Regulated Expression of Exon v6 Containing Isoforms of CD44 in Man: Downregulation during Malignant Transformation of Tumors of Squamocellular Origin

Marko Salmi,*‡ Kristiina Grön-Virta,‡§ Petri Sointu,* Reidar Grenman,‖ Hannu Kalimo,¶ and Sirpa Jalkanen*‡

*National Public Health Institute,‡ MediCity Unit, and §Centre for Biotechnology, BioCity, the ‖Department of Otorhinolaryngology, Turku University Central Hospital, and the ¶Department of Pathology, University of Turku, 20520 Turku, Finland

Abstract. CD44 is a family of glycoproteins involved in cell–cell and cell–matrix interactions. In addition to the major 90-kD form present on most hematopoietic cells, larger 140–230 kD forms are found on keratinocytes and carcinoma cell lines. These bigger isoforms of CD44 arise by alternative splicing that results in insertion of one or more of the "variant" exons into the extracellular part of the 90-kD constant form of the molecule. In rat, v6 (variant exon v6) containing form of CD44 confers metastatic potential to carcinoma cells, and therefore, it is of interest to study the distribution of this isoform in humans. We raised antibodies against a synthetic peptide containing a sequence encoded by the exon v6. A mAb thus obtained (designated Var3.1) strongly reacted with the plasma membranes of squamous cells in upper layers of skin and tonsil surface epithelia. Weaker staining was seen in germinal centers, vascular endothelia and enterocytes. Exon v6 containing forms of CD44 (CD44v6) were absent from tissue leukocytes and connective tissue components. In comparison, Hermes-3 epitope (on the constant part) containing forms of CD44 were preferentially localized in basal layers of epithelia, present on the surface on most leukocytes and connective tissue cells, and undetectable on the luminal surface of high endothelial venules. In benign neoplasms, epithelial cells stained with mAb Var3.1 like in normal tissues. In contrast, immunostaining of 30 squamous carcinoma specimens (both primary and metastatic lesions) revealed that malignant transformation resulted in downregulation or disappearance of Var3.1 epitope, but in majority of cases, not in diminished synthesis of the Hermes-3 epitope. Biochemical analyses showed that mAb Var3.1 recognized two major forms of CD44 (220 and 300 kD). In conclusion, epitopes on exon v6 and constant part of CD44 are differentially synthesized and regulated during normal and malignant growth of cells in man.

CELL–cell and cell–matrix interactions are of fundamental importance to multicellular organisms in controlling organized growth, differentiation, and migration of cells. CD44 is one of the molecules known to be involved in these adhesion-dependent processes. In man, it was independently discovered by several groups using antibodies against molecules referred to as brain-granulocyte-T lymphocyte antigen, human medullary thymocyte antigen, Lutheran inhibitor related antigen, p85, phagocytic glycoprotein-1, Hermes-antigen, extracellular matrix receptor type III, and hyaluronate receptor (5, 7, 16, 23, 28, 36, 55, 56). Later, these antibodies have been shown to identify the same CD44 cluster (6, 10, 41, 44). CD44 is a multifunctional glycoprotein (for review see references 17, 18) involved at least in lymphocyte-endothelial cell interactions (26, 28, 42, 43), adhesion of cells to extracellular matrix proteins (2, 5, 27, 40, 56), lymphohematopoiesis (39), homotypic adhesion (3, 51), T cell activation and adherence (1, 8, 15, 22, 32, 45, 48, 49), cytokine release (57), metastasis formation (see below), and lateral movement of cells (25). CD44 is widely distributed on several hematopoietic and nonhematopoietic cells including all subsets of leukocytes, erythrocytes, many types of epithelium, mesenchymal elements like fibroblasts and smooth muscle cells, and glial cells of the central nervous system (5, 7, 16, 23, 36, 37, 43, 55). Most hematopoietic cells, fibroblasts, and glial cells predominantly express a 90-kD form of CD44. At least lymphocytes also have a minor 180-kD form which represents a chondroitin sulfate modification of the 90-kD backbone (29). In contrast, CD44 antigen in epithelial cell lines is considerably larger (140–160 kD), and still larger forms up to 230-kD have been described (4, 41, 43). Recently, the molecular basis of these biochemically distinct forms has been

Please address all correspondence to Marko Salmi, National Public Health Institute, Kiinamyllynkatu 13, SF-20520 Turku, Finland.

resolved. Molecular cloning of human CD44 cDNA from lymphoid lines revealed an integral membrane glycoprotein, which has an NH_2-terminal extracellular region, short hydrophobic transmembrane region, and a cytoplasmic tail (12, 52). Later, an epithelial 150-kD form was cloned from keratinocytes and carcinoma cell lines (4, 53). It contains an additional stretch of 132 amino acids inserted in the membrane proximal part of the common peptide backbone. Forms containing the same sequence or a shorter part of it were also found in hematopoietic cells (9). In the rat, and subsequently in man, altogether five extra "domains" of CD44 have been identified (13, 20, 24, 33). More recently, these "domains" have been reported to be comprised of at least ten distinct exons named v1–v10 (1). Finally, genomic cloning revealed that human CD44 contains at least 19 exons, 12 of which can be alternatively spliced (47). CD44 molecules containing one or more (in different combinations) of these alternatively spliced exons within the common backbone are designated as variant forms to distinguish them from the major 90-kD lymphocyte form (standard). In this paper, we will use the term exon v6 (the sixth variant exon of CD44) that corresponds to nucleotides 1140–1267 of the largest known form of human CD44 cDNA (20), and to the tenth exon in the genomic structure of CD44 (47). Exon v6 together with exon v7 (nucleotides 1268–1397 in Ref. 20) form the structure that has formerly been called domain 3 of CD44 (13). For simplicity, the term exon v6 will be used to refer both to the DNA sequence of exon v6 and to the protein that it encodes.

CD44 isoforms play important and distinct roles in tumor metastasis. The standard 90-kD lymphocyte form apparently contributes to the metastatic capacity of non-Hodgkin lymphomas in man (21, 30). The lymphocyte form, but not the 150-kD epithelial form (containing exons v8–10), also enhances local tumor formation and metastatic proclivity of transfected lymphoma cells in a nude mouse model (54). On the other hand, expression of the epithelial form is increased in carcinoma cell lines, which may suggest a role for the 150-kD molecule in tissue invasiveness (52). Finally, in a rat model, exon v6 has been reported to confer metastatic potential to rat adenocarcinoma cells (13). In man, specific analysis of the expression pattern of these variant forms has been limited to the RNA level in cell lines (20) since there has been no mAbs available that can distinguish between the standard and variant CD44. It is currently not known what kind of tissue distribution the variant forms exhibit in man and whether they are expressed on the cell surface. Moreover, it is not clear how expression of different isoforms is regulated during normal cell differentiation and what kind of changes take place in malignant transformation.

To address these questions, we produced mAbs against exon v6 of the human variant CD44. Distribution of the exon v6 containing forms of CD44 (CD44v6)[1] in normal tissues and tumors was determined, and compared to that of the forms recognized by mAb Hermes-3. Also, biochemical properties of CD44v6 were analyzed. The results may be helpful in understanding the biological role of CD44v6 in man.

1. *Abbreviation(s) used in this paper*: CD44v6, exon v6 containing forms of CD44; IPTG, isopropyl β-D-thiogalactopyranoside; pGEX-2T-Var, plasmid GEX-2T with exon v6 containing insert.

Materials and Methods

Production of mAb against Human Exon v6 of Variant CD44

A synthetic peptide representing a 16-amino acid sequence from the exon v6 of the human variant CD44 (STTEETATQKEQWFGN, ref 20; an additional COOH-terminal cysteine was included for coupling purposes) was prepared using an automated peptide synthesizer (model 431 A, Appl. Biosystems Inc., Foster City, CA). Purification of the peptide was carried out with a preparative HPLC (Appl. Biosystems Inc.) using a reverse phase column and its purity was confirmed by an analytical HPLC. The peptide was also independently sequenced (model 477A equipped with an online PTH amino acid analyzer 120A, Appl. Biosystems Inc.) and analyzed with desorption time-off-flight mass analyzer (BioIon^{TM20} Biopolymer Mass Analyzer, Appl. Biosystems) and found to be correct. One hundred microgram peptide in incomplete Freund's adjuvant was injected into the footpads of specific pathogen free Balb/c mice three times at one week intervals. After sacrificing, lymphocytes from popliteal lymph nodes were isolated and fused with NS-1 myeloma cells using standard procedures. Hybridoma supernatants were tested in ELISA (see below) using the synthetic peptide as an antigen and a positive hybridoma (designated Var3.1) was subcloned twice by limiting dilution. The isotype of mAb Var3.1 is Ig G_1.

Production of anti-CD44 mAbs of Hermes-series has been described (26). Hermes-3 recognizes an epitope in the proximal extracellular part of the constant region of CD44 (12). 3G6 and 11G2, mouse mAbs against chicken T cells, were used as negative controls. All antibodies were used as serum-free supernatants or as $(NH_4)_2SO_4$ precipitated concentrates.

ELISA

The synthetic peptide from the exon v6 (STTEETATQKEQWFGN-C) and control peptides (TQSEAWTFTQENKTEG, a scrambled version of the exon v6 specific peptide and DELPQVTLPHPNLHGPEILDVPST, an irrelevant peptide) were absorbed to the bottom of microtiter wells (Dynatech Labs. Inc., Chantilly, VA) overnight at 37°C (10 μg/well). After washings, the remaining binding sites were blocked with 1% gelatin, and after washings the primary antibodies were added for 2 h. Alkaline phosphatase-conjugated goat anti-mouse IgG and IgM (Tago, Inc., Burlingame, CA) was used as the second stage antibody, and *p*-nitrophenylphosphate as the substrate. Absorbances were read in Multiscan (Labsystems, Helsinki, Finland) at 405 nm.

Construction and Analysis of pGEX-2T-fusion Proteins Containing Exon v6 of Human Variant CD44

Exon v6 containing form of CD44 was amplified from HaCaT cells by reverse transcriptase PCR and cloned into pGEX-2T vector (50) for fusion protein production. HaCaT RNA was isolated by the guanidine isothiocyanate–phenol extraction method. First strand cDNA synthesis was carried out using 1.5 μg total RNA, oligo(dT) primer, and M-MLV reverse transcriptase according to the instructions of the manufacturer (Perkin Elmer Cetus, Norwalk, CT). Because HaCaT cells have several forms of CD44 (data not shown), existence of v6 in the PCR product was ensured by using two sets of primers in two separate PCR amplification reactions as schematically illustrated in Fig. 2 *A*. In one reaction (Ia), primers A and B were used and in the other reaction (Ib), primers C and D were employed. Thereafter, the products were combined in a reaction (II) using primers E and F which contained BamHI and EcoRI tails, respectively. The presence of exon v6 in the 0.95-kb PCR product was confirmed by DNA sequencing.

The 0.95-kb fragment (including variant exons v6–10) was isolated from 1.5% agarose gel, digested with BamHI and EcoRI, and ligated using T4 ligase (Stratagene, La Jolla, CA) into pGEX-2T expression vector (46, 50). Hereafter, the plasmid pGEX-2T with the 0.95-kb v6 containing insert is called pGEX-2T-Var. Bacteria (*E. coli* DH5α strain) were transformed with pGEX-2T-Var using $CaCl_2$ method. Production of fusion protein was induced with isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h, after which bacteria were pelleted and lysed in Laemmli's sample buffer containing 2% SDS. Samples from whole cell lysates of pGEX-2T-Var and pGEX-2T (control) transformants were run using a 5–12.5% SDS-PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Schleicher-Schuell, Dassel, Germany) by blotting 1.5 h at 1A in a Transphor apparatus (Hoefer Sci. Instrs., San Francisco, CA). The membranes were then soaked for 48 h in PBS containing 0.1% Tween-20 and 1% non-fat milk powder. After washings in PBS, membranes were cut into strips and

incubated overnight with primary antibodies. After washing, a 3-h incubation in PBS containing peroxidase-conjugated rabbit anti-mouse Ig (Dakopatt A/S, Glostrup, Denmark) was performed. After extensive washing, the membranes were developed in PBS containing 16% methanol, 0.5 mg/ml 4-chloro-1-naphthol (Sigma Chem. Co., St. Louis, MO) and 0.01% hydrogen peroxide. Same amount of samples were also run on parallel gels which were subsequently fixed in 40% methanol and 10% acetic acid, and stained with Coomassie brilliant blue. Alternatively, after IPTG induction and pelleting, the bacteria were resuspended in PBS containing 1% NP-40, 10 mM EDTA, 1 mM PMSF, and 1% aprotinin. Cells were lysed by three cycles of freezing and thawing. After centrifugation, supernatants were collected and added to glutathione-agarose beads (Glutathione Sepharose 4B, Pharmacia, Uppsala, Sweden) that were preequilibrated in the lysis buffer. After a 10-min incubation at room temperature, beads were washed three times with PBS and once with washing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), and resuspended in thrombin cleavage buffer (3 mM $CaCl_2$ in washing buffer). Thrombin (from human plasma, Sigma Chem. Co.) was added for 2 h at room temperature. Thereafter, samples were resolved in SDS-PAGE and blotted onto Hybond-C Super nitrocellulose membrane (Amersham Intl., Buckinghamshire, England) using Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad Labs., Hercules, CA) and Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2). The membrane was blocked in PBS containing 10% non-fat milk powder and 0.3% Tween-20 for 1 h at room temperature. Immunodetection was done using enhanced chemiluminescence system (ECL Western blotting detection system, Amersham Intl.) according to the instructions of the manufacturer. Peroxidase-conjugated affinity-purified anti-mouse Ig (Amersham Intl.) was used as the second-stage reagent. Light emission was detected with Hyperfilm-ECL (Amersham Intl.).

Purification of CD44 and Western Blotting

PBL from leukopheresis samples of patients suffering from rheumatoid arthritis were used for CD44 antigen isolation as previously described (27). Briefly, lymphocytes (25-ml packed cells) were lysed in lysis buffer (1% NP-40, 0.15 M NaCl, 0.01 M Tris, 1.5 mM $MgCl_2$, and 1 mM PMSF, pH 7.0). The clarified lysate was applied first to a Sepharose CL-4B (Pharmacia) column, and then sequentially to three CnBr-activated Sepharose-4B (Pharmacia) columns derivatized with normal mouse serum, with irrelevant mAb and with Hermes-3 mAb (5 mg/ml, 3 ml column volume). The column was washed extensively with the lysis buffer. Thereafter, the material bound to the Hermes-3 column was eluted with 50 mM triethylamine and lyophilized. Isolated CD44 was subjected to SDS-PAGE and blotting as described above for fusion proteins with the exception that membranes were blocked in 0.1% Tween-20 for 3 h and 5% AB-serum (Finnish Red Cross, Helsinki, Finland) was added with the second-stage antibody.

Immunohistochemistry and Immunoelectronmicroscopy

Tissue distribution of the different forms of CD44 was determined using immunoperoxidase staining. Surgical and skin punch biopsy specimens were snap frozen in liquid nitrogen. Five μm frozen sections were cut, air-dried, and acetone fixed. Sections were overlaid with mAb supernatants and incubated for 30 min at room temperature in a humidified chamber. After two washings in PBS, peroxidase-conjugated rabbit anti-mouse Ig in PBS containing 5% AB-serum was added. Finally, the reaction was developed by adding 3,3-diaminobenzidine (Polysciences, Inc., Warrington, PA) in PBS containing 0.03% hydrogen peroxide for 3 min. After staining, the sections were counterstained in hematoxylin (Sigma Chem. Co.), dehydrated, cleared in xylene, and permanently mounted in DePex (BDH Limited, Pool, Dorset, England).

For immunoelectronmicroscopy, samples from human tonsils were snap-frozen in freon 22 chilled with liquid nitrogen. About 5-μm frozen sections were stained immediately using sequential incubations with mAb Var3.1, biotinylated horse anti-mouse Ig, and avidin DH-biotinylated horseradish peroxidase H complex (Vectastain ABC Kit; Vector Labs., Burlingame, CA). The reactions were followed by fixation in phosphate buffered 2% glutaraldehyde. Thereafter, a representative section was examined light microscopically, and an appropriate area was selected. The corresponding area in parallel sections was trimmed, sections were postfixed in phosphate buffered 2% osmium tetroxide, dehydrated, and embedded in epon at the open end of an inverted BEEM capsule. Thin sections were double-stained with uranyl acetate and lead citrate, and then examined in a JEM 100 electron microscope. Slides processed without the primary or secondary antibody, with and without the double staining, served as controls.

Cells and Cell Lines

Human PBL were isolated from healthy adult volunteers using Ficoll-gradient (Ficoll-Hypaque, Pharmacia) centrifugation. PBL were used fresh, or to obtain activated blast cells, stimulated with combination of 10 μg/ml PHA and 1:100 PWM (GIBCO, BRL, Gaithersburg, MD) for 3 d at 37°C in RPMI 1640 (GIBCO BRL) supplemented with 10% FCS, 4 mM L-glutamine, 10 mM Hepes, 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml). Human cell lines HeLa (epithelioid carcinoma), KG-1, KG-1a, K-562, U937 (leukemic cells), A549 (lung carcinoma), Tera-1 (HTB 105, embryonal carcinoma), and T-47 D (HTB 133, ductal carcinoma of breast) were obtained from American Type Culture Collection (Rockville, MD). U1690 (human lung carcinoma cell line) was a kind gift from Dr. H. Hirvonen (Department of Medical Biochemistry, Turku University, Finland) and HaCaT, a spontaneously immortalized non-tumorigenic keratinocyte line, was a kind gift from Professor N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany). All cell lines were cultured in Dulbecco's modified minimal essential medium (GIBCO BRL) supplemented with 10% FCS, 10 mM Hepes, and antibiotics. Adherent cells were passaged using trypsin-EDTA (Boehringer-Mannheim, Germany).

Immunofluorescence Staining

Blood cells and adherent cell lines (detached with 5 mM EDTA in Ca^{2+}, Mg^{2+}-free HBSS) were stained in suspension. Cells were stained unfixed or after fixation and permeabilization (1% formaldehyde in PBS (10 min) followed by −20°C acetone (5 min) followed by two washings in PBS). Cells were incubated with primary antibodies for 20 min at 4°C and washed twice in PBS containing 5% FCS and 1 mM sodium azide. Next, FITC-conjugated sheep anti-mouse IgG (Sigma Chem. Co.) in PBS containing 5% human AB-serum was added for 20 min. Thereafter, the cells were washed twice and fixed in PBS containing 1% formaldehyde. Analyses were done using a FACScan cytometer (Becton Dickinson, Mountain View, CA). For immunofluorescence microscopy, cells were spun on microscopic slides with Cytospin 2 cytocentrifuge (Shandon Southern, Surrey, England) and mounted in glycerol containing 10% PBS. Alternatively, adherent cells were grown on glass coverslips, and processed without detachment for immunofluorescence microscopy as described above.

When studying the detergent resistance of Var3.1 and Hermes-3 epitopes, HaCaT cells grown on glass slides and cytocentrifuge preparations of PBL were used. Cells were first fixed and permeabilized as described above. Thereafter, cells were incubated in PBS with or without 0.5% NP-40 for 5 min at 4°C and washed twice. Next, the cells were stained for immunofluorescence as described above, and analyzed using fluorescence mi-

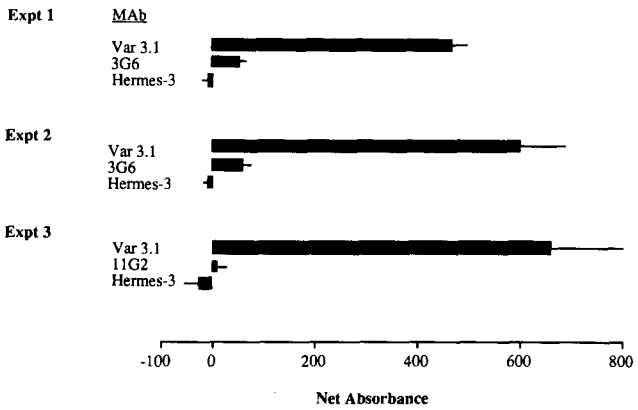


Figure 1. mAb Var3.1 is specific for human exon v6 of CD44. Binding of mAbs Var3.1, Hermes-3, and negative controls (3G6 and 11G2) to v6 specific peptide (STTEETATQKEQWFGN), and either to an irrelevant peptide (DELPQVTLPHPNLHGPEILDVPST, Expts. *I* and *II*) or to a scrambled version of v6 specific peptide (TQSEAWTFTQENKTEG, Expt. *III*) was determined. Results are presented as net absorbances (mean ± SD) from triplicate samples of three independent ELISA experiments (net absorbance = absorbance to v6 specific peptide − absorbance to control peptide).

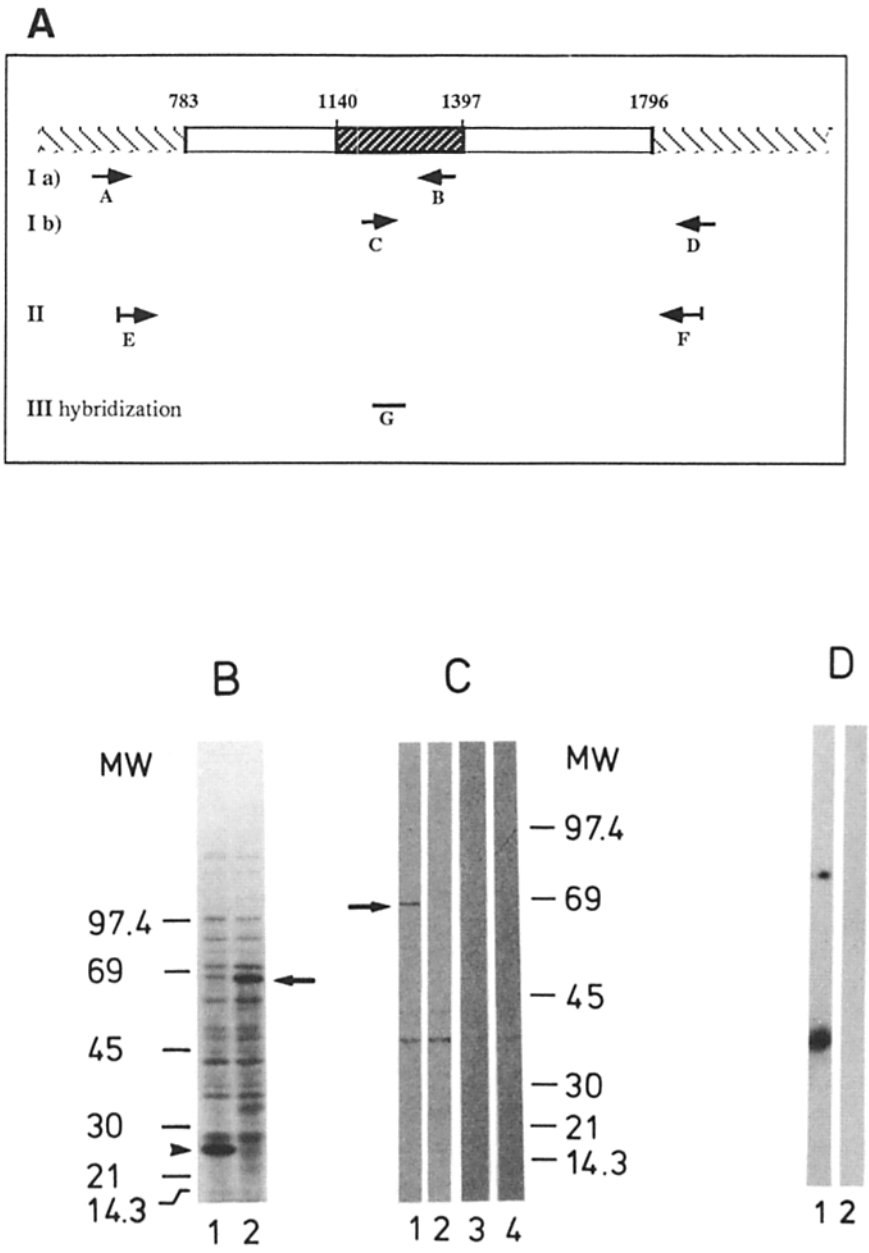


Figure 2. mAb Var3.1 recognizes recombinant proteins carrying exon v6. (*A*) Schematic representation of the PCR strategy used to amplify CD44v6 from HaCat cells (see text for details). Box represents the variant part of CD44 and exons v6 and v7 are highlighted as a darkened area. Primers were as follows: *A*, 5′ CAATTACCATAACTATTGTTAACCG 3′; *B*, 5′ AATCAGTCCAGGAACTGTCCT 3′; *C*, 5′ GGCAACAGATGGCATGAGGG 3′; *D*, 5′ AGTGGTATGGGACCCCCACTGGGG 3′; *F*, 5′ ATAGGATCCAACCGTGATGGCACCCGCT 3′; *F*, 5′ TATGAATTCGGAATGTGTCTTGGTCTC 3′. Probe G: 5′ GCTGTCCCTGTTGTCGAATG 3′. Numbering of nucleotides is based on data presented in references 19, 20, 52. (*B*) Coomassie blue staining of the whole cell lysates of transformed bacteria after IPTG induction. (Lane *1*) Cells transformed with pGEX-2T (*arrowhead*: the ~28-kD product of the parent vector); (lane *2*) cells transformed with pGEX-2T-Var (*arrow*: the ~60-kD fusion protein containing v6). (*C*) Immunoblotting of the same lysates. Whole cell lysates of IPTG-induced bacteria transformed with pGEX-2T-Var (lanes *1* and *3*) and with pGEX-2T (lanes *2* and *4*) were stained with mAbs Var3.1 (lanes *1* and *2*) and 3G6 (negative control, lanes *3* and *4*). mAb Var3.1 stains the ~60-kD fusion protein (lane *1*, *arrow*) but not the product of pGEX-2T (lane *2*). Both mAbs nonspecifically reacted with a ~38-kD molecule. (*D*) Immunoblotting of the purified fusion protein. Affinity-purified fusion protein from IPTG-induced bacteria transformed with pGEX-2T-Var was cleaved with thrombin before immunoblotting. mAb Var3.1 (lane *1*) but not 11G2 (lane *2*) stains the v6 containing 33-kD cleavage product. The less prominent ~60-kD mAb Var3.1-reactive band represents uncleaved fusion protein. *MW*, molecular weight standards in kD.

croscopy. Similar results were obtained, when the cells were first stained and thereafter treated with the detergent.

PCR Amplification of Lymphocyte v6

Total RNA was isolated from PBL obtained from blood donors and from HaCaT cells using guanidine isothiocyanate method and reverse transcribed to cDNA. To study the presence of exon v6 containing mRNA in these cells, primers B and C (see Fig. 2 *A*) were used for PCR. The PCR products were separated in 1.5% agarose gel, blotted onto nylon membranes (Zeta-Probe, BioRad Labs.), hybridized to a P^{32}-labeled oligonucleotide probe from exon v6 (probe G in Fig. 2 *A*) according to standard procedures for Southern blotting (46). As controls, parallel reactions were performed that contained all the reagents except reverse transcriptase.

Results

Establishment of the Human Variant Exon v6-Specific Monoclonal Antibody

Exon v6 has been reported to play a crucial role in the development of metastatic deposits of rat adenocarcinoma cells (13). To study the expression of CD44v6 in man, mAbs against a synthetic polypeptide from the exon v6 were produced. The hybridomas were screened by using ELISA. Supernatant from one hybridoma recognized the peptide used for immunization, but not a scrambled version of the same

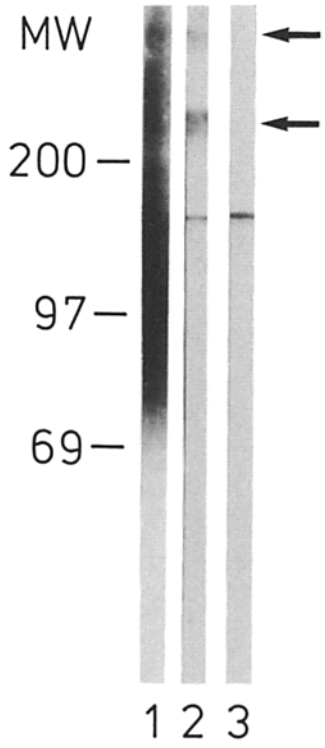


Figure 3. Molecular weight of CD44v6. Hermes-3 reactive material was isolated from a leukopheresis sample using an affinity column, resolved in SDS-PAGE, and blotted. Reactivity with mAbs Var3.1 and Hermes-3 was analyzed using immunoperoxidase method (see Materials and Methods) (Lane *1*) Hermes-3; (lane *2*) Var3.1 (lane *3*) 3G6 (negative control). Arrows point to the two major forms of CD44 recognized by mAb Var3.1. Molecular weight standards (kD) are indicated on the left.

peptide or another unrelated peptide (Fig. 1). This exon v6 specific mAb (designated mAb Var3.1) was selected for further studies after subcloning.

The specificity of mAb Var3.1 was demonstrated by showing that it reacted with a recombinant protein containing human v6. Since HaCaT cells express several forms of CD44, inclusion of v6 in the construct was ensured by making the PCR reaction in two steps (Fig. 2 *A*). First, variant CD44 was produced in two reactions in which the primer C was from exon v6 (and both B and C from the sequence that was formerly called domain 3, ref. 20) and thus determined the specificity of the reaction. Next, these two PCR products were joined by using primers from the constant part of CD44. The 0.95-kb PCR product was then cloned into pGEX-2T expression vector and *E. coli* were transformed with the construct. After IPTG induction, proteins from whole bacterial lysates were separated in SDS-PAGE and subjected to Coomassie staining (Fig. 2 *B*). Bacteria transformed with pGEX-2T-Var produced an IPTG-inducible ~60-kD molecule, while pGEX-2T encoded for an inducible ~28-kD molecule. Next, whole cell lysates of transformed bacteria were subjected to SDS-PAGE and Western blotting (Fig. 2 *C*). It was found that mAb Var3.1 reacted with the ~60-kD fusion protein but not with the product of the parent vector alone. A negative control mAb failed to stain the

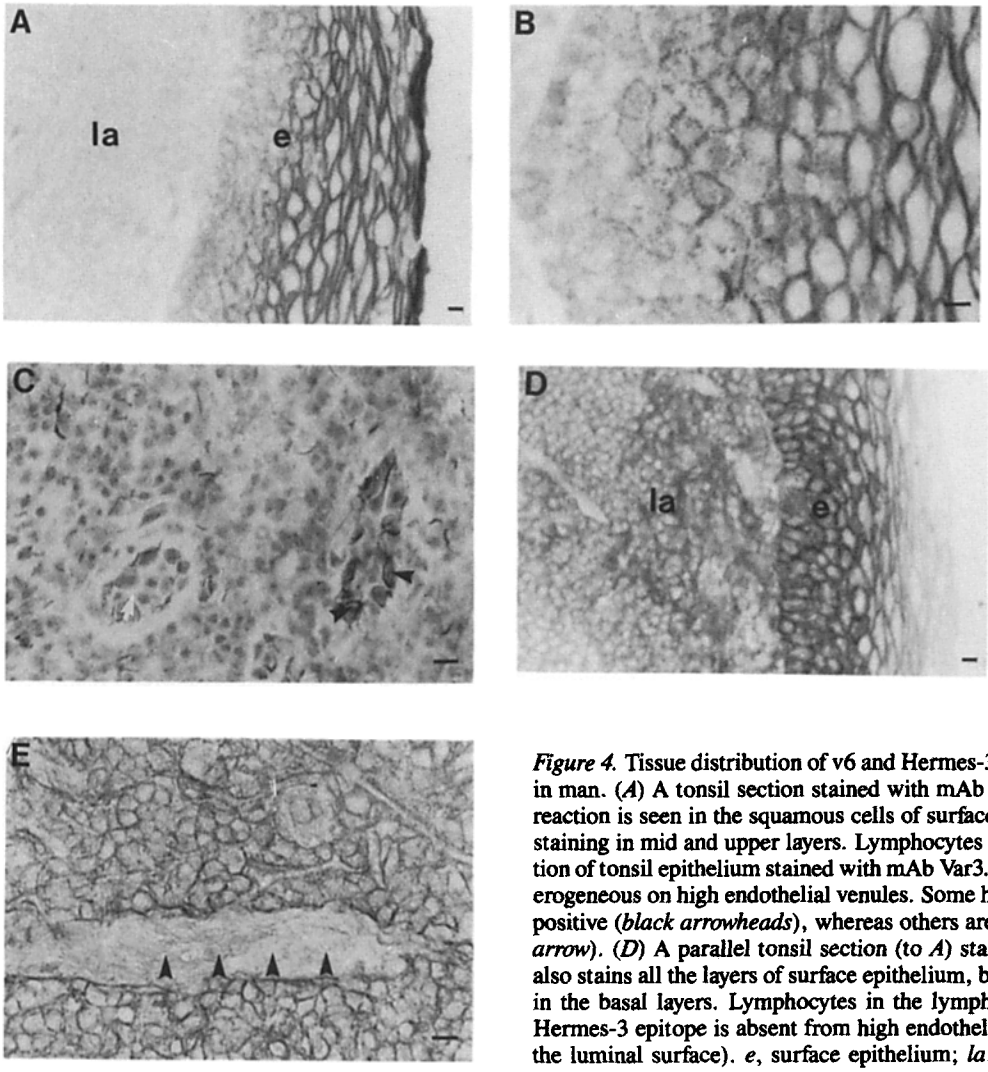


Figure 4. Tissue distribution of v6 and Hermes-3 epitope containing forms of CD44 in man. (*A*) A tonsil section stained with mAb Var3.1. Positive immunoperoxidase reaction is seen in the squamous cells of surface epithelium. Note the predominant staining in mid and upper layers. Lymphocytes are negative. (*B*) Higher magnification of tonsil epithelium stained with mAb Var3.1. (*C*) Expression of CD44v6 is heterogeneous on high endothelial venules. Some high endothelial venules are brightly positive (*black arrowheads*), whereas others are negative or weakly positive (*white arrow*). (*D*) A parallel tonsil section (to *A*) stained with Hermes-3. This antibody also stains all the layers of surface epithelium, but the expression is most prominent in the basal layers. Lymphocytes in the lymphatic area are brightly positive. (*E*) Hermes-3 epitope is absent from high endothelial venules (*arrowheads* pointing to the luminal surface). *e*, surface epithelium; *la*, lymphoid area. Bar, 15 μm.

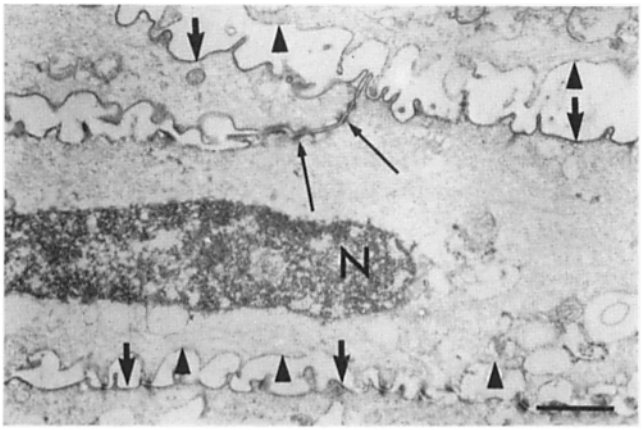


Figure 5. CD44v6 is expressed on the surface of squamous epithelial cells. In immunoelectronmicroscopy, plasma membranes of the squamous epithelial cells in human tonsil stain darkly with mAb Var3.1. In most cells, the more superficial surface (*short arrows*) stain more strongly than the basal surface (*arrowheads*). Desmosomes (*long arrows*); nucleus, *N*. Avidin-biotin peroxidase method. Bar, 1 μm.

~60-kD molecule. In additional experiments, the glutathione-S-transferase carrier was removed from glutathione-agarose bound fusion proteins by thrombin cleavage. The released v6-containing fragment was then analyzed in immunoblotting. mAb Var3.1 reacted with the 33-kD protein containing v6, whereas control mAbs did not react with this molecule (Fig. 2 *D*). Together, the results of ELISA and fusion protein assays unambiguously show that mAb Var3.1 recognizes exon v6 of CD44 in man.

Immunoblotting analyses of Hermes-3 purified CD44 antigen from leukopheresis samples revealed that under non-reducing conditions, mAb Var3.1 recognized two major bands (~220 and 300 kD) and one faint bigger band (Fig. 3). This experiment shows that mAb Var3.1 recognizes an epitope of purified CD44. mAb Hermes-3 stained proteins of very variable sizes (70–300 kD) from the purified CD44 material (Fig. 3).

Expression of CD44v6 in Normal Tissues

Immunoperoxidase staining of frozen sections from normal human tissues was performed to study the tissue distribution of CD44v6. In the tonsil, surface epithelium intensely stained with mAb Var3.1 (Fig. 4, *A* and *B*). Reactivity was particularly strong in the mid and upper layers of stratified squamous epithelium (upper stratum spinosum and stratum granulosum), while cells in the basal layers exhibited fainter staining. Reticulated crypt epithelium also stained positively with mAb Var3.1. Tonsillar lymphocytes were practically nonreactive with mAb Var3.1, as were connective tissue components (Fig. 4 *A*). In germinal centers, mAb Var3.1 faintly reacted with cells of dendritic morphology. Luminal surface of some blood vessels, including high endothelial venules, also stained with mAb Var3.1 (Fig. 4 *C*). In comparison, expression of the Hermes-3 epitope on tonsillar surface epithelium was most pronounced basally and notably less was seen in upper layers (Fig. 4 *D*). mAb Hermes-3 intensely reacted with practically all lymphocytes outside the germinal centers, and fibroblasts were strongly positive in the septae. On the other hand, endothelial lining of most vessels and all high endothelial venules was Hermes-3 negative (Fig. 4 *E*).

Table I. Cell and Tissue Distribution of Var3.1 and Hermes-3 Epitopes of CD44

	Var3.1	Hermes-3
Blood cells (surface)		
PBL	−	++
Granulocytes	−	++
Monocytes	−	++
Tonsil		
Lymphocytes	−	++
Dendritic-like cells in germinal centers	+	+
HEV	+	−
Surface epithelium	++	++
Skin		
Stratum basale	+	++
Stratum spinosum	+	++
Stratum granulosum	++	+
Dermal fibroblasts	−	++
Hair follicles	++	++
Sweat glands	+	++
Intestine		
Lymphoid cells	−	++
Enterocytes	−/+	++
Smooth muscle	−	++
Brain		
Neurons	−	−
Glial cells	−	++

Blood cells were surface-stained for immunofluorescence and analyzed with FACS. Frozen sections of tissues were stained using immunoperoxidase staining. Intensity of staining was scored as follows: −, negative; −/+, weak; +, moderate; ++, strong. *HEV*, high endothelial venules.

In EM, the plasma membrane of the superficial squamous cells stained homogeneously dark in peroxidase preparations, and the staining was usually more intense on the superficial side of the cell (Fig. 5).

Expression patterns obtained by mAbs Var3.1 and Hermes-3 were clearly distinct in several other tissues in addition to tonsil (Table I). In the skin, CD44v6 was preferentially localized in the upper layers of the epidermis, except in keratinized surface layer that was negative. In the dermis, fibroblasts and other stromal elements were negative, while hair follicles and sweat glands stained positively with mAb Var3.1. Expression of the Hermes-3 epitope in the epidermis was most prominent basally, and in the dermis, Hermes-3 epitope was abundantly present in fibroblasts. In the intestine, enterocytes and dendritic cells of Peyer's patches showed weak reactivity with mAb Var3.1, whereas CD44v6 was absent from other structures. mAb Hermes-3 stained enterocytes much more strongly, and the Hermes-3 epitope was also present in lymphoid, smooth muscle, and connective tissue cells of the gut. CD44v6 was not present on neurons or glial cells of brain white or gray matter. Peripheral nerves also lacked this molecule. In contrast, glial cells of white matter were intensely positive with mAb Hermes-3.

Intracellular Localization of CD44v6 in Blood Leukocytes and Cell Lines

CD44v6 was absent from the surface of PBL, monocytes, and granulocytes as determined by FACS-analysis (Fig. 6).

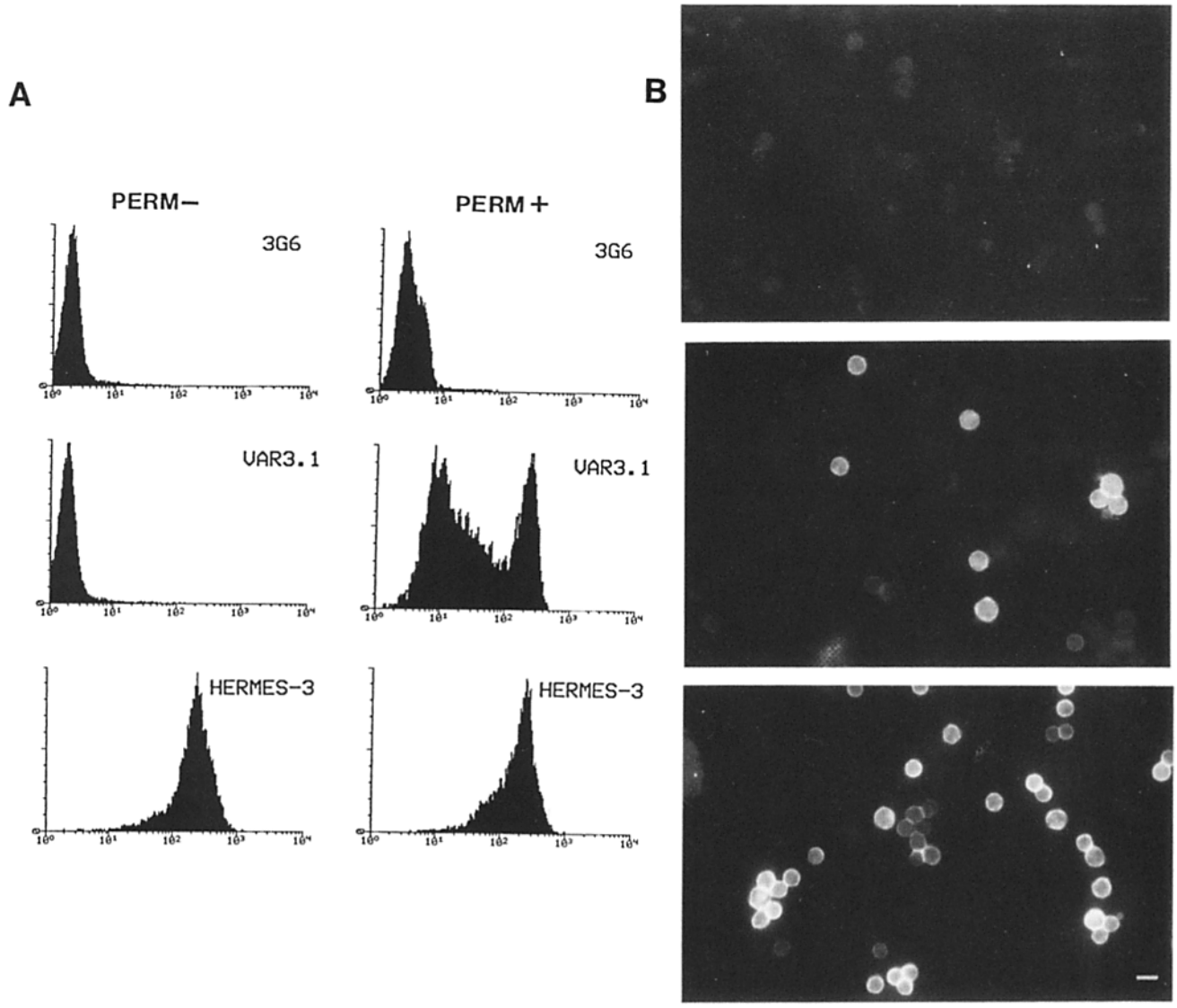


Figure 6. Expression of CD44v6 on blood lymphocytes. (*A*) Fresh PBL (*PERM-, left column*) do not express CD44v6 on their surface, but are Hermes-3 bright. When the cells are permeabilized with 1% formaldehyde and acetone before staining (*PERM +, right column*), many cells become CD44v6 positive. x-axis is relative fluorescence on a log scale; y-axis is cell number. (*B*) In immunofluorescence microscopy, permeabilized PBL show intracellular staining for CD44v6, which preferentially is localized in the periphery of cells. (*Top*) mAb 3G6 (negative control); (*middle*) mAb Var3.1; (*bottom*) mAb Hermes-3. Bar, 10 μm.

PWM/PHA-induced immunoblasts and plasma cells did not express this form of CD44 either. However, when the cells were subjected to permeabilization before the immunofluorescence staining, majority of both unactivated and activated lymphocytes expressed Var3.1 epitope (Fig. 6 *A*). Number of positive cells ranged between 50 and 100% in different individuals. In fluorescence microscopy, positive reactivity was preferentially localized in the periphery of the cells, and fainter diffuse staining was detectable throughout the cytoplasm (Fig. 6 *B*). Existence of CD44v6 in normal PBL was confirmed by showing the presence of v6 specific mRNA in these cells (Fig. 7). In contrast to mAb Var3.1, the Hermes-3 epitope was abundantly expressed on surfaces of all human blood leukocyte subtypes as confirmed in FACS and immunofluorescence microscopy analyses (Fig. 6).

We also tested several cultured cell lines for mAb Var3.1 positivity. Human keratinocyte (HaCaT), epithelial carcinoma (HeLa, U1690, A549), and hematopoietic (KG-1, KG-1a, K562, U937) cell lines all lacked CD44v6 on their surface. However, all of them tested after permeabilization (HaCaT, HeLa, U1690) showed clear intracellular staining with mAb Var3.1. Nonspecific "stickiness" of mAb Var3.1 was ruled out by showing that two carcinoma cell lines (Tera-1 and T-47D) were negative with this mAb also intracellularly. In contrast to CD44v6, all cell lines (except Tera-1 and T-47D) displayed Hermes-3 reactivity both on the cell surface and in the cytoplasm (data not shown).

Molecular Form and Detergent Solubility of CD44v6

Since CD44 is known to associate with cytoskeleton, we determined whether CD44v6 would also be linked to cytoskeletal proteins. Permeabilized HaCaT cells were incubated

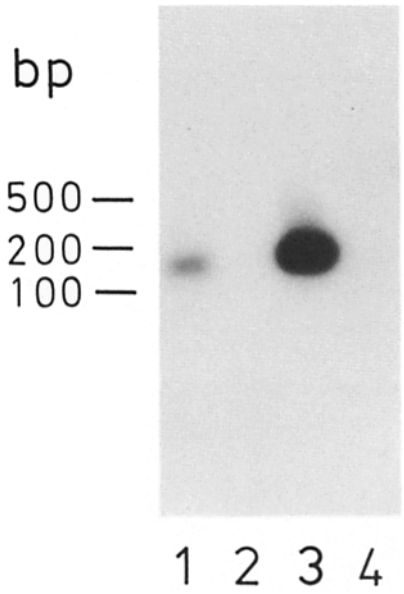


Figure 7. v6-specific RNA is present in human PBL. RNA was isolated from PBL and HaCat cells, reverse transcribed to cDNA, PCR amplified with primers B and C (see Fig. 2 *A*), separated in agarose gel, transferred onto nylon membrane, and hybridized with a v6 specific probe (probe G, Fig. 2 *A*). (Lane *1*) lymphocytes; (lane *3*) HaCat cells. Lanes *2* (lymphocyte) and *4* (HaCaT) represent negative control reactions which were identical to those seen in lanes *1* and *3* with the exception that no reverse transcriptase was added into the cDNA synthesis reaction.

in PBS with or without 0.5% nonionic detergent NP-40 and stained for immunofluorescence (Fig. 8). Immunofluorescence microscopy showed that a considerable amount of CD44v6 was in NP-40 insoluble form. In contrast, significant amount of Hermes-3 containing form of CD44 disappeared during the NP-40 treatment. Similar results were obtained when PBL were analyzed (data not shown).

No molecular mass for CD44v6 was obtained from NP-40 lysates of HaCaT cells in Western blotting or in immunoprecipitations after labeling with [^{35}S]methionine, [^{35}S]cysteine, [^{35}S]sulphate, and [^{14}C]glucosamine, probably due to the poor NP-40 solubility of CD44v6 in these cells. Moreover, mAb Var3.1 also appears to be a poorly precipitating antibody. However, in immunoblotting of SDS solubilized HaCaT cells, a faint ~200-kD band was seen (data not shown).

Downregulation of CD44v6 in Human Neoplasms

The role of different CD44 forms in spread of malignancies is currently under dispute. Therefore, we stained 37 samples from benign (7 papillomas) and malignant (total 30: 5 metastatic, 5 grade III, 10 grade II and 10 grade I head and neck squamous cell carcinomas) epidermal tumors for expression of CD44v6. These experiments showed that all epidermal cells in benign neoplasms stained with mAb Var3.1 like their normal counterparts in the neighboring healthy tissue. In contrast, expression of CD44v6 was downregulated in all carcinoma samples in the malignant areas. In general, better differentiated carcinomas displayed more intense mAb Var3.1 reactivity than the more undifferentiated ones. As examples, staining patterns of a benign papilloma and a squamocellular carcinoma are shown in Fig. 9. All distant metastatic lesions of squamocellular carcinomas were practically negative with mAb Var3.1. In contrast, Hermes-3 brightly stained all benign and majority of the malignant cell types, including the metastatic deposits, in these specimens (Fig. 9).

Discussion

Differential Localization of Var3.1 and Hermes-3 Epitopes of CD44

In this paper, we describe production of a novel mAb against exon v6 of human CD44. Specificity of this mAb Var3.1 was confirmed by showing that it recognizes (*a*) a synthetic peptide from exon v6, (*b*) recombinant protein containing v6, and (*c*) isolated CD44 antigen. Anti-CD44 antibodies generated so far bind to epitopes on three different clusters of the standard lymphocyte form (12, 35). Thus, mAb Var3.1 is the first mAb reported that can discriminate CD44v6 from those forms of CD44 that do not possess exon v6 (while revising this manuscript, a MoAb 17 against an undefined variant exon of CD44 [14] and a polyclonal antibody against v6 [Ref. 19] have been described). mAb Var3.1 enabled us to analyze the expression of this exon in normal and malignant human tissues. CD44v6 was present in different types of epithelial cells, dendritic cells, and endothelial cells of blood vessels. Most abundant expression was seen in squamous epithelial cells, and the Var3.1 epitope appeared to be concentrated on the superficial side of the cells. Comparison of the staining patterns between mAb Var3.1 and Hermes-3, which binds to the constant part of the CD44 molecule, revealed several interesting features (summarized in Table I). At squamous surface epithelia, their reactivity profiles were different: mAb Var3.1 stained most intensely the cells in the mid and upper layers of the epithelium, while the basal layers displayed the brightest reactivity with mAb Hermes-3. Connective tissue components were strongly reactive with Hermes-3 but did not stain with mAb Var3.1. High endothelium of blood vessels, on the other hand, was mAb Var3.1-positive, but Hermes-3-negative. CD44v6 was not present on lymphoid cells of secondary lymphatic organs or on the surface of peripheral blood lymphocytes. All these leukocyte populations, however, stained brightly with mAb Hermes-3. Thus, the expression of exon v6 is restricted to few specialized cell types, whereas Hermes-3 epitope is present on a wide variety of cells.

Hermes-3 negative, Var3.1 positive phenotype of some high endothelial venules provides evidence that forms of CD44 exist which do not react with mAb Hermes-3. This means that new isoforms of CD44 that do not contain the entire constant region are likely to be found. Alternatively, this finding could be explained by different affinities of mAbs Var3.1 and Hermes-3 (highly unlikely on the basis of comparable staining intensities) or by cross-reaction of mAb Var3.1 with some other molecule(s).

In contrast to the expression on the surface of epithelial cells in vivo, peripheral blood leukocytes and several epithelial cell lines only expressed CD44v6 intracellularly. It apparently distributed both as a membrane-associated form and diffusely in the cytoplasm. The acetone treatment per se

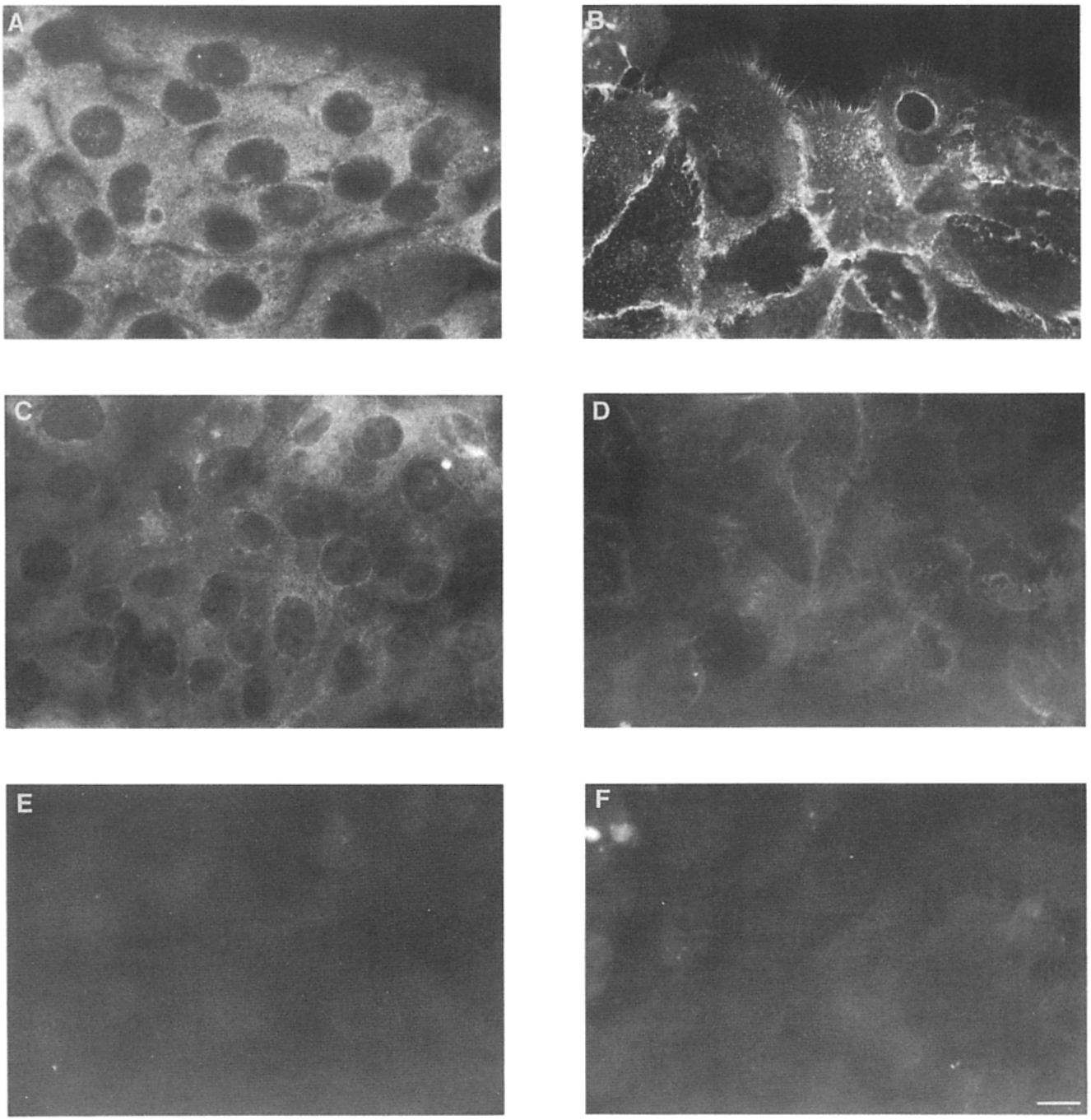
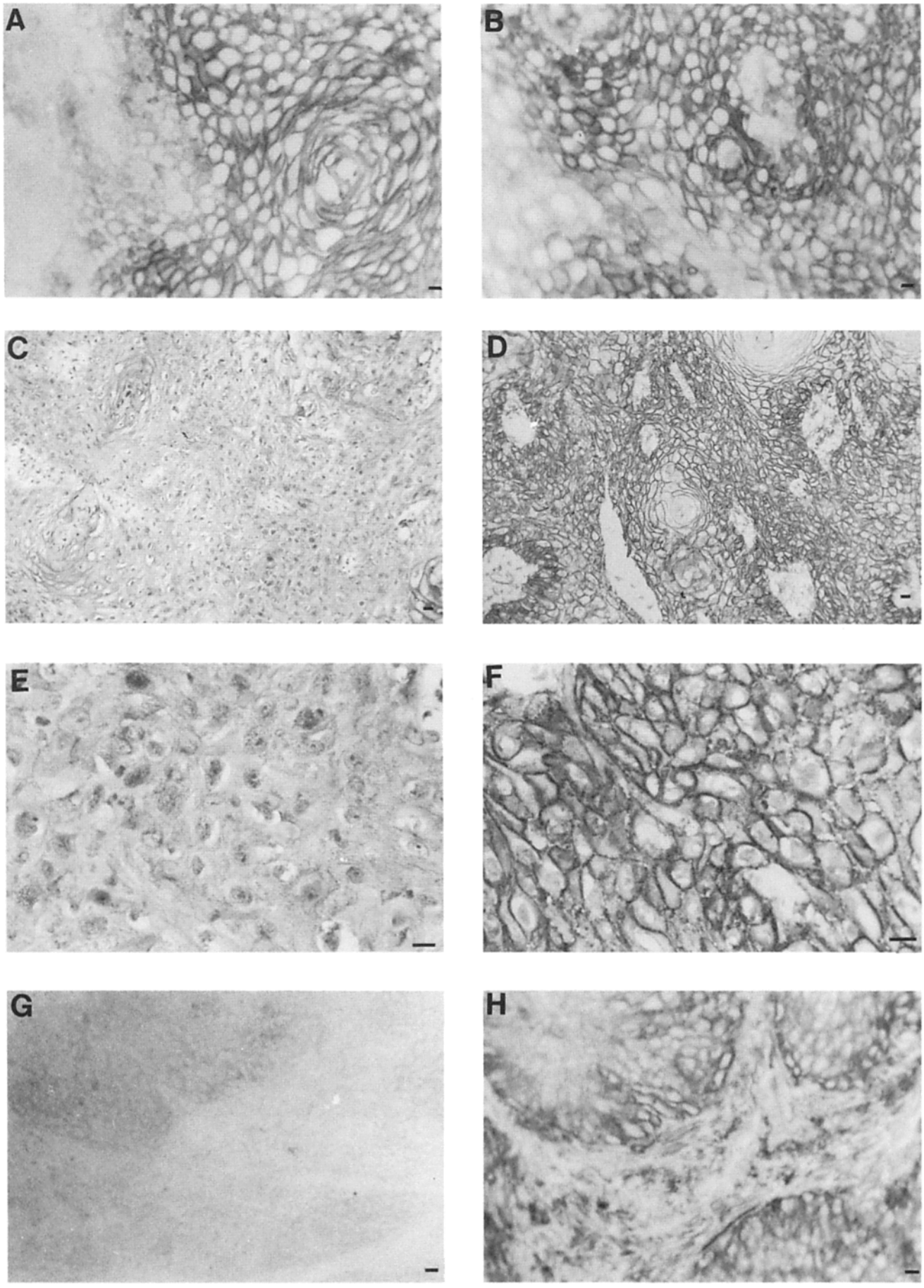


Figure 8. CD44v6 is associated with the cytoskeleton. Fixed and permeabilized HaCaT cells were treated without (*A–B*) or with (*C–F*) 0.5% NP-40 before immunofluorescence staining with mAb Var3.1 (*A* and *C*), Hermes-3 (*B* and *D*), and 3G6 (negative control, *E* and *F*). Significant amount of the Var3.1 reactive material was resistant to NP-40 treatment, while Hermes-3 staining was greatly diminished after the treatment. Bar, 10 μm.

(used for permeabilization) was not necessary for accessibility of Var3.1 epitope to mAb, since mAb Var3.1 stainings produced identical reaction patterns on acetone-fixed and nonfixed cryostat sections of tonsil. Therefore, acetone does not unmask the Var3.1 epitope by dissolving some lipid constituents of the cell membrane. We observed that considerable amount of CD44v6 was in an NP-40-insoluble form, and thus, it is most probably linked to cytoskeletal proteins. The cytoplasmic tail of the standard CD44 is known to be associated with ankyrin, which links transmembrane proteins to actin and fodrin in mouse T-lymphoma cells (31). CD44 also colocalizes with vimentin in WI-38 and with actin in 3T3 cells (5, 34). Furthermore, A3D7 and Hermes-1 (other anti-CD44 antibodies against the constant part) reactive material has been shown to exist in the NP-40 insoluble form in human T cells (11). Our results suggest that CD44v6 can, at least partly, account for these previously described detergent insoluble forms of CD44.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Exon v6 Is Downregulated in Malignant Squamocellular Tumors in Man

In tumors, expression of CD44v6 was not altered in benign epithelial neoplasms. In contrast, malignant transformation was associated to the downregulation of CD44v6. Importantly, the variant CD44v6 was practically absent from the metastatic cells. On the other hand, majority of malignancies remained Hermes-3 positive. These observations hold true in the material of 37 epithelial tumors analyzed in this work, but these results warrant further studies on the expression of CD44v6 on more cases and on other types of carcinomas. Our results suggest that exon v6 would not be responsible for invasive growth and metastasis formation of epithelial squamous carcinomas. Rather, its expression seems to be associated with the regulated, normal differentiation and proliferation of epithelial cells, and its expression seems to be silenced during malignant transformation.

Present results are in agreement with the works reporting that the standard lymphocyte form of CD44 is important in the invasion and metastasis formation. In a recent study on distinct effects of standard and epithelial CD44 isoforms (containing exons g8–10) on tumor growth in an in vivo model, the standard 80–90-kD form, but not the 150-kD form, enhanced tumor invasiveness and metastatic activity (54). In studies of non-Hodgkin lymphomas, surface expression of Hermes-3 correlated positively to the prevalence of metastasis (21, 30). Since we were unable to detect exon v6 on the surface of any leukocyte subset or line, standard form is the most likely candidate in mediating the metastatic behavior of non-Hodgkin lymphomas as well. On the other hand, Günthert et al. found in their rat model that expression of the variant form of CD44 on a rat carcinoma cell line resulted in the acquisition of metastatic properties (13). Specifically, the rat homologue of human exon v6 was implicated to have a central role in this process. In this context it should be noted that the antibodies against the rat exon v6 (1.1.ASML, ref. 13, not cross-reactive with human exon v6) and against the human exon v6 (Var3.1) should be reactive with the homologous or physically close epitopes. The 1.1ASML epitope has been mapped within an amino acid sequence of rat exon v6 that only differs by three amino acids from the corresponding human peptide in v6 that was used for immunization. Very recently, using polyclonal antibody against v6, Heider et al. have shown that expression of v6 is upregulated in precancerous and malignant lesions of human colonic epithelium (19). Most likely, the different carcinoma material accounts for the majority of the differences observed, since we used carcinomas evolving from human keratinocytes, while adenocarcinomas were used in other studies (13, 19). In fact, our preliminary studies with adenocarcinoma specimens indicate that in certain cases mAb Var3.1 epitope is upregulated during malignant transformation. It has been also reported that several alternatively spliced large molecular weight variants were overproduced in malignant tumors of breast and colonic tissue in man when analyzed by PCR and hybridization (38). Thus, the role of CD44 in tumor metastasis may be dependent on the species, type of carcinoma, or host microenvironments.

In conclusion, we have shown that the expression of the Var3.1 and Hermes-3 epitope containing forms of CD44 in human tissues and cells differs dramatically. Most notably, CD44v6 is preferentially epithelial in distribution, intracellular in lymphoid and epithelial cell lines, and greatly diminished or absent in malignant squamous epithelial tumors. mAb Var3.1 will offer a possibility to study the regulation of expression of a certain form of CD44 during cell differentiation. It will also prove helpful in dissecting the role of different isoforms in multiple functions of CD44. Finally, it may be useful in determining the malignant transformation of epithelial cells.

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References

1. Arch, R., K. Wirth, M. Hofmann, H. Ponta, S. Matzku, P. Herrlich, and M. Zöller. 1992. Participation in normal immune responses of a metastasis-inducing splice variant of CD44. *Science (Wash. DC).* 257: 682–685.
2. Aruffo, A., I. Stamenkovic, M. Melnick, C. B. Underhill, and B. Seed. 1990. CD44 is the principal cell surface receptor for hyaluronate. *Cell.* 61:1303–1313.
3. Belitsos, P. C., J. E. K. Hildreth, and J. T. August. 1990. Homotypic cell aggregation induced by anti CD44(Pgp-1) monoclonal antibodies and related to CD44(Pgp-1) expression. *J. Immunol.* 144:1661–1670.
4. Brown, T. A., T. Bouchard, T. St. John, E. Wayner, and W. G. Carter. 1991. Human keratinocytes express a new CD44 core protein (CD44E) as a heparan-sulfate intrinsic membrane proteoglycan with additional exons. *J. Cell Biol.* 113:207–221.
5. Carter, W. G., and E. A. Wayner. 1988. Characterization of the class III collagen receptor, a phosphorylated, transmembrane glycoprotein expressed in nucleated human cells. *J. Biol. Chem.* 263:4193–4201.
6. Culty, M., K. Miyake, P. W. Kincade, E. Sikorski, E. C. Butcher, and C. Underhill. 1990. The hyaluronate receptor is a member of the CD44 (H-CAM) family of cell surface glycoproteins. *J. Cell Biol.* 111:2765–2774.
7. Dalchau, R., J. Kirkley, and J. W. Fabre. 1980. Monoclonal antibody to a human brain-granulocyte-T lymphocyte antigen probably homologous to the W 3/13 antigen of the rat. *Eur. J. Immunol.* 10:745–749.
8. Denning, S. M., P. T. Le, K. H. Singer, and B. F. Haynes. 1990. Antibodies against the CD44 p80, lymphocyte homing receptor molecule augment human peripheral blood T cell activation. *J. Immunol.* 144:7–15.
9. Dougherty, G. J., P. M. Lansdorp, D. L. Cooper, and R. K. Humphries. 1991. Molecular cloning of CD44R1 and CD44R2, two novel isoforms of the human CD44 lymphocyte "homing" receptor expressed by hemopoietic cells. *J. Exp. Med.* 174:1–5.
10. Gallatin, W. M., E. A. Wayner, P. A. Hoffman, T. St. John, E. C. Butcher, and W. G. Carter. 1989. Structural homology between lymphocyte receptors for high endothelium and class III extracellular matrix receptor. *Proc. Natl. Acad. Sci. USA.* 86:4654–4658.
11. Geppert, T. D., and P. E. Lipsky. 1991. Association of T cell-surface molecules with the cytoskeleton. *J. Immunol.* 146:3298–3305.
12. Goldstein, L. A., D. F. H. Zhou, L. J. Picker, C. N. Minty, R. F. Bar-

Figure 9. Expression of CD44v6 in tumors. A benign cutaneous papilloma is positive with both mAb Var3.1 (*A*) and mAb Hermes-3 (*B*). A squamocellular carcinoma of the skin displays greatly diminished expression of CD44v6 (*C*), but remains brightly Hermes-3 positive (*D*). (*E*) Higher magnification from *C*. (*F*) Higher magnification from *D*. (*G*) Metastatic cells from squamocellular carcinoma are practically mAb Var3.1 negative, but (*H*) they still contain the Hermes-3 epitope. Bar, 15 μm.

gatze, J. F. Ding, and E. C. Butcher. 1989. A human lymphocyte homing receptor, the Hermes antigen, is related to cartilage proteoglycan core and link proteins. *Cell.* 56:1063–1072.
13. Günthert, U., M. Hofmann, W. Rudy, S. Reber, M. Zöller, I. Haussmann, S. Matzku, A. Wenzel, H. Ponta, and P. Herrlich. 1991. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell.* 65:13–24.
14. Haggerty, J. G., R. H. Bretton, and L. M. Milstone. 1992. Identification and characterization of a cell surface proteoglycan on keratinocytes. *J. Invest. Dermatol.* 99:374–380.
15. Hale, L. P., K. H. Singer, and B. F. Haynes. 1989. CD44 antibody against In(Lu)-related p80, lymphocyte-homing receptor molecule inhibits the binding of human erythrocytes to T cells. *J. Immunol.* 143:3944–3948.
16. Haynes, B. F., E. A. Harden, M. J. Telen, M. E. Hemler, J. L. Strominger, T. J. Palker, R. M. Scearce, and G. S. Eisenbarth. 1983. Differentiation of human T lymphocytes 1. Acquisition of a novel human cell surface protein (p80) during normal intrathymic T cell maturation. *J. Immunol.* 131:1195–1200.
17. Haynes, B. F., H.-X. Liao, and K. L. Patton. 1991. The transmembrane hyaluronate receptor (CD44): multiple functions, multiple forms. *Cancer Cells (Cold Spring Harbor).* 3:347–350.
18. Haynes, B. F., M. J. Telen, L. P. Hale, and S. M. Denning. 1989. CD44-a molecule involved in leukocyte adherence and T-cell activation. *Immunol. Today.* 10:423–428.
19. Heider, K.-H., M. Hofmann, E. Horst, F. van den Berg, H. Ponta, P. Herrlich, and S. T. Pals. 1993. A human homologue of the rat metastasis-associated variant of CD44 is expressed in colorectal carcinomas and adenomatous polyps. *J. Cell Biol.* 120:227–233.
20. Hofmann, M., W. Rudy, M. Zöller, C. Tolg, H. Ponta, P. Herrlich, and U. Günthert. 1991. CD44 splice variants confer metastatic behavior in rats: homologous sequences are expressed in human tumor cell lines. *Cancer Res.* 51:5292–5297.
21. Horst, E., C. J. L. M. Meijer, T. Radaszkiewicz, G. J. Ossekoppele, J. H. J. M. van Krieken, and S. T. Pals. 1990. Adhesion molecules in the prognosis of diffuse large-cell lymphoma: expression of a lymphocyte homing receptor (CD44), LFA-1(CD11a/18), and ICAM-1(CD54). *Leukemia (Basingstoke).* 4:595–599.
22. Huet, S., H. Groux, B. Caillou, H. Valentin, A. M. Prieur, and A. Bernard. 1989. CD44 contributes to T cell activation. *J. Immunol.* 143: 798–801.
23. Isacke, C. M., C. A. Sauvage, R. Hyman, J. Lesley, R. Schulte, and I. S. Trowbridge. 1986. Identification and characterization of the human Pgp-1 glycoprotein. *Immunogenetics.* 23:326–332.
24. Jackson, D. G., J. Buckley, and J. I. Bell. 1992. Multiple variants of the human lymphocyte homing receptor CD44 generated by insertions at a single site in the extracellular domain. *J. Biol. Chem.* 267:4732–4739.
25. Jacobson, K., D. O'Dell, B. Holifield, T. L. Murphy, and J. T. August. 1984. Redistribution of a major cell surface glycoprotein during cell movement. *J. Cell Biol.* 99:1613–1623.
26. Jalkanen, S. T., R. F. Bargatze, J. de los Toyos, and E. C. Butcher. 1987. Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85–95-kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal, or synovial endothelial cells. *J. Cell Biol.* 105:983–990.
27. Jalkanen, S., and M. Jalkanen. 1992. Lymphocyte CD44 binds to the C-terminal heparin binding domain of fibronectin. *J. Cell Biol.* 116: 817–825.
28. Jalkanen, S., R. F. Bargatze, L. R. Herron, and E. C. Butcher. 1986. A lymphoid cell surface glycoprotein involved in endothelial cell recognition and lymphocyte homing in man. *Eur. J. Immunol.* 16:1195–1202.
29. Jalkanen, S., M. Jalkanen, R. Bargatze, M. Tammi, and E. C. Butcher. 1988. Biochemical properties of glycoproteins involved in lymphocyte recognition of high endothelial venules in man. *J. Immunol.* 141:1615–1623.
30. Jalkanen, S., H. Joensuu, K.-O. Söderström, and P. Klemi. 1991. Lymphocyte homing and clinical behavior of non-Hodgkin's lymphoma. *J. Clin. Invest.* 87:1835–1840.
31. Kalomiris, E. L., and L. Y. W. Bourguignon. 1988. Mouse T lymphoma cells contain a transmembrane glycoprotein (GP85) that binds ankyrin. *J. Cell Biol.* 106:319–327.
32. Koopman, G., Y. van Kooyk, M. de Graaff, C. J. L. M. Meijer, G. C. Figdor, and S. T. Pals. 1990. Triggering of the CD44 antigen on T lymphocytes promotes T cell adhesion through the LFA-1 pathway. *J. Immunol.* 145:3589–3593.
33. Kugelman, L. C., S. Ganguly, J. G. Haggerty, S. M. Weissman, and L. M. Milstone. 1992. The core protein of epican, a heparan sulfate proteoglycan on keratinocytes, is an alternative form of CD44. *J. Invest. Dermatol.* 99:381–385.
34. Lacy, B. E., and C. B. Underhill. 1987. The hyaluronate receptor is associated with actin filaments. *J. Cell Biol.* 105:1395–1404.
35. Letarte, M. 1986. Human p85 glycoprotein bears 3 distinct epitopes defined by several monoclonal antibodies. *Mol. Immunol.* 23:639–644.
36. Letarte, M., S. Iturbe, and E. J. Quakenbush. 1985. A glycoprotein of molecular weight 85,000 on human cells of B-lineage: detection with a family of monoclonal antibodies. *Mol. Immunol.* 22:113–124.
37. Lucas, M. G., A. M. Green, and M. J. Telen. 1989. Characterization of the serum In(Lu)-related antigen: identification of a serum protein related to erythrocyte p80. *Blood.* 73:596–600.
38. Matsumura, Y., and D. Tarin. 1992. Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Lancet.* 340:1053–1058.
39. Miyake, K., K. L. Medina, S.-I. Hayashi, S. Hamaoka, T. Ono, and P. W. Kincade. 1990. Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures. *J. Exp. Med.* 171: 477–488.
40. Miyake, K., C. B. Underhill, J. Lesley, and P. W. Kincade. 1990. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J. Exp. Med.* 172:69–75.
41. Omary, M. B., I. S. Trowbridge, M. Letarte, M. F. Kagnoff, and C. M. Isacke. 1988. Structural heterogeneity of human Pgp-1 and its relationship with p85. *Immunogenetics.* 27:460–464.
42. Oppenheimer-Marks, N., L. S. Davis, and P. E. Lipsky. 1990. Human T lymphocyte adhesion to endothelial cells and transendothelial migration. Alteration of receptor usage relates to the activation status of both the T cell and the endothelial cell. *J. Immunol.* 145:140–148.
43. Picker, L. J., M. Nakache, and E. C. Butcher. 1989. Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. *J. Cell Biol.* 109:927–938.
44. Picker, L. J., J. de los Toyos, M. J. Telen, B. F. Haynes, and E. C. Butcher. 1989. Monoclonal antibodies against the CD44[In(Lu)-related p80], and Pgp-1 antigens in man recognize the Hermes class of lymphocyte homing receptors. *J. Immunol.* 142:2046–2051.
45. Rothman, B. L., M.-L. Blue, K. A. Kelley, D. Wunderlich, D. V. Mierz, and T. M. Aune. 1991. Human T cell activation by OKT3 is inhibited by a monoclonal antibody to CD44. *J. Immunol.* 147:2493–2499.
46. Sambrook, J., E. F. Fritsch, and T. Maniatis. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1989.
47. Screaton, G. R., M. V. Bell, D. G. Jackson, F. B. Cornelis, U. Gerth, and J. I. Bell. 1992. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc. Natl. Acad. Sci. USA.* 89:12160–12164.
48. Seth, A., L. Gote, M. Nagarkatti, and P. S. Nagarkatti. 1991. T-cell-receptor-independent activation of cytolytic activity of cytotoxic T lymphocytes mediated through CD44 and gp90^{MEL-14}. *Proc. Natl. Acad. Sci. USA.* 88:7877–7881.
49. Shimizu, Y., G. A. van Seventer, R. Siraganian, L. Wahl, and S. Shaw. 1989. Dual role of the CD44 molecule in T cell adhesion and activation. *J. Immunol.* 143:2457–2463.
50. Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. *Gene (Amst.)* 67:31–40.
51. St. John, T., J. Meyer, R. Idzerda, and W. M. Gallatin. 1990. Expression of CD44 confers a new adhesive phenotype on transfected cells. *Cell.* 60:45–52.
52. Stamenkovic, I., M. Amiot, J. Pesando, M, and B. Seed. 1989. A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. *Cell.* 56:1057–1062.
53. Stamenkovic, I., A. Aruffo, M. Amiot, and B. Seed. 1991. The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:343–348.
54. Sy, M. S., Y.-J. Guo, and I. Stamenkovic. 1991. Distinct effects of two CD44 isoforms on tumor growth in vivo. *J. Exp. Med.* 174:859–866.
55. Telen, M. J., G. S. Eisenbarth, and B. F. Haynes. 1983. Regulation of expression of a novel erythrocyte surface antigen by the inhibitor Lutheran In(Lu) gene. *J. Clin. Invest.* 71:1878–1886.
56. Underhill, C. B., S. J. Green, P. M. Tarone, and G. Comoglio. 1987. The hyaluronate receptor is identical to a glycoprotein of Mr 85,000 (gp85) as shown by a monoclonal antibody that interferes with binding activity. *J. Biol. Chem.* 262:13142–13146.
57. Webb, D. S. A., Y. Shimizu, G. A. van Seventer, S. Shaw, and T. L. Gerrard. 1990. LFA-3, CD44, and CD45: physiologic triggers of human monocyte TNF and IL-1 release. *Science (Wash. DC).* 249:1295–1297.