-selectin on HDMEC but not on HUVEC in response to both IL-1β and TNF-α have also been recently reported by Sepp et al. (33).

The observation that the intensity of expression of E-selectin varies in a given HDMEC population suggests that not all endothelial cells respond to stimuli in an identical manner. Since the endothelial cells used in these studies were isolated from the capillary, arteriolar, and venular segments of the skin microvasculature, a uniform staining of the population at physiological levels of cytokines would not be expected. The concentrations of cytokines used in the present studies are believed to be in a normal range and able to distinguish between highly sensitive cells, such as those from the venular segment, from the less sensitive cells from the arteriolar segment.

In the HUVEC population, a small subset of cells exists that expresses E-selectin constitutively as demonstrated by both FACS® (Fig. 4 B) and RT-PCR (data not shown) analysis. This could be due to the inherent characteristics of a fetal cell population. When E-selectin cDNA was amplified using a rate limiting number of PCR cycles and analyzed by PCR–IA, differences in the response to IL-10 compared with IL-1β and TNF-α became evident (Fig. 8). Untreated HDMEC express the lowest detectable levels of E-selectin, a finding consistent with reports from other laboratories (3, 4, 13, 30). It is of interest that IL-10, IL-1β, and TNF-α induce comparable levels of E-selectin cDNA in HDMEC (Fig. 8), whereas FACS® analysis indicated minor variations in the amount of synthesis of E-selectin by TNF-α (Fig. 4). E-selectin mRNA levels in HUVEC treated with IL-1β and IL-10 are comparable, whereas for TNF-α, a significantly higher level of E-selectin mRNA is

Figure 3. Localization of E-selectin on HDMEC treated with IL-10. HDMEC grown on glass coverslips were treated with IL-10 (200 U/ml) for 0 (A), 4 (B), 24 (C), and 48 h (D). The untreated cells do not express detectable E-selectin. The expression of E-selectin occurs as early as 4 h and persists for at least 24 h. At the end of 48 h, E-selectin cannot be detected. The cells were stained with a primary mAb to E-selectin and FITC-conjugated secondary Ab and analyzed by fluorescence microscopy. (×320).

HUVEC, treated either with IL-1β or IL-10, a comparable amount of E-selectin mRNA was detected and TNF-α appeared to be the most potent inducer of E-selectin mRNA.
expressed, a finding consistent with that from other laboratories (3, 4, 13).

In vivo, E-selectin is expressed by endothelium in allergic cutaneous inflammation and has been reported to mediate the adhesion of eosinophils in vitro (34, 35). Whereas normal skin does not express detectable levels of IL-10, skin injured by tape stripping or by application of a strong Ag (e.g., poison ivy) strongly expresses IL-10 by 18–24 h (36). Fehr et al. (37) observed that T lymphocytes activated during the induction phase of contact hypersensitivity by DNFB secrete IL-10 and proliferate in a class II MHC-restricted manner. The intradermal injection of a contact allergen that induces IL-1β mRNA also results in the production of IL-10 and the eventual induction of a primary immune response in the skin of BALB/c mice (38). All of the above findings suggest that IL-10 may be one of the cytokines required in the induction phase of allergy.

In Th2-type inflammatory skin diseases, IL-10 was identified throughout all levels of epidermis and in the cytoplasm of keratinocytes in the basal layers and in the cell membranes of the Malpighian layer (36). T lymphocytes derived from a Sezary syndrome patient were also immunoreactive for IL-10 (36), and T lymphocytes obtained from skin lesions of mucocutaneous and diffuse mucocutaneous forms of American cutaneous leishmaniasis produced...
IL-10 mRNA abundantly (39). Resident cells in the skin rather than infiltrating leukocytes appear to be the source of cytokines that mediate endothelial activation (17, 38).

In animal models of lupus erythematosus, the onset of the disease was significantly delayed by the administration of neutralizing Abs against IL-10 and was increased when IL-10 was continuously administered (40). The induction of E-selectin by IL-10 is likely to contribute to the infiltration of cells that express sLex, a ligand for E-selectin, and ultimately result in damage to the microvasculature in these diseases (41). IL-10 has been reported to inhibit chemotactic responses of CD4+ but not CD8+ T lymphocytes towards IL-8 (42, 43), which might lead to a focusing of T lymphocytes in an area of T lymphocyte activation. These mechanisms may begin to explain the specific and continuous localization of T lymphocytes in the dermal microenvironment in allergic and autoimmune disorders.

The induction of cell adhesion molecules may also have a therapeutic potential. In breast carcinomas treated with intrallesional injections of IFN-α and IFN-γ, complete or partial regressions were associated with activated endothelium expressing HLA-DR, ICAM-1, and infiltration of dermis and tumors by T lymphocytes (44). These observations suggest that potentiation of a cell-mediated immunological response in neoplastic tissues may help to inhibit tumor cell growth (44). Cytokines such as IL-10 that are antiproliferative and induce cell adhesion molecules, may play a role in controlling tumor growth by these mechanisms.

Leukocyte recruitment is dependent not only on ligands expressed on leukocytes and molecules inducible on endothelial cells but also on processes active during transendothelial migration into the extracellular spaces. Since the mechanisms involved are as yet unknown, much remains to be explored to further our understanding of the fascinating but complex interaction of circulating neutrophils and the microvascular endothelium during the induction of the acute inflammatory response. IL-10, in addition to its immunoinhibitory properties, is a potent recruitment signal for leukocyte migration in vivo. It is possible that IL-10 could induce other cell adhesion molecules on HDMEC. The ability to isolate HDMEC from 4-mm punch biopsies of normal and inflamed skin should make such studies feasible (45).

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