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HUMAN DERMAL MICROVASCULAR ENDOTHELIAL CELLS EXPRESS THE 140-KD ISOFORM OF NEURAL CELL ADHESION MOLECULE

Hitoshi Mizutani[1], William Roswit[2], John Hemperly[3], Thomas Lawley[4], Carolyn Compton[5], Robert Swerlick[4], and Thomas S. Kupper[6*]

[1] Mie University, Mie 514, Japan

[2] Washington University, St. Louis, MO

[3] Becton Dickinson, Research Triangle,NC

[4] Emory University, Atlanta, GA

[5] Massachusetts General Hospital
and [6] Brigham and Women's Hospital / Harvard Medical School, Boston, MA

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It has only recently been appreciated that the level of gene expression of cell surface markers can be different in endothelial cells derived from different anatomical sites, and that these differences can persist in vitro. In this study, we identify an immunoglobulin gene superfamily member, neural cell adhesion molecule (NCAM), that is expressed on the cell surface of human dermal microvascular endothelial cells but not on umbilical vein, pulmonary vein, aorta, or pulmonary artery derived endothelial cells. By western blot analysis, we identified the 140 kD isoform of NCAM on the surface of human dermal microvascular endothelial cells (HDMEC) derived from dermis. Isolates of HDMEC from human foreskin reproducibly expressed high levels of cell surface immunoreactive protein. In contrast, endothelial cells from large vessels never expressed NCAM constitutively and could not be induced to express NCAM by three proinflammatory cytokines. Western blot analysis of membrane preparations of HDMEC indicated that NCAM protein migrated as a single species with a molecular mass of 140 kD. RT-PCR identified NCAM mRNA in HDMEC cells. The potential for expression of NCAM on small vessels in skin can be interpreted in different ways. Members of the immunoglobulin gene family, including ICAM-1, ICAM-2, and VCAM-1, can be expressed on the cell surface of all endothelial cells and serve as adhesion molecules for leukocytes. It is also possible that, by analogy, NCAM serves as a ligand for a receptor on leukocytes, particularly those that also express NCAM (e.g., natural killer cells). Alternatively, it is possible that NCAM expression permits endothelial cell-cell adhesion, enhancing the structural integrity of microvessels or facilitates neural interactions with microvascular endothelium.

* To whom correspondence should be addressed.

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Endothelial cells comprise the interface between the intravascular and extravascular space, and the endothelial cells that line the internal walls of large vessels express a defined subset of cell surface markers (1). Endothelial cells that line the walls of small vessels and capillaries are remarkably similar phenotypically, particularly when grown in vitro (2). However, it has become clear that differences in the expression of certain genes between large vessel and small vessel (microvascular) endothelial cells can be readily demonstrated (3,4,5,6). This has been investigated most extensively using microvascular endothelial cells derived from human dermis, hereafter termed HDMEC's (7). For example, HDMEC's express higher constitutive levels of ICAM-1, both in vivo and in vitro, than do human umbilical vein endothelial cells (HUVEC), a prototype cell for large vessel endothelium (4). Certain cell surface markers, such as CD36, are expressed on cultured HDMEC's but not on cultured HUVEC (3). CD36 has recently been identified as a co-receptor for *Plasmodium falciparum* on microvascular endothelial cells (3,8). Other studies have shown that microvascular endothelial cells behave differently than do HUVEC when grown on extracellular matrix components. In particular, HDMEC rapidly form tubes resembling capillaries in matrigel, and form ring like structures when grown on laminin substrates (7).

In the present study, we report on the constitutive expression of NCAM by HDMEC. NCAM, a member of the immunoglobulin gene superfamily, is expressed principally by cells of the central and peripheral nervous system (9,10). It is best understood as a molecule that mediates homophilic adhesion, resulting in aggregation of cells bearing NCAM on their surface (10,11,12,13). Control of NCAM gene expression is complex, and multiple alternatively spliced forms have been identified (14). Principal forms identified, all of which are derived from alternative splicing, are 180 kD, 140kD, and a 120 kD form that can be readily shed from the cell surface (15). Post-translational modifications, including polysialylation, appear to be important in modifying function (16). We report that HDMEC, but not HUVEC or other large vessel endothelial cells, express the 140 kD form of NCAM on their cell surface constitutively in vitro. Cytokines that can induce the expression of other members of the immunoglobuilin gene superfamily (e.g., ICAM-1, VCAM-1) do not influence levels of cell surface NCAM, as determined by FACS analysis, on any endothelial cell tested. Potential roles of NCAM in the cutaneous microenvironment are discussed.

MATERIALS AND METHODS

Antibodies:

Monoclonal antibodies specific for NCAM, including 14.2 and Leu 19, have been previously described (17,18), as has the polyclonal rabbit antiserum to NCAM.

Cells:

Human dermal microvascular endothelial cells HDMEC were isolated from human neonatal foreskins as described previously (3,7). Briefly, foreskins were cut into small pieces, treated with 0.3% trypsin (Sigma Chemical Co., St. Louis, MO) and 1% EDTA (Sigma) and individual segments were compressed with a scalpel blade to express microvascular fragments. The microvascular segments were layered on to a 35%

Percoll (Pharmacia AB, Sweden) gradient in Hank's balanced salt solution (HBSS) and spun at 400 x g for 15 minutes at room temperature. The fraction with a density less than 1.048g per ml, was applied to gelatin (Sigma) precoated tissue culture dishes and cultured in MCDB 131 (Clonetics Corp., San Diego, CA) with epidermal growth factor 5 ng/ml (Clonetics), hydrocortisone acetate 1 ug/ml (Sigma), dibutyryl cyclic AMP 5 x 10^{-5} M (Sigma), penicillin 100 U/ml, streptomycin 100 µg/ml, ciprofloxacillin 10 µg/ml (Miles Laboratories, Westhaven, CT), amphotericin B 250 µg/ml (Sigma), and 30% human serum (Irvine Scientific, Santa Ana, CA). The resulting cell cultures were consistently 100% pure, as assessed by morphological and immunochemical criteria. Experiments were conducted with endothelial cells at passages 2-8.

Human umbilical vein endothelial cells, as well as pulmonary vein and pulmonary artery endothelial cells, were obtained from Clonetics Corporation as frozen pellets and were reconstituted and grown in Endothelial Cell Growth Medium (Clonetics). Melanocytes were grown in Melanocyte Growth Medium (MCDB 153 containing 10ng/ml PMA), and dermal fibroblasts were obtained from tissue explants and grown in DMEM 10% FCS.

Western Blotting:

Western blot analysis was performed as described previously in detail (19). Samples were loaded on to a 10% polyacrylamide gel containing 0.1% SDS under reducing conditions. After electrophoresis, proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA) in a transblot cell apparatus (Bio-Rad). The blot was blocked with dry milk and then incubated for 1 hour with monoclonal anti NCAM antibody (14.2) diluted in 0.05% Tween 20, 10 mM tris-buffer (pH 8.0). After washing in TBST, alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Promega Biotec, Madison, WI) was added and the blot was developed and photographed. Molecular weight markers are 200 kD, 116,250 D, and 97,400 D (myosin, b-galactosidase, phosphorylase B).

FACS Analysis:

Approximately 0.5 to 1.0 X 10^6 cells per tube were stained for flow cytometry using the following mAb: 14-2 (mouse anti human NCAM) and Leu 19 (mouse anti human leukocyte antibody, reactive with NCAM). Secondary reagents used were FITC-labeled goat anti-mouse Ig . After staining was completed, 10 to 20 ml of propidium iodide (Sigma) solution (5 mg/ml) was added to each tube to allow electronic exclusion of dead cells from the analyzed population. For each sample, 10,000 events gated on the basis of forward and size scatter were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) utilizing LYSYS II software (Version 1.1).

RT-PCR:

RT-PCR was based on the one-step protocol described by Goblet, et. al. (20). Briefly, 2 µg poly(A)+ RNA and 300 ng of each primer in 66 ul water were incubated at 65 C for 15 minures and cooled on ice. Then 33 ul of 3XRT-PCR reagent mix [3XPCR buffer: 150 mM KCL, 30 mM tTrisCL (pH 8.3), 4.5 mM MgCl2, 0.03% gelatin; 600 uM dNTPs; 200 units m-MLV reverse transcriptase; 4 units RNasin (Promega, Madison, WI); 2.5 units AmpliTaq (Perkin-Elmer Cetus, Norwalk, CT) was added and the reaction was incubated at 37 C for 30 minutes, followed by forty cycles of 94 C for 1 minute; 40 C for 2 minutes; 72 C for 2 minutes. To amplify putative reverse transcribed NCAM cDNA, we utilized human NCAM cytoplasmic primers A (5' CCAATGGAGAAAAGCT 3'), corresponding to position 316-333 and B (5' ATTGCTGAGAACAA 3'), corresponding to positions 1005-1022), respectively.

RESULTS AND DISCUSSION

Several different cell types resident to skin were grown in pure culture, including keratinocytes, melanocytes, fibroblasts, and HDMEC. Lysates from these cells were

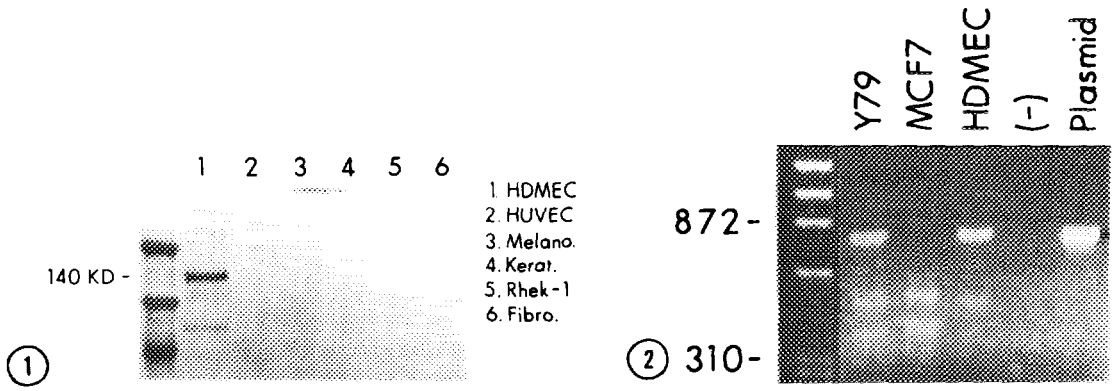
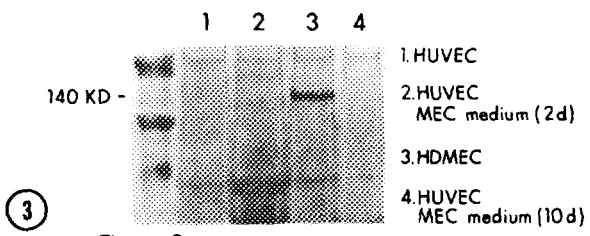


Figure 1.
Western blot analysis of lysates from HDMEC, HUVEC, human melanocytes, human keratinocytes, RHEK-1 (an SV40 transformed human keratinocytes line), and human dermal fibroblasts. Molecular weight markers are 200 kD, 116,250 D, and 97,400 D (myosin, b-galactosidase, phosphorylase B).

Figure 2.
RT-PCR analysis of mRNA derived from HDMEC, Y79 retinoblastoma cells, MCF7 cells. The last two lanes contain no cDNA and NCAM 140 plasmid cDNA, respectively. The predicted size of the PCR product is 706 bp.

analyzed using Western blot with a monoclonal antibody that recognizes all isoforms of NCAM (Figure 1). Of the cells tested, only human dermal microvascular endothelial cells (HDMEC) showed a protein species reactive with this antibody. This protein species migrated as a single band with an apparent molecular weight of 140 kD. Interestingly, lysates of human umbilical vein endothelial cells did not show a reactive protein species; this result was highly reproducible. To our surprise, melanocytes, a cell derived from embryonic neural crest, were also negative for NCAM expression (Figure 1).

To determine whether this putative 140 kD NCAM protein was translated from authentic NCAM mRNA, RNA was extracted and subjected to reverse transcription, followed by amplification with primers specific for human NCAM. Y79, a retinoblastoma cell line known to express NCAM (21), and MCF7, an adherent cell line known not to express NCAM, served as positive and negative controls, respectively. HDMEC cells and Y79 retinoblastoma cells, but not MCF7 cells, contained readily demonstrable NCAM mRNA, as judged by RT-PCR (Figure 2). In other studies, we were unable to detect NCAM mRNA in HUVEC. The presence in HDMEC of an mRNA species whose reverse transcribed cDNA product is indistinguishable from that of Y79 NCAM mRNA or NCAM plasmid cDNA, coupled with the observation of a 140 kD protein species reactive with a monoclonal antibody to human NCAM, strongly suggested that HDMEC express the 140 kD isoform of NCAM.



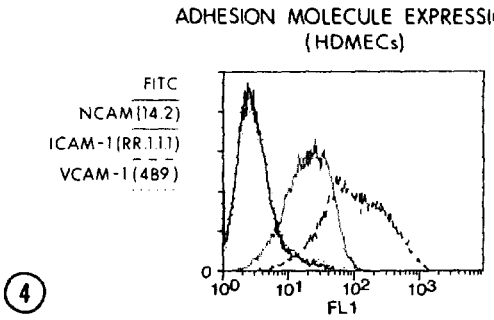


Figure 3.
Western blot analysis of lysates from HUVEC grown in conventional medium (Lane 1) or in HDMEC medium (see Materials and Methods section) for 2 days (Lane 2) and 10 days (Lane 4). HDMEC is shown in Lane 3 for comparison.

Figure 4.
Flow cytometric analysis of HDMEC cells with antibodies to ICAM-1 (R 1.1.1), VCAM-1 (4B9), and NCAM (14.2), or FITC labelled secondary antibody only (solid line).

While several differences exist between HUVEC and HDMEC, they are also grown under significantly different conditions in vitro. It was therefore formally possible that expression of NCAM was facilitated by factors present in HDMEC medium. For the next experiment, HUVEC were grown in the more enriched HDMEC medium, and then extracts of cells were analyzed for NCAM protein expression. Despite the fact that HUVEC could be cultured in HDMEC medium successfully for several days, these cells never expressed immunoreactive NCAM protein (Figure 3). The reciprocal experiment could not be performed, as HDMEC could not successfully be cultured in the medium used to grow and expand HUVEC. These data indicated that the constitutive expression of NCAM by HDMEC was not solely related to the culture medium in which the cells were grown.

Subsequent experiments involving flow cytometry indicated that NCAM was expressed on the cell surface on HDMEC and could be detected by two distinct antibodies, 14.2 (Figure 4) and Leu 19 (data not shown). Unlike levels of ICAM-1, levels of cell surface NCAM could not be altered by primary cytokines (not shown). Thus, with regards to cytokine responsiveness, NCAM expression resembles that of ICAM-2 more closely than ICAM-1 (22). HUVEC cells were also examined by flow cytometry, and the absence of NCAM expression seen in the Western blot experiments was confirmed by this methodology (Figure 5 A,B). Several attempts were made to induce expression of NCAM on HUVEC with cytokines known to enhance gene expression of other immunoglobulin superfamily members. In Figures 5 A and B a representative experiment is shown wherein HUVEC were stimulated with IL-1α. Despite enhanced expression of ICAM-1, VCAM-1, and E-selectin, no de novo expression of NCAM could be detected. Similar results were seen when other large

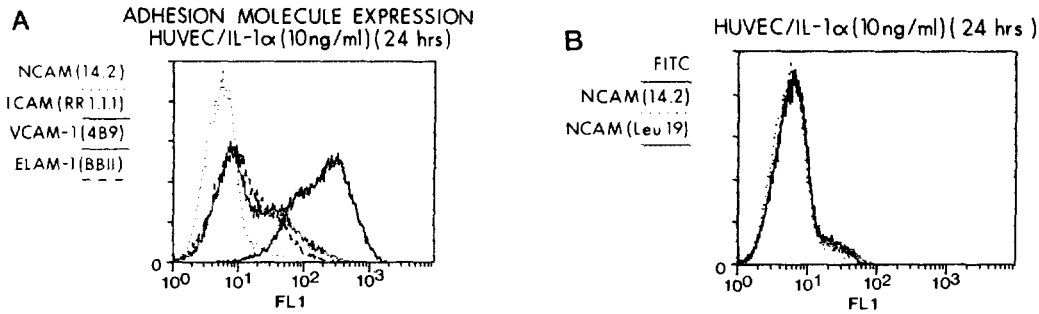


Figure 5.

A. Flow cytometric analysis of HUVEC cells stimulated with IL-1 alpha 24 hours previously. Antibodies to ICAM-1 (R 1.1.1), VCAM-1 (4B9), and E-selectin (BB11) were used.

B. Same cells as shown in 5A, but analyzed with two antibodies to human NCAM (14.2 and Leu-19). FITC control is shown as a solid line.

vessel endothelial cells were tested . Neither pulmonary artery nor pulmonary vein endothelial cells exhibited constitutive NCAM expression, nor could NCAM be induced on these cells by inflammatory cytokines (not shown).

NCAM has been implicated in a large number of adhesion interactions and biological processes (9,10,11), most of them related to neuronal or neuromuscular development (9,10). Its distribution in adult tissues has been previously limited to cells in the nervous system, muscles, chromaffin cells, and various transformed cell lines. A single report indicates that NCAM can be expressed by vasculature in the corpus luteum (23). The expression of NCAM on cultured dermal microvascular endothelial cells in vitro, suggests roles for this molecule that do not involve the central or peripheral nervous system. The absence of constitutive NCAM expression on large vessel endothelial cells, and the inability of several primary cytokines to upregulate NCAM expression on these cells, suggests that the whatever the function of NCAM, it is not central to endothelial cell survival in general.

Our attempts at showing that NCAM on HDMEC could serve as a leukocyte adhesion molecule for NCAM positive leukocytes have been equivocal, at least in part because of the presence of multiple known interactions between leukocytes and endothelial cells unrelated to NCAM (data not shown). However, it is attractive to speculate that this may be a role of endothelial NCAM in vivo. Other putative roles for NCAM on these cells can be readily imagined. It is possible that this homophilic interaction provides additional strength to the cell-cell interactions in these microvessels. Finally, whether NCAM expression influences neural interactions with vessels in dermis is also completely obscure. Future experiments that address the role of NCAM in HDMEC, both in vivo and in vitro, will center around these provocative possibilities.

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