Expression and Modulation of CD44 Variant Isoforms in Humans

Charles R. Mackay,* Hans-Joachim Terpe,‡ Reinhard Stauder,*§ Wendy L. Marston,* Heike Stark,† and Ursula Günthert*

• The Basel Institute for Immunology, CH-4005 Basel, Switzerland; ‡ Institut für Pathologie, Klinik der Justus-Liebig-Universität Giessen, D-35385 Giessen, Germany; § Abt. für Immunhämatologie und Onkologie, Universitätsklinik für Innere Medizin, A-6020 Innsbruck, Austria

Abstract. CD44 is a ubiquitous surface molecule that exists as a number of isoforms, generated by alternative splicing of 10 "variant" exons. Little is known about the expression and function of the variant isoforms, except that certain isoforms may play a role in cancer metastasis. We produced mAbs against CD44 variant regions encoded by exons 4v, 6v, and 9v, by immunizing mice with a fusion protein spanning variant exons 3v to 10v. A comprehensive analysis of human tissues revealed that CD44 variant isoforms were expressed widely throughout the body, principally by epithelial cells. However there was differential expression of CD44 variant exons by different epithelia. Most epithelia expressed exon 9v, but much fewer expressed 6v or 4v. The regions of epithelia that expressed the highest levels of the variant isoforms were the generative cells, particularly the basal cells of stratified squamous epithelium, and of glandular epithelium. CD44 variant isoforms were also expressed differentially by leukocytes, with CD44-9v expressed at very low levels and CD44-6v and 4v virtually absent. However, CD44-9v and CD44-6v were the main variants that were transiently upregulated on T cells after mitogenic stimulation and on myelomonocytic cell lines by TNFα and IFNγ treatment. Some epithelial cell lines could preferentially upregulate CD44-6v upon IFNγ incubation. These results show that CD44 variant isoforms are expressed much more widely than first appreciated, and that expression of the variant isoforms on some cell types can be modulated by particular cytokines.

CD44 is a widely expressed cell surface glycoprotein that serves as an adhesion molecule in cell–substrate and cell–cell interactions, including lymphocyte homing, hemopoiesis, cell migration, and metastasis (for reviews see Haynes et al., 1991; Underhill, 1992; Günthert, 1993; Lesley et al., 1993). CD44 also has other functions that relate to lymphocyte activation (Haynes et al., 1989) and the binding of certain cytokines to endothelium (Tanaka et al., 1993). CD44 is a proteoglycan with an NH2-terminal region that is structurally related to several hyaluronate binding proteins (Underhill, 1992). CD44 is known to bind hyaluronate and collagen (Carter and Wayner, 1988; Aruffo et al., 1990; Culty et al., 1990; Miyake et al., 1990) and a chondroitin sulfated form of CD44 binds fibronectin (Jalkanen and Jalkanen, 1992). Additional ligands for CD44 may well exist. The numerous functions and molecular interactions of CD44 probably relate to its complex structure. In addition to the "standard" 85–95-kD form (CD44s), several larger "variant" isoforms exist (CD44v) that are generated by alternative splicing of at least 10 exons (Screaton et al., 1992; Günthert, 1993). PCR and Northern analyses have demonstrated that certain combinations of the variant exons are expressed on epithelial cells, carcinomas, and some hemopoietic cells (Brown et al., 1991; Dougherty et al., 1991; Günthert et al., 1991; Hofmann et al., 1991; Stamenkovic et al., 1991; Cooper et al., 1992; He et al., 1992; Jackson et al., 1992; Kugelman et al., 1992; Screaton et al., 1992).

Little is known about the function or regulation of CD44v molecules. Expression of CD44v in some tissues appears to relate to tumor progression, particularly the metastatic potential of some cancers (Reber et al., 1990; Günthert et al., 1991; Matsumura and Tarin, 1992; Heider et al., 1993; Hofmann et al., 1993; Koopman et al., 1993; Rudy et al., 1993; Seiter et al., 1993; Tanabe et al., 1993). In rats, a region of CD44 encoded by variant exon 6 (referred to hereafter as CD44-6v) conferred metastatic potential to nonmetastasizing pancreatic carcinoma cells (Günthert et al., 1991; Rudy et al., 1993). Moreover, a mAb recognizing rat

1. Abbreviations used in this paper: APAAP, alkaline phosphatase anti-alkaline phosphatase; CD44s, standard CD44; CD44v, variant CD44; HPRT, hypoxanthine phosphoribosyltransferase; PE, phycoerythrin.
CD44-6v (Matzku et al., 1989) blocked invasion of these carcinoma cells into the draining lymph nodes and the lung, possibly by interfering with the binding of CD44-6v to a ligand in these tissues (Reber et al., 1990; Seiter et al., 1993). An analysis of human breast and colon carcinoma using reverse transcribed PCR revealed a gross over-production of CD44-6v, or CD44-8v-10v, in contrast to normal tissues in which only the standard form was detected (Matsumura and Tarin, 1992; Heider et al., 1993; Koopman et al., 1993; Tanabe et al., 1993). In humans and rats, CD44-6v is transiently expressed on B cells, T cells and macrophages after stimulation (Arch et al., 1992; Koopman et al., 1993).

The ubiquitous expression of CD44, its complex structure and its involvement in tumor progression underscores a need to determine the expression, regulation and function of CD44 isoforms. This paper reports on the differential expression and modulation of a number of CD44v isoforms, assessed using a panel of newly generated isoform-specific mAbs.

**Materials and Methods**

**Cells and Tissues**

The characteristics and culture requirements of most of the cell lines used in this study have been described previously (Hofmann et al., 1991). Primary fibroblasts derived from human neonatal foreskin were provided by Professor W. Müller, Kinderklinik, Basel. All other cell lines originated from the American Type Culture Collection (Rockville, MD). Normal human tissues were obtained immediately postmortem, or from biopsies or resected tissues after surgery. Tissues for immunohistological staining were snap frozen in liquid nitrogen and stored at -70°C until use.

**Monoclonal Antibodies**

A panel of mAbs was produced against the variant regions of human CD44 by immunizing mice with a fusion protein corresponding to exons 3v to 10v of the variant region (Güntert, 1993). Mice were immunized i.p. with 100 μg of fusion protein, three times over a period of 6 wk. 4 d after the last immunization, the spleen was taken and cell fusion performed using the cell line SP2/O, as described (Harlow and Lane, 1988). The mAbs 11.24 and 11.31 are IgG1 isotype, and mAbs 11.9 and 11.10 are IgG2a isotype. Other mAbs used in this study included anti-CD44 standard mAbs 25-32 (Mackay et al., 1988), F10-44-2 (Planagan et al., 1989) (Serotec, Oxford, UK) and Hermes-3 (Jalkanen et al., 1987) (kindly provided by Dr. Sirpa Jalkanen). A mAb termed 14-14 specific for α6 integrin was produced in our laboratory. The ICAM-1 specific mAb RR/I was a gift of Dr. T. Springer. Phycoerythrin-labeled antibody to CD20 was purchased from Becton Dickinson. A mAb termed 14-14 specific for α6 integrin was produced in our laboratory. A mAb termed 14-14 specific for α6 integrin was produced in our laboratory. A mAb termed 14-14 specific for α6 integrin was produced in our laboratory. A mAb termed 14-14 specific for α6 integrin was produced in our laboratory.

**Immunofluorescent Staining and Flow Cytometry**

For one color immunofluorescence staining, 10^6 cells were reacted with 50 μl of hybridoma supernatant for 30 min at room temperature (RT). The mAbs 11.10 and 11.9 were found to stain more effectively at RT. Cells were then washed once and resuspended in 50 μl of FITC goat anti-mouse Ig using 20 μl of 10% normal mouse serum, and then adding an appropriate amount of PE-conjugated mAb. After 10 min at 4°C, the cells were washed twice and analyzed using the FACS®. Monocytes and granulocytes were identified within human blood buffy coat cells by their forward angle and side scatter profile. T cells and B cells were identified with anti-CD3-PE and anti-CD20-PE, respectively. CD45RO and CD45RA T cells were identified using anti-CD45RA-PE together with biotinylated anti-CD3 and CY-Chrome-labeled streptavidin (PharMingen, San Diego, CA).

**In vitro Stimulation of Cells with Cytokines or Mitogens**

In vitro stimulation of human T cells or T cell clones with phytohemagglutinin (PHA) or in the mixed leukocyte reaction (MLR) followed previously published procedures (Lanzavecchia, 1985). The effect of various cytokines on the expression of CD44 was assessed using the following concentrations: TNFα and TGFβ at 10 ng/ml, IFNγ, GM-CSF, IL-4 at 250 U/ml, and IL-1β at 50 U/ml. TNFα and IFNγ were obtained from the central research department of Hoffmann La Roche, from Drs. Werner Lesslauer and Gianluigi Baricco. GM-CSF, IL-4, TGF-β and IL-1β were purchased from British Biotechnology (Owen, UK).

**Fusion Proteins, Synthetic Peptides, and ELISA**

The CD44v region covering exons 3v to 10v was cloned from cDNA via PCR amplification. The two PCR primers (5′ GTACGCTTCATGAGAAGGC- CCTTGCAGGCT, and 5′ CTGTAAGAAGGTTGAGTACTG) correspond to positions 1 to 30 and positions 1014 to 985, respectively, of the exons 3v to 10v, as described (Hofmann et al., 1991). To insert the PCR product directly into the glutathione transferase fusion vector pGEX-2T (Smith and Johnson, 1988), EcoRI sites were added at the 5′ ends of the primers. The resulting plasmid pGEX-CD44(3v-10v) codes for a fusion protein of ~85 kD. Subclones were constructed by using appropriate restriction sites and inserted into the pGEX expression system: fragment 1 (position 2-320), fragment 2 (position 377-583), fragment 3 (position 638-848), fragment 4 (position 1-244), fragment 5 (position 256-476), fragment 6 (position 483-830), fragment 7 (position 830-1014), fragment 8 (position 320-483), fragment 9 (position 144-486), fragment 10 (position 486-720), fragment 11 (position 699-916) (see Fig. 1 A). Fusion proteins were prepared after isopropyl-β-D-thiogalacto-pyranoside induction of the tac promoter as described (Smith and Johnson, 1988). Briefly, cells were resuspended in PBS containing 1% Triton X-100, 0.5% Tween 20, 0.03% SDS (PBS-TTs) and sonicated. Glutathione Sepharose-4B was added to the bacterial lysate. After washing the affinity matrix several times with PBS-TTS, elution was performed with PBS containing 5 mM glutathione. Aliquots of the preparations were run on 10% SDS polyacrylamide gels (Laemmli, 1970) and stained with Coomassie blue for control of purity. Synthetic peptides were constructed as 16- or 20-mer overlapping sequences covering human exons 3v to 10v (Hofmann et al., 1991). All peptides were synthesized on a Rink amide am resin (Novabiochem USA, La Jolla, CA) using the ABIMED Ams 422 Multiple Peptide Synthesizer, utilizing Fmoc-chemistry. The amino acid sequences of the peptides were as follows: (1) GTSNTISAGWEPNEENEDE; (2) WERNENEDERDRLS; (3) NEDERDRLSFSGSIDDDDE; (4) IDDDDEDISSTISTTPPP; (5) PRAFDHTKQNQDWTQWNPSH; (6) NPSHSNPVGLLQTTTRMTDV; (7) RMTVDVRGDNTTAYEGN; (8) GTDAEGYNWNPEAHPLLIIH; (9) EAHPPLLIEHEHHEE; (10) HHEEEETPHSTIQATPS; (11) IQATPSSTTEETATQKEQW; (12) EETAQTEKQOWGWRHEGYR; (13) NWRHEGYRQTPREDHS; (14) TRPESDHTGASTAAASHT; (15) TAAASHTSHMPQQRKT; (16) TSHMPQQTRESPDSSWTD; (17) EDSSWTFDFNPNSHPHM; (18) PTANPNTGLVEDLDRT; (19) SFTSPSHSLEEDKDHP; (20) IFTQSTYPHTKSREKST; (21) VTS-AKTGSGFVATVTV.

ELISA was performed by coating fusion proteins from the pGEX subclones onto 96-well microtiter plates, at a concentration of 5-10 μg/ml, for at least 4 h at 4°C. After washing, the wells were then incubated with anti-CD44 antibodies for 2 h at room temperature, washed with PBS and incubated with HRP-coupled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The substrate used for the color reaction was 2,2′-azino-di-[3-ethylbenz-thiazoline-6-sulfonic acid], which was measured at 405 nm in an ELISA reader. For a more defined localization of the epitope specificity of the CD44v-specific mAbs, fusion protein pGEX-CD44v was coated onto the wells, and 16-20-mer synthetic peptides at a concentration of 10 μg/ml were used to block reactivity of the mAbs.

**Immunohistochemistry**

5-μm frozen sections were prepared and immunostained using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (Cordell et al., 1984). Briefly, sections were air-dried, dewaxed in xylene for 5 min, followed by washes in 100% acetone and were then incubated, successively, with hybridoma supernatant diluted 1:30 to 1:100 for 30 min, anti-mouse Ig, APAAP complex (Dakopatts, Denmark), and the substrate for the color reaction (Naphthol AS-BI phosphate/New
mAbs of irrelevant specificities were used as a first step negative controls. The immunostaining results were evaluated semi-quantitatively and classified as follows: strong expression, ++; moderate expression, +++; weak expression, +; and no detectable expression, −.

**Immunoprecipitation and SDS-PAGE**

Immunoprecipitation of radio-labeled molecules and SDS-PAGE followed standard procedures (Harlow and Lane, 1988). Briefly, HKPII cells were labeled with [35S]methionine (Amersham) by incubation for 4 h with 1 mCi in a 75 ml tissue culture flask. The lysis buffer used to solubilize cells consisted of 2% NP-40 (Sigma Chemical Co.) in 20 mM Tris-HCl pH 8, 1 mM MgCl2, 150 mM NaCl, and 0.1 mM PMSF. Protein G-Sepharose (Pharmacia) were used for pre-clearing lysates and for isolating precipitated material. For immunoblotting, cells were incubated on ice in a buffer containing 10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 1 mM PMSF, and 10 µg/ml Aprotinin (Sigma Chemical Co.). The cell lysate was mixed vigorously and centrifuged at 13,000 g for 10 min. The supernatant was adjusted to gel sample buffer, boiled for 5 min, and proteins were separated on 7.5-15% gradient polyacrylamide SDS gels under reducing conditions (Laemmli, 1970). Precipitated material. For immunoblotting, cells were incubated on ice in a buffer containing 10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 1 mM PMSF, and 10 µg/ml Aprotinin (Sigma Chemical Co.). The cell lysate was mixed vigorously and centrifuged at 13,000 g for 10 min. The supernatant was adjusted to gel sample buffer, boiled for 5 min, and proteins were separated on 7.5-15% gradient polyacrylamide SDS gels under reducing conditions (Laemmli, 1970).

**Semiquantitative Reverse Transcribed PCR**

The myelomonocytic cell lines THP-1 and U937 were treated with TNFα and IL-4 for 24 h as described above. Approximately 10⁶ cells were taken for RNA preparation using the method of Chomczynski and Sacchi (1987) and with CD44 variants, immunoprecipitations were performed using lysates prepared from the HKPII keratinocyte cell line, which expresses predominantly CD44v regions 3v-10v (Hofmann et al., 1991). Immunoprecipitation of CD44 from an [35S]methionine lysate of HKPII using Hermes-3, 25.32, or F10-44-2 (anti-CD44s mAbs which recognize epitopes in the 5' and 67bp, because the primers used have additional 10-bp extensions at their 5' ends for easier cloning.

The PCR reactions were done as follows: 96°C for 5 s, 50°C (HPRT) or 60°C (CD44) for 15 s, and 72°C for 60 s over 30 cycles, followed by 72°C for 10 min in an Ams Biotechnology thermal cycler. Controls were treated identically, except for replacing the cDNA by water. 7-µl aliquots of the 30-µl reactions were separated on 1.2% agarose gels, followed by alkaline blotting onto Hybond N+ membranes (Amersham). Membranes were then hybridized under stringent conditions (65°C) with the following α-32P dCTP labeled human CD44 probes: exons 5v + 6v, positions 242-467, exon 6v, positions 361-467, exons 8v, 9v + 10v, positions 623-980 (Hofmann et al., 1991) and exons 5s + 6s, positions 561-825 (Stamenkovic et al., 1989). After a short exposure toHyperfilm-MP (Amersham), the amount of radioactivity bound was scanned using a phosphoimager.

**Results**

**Production of mAbs Against Human CD44 Variant Regions**

A scheme of the full length human CD44 molecule, illustrating the organization of the variant exons, is shown in Fig. 1 A. A bacterially synthesized fusion protein was constructed in the pGEX expression system encoding exons 3v-10v. This protein was used to immunize Balb/c mice, from which a number of CD44v-specific mAbs were derived. The specificity of the various mAbs for the different exon products was determined by ELISA, using smaller fusion proteins encoded by the variant exons (Fig. 1 A). A more precise determination of the epitope specificities was achieved by blocking the reactivity of the antibodies for the fusion protein CD44-(3v-10v) with 16-20-mer peptides corresponding to specific regions of the CD44v sequence (Fig. 1, A and B). A large number of variant-specific mAbs were generated, but only a proportion of these reacted with the fully processed CD44v regions, as revealed by flow cytometry of human cells (see below). This presumably relates to posttranslational modifications or folding of the CD44 molecule synthesized in eukaryotes, which leads to a loss of specificity of some of the fusion protein-reactive mAbs. The specificity of those mAbs reactive with naturally expressed CD44v regions is mAb 11.10: exon 4v, 11.9 and 11.31: exon 6v; and 11.24: exon 9v (Fig. 1 A). The sequences of the peptides that blocked the reactivities of these antibodies in the ELISA were, respectively, No. 5: PRAFDHTKQNDWQTWNPSH (positions 47-66); No. 12: EETAQKKEQFWGNWHEYG (positions 129-148) and No. 19: SFSTSSHEGLEEDKDH (positions 247-262, from Hofmann et al., 1991). The epitopes for mAb 11.9 and 11.31 are located in the same region as for mAb 1.1 ASML, known to block metastasis formation in the rat (Seiter et al., 1993).

To confirm the reactivity of mAbs 11.10, 11.9, and 11.24 with CD44 variants, immunoprecipitations were performed using lysates prepared from the HKPII keratinocyte cell line, which expresses predominantly CD44v regions 3v-10v (Hofmann et al., 1991). Immunoprecipitation of CD44 from an [35S]methionine lysate of HKPII using Hermes-3, 25.32, or F10-44-2 (anti-CD44s mAbs which recognize epitopes in the 5' and 67bp, because the primers used have additional 10-bp extensions at their 5' ends for easier cloning.

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**CD44 Variants are Expressed Differentially by Various Epithelia**

Previous studies using PCR, Northern blot analysis, and mAbs against CD44-6v have shown that some normal tissues express CD44v isoforms, although results have been contradictory (Brown et al., 1991; Dougherty et al., 1991; Günther et al., 1991; Stamenkovic et al., 1991; Matsumura and Tarin, 1992; Heider et al., 1993; Salmi et al., 1993). An analysis of different tissues using the variant-specific mAbs and APAAP immunohistochemical staining showed in fact that CD44v isoforms were expressed widely (Fig. 2 and Table I). There were three main findings. First, the most intense and extensive expression of variant isoforms was by epithelium, and epithelium from most tissues expressed

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Figure 1. (A) Schematic presentation of the standard and the variant CD44 regions. At amino acid 223 in human CD44, up to 381 additional amino acids may be present, due to alternative splicing of 9 or 10 variant exons. The region encoding exons 3v-10v was isolated by reverse transcribed PCR and inserted into pGEX-2T to produce a glutathione transferase fusion protein which was used as an immunogen to produce mAbs. Subclones were generated and pGEX fusion proteins synthesized (fragments numbered 1-11, shown at the bottom) to determine the specificity of anti-variant mAbs in an ELISA. The exact positions of fragments 1 to 11 are indicated in Materials and Methods. The mAbs that were reactive with individual fragments in the ELISA are indicated at the left hand side. (B) Synthetic peptides encoding specific
CD44-9v. Other cell types including stromal elements, connective tissue, blood vessels and muscle expressed only CD44s as previously described (Mackay et al., 1988; Flanagan et al., 1989; Picker et al., 1989). Second, the variant exons were expressed differentially in the various epithelial tissues, in that most epithelia expressed CD44-9v whereas fewer epithelial tissues expressed 6v or 4v. CD44v were expressed most intensively in stratified squamous epithelium, particularly in oesophagus, skin and tonsil (Table I and Fig. 2 A). The generative epithelial cells expressed the highest levels of CD44v. Thus the basal cells of stratified squamous epithelium such as oesophagus and skin expressed high levels of all of the CD44v exon products. Epithelium of the skin stained intensely for 9v and 6v but slightly less for 4v (Fig. 2 B). The generative cells of intestinal epithelium, situated in the lower part of the crypts, expressed CD44-9v but not 4v or 6v (Fig. 2 C). The base of glands of the stomach showed a similar pattern of expression (not shown).

Staining of epithelial cell lines with anti-CD44v mAbs, using FACS® analysis, showed that most epithelial cell lines expressed CD44-9v. In general, the expression of CD44v on cell lines detected by flow cytometry correlated with the expression of CD44v in tissues by immunohistochemistry. Thus, squamous epithelial cell lines (i.e., HPKII) expressed CD44-4v, -6v and -9v, whereas cell lines derived from glandular epithelium usually expressed only CD44-9v. One colon carcinoma line (HT29) expressed CD44-9v, while others were negative (COLO 320) (Hofmann et al., 1991), possibly reflecting the differential expression of CD44-9v within intestinal epithelium (see Table I). A neuroblastoma line (SH-EP) and an astrocytoma line (1321 N1) expressed low levels of CD44-9v, and were 6v- and 4v-negative. Other cell types such as primary fibroblasts, a melanoma cell line (MeWo) and cultured endothelial cells were positive only for CD44s (not shown).

Immunocytochemical staining of growing HPKII keratinocytes revealed a distinct distribution of CD44v: the spreading edge of the culture expressed high levels of the variant isoforms, particularly in the filopodia (not shown).

**Leukocytes Express Predominantly CD44-9v and not 6v**

Fig. 3 shows the expression of CD44v isoforms on various leukocyte types, as revealed by FACS® analysis with mAbs 11.24, 11.9, and 11.10. CD44-9v isoforms were weakly expressed on T and B cells, monocytes and granulocytes, but not on thymocytes. Only monocytes expressed CD44-6v and -4v isoforms at low levels. However, after activation with PHA or in the MLR, CD44-9v and to a lesser extent CD44-6v were upregulated on T cell lines (Fig. 3). Only a minor proportion of the CD44 molecules on activated T cells and other leukocytes contained variant sequences, since intense staining was observed with antibodies to CD44s, but much lesser staining with mAbs 11.24 or 11.9 (Fig. 3). Upregulation of CD44-9v and CD44-6v on stimulated T cells occurred within 24 h and was transient, since expression of these determinants decreased to pre-stimulation levels by day 6; other activation markers such as CD26 and IL-2R were expressed for much longer periods. In addition, CD45RO+ T cells which are memory/previously activated cells, expressed slightly higher levels of CD44-9v than did CD45RA+ (naive) T cells (not shown). In contrast to peripheral T cells, thymocytes did not express CD44-9v or CD44-6v at all. It has been suggested that CD44 is an adhesion molecule for pro T cell homing to the thymus (Hyman et al., 1986), but gating on early stage CD4+CD8- thymocytes revealed that they were CD44-9v- and 6v- although mostly CD44s (not shown).

**TNFα and IFNγ modulate CD44 Isoforms on Monocytic Cell Lines**

CD44 expression on various cell lines was examined after their incubation with different cytokines. TNFα has been reported to upregulate CD44 on human endothelial cells (Mackay et al., 1993), and using anti-CD44s mAbs we found a similar upregulation of CD44 on the myelomono- cytic cell lines THP-1 (Fig. 4 A) and U937. On myelomonocytic cell lines, IFNγ also modulated CD44 expression, whereas IL-1, IL-4, GM-CSF, or TGFβ had little effect (Fig. 4 A). More significant was the fact that CD44v isoforms were differentially upregulated on myelomonocytic cell lines. Both TNFα and IFNγ modulated CD44-9v and -6v expression, and their upregulation was much more apparent than that of CD44s. Moreover, TNFα preferentially upregulated CD44-9v, whereas IFNγ preferentially upregulated CD44-6v (Fig. 4 A). However the upregulation of CD44v on THP-1 by TNFα or IFNγ was less than that of ICAM-1, an adhesion molecule well characterized for its upregulation in response to inflammatory cytokines (Springer, 1990). The effect of regions of CD44 (3v-10v) were used to localize the exact epitope positions for mAbs 11.24, 11.9, and 11.10. 21 different peptides were used to block the reactivity in the ELISA of the various mAbs against fusion protein CD44 (3v-10v). The regions of CD44 to which the 21 peptides correspond are indicated in Fig. 1 A and their sequences are given in Materials and Methods. The specificities of the different mAbs are also indicated in A. The epitope for mAb 25.32 (anti-standard) has been localized to exon 5s, using a fusion protein encoding this region (not indicated). (C) Immunoprecipitation of 35S-labeled proteins from an HPKII keratinocyte cell lysate. The lysate was prepared as described in Materials and Methods and aliquots were incubated with the indicated mAbs. Immunoprecipitated material was separated on 7.5-15% SDS polyacrylamide gradient gels.
Figure 2. Expression of CD44v in human epithelial tissues. Oesophagus (A), skin (B), or small intestine (C) were stained with mAbs to CD44s (25.32), CD44-9v (11.24), CD44-6v (11.9), and CD44-4v (11.10) using the APAAP technique. A more extensive description of the reactivity of these mAbs is given in Table I. Bars, 65 μm.
| Epithelial tissue within | mAb 25.32 (CD44s) | mAb 11.24 (CD44-9v) | mAb 11.9 (CD44-6v) | mAb 11.10 (CD44-4v) |
|-------------------------|------------------|------------------|------------------|------------------|
| Skin                    |                  |                  |                  |                  |
| Epidermis               | ++ +             | ++ +             | ++ +             | ++ +             |
| Hair follicles          | ++ +             | ++ +             | ++ +             | ++ +             |
| Sweat glands            | ++ +             | ++ +             | ++ +             |                  |
| Small intestine         | ++ +             |                  |                  |                  |
| Large intestine         | ++ +             | ++ +             |                  |                  |
| Stomach                 | ++ +             |                  |                  |                  |
| Pancreas                |                  |                  |                  |                  |
| Acini                   |                  |                  |                  |                  |
| Ducts                   | ++ +             | ++ +             |                  |                  |
| Kidney                  |                  |                  |                  |                  |
| Tubular region          |                  |                  |                  |                  |
| Liver                   |                  |                  |                  |                  |
| Bile ducts              | ++ +             | ++ +             |                  |                  |
| Lung                    |                  |                  |                  |                  |
| Bronchial epith.        | ++ +             | ++ +             | ++ +             | ++ +             |
| Oesophagus              | ++ +             | ++ +             | ++ +             |                  |
| Thyroid gland           | ++ +             | ++ +             |                  |                  |
| Salivary gland          | ++ +             | ++ +             |                  |                  |
| Mammary gland           | ++ +             | ++ +             |                  |                  |
| Adrenal gland           |                  |                  |                  |                  |
| Ovary                   |                  |                  |                  |                  |
| Endometrium             | ++ +             | ++ +             |                  |                  |
| Epididymis              | ++ +             | ++ +             |                  |                  |
| Prostate gland          | ++ +             | ++ +             |                  |                  |
| Urinary tract           | ++ +             | ++ +             |                  |                  |
| Tonsil                  | ++ +             | ++ +             | ++ +             | ++ +             |
| Thymus                  | ++ +             | ++ +             |                  |                  |
| Hassall's Corpuscles    | +                | ++ +             |                  |                  |

The effect of cytokines on CD44 expression was also examined on epithelial cell lines. IFNγ was the only cytokine that had a marked effect on CD44 or ICAM-1 expression on epithelial cell lines. Although epithelial cell lines varied in their response to IFNγ, a common effect was an upregulation of CD44-6v, and a downregulation or maintenance of other isoforms and CD44s expression. This is shown for the large lung cell carcinoma LCLC97, the colon carcinoma HT29, and the keratinocyte line HPKII (Fig. 5). By FACS* analysis, CD44s expression on the cell lines was usually downregu-
pression of α6 and α2 integrins were unaffected (not shown).

Activated T

T cells

Thymocytes

Monocytes

Granulocytes

Fluorescence intensity

CD44

CD44-9v

CD44-6v

CD44-4v

Actiuated  T

B cells

CD3

CD20

Figure 3. FACS® analysis of human leukocytes stained with mAbs specific for CD44-4v (11.10), CD44-6v (11.9), CD44-9v (11.24), and CD44s (25.32). T cells, B cells, a PHA-stimulated T cell clone, thymocytes, monocytes, and granulocytes were stained with the various mAbs, and in each plot staining with the individual antibody is indicated. T cells and B cells were gated electronically after two-color immunofluorescence staining, using PE-labeled mAbs to CD3 and CD20. Monocytes and granulocytes were identified according to their forward angle and side scatter.

lated ~2–5-fold by the treatment, as was to a lesser extent CD44-9v, whereas CD44-6v was upregulated ~3–5-fold (Fig. 5 A). ICAM-1 was included as a control, and was consistently upregulated 5–10-fold by IFNγ, whereas the expression of α6 and α2 integrins were unaffected (not shown). A similar effect for IFNγ treatment on CD44 expression was seen with other epithelial cell lines, including HaCaT (keratinocyte line), and RPMI7451 which was also analyzed by RT PCR (and FACS® not shown) neither up- nor downregulated CD44 or CD44v upon TNFα and IFNγ treatment (Fig. 5 B), illustrating that CD44 was not modulated on all epithelial lines by IFNγ.

Discussion

CD44 is a complex molecule that has been implicated in many functions. In this report, we studied the expression and modulation of the variant isoforms of human CD44 by generating mAbs against a fusion protein encoding the variant exons 3v–10v. This study shows that CD44 variant isoforms are expressed widely throughout the body, that CD44v exon products are expressed on epithelium in a differential manner, and that expression of CD44 and CD44 variants can be modulated by certain cytokines.

The extensive expression of CD44v isoforms within many normal epithelia most likely serves an adhesive function relating to the interaction and migration of the resident cells. There is an ongoing process of epithelial cell regeneration, differentiation, and migration, especially during embryogenesis and wound healing, which involves changes in surface phenotype and adhesive properties of cells (Weigel et al., 1986; Woodley et al., 1986; Adams and Watt, 1990). Within epithelial tissues, CD44v isoforms were expressed most intensely by the generative cells, such as the basal cells of squamous epithelia, or the cells of glandular epithelium. These epithelial cells have a rapid rate of proliferation (Chang and Leblond, 1974; Watt, 1984; Gordon, 1989), suggesting that CD44v expression may somehow correlate with proliferation. A previous study also noted the close relationship between expression of hyaluronate receptors and proliferation by epithelial cells (Alho and Underhill, 1989; Brown et al., 1991). In addition, expression of proteoglycans is known to relate to cellular proliferation (Hardingham and Leblond, 1974; Watt, 1984; Gordon, 1989), suggesting that CD44v expression may somehow correlate with proliferation. A previous study also noted the close relationship between expression of hyaluronate receptors and proliferation by epithelial cells (Alho and Underhill, 1989; Brown et al., 1991). In addition, expression of proteoglycans is known to relate to cellular proliferation (Hardingham and Leblond, 1974; Watt, 1984; Gordon, 1989), suggesting that CD44v expression may somehow correlate with proliferation. A previous study also noted the close relationship between expression of hyaluronate receptors and proliferation by epithelial cells (Alho and Underhill, 1989; Brown et al., 1991). In addition, expression of proteoglycans is known to relate to cellular proliferation (Hardingham and Leblond, 1974; Watt, 1984; Gordon, 1989), suggesting that CD44v expression may somehow correlate with proliferation.
isoforms in abnormal amounts and/or compositions may accompany malignant transformation. A second possibility is that the generative cells of epithelia which express the highest levels of CD44v isoforms are the cells that most often undergo malignant transformation. The uncontrolled growth of these cells coupled with the expression of CD44 isoforms and possibly other adhesion molecules and proteases might render them more invasive and metastatic. Hence the alteration in adhesive properties that CD44v isoforms confer on cancer cells would relate to the normal developmental processes that occur within epithelia and other tissues by proliferating and differentiating cells. Interestingly, other molecules involved in adhesion, proliferation and tissue regeneration have also been implicated in the metastatic spread of cancer cells (for review see Birchmeier et al., 1991; Van Roy and Mareel, 1992). CD44v isoforms are certainly not expressed on all metastasizing cancer cells, since all cases of malignant melanomas studied by us (our own unpublished observations) express only CD44s. CD44-6v shows a much more restricted expression in nonmalignant tissues compared with CD44-9v, however it is upregulated in some malignant tissues which normally express only low levels of CD44-6v (Matsumura and Tarin, 1992; Heider et al., 1993). Recently, a downregulation of CD44v has been shown to accompany malignant transformation of some squamous epithelia (Salmi et al., 1993).

We found that most leukocyte cell types express very low levels of CD44v. Exon products of 4v and 6v were virtually absent from lymphocytes, and likewise Koopman et al. (1993) and Salmi et al. (1993) found very little expression of CD44-6v on leukocytes. In our study, CD44-9v was found to be the exon expressed most abundantly, although we estimate that CD44-9v still comprises not more than 5% of total CD44, since staining with anti-CD44-9v was generally two logs lower than with anti-CD44s. On T cells, expression of CD44-9v and CD44-6v increased after stimulation, and this induction was rapid and transient. A study in rats showed that CD44-6v but not other CD44v exons were induced on T cells after stimulation (Arch et al., 1992). In our study, CD44-9v was more readily induced than CD44-6v, and it was only the most acutely activated T cells that expressed CD44-6v. The expression of CD44v on activated T cells might endow them with properties similar to those of metastasizing cancer cells. Some metastasizing cancer cells show remarkable tissue-selective migration patterns (Nicolson, 1991), similar to the tissue selective migration patterns of activated T cells (Mackay, 1993).

CD44 is another adhesion molecule that is modulated by TNFα and IFNγ. On myelomonocytic cell lines, CD44 upregulation was more modest compared with ICAM-1, an adhesion molecule well characterized for its upregulation in response to inflammatory cytokines (Springer, 1990). A recent study using human endothelial cells also showed that CD44 was upregulated by TNFα (Mackay et al., 1993) and, in addition, TNFα has been reported to upregulate a molecule structurally related to CD44 on fibroblasts (Lee et al., 1992). On myelomonocytic cell lines such as U937 and THP-1, TNFα as well as IFNγ induced CD44-9v and -6v, and on some epithelial cell lines IFNγ upregulated CD44-6v. In other studies using RNA expression analyses, we found that TNFα induced several CD44v isoforms in murine stromal cell lines (Gutierrez-Ramos, J. C., U. Günther, and C. Mackay, manuscript in preparation). TNFα also upregu-
Figure 5. (A) Modulation of CD44 variant isoforms on epithelial cells by IFN-α. IFN-γ was added to cultures of LCLC97 cells (top), HT29 cells (middle), and HPKII cells (bottom) for 48 h and cells were then stained for CD44s (mAb Hermes-3), CD44-9v (mAb 11.24), and CD44-6v (mAb 11.9) and analyzed on the FACS®. The profile for IFN-γ treated cells is indicated in each plot. (B) RT-PCR on IFN-γ and TNF-α stimulated epithelial cells. LCLC97 and RPMI7451 cells were treated with TNF-α or IFN-γ for 24 h. RNA and cDNA were prepared as described in Materials and Methods. Quantitation of cDNA was performed by standardizing for equal amounts of HPRT cDNA. The upper three panels show CD44 PCR products, blotted onto Hybond N+ membranes and hybridized to the indicated probes. The two lower panels are ethidium bromide stained agarose gels, containing either the CD44 specific PCR products (fourth panel) or the HPRT equilibration products (bottom panel).
lated CD44-9v on cultured human dendritic cells, in a similar manner to that described here for myelomonocytic cell lines (Sallustio, F., and A. Lanzavecchia, manuscript submitted for publication). The basis for CD44v upregulation on some cell lines and not others is unknown. The modulation of CD44 expression by TNFα or IFNγ might also relate to epithelial migration and cancer metastasis. TNFα has cytotoxic effects on some tumor cell types (Vassalli, 1992), but in other cases it promotes cancer growth and metastasis (Malik, 1992; Orosz et al., 1993). TNFα also stimulates epithelial cell motility in vitro (Rosen et al., 1991), which has led to speculation that this cytokine may play a role in wound healing, or the invasiveness of some carcinomas. Moreover, IFNγ not only modulates the interactions of leukocytes with endothelium, but also plays an essential role in regulating neutrophil/epithelial adhesions in transmigration due to its effects on epithelial cells (Kvale et al., 1992; Colgan et al., 1993).

In this report we have compared the expression and modulation of three important regions of CD44, the metastatic form (containing exon 6v), the epithelial form (containing exon 9v), and a rarely expressed region containing exon 4v. Antibodies against these different CD44v isoforms should prove invaluable for further analysis of CD44v expression, regulation, and function.

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