BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **245,** 172–176 (1998)
ARTICLE NO. RC988295

CD44 Isoforms, Including the CD44 V3 Variant, Are Expressed on Endothelium, Suggesting a Role for CD44 in the Immobilization of Growth Factors and the Regulation of the Local Immune Response

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Received February 13, 1998

Endothelium plays a central role in the regulation of site and inflammation specific leukocyte migration. Some of the mediators involved in leukocyte migration, such as chemokines, can bind to heparan sulfate on the endothelium resulting in immobilization near their sites of production. Because CD44 variants expressing V3 have been shown to carry heparan sulfate side chains and to interact through these side chains with heparan sulfate binding growth factors, we investigated the expression of CD44 variants on endothelium. We found a strong expression of V5, V7-8 and V10 CD44 variants and a weaker expression of V3 and V6 CD44 variants on endothelium by using immuno-histochemistry and by FACS analysis. Expression of CD44 V3 variants was confirmed at both the protein and mRNA levels by Western blotting and by reverse transcriptase-PCR respectively. Expression of CD44 variants was unaffected by IL-1β, IL-8, TNFα, IFNγ or IL-4 treatment, indicating either constitutive expression of these variants or involvement of other cytokines in their regulation.

Endothelial cells play a central role in the regulation of leukocyte migration from the blood to the tissues, both during lymphocyte trafficking through the lymphoid organs and during migration to sites of inflammation (1-3). A three step model has been proposed to explain the regulation of site specific and inflammation specific leukocyte migration. These steps involve 1) transient interaction of leukocytes with the endothelium, regulated by selectin adhesion receptors, 2) leukocyte activation, by chemokines or other inflammatory mediators, 3) activation dependent firm adhesion, involving integrin adhesion receptors (1-3). While many of the molecules involved in transient as well as firm adhesion have been defined, the mechanisms regulating cell activation are less well defined. Recently, the chemokine MIP1β was found to bind to CD44 via heparan sulfate and this bound MIP1β was shown to promote adhesion of T-cells to VCAM-1 via activation of the integrin molecule VLA-4 on the T-cell (4). These studies suggest an important role for CD44 in binding and presentation of heparan sulfate binding growth factors, thereby forming an important intermediary in the cell activation phase of leukocyte migration. CD44 comprises a family of adhesion molecules that are all generated from a single gene on chromosome 11 (reviewed 5-7). Genomic cloning of CD44 has revealed that there are 19 exons, 10 of which can be alternatively spliced. Besides the major "standard" CD44 transcript, where all variable exons are spliced out, at least 18 different variant CD44 transcript have been defined carrying combinations of variable exons in addition to the standard exons (5-12). Further heterogeneity of the CD44 molecule is generated through differential use of numerous N-linked and O-linked glycosylation sites as well as glycosaminoglycan attachment sites (13-16). Recently, it was shown that among the glycosaminoglycan (GAG) attachment sites in CD44 only the GAG site in exon 3 has the optimal SGSG consensus motif for GAG binding, and that only CD44 variants containing V3 carry heparan sulfate side chains (11,14). In addition, several heparin binding growth factors, i.e. basic-fibroblast growth factor, heparin binding epidermal growth factor, amphiregu-

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0006-291X/98 $25.00

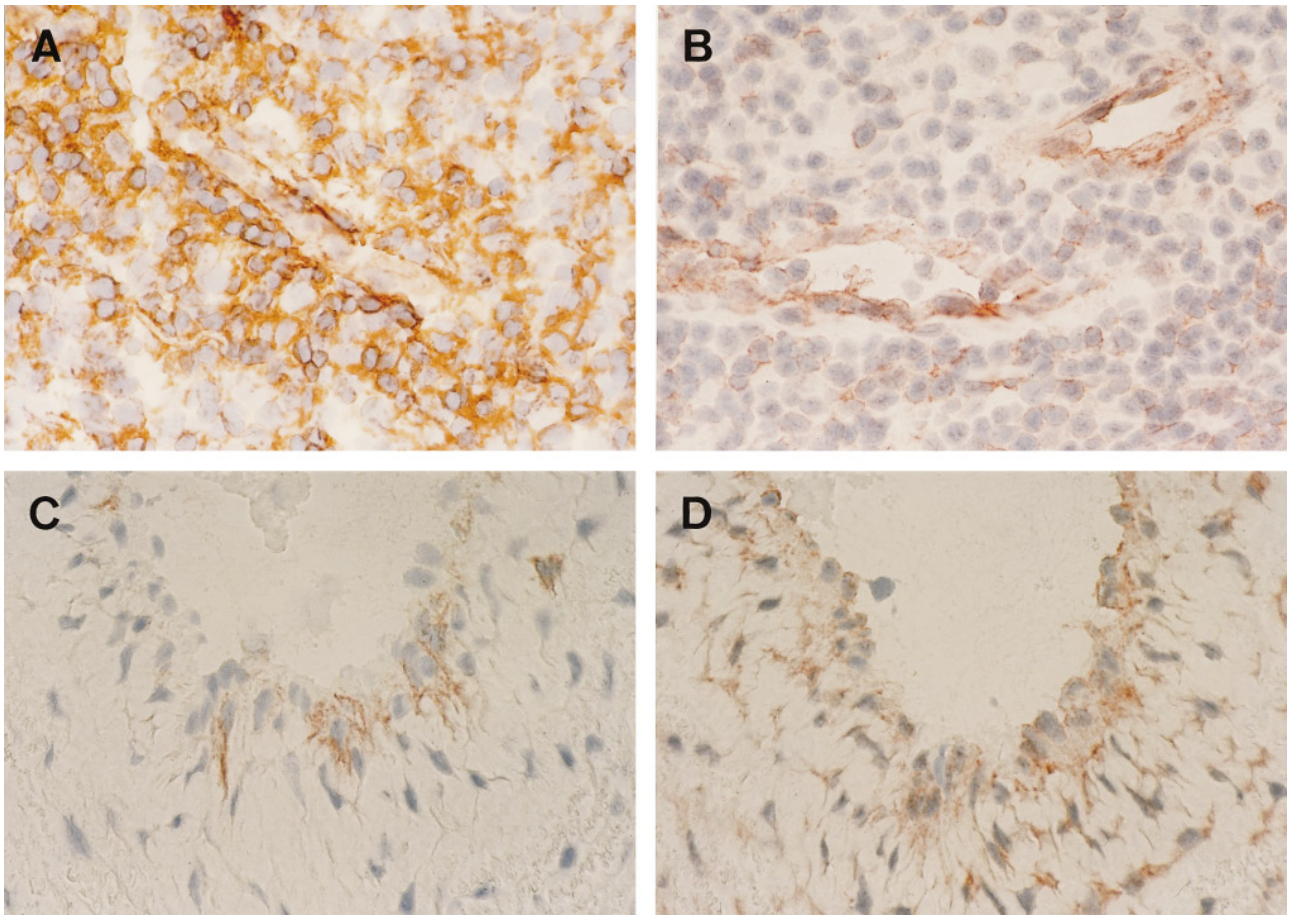

FIG. 1. CD44 standard and variant expression on capillaries in tonsil tissue and on umbilical vein. Expression of standard CD44 (NKI-P1) (A, C) and CD44 variant V10 (VFF16) (B, D) is shown on capillaries in tonsil tissue (A, B) and on umbilical vein (C, D). Magnification ×600.

lin, were shown to bind to V3 containing CD44 variants (15). The growth factor binding function of CD44 will be particularly important on the endothelium, which forms the first contact site between the tissue and leukocytes in the process of immune surveillance. In the present article we therefore explored the expression of CD44 variants on endothelium.

MATERIALS AND METHODS

Antibodies. MAb used were NKI-P1 (IgG1) and Hermes 3 (IgG2a), directed against the standard form of CD44 (16,17); BBA11 (IgG2b, British Biotechnology, Oxon, England) directed against variant V3; VFF8 (IgG1) directed against V5 (18); VFF7 (IgG1) and VFF18 (IgG1) directed against V6 (18, 19); VFF15 (IgG1) and VFF17 (IgG2b) directed against V7-8 (18); VFF16 (IgG1) directed against V10 (18); BBA3 (IgG1) reactive with ICAM-1 (CD54, British Biotechnology); BBA5 (IgG1) reactive with VCAM-1 (CD106, British Biotechnology).

Immuno-histochemistry. Expression of standard and variant CD44 mAb on tonsil tissue and umbilical cord tissue was determined on cryostat sections as previously described (19). In brief, sections were fixed in acetone for 10 min. and incubated with methanol containing 0,3% (v/v) H_2O_2 (Sigma, St. Louis, MO) for 30 min. to inactivate endogenous peroxidases. Tissue sections were washed in PBS and then preincubated with normal goat serum (10% in PBS) followed by 1 hour incubation with the primary antibody. Subsequently, the sections were incubated with biotinylated Goat anti-Mouse antibody (DAKOPATTS, Glostrup, Denmark) for 30 min. For detection a streptavidine-biotin-peroxidase complex (DAKO) was used. All antibodies were titrated to give optimal staining results. Peroxidase (HRP) activity was detected using H_2O_2 (0,03%) and 3-amino-9-ethylcarbazole (AEC, Sigma).

Endothelial cells. HUVEC were isolated according to the method of Jaffe et al (20), and cultured on 1% gelatine coated plastic, in medium consisting of RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% heat inactivated FCS (Integro BV, Zaandam, The Netherlands), 10% normal human serum (Biowhittaker, Walkersville, MA), 2mM glutamine (GIBCO) and antibiotics. For studying the role of cytokines in the regulation of CD44 variant expression HUVEC were stimulated for 2, 4 or 24 hours with Il-4 (50 U/ml) (Genzyme, Cambridge, MA), IL-1β (100 U/ml, Genzyme), IL-8 (80 ng/ml, Becton Dickinson, Mountain View, CA), or for 2, 4, 24 and 48 hours with IFNγ (100 U/ml, Genzyme) or TNFα (100 U/ml, Genzyme).

FACS analysis. For studying the expression of standard and variant CD44 mAb on HUVEC, cells were sequentially incubated (PBS containing 1% BSA) with appropriate dilutions of the different mAb and phycoerythrin (PE) labelled Goat anti-Mouse Immunoglobulins (Southern Biotechnology Associates Inc., Birmingham, LA), for 30

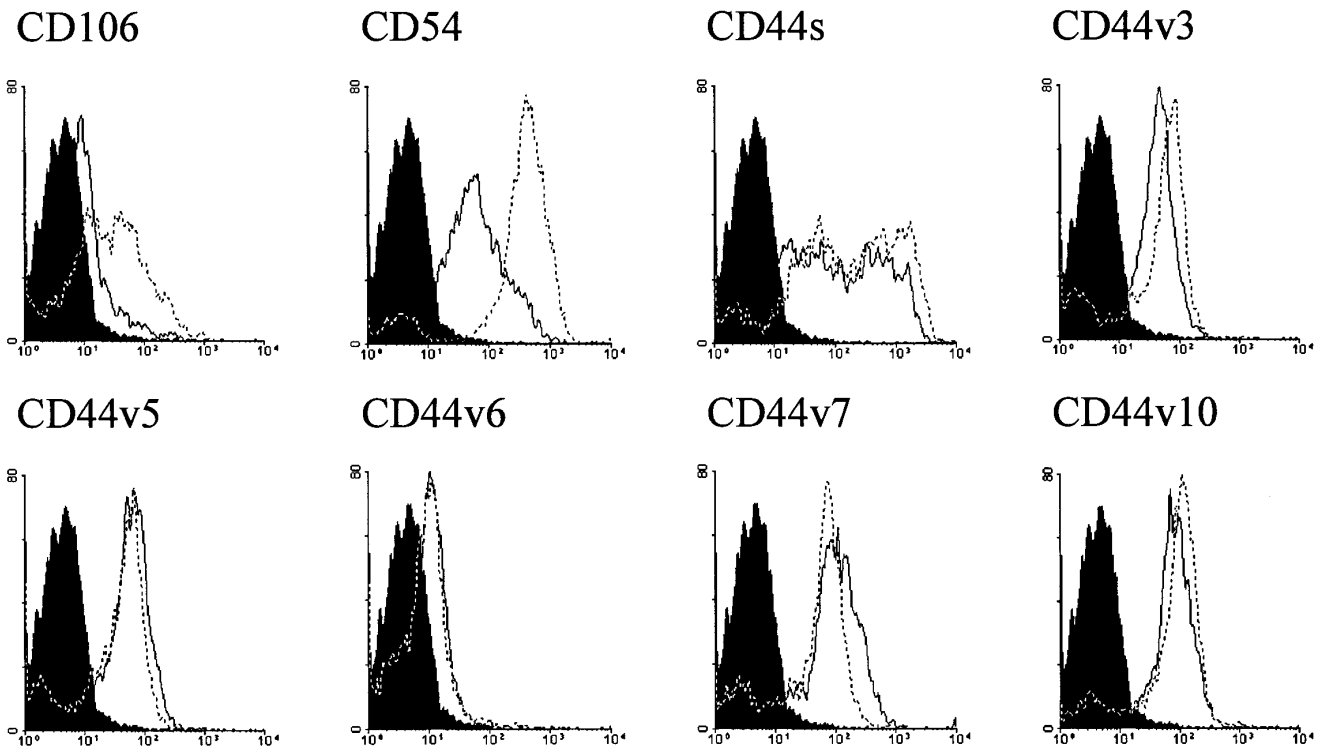

FIG. 2. Expression of CD44 variants on HUVEC, determined by FACS analysis. Expression of CD106 (VCAM-1), CD54 (ICAM-1), standard CD44 (NKI-P1), CD44 V3 (BBA11), CD44 V5 (VFF8), CD44 V6 (VFF18), CD44 V7-8 (VFF17), CD44 V10 (VFF16) on unstimulated HUVEC (closed line) or HUVEC stimulated with IL-1β (100 U/ml, 24 hours) (stippled line) is shown. Control is black diagram.

min. at 0 °C. Fluorescence intensity was measured by FACScan (Becton Dickinson).

Immuno precipitation. Immuno precipitation and Western blotting were performed as described (21). In brief, cells were washed twice in ice cold PBS and lysed in NP-40 lysis buffer, containing 50 mM Tris, 150 mM NaCl, 1% NP-40 (Fluka, Buchs, Switzerland), 10 μg/ml sodium orthovandate (Sigma), 5 mM NaF, 2 mM EDTA, pH 8,0. The insoluble nuclear material was removed by centrifugation at 14000 rpm at 4 °C for 20 min. The supernatant was precleared by incubation with protein A sepharose for 45 min. Total CD44 or CD44 variants containing the exon V3 encoded domain were precipitated using protein A sepharose coated with Hermes 3 or BBA11 mAb respectively. Uncoated protein A sepharose beads served as control. Proteins were resolved on 8% SDS-PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a transblot apparatus (BioRad). Nonspecific interactions were blocked with PBS containing 5% dry milk. Subsequently the membranes were incubated at room temperature with the anti-CD44 mAb Hermes 3, followed by peroxidase-conjugated Rabbit anti-Mouse (DAKO), for 1 hour each. After each individual antibody incubation, the membranes were washed with PBS containing 0,3% Tween 20 (Sigma). Signals were developed with the ECL (enhanced chemiluminescence)-system (Amersham, Paris, France).

RNA Isolation and RT-PCR. Total RNA was isolated with RNAsol (Cinna/Biotex Laboratories, Houston, TX) according to manufacturers description. First-strand cDNA synthesis was performed on total RNA by a standard reverse transcription reaction, using Moloney leukemia virus reverse transcriptase (Gibco BRL/Life Technologies) and p(dN)6 random hexamers (Pharmacia Biotech). PCR was performed with Taq DNA Polymerase (Gibco BRL/Life Technologies), 100 μM dNTPs (Pharmacia Biotech) and 2 mM MgCl2 in 1× PCR Buffer (both Gibco BRL/Life Technologies). Primers used were 5′PCR (5′-CAGACCTGCCCAATGCCTTTGATGGACC-3′) in combination with 3′PCR (5′-CAAAGCCAAGGCCAAGAGGGATGCC-3′), or in combination with v3f (5′-CCTGATCCCGAAAAACTGAGG-3′). PCR was started with a 5 min denaturation step at 95 °C, after which amplification was performed in 35 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 1 min and elongation at 72 °C for 2 min. After a final elongation step for 10 min at 72 °C, samples were cooled on ice and analysed by electrophoresis in a 1.5% agarose TBE-gel containing ethidium bromide.

RESULTS AND DISCUSSION

Using mAb directed against the standard portion of CD44 we and others have previously shown a strong in situ CD44 expression on leukocytes, keratinocytes and a weak expression on capillaries (17,22-24). We now studied the presence of CD44 variants on endothelium by immuno-histochemistry using a panel of anti-CD44 variant mAb. On capillaries in tonsil tissue, we observed a strong expression of V7-8 (VFF15, VFF17) and V10 (VFF16) splice variants, a moderate expression of V5 (VFF8) and no expression of V3 (BBA11) and V6 (VFF7,VFF18) (Fig. 1 and not shown). Identical expression patterns were observed on the endothelium lining the umbilical vein (Fig. 1 and not shown). Further studies on CD44 variant expression patterns and its regulation through cytokines were therefore per-

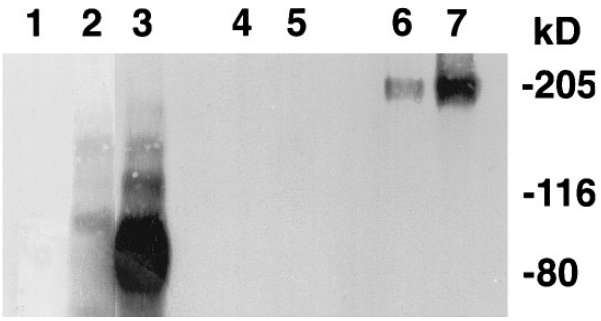

FIG. 3. Molecular characterization of CD44 proteins expressed on HUVEC. CD44 variants were precipitated from HUVEC, separated by electrophoresis on 8% SDS-PAGE and stained with Hermes 3 mAb. Lane 1, HUVEC control precipitate with unlabelled beads; lane 2, HUVEC precipitate using BBA11 (anti-V3) labelled beads; lane 3, HUVEC precipitate using Hermes3 (anti-sCD44) labelled beads, showing a dominant 90 kD standard CD44 band and bands of higher molecular weight, some of which similar to bands found in the BBA11 precipitate (lane 2); lane 4, BBA11 labelled beads; lane 5, Hermes 3 labelled beads; lane 6, CD44V3-10 transfected Namalwa cells precipitated with BBA11 labelled beads; lane 7, CD44V3-10 transfected Namalwa cells precipitated with Hermes 3 labelled beads.

formed on cultured HUVEC. FACS analysis confirmed the expression of CD44 V5, V7 and V10 variants on endothelium and in addition showed a low expression of V3 and V6 variants, which probably fell below immuno-histochemical detection levels (Fig. 2). Culturing the cells for 2, 4 or 24 hours in the presence of IL-1β, IL8, IL4, or for 2, 4, 24 or 48 hours in the presence of IFNγ or TNFα did not alter the expression of any of the CD44 variants (Fig. 2 and not shown). ICAM-1 (CD54) and VCAM-1 (CD106) served as controls and were upregulated following IFNγ (ICAM-1), TNFα (ICAM-1, VCAM-1) and IL-1β (ICAM-1, VCAM-1) stimulation. Interestingly, expression of CD44 variants on leukocytes and epithelial cell lines was previously shown to be modified by IFNγ and TNFα treatment (25), which implies that the regulation of CD44 variant expression is cell type dependent. During inflammation the different cell types involved, i.e. epithelial cells, leukocytes, endothelial cells, may therefore respond differently to the same inflammatory cytokine.

Expression of CD44 variants on endothelium was studied further at the protein and mRNA level by immuno precipitation and reverse transcription (RT)-PCR analysis respectively. Precipitation of all CD44 variants from HUVEC by using the anti-standard CD44 mAb Hermes 3 revealed the presence of multiple high molecular weight variants besides the dominant 90 kD standard form (Fig. 3). Some of these bands were also seen when V3 containing CD44 molecules were selectively precipitated using the BBA11 anti-CD44V3 mAb (Fig. 3). These V3 containing variants had lower molecular weights than the CD44V3-V10 variant precipitated from CD44V3-10 transfected Namalwa cells (Burkitt lymphoma cell line). This reduction in molecular weight may either be caused by splicing out of some of the other variant exons, or by a reduced glycosylation of CD44 in HUVEC in relation to Namalwa. RT-PCR analysis, using primers corresponding to sequences of the 5′ and the 3′ standard region (19), showed expression of standard and variant CD44 transcripts on endothelium (Fig. 4A). PCR using primers for the 5′ standard region in combination with 3′ primers specific for CD44V3 revealed the presence of CD44V3 containing transcripts in endothelium, but not in the control cell line Raji, a CD44 negative Burkitt lymphoma cell line (Fig. 4B).

Expression of CD44 on endothelium has been well documented (17,22). However, its function on endothelium has remained obscure. Here we describe expression of variant CD44 molecules, including V3 variants, on endothelium by using immuno-histochemistry, FACS analysis, immune precipitation and RT-PCR. Thus, CD44 variants on endothelium may function as a binding site for heparin binding growth factors and thereby form an important intermediary in the multistep cell migration model (1-3). In contrast to our results, Bennett et al. (15) and Mackay et al (25) did not observe expression of CD44 variants on endothelium.

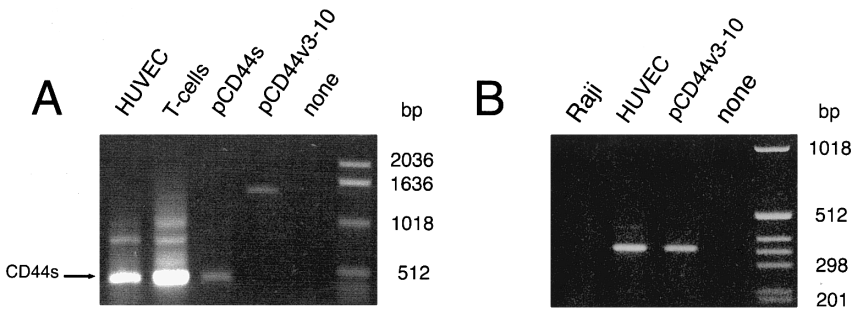

FIG. 4. CD44 RNA transcripts in HUVEC. Detection of RNA transcripts encoding standard and variant CD44 was performed by RT-PCR using (A) 5′ and 3′ primers specific for standard CD44, resulting in detection of all transcripts or (B) a 5′ primer specific for standard CD44 in combination with a 3′ primer specific for V3, resulting in detection of only transcripts containing V3. PCR was performed on HUVEC and in A on anti-CD3 stimulated PBL (T-cells) and plasmids encoding either standard CD44 (pCD44s) or CD44V3-10(pCD44V3-10) and in B on Raji cells (a CD44 negative Burkitt lymphoma cell line) and pCD44V3-10.

Although we can only speculate on the cause of these differences, the sensitivity of the detection methods used and differences in the anti-CD44 variant mAb used may play a role. For instance, in our FACS analysis we used phycoerythrin instead of FITC labelled antibodies, which are less sensitive. Lokeshwar et al. (26) recently found expression of CD44 variants containing exon V10 in bovine endothelial cells. However, in contrast to our results no other variants were observed and no glycosaminoglycan modifications were seen. Whether these differences represent a species specific phenomenon or depend on the activation status of the endothelium remains to be determined. Specifically, expression of CD44V3 and the amount of heparan sulfate attached to this domain may form an important site for regulation of endothelial cell function.

ACKNOWLEDGMENTS

This study was carried out under the projects of ODP/DA/2 and ODP/DE/1 of the Van Loghem Immunology Institute, Academic Medical Center, University of Amsterdam, Amsterdam. We thank the IRCC S. Mateo Hospital for the award of a visitry fellowship and Dr. O.J. de Boer for his generous supply of cultured endothelial cells.

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