Expression and Modulation of CD44 Variant Isoforms in Humans

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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 NH_2-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)[1] 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

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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, Kinderspital, 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 (Günthert, 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 IgG_1 isotype, and mAbs 11.9 and 11.10 are IgG_{2a} isotype. Other mAbs used in this study included anti-CD44 standard mAbs 25–32 (Mackay et al., 1988), Fl0-44-2 (Flanagan 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/1 was a gift of Dr. T. Springer. Phycoerythrin-labeled antibody to CD20 was purchased from Becton Dickinson (Mountain View, CA).

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 (Cappel Laboratories, Cochranville, PA). After 10 min at 4°C, the cells were washed twice and analyzed using a FACS® (Becton Dickinson). Propidium iodide was used to identify and exclude dead cells from analysis. For two-color immunofluorescence, cells were stained as described above, and were then stained with phycoerythrin (PE)-conjugated mAb by first blocking unoccupied sites on the FITC goat anti-mouse Ig using 20 ml 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®. Electronic gating was performed to analyze cells of interest. 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 and other adhesion molecules was determined by incubating various cell lines in 25-ml tissue culture flasks. Cells were treated for 1, 3, or 6 d with recombinant cytokines at the following concentrations: TNFα and TGFβ at 10 ng/ml, IFNγ, GM-CSF, IL-4 at 250 U/ml, and IL-1 at 50 U/ml. TNFα and IFNγ were obtained from the central research department of Hoffmann La Roche, from Drs. Werner Lesslauer and Gianni Garotta. GM-CSF, IL-4, TGF-β and IL-1 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′ GTACGTCTTCAAATACCATCTCAGCAGGCT, and 5′ CTGATAAGGAACGATTGACATTAGAGTTGG) 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 638–1014), 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 beads (Pharmacia, Freiburg, FRG) were 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) GTSSNTISAGWEPNEENEDE; (2) WEPNEENEDERDRHLS; (3) NEDERDRHLSFSGSGIDDDE; (4) IDDDEDFISSTISTTP; (5) PRAFDHTKQNQDWTQWNPSH; (6) NPSHSNPEVLLQTTTRMTDV; (7) RMTDVDRNGTTAYEGN; (8) GTTAYEGNWNPEAHPPLIHH; (9) EAHPPLIHHEHHEEEE; (10) HHEEEETPHSTSTIQATPSS; (11) IQATPSSTTEETATQKEQWF; (12) EETATQKEQWFGNRWHEGYR; (13) NRWHEGYRQTPREDSH; (14) TPREDSHSTTGTAAASAHTS; (15) TAAASAHTSHPMQGRT; (16) TSHPMQGRTTPSPEDSSWTD; (17) EDSSWTDFFNPISHPM; (18) PTANPNTGLVEDLDRT; (19) SFSTSHEGLEEDKDHP; (20) LEGYTSHYPHTKESRT; (21) VTSAKTGSFGVTAVTV.

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-CD44v 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-CD44(3v-10v) 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, fixed in acetone for 5 min 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 (Napthol AS-BI phosphate/New

Fuchsin; Sigma Chemical Co., Deisenhofen, FRG). 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, HPKII cells were labeled with [^{35}S]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 $MgCl_2$, 150 mM NaCl, and 0.1 mM PMSF. Protein A- and Protein G-Sepharose (Pharmacia) were used for preclearing 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).

Semiquantitative Reverse Transcribed PCR

The myelomonocytic cell lines THP-1 and U937 were treated with TNFα and IFNγ for 24 h as described above. Approximately 10^6 cells were taken for RNA preparation using the method of Chomczynski and Sacchi (1987) including modifications by Keller et al. (1993). cDNA synthesis was performed using random hexanucleotide primers (Promega, Heidelberg, FRG) and mouse mammary leukaemia virus reverse transcriptase (Promega). The relative expression of hypoxanthine phosphoribosyltransferase (HPRT) was used for standardising the reaction. The following primers were used: CCAAAGATGGTCAAGGTCGC (5′ sequence; positions 448–467) and CTGCTGACAAAGATTCACTGG (3′ sequence; positions 1292–1272), yielding a HPRT cDNA product of 0.84 kb.

Based on densitometric scanning of the HPRT products, the amount of cDNA for the CD44 reactions was adjusted, according to the procedure from Keller et al. (1993). CD44 PCR was performed using the primers GCACAGACAGAATCCCTGCTACC, 5′ sequence, positions 759–781 from Stamenkovic et al. (1989) and GGGGTGGAATGTGTCTTGGTCTC, 3′ sequence, positions 805–783 from Stamenkovic et al. (1989), which are located in the CD44s sequence directly flanking the variant exons. Depending on the composition and number of variant exons expressed, products of different lengths and quantity were produced. CD44s yields a product of 67 bp, 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-^{32}P 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 to Hyperfilm-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: PRAFDHTKQNQDWTQWNPSH (positions 47–66); No. 12: EETATQKEQWFGNRWHEGYR (positions 129–148) and No. 19: SFSTSHEGLEEDKDHP (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 HPKII keratinocyte cell line, which expresses predominantly CD44v regions 3v–10v (Hofmann et al., 1991). Immunoprecipitation of CD44 from an [^{35}S]methionine lysate of HPKII using Hermes-3, 25.32, or F10-44-2 (anti-CD44s mAbs which recognize epitopes in the 5′ standard region and precipitate all isoforms) showed that CD44 was expressed as multiple forms, migrating at 130, 190, and 250–300 kD (Fig. 1 *C*). Precipitation of CD44 from ^{125}I-lactoperoxidase surface-labeled HPKII revealed the same bands (not shown). Hermes-3, 25.32 and F10-44-2 showed slightly different precipitation patterns, presumably because of different affinities for the distinct sizes. The variant-specific mAbs 11.24 (9v-specific), 11.10 (4v-specific), and 11.9 (6v-specific) precipitated molecules of similar size to the CD44s-specific mAb but with varying intensities: 11.24 precipitated the 130-, 190-, and 250–300-kD components, 11.10 predominantly the 130-kD component, and 11.9 a 250–300-kD component (Fig. 1 *C*).

Western blot analyses with mAb 11.24 and 11.10 using protein extracts from different cell lines confirmed the immunoprecipitation data (not shown). mAb 11.31 and 11.9 did not react with denatured CD44 proteins.

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; Günthert 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

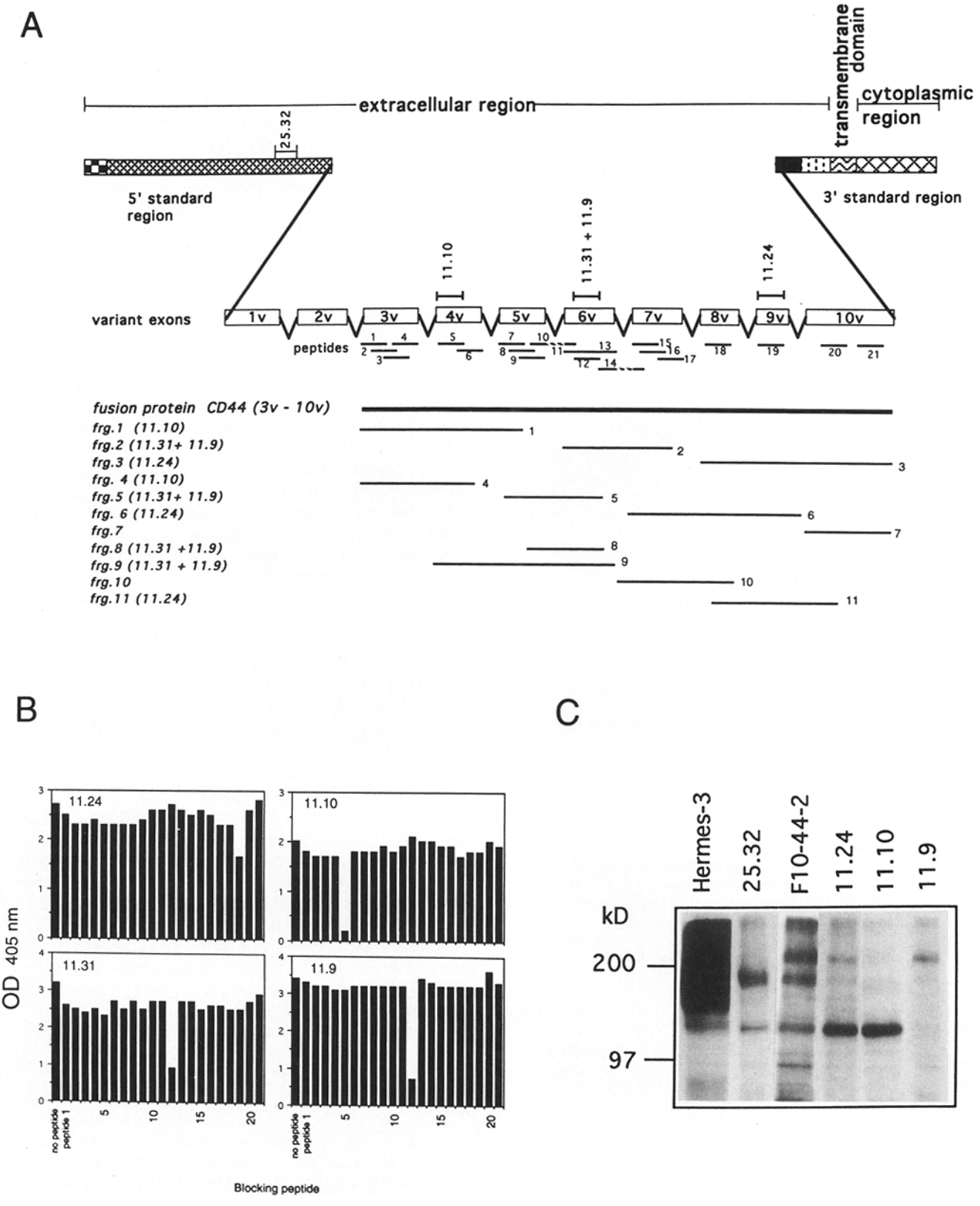


Figure 1. (*A*) Schematic presentation of the standard and the variant CD44 regions. At amino acid 223 in human CD44, up to 381 additional amino acids may be present, due to alternative splicing of 9 or 10 variant exons. The region encoding exons 3v–10v was isolated by reverse transcribed PCR and inserted 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* and *B*). Third, within an epithelial layer (e.g., oesophagus), the different CD44v isoforms had a non-uniform expression; exon 4v products were expressed to a lesser degree than exon 6v products, which in turn were expressed less than exon 9v containing isoforms (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 NI) 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 and 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 $CD44\text{-}9v^-$ and $6v^-$ although mostly $CD44^{hi}$ (not shown).

Immunohistochemical analysis of thymus tissue sections confirmed that CD44v molecules were not expressed on thymocytes, although epithelial cells within Hassall's corpuscles were stained by all of the variant-specific mAbs (Table I). Staining of various lymphoid tissues revealed an absence of staining of CD44v on lymphocytes, presumably because the APAAP technique is not as sensitive as FACS® analysis. However plasma cells in the bone marrow were clearly stained by 11.24, and pulmonary macrophages were stained by mAbs 11.24 and 11.9 (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 myelomonocytic 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. 4A). 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 11.10, 11.31, 11.9, and 11.24. 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 ^{35}S-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 polyacylamide gradient gels.

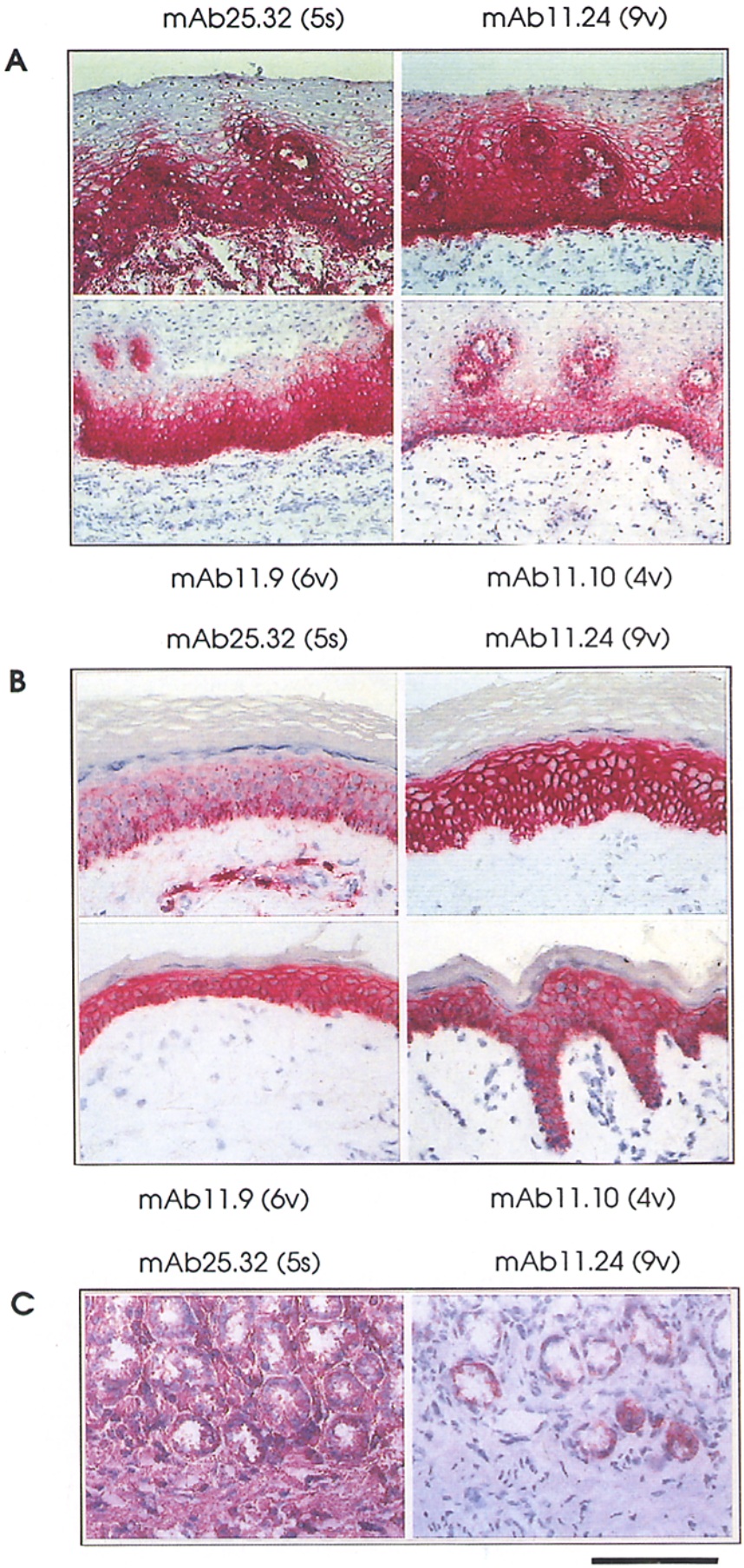


Figure 2. Expression of CD44v in human epithelial tissues. Oesophagus (*A*), skin (*B*), or small intestine (*C*) were stained with mAbs to CD44s (25.32), CD44-9v (11.24), CD44-6v (11.9), and CD44-4v (11.10) using the APAAP technique. A more extensive description of the reactivity of these mAbs is given in Table I. Bars, 65 μm.

Table I. Differential Expression of CD44 Isoforms within Epithelia from Various Human Tissues

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	+++	+ (base of crypts)	−	−
Large intestine	+++	++ (base of crypts)	−	−
Stomach	+++	++ (base of glands)	−	−
Pancreas				
Acini	−	−	−	−
Ducts	+++	+++	−	−
Kidney				
Tubular region	+ (some)	+ (some)	−	−
Liver				
Bile ducts	++	+++ (some)	−	−
Lung				
Bronchial epith.	+++	+++	+	−
Oesophagus	+++	+++	+++	+++ (basal layer)
Thyroid gland	+++	++	−	−
Salivary gland	+++	+++	−	−
Mammary gland	+++	+++ (basal layer)	+ (basal layer)	−
Adrenal gland	−	−	−	−
Ovary	−	−	−	−
Endometrium	+++	++ (basal layer)	−	−
Epididymis	+++	+++ (basal layer)	−	−
Prostate gland	+++	+++	+	−
Urinary tract	+++	+++	−	−
Tonsil	+++	+++	++	++
Thymus	+++	+	−	−
Hassall's Corpuscles	+	++	+	+

the cytokines on THP-1 was also examined after 1, 3, and 6 d of exposure, showing a slight decrease at day 6 as compared to the values after 24 h (Fig. 4 *A*). No modulation of CD44-4v was observed (not shown).

The modulating effect of TNFα and IFNγ on THP-1 and U937 was confirmed by reverse transcribed PCR (Fig. 4 *B*). This allowed a better assessment of the upregulation of CD44v in comparison to CD44s. As shown in Fig. 4 *B*, CD44s was not induced in THP-1 or U937 by TNFα or IFNγ, as tested using a probe for CD44s. However CD44-9v and 6v were both markedly upregulated by TNFα and IFNγ. A semiquantitative evaluation of the PCR products revealed an increase for the epithelial variants, most likely comprising exons 8v, 9v + 10v (larger band) and exons 9v + 10v or 8v + 10v (smaller band) by 2–3 fold as compared to the non-treated cells. Modulation of these exons by TNFα and IFNγ was less prominent in U937 cells than in THP-1 cells (Fig. 4 *B*). The probe comprising exons 5v + 6v detected predominantly a small band of 200 bp, which was also upregulated by TNFα and IFNγ 2–3-fold in both cell lines. This product represents only exon 6v as revealed by sequencing. In addition to this variant, THP-1 cells also produced larger bands, comprising variant sequences that included exons 7v and/or 6v. These larger bands were also upregulated 2–3-fold especially upon IFNγ stimulation.

Modulation of CD44 on Epithelial Cell Lines by INFγ

The effect of cytokines on CD44 expression was also examined on epithelial cells. 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-

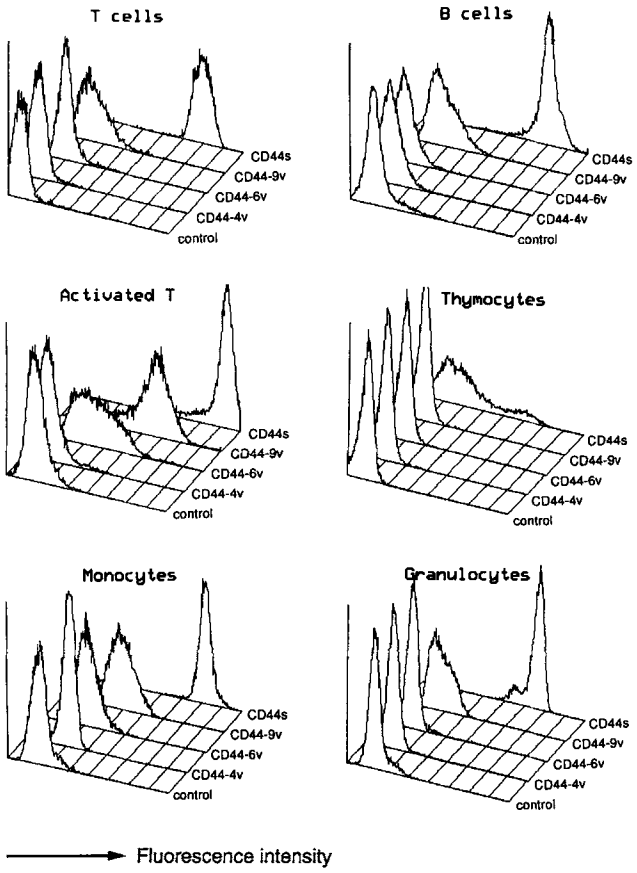


Figure 3. FACS® analysis of human leukocytes stained with mAbs specific for CD44-4v (11.10), CD44-6v (11.9), CD44-9v (11.24), and CD44s (25.32). T cells, B cells, a PHA-stimulated T cell clone, thymocytes, monocytes, and granulocytes were stained with the various mAbs, and in each plot staining with 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 WI-38 (lung epithelial line, not shown). The characteristic pattern of CD44 modulation observed by FACS® staining was also confirmed by RT PCR. As shown in Fig. 5 *B*, LCLC97 cells upregulated an exon 6v containing RNA, but downregulated CD44s. This effect was most prominent after 24 h of IFNγ treatment, but was not apparent upon incubation with TNFα, apart from a slight downregulation of CD44s. Expression of the epithelial variant (exons 8v, 9v, and 10v containing isoforms) was also slightly downregulated by the cytokine treatment, paralleling the FACS® data. In contrast to LCLC97 cells, the cholangiocarcinoma line 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 Fosang, 1992; Wight et al., 1992). The generative cells in the crypts of the small intestine give rise to progeny that migrate out of the crypts and move to the top of the villus (Gordon, 1989). Similarly, the generative cells of the basal layer of squamous epithelium, such as in the skin and in the oesophagus, produce cells that differentiate and migrate upwards (Watt, 1984). In this study we did not use mAbs to exon products other than CD44-4v, 6v and 9v. However since certain exons are expressed together in domains (Hofmann et al., 1991; Stamenkovic et al., 1991; Günthert, 1993), we expect that 6v will show a similar pattern of expression to that of 7v (the "metastatic form"), and 9v will show similar patterns of expression to that of 8v and 10v (the "epithelial form").

Many studies have found an association between CD44v expression and malignant transformation or cancer metastasis (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; Salmi et al., 1993; Seiter et al., 1993; Tanabe et al., 1993). In certain human cancers, cells appeared to express an abnormal profile of CD44v molecules, for instance, 6v expression on colon carcinomas (Heider et al., 1993), breast cancers (Matsumura and Tarin, 1992), or lymphomas (Koopman et al., 1993). Two possibilities might account for the expression of CD44v isoforms on metastasizing cells. First, expression of CD44v

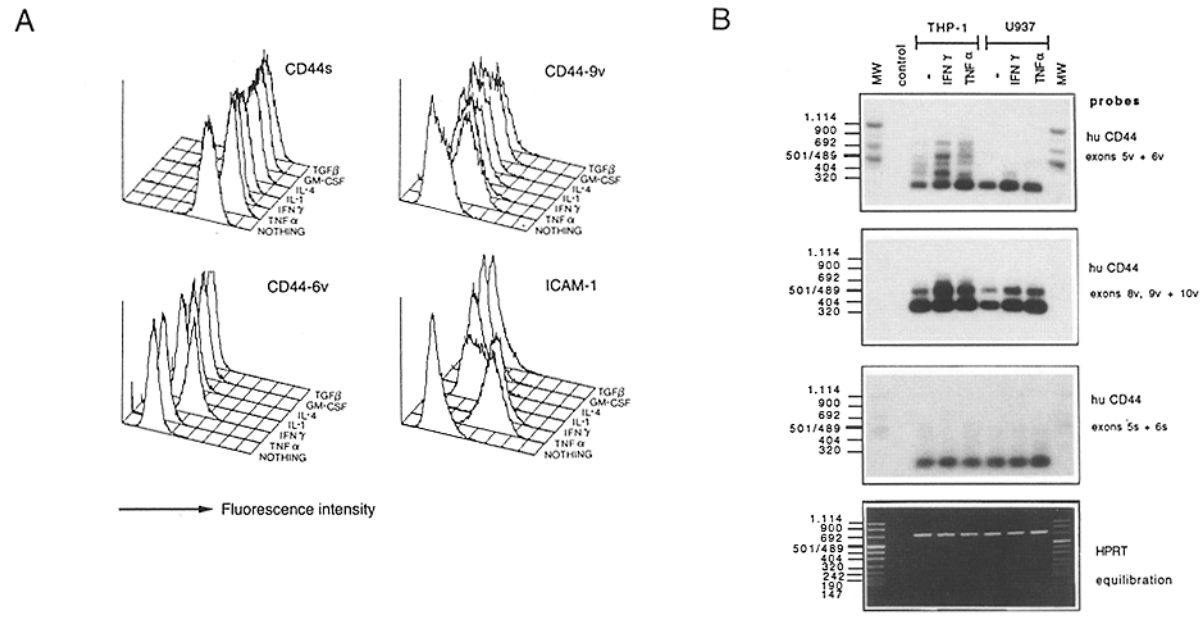
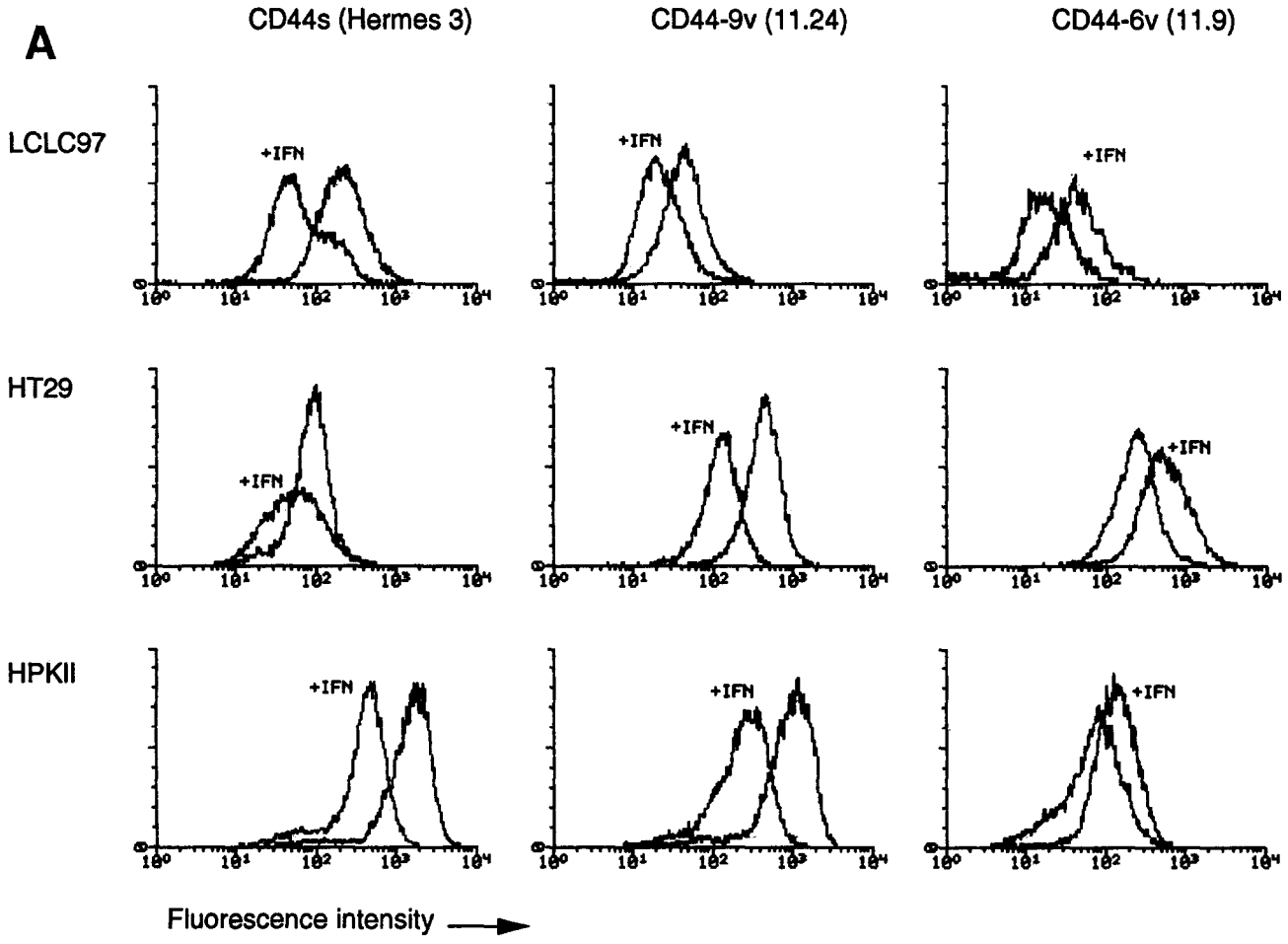


Figure 4. (*A*) Upregulation of CD44 variant isoforms on the myelomonocytic cell line THP-1 by TNFα and IFNγ. Various cytokines were added to cultures of THP-1 cells for 24 h and then stained for CD44s (mAb 25.32), CD44-9v (mAb 11.24), CD44-6v (mAb 11.9) and ICAM-1 (mAb RR/1) and analyzed on the FACS®. (*B*). RT-PCR on TNFα and IFNγ stimulated myelomonocytic cell lines. THP-1 and U937 cells were treated with TNFα and IFNγ 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 (*lower panel*). CD44 PCR products were separated on agarose gels, blotted onto Hybond N^+ membranes and hybridized to the respective CD44 probes. Primers used and the positions of the probes are indicated in Materials and Methods.

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 CD44-6v was 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. Günthert, and C. Mackay, manuscript in preparation). TNFα also upregu-



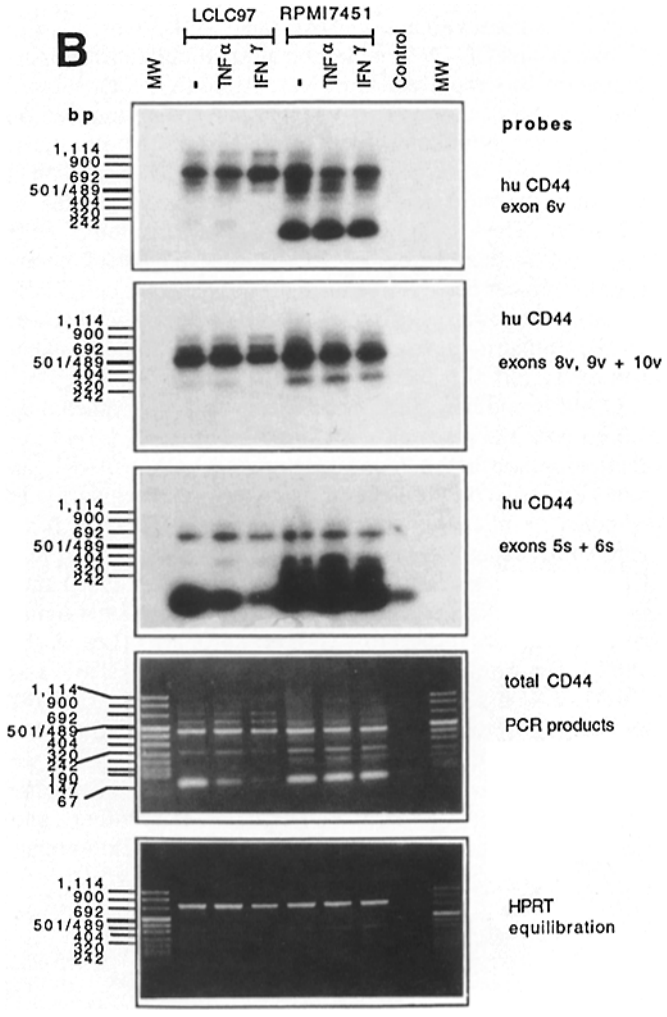


Figure 5. (*A*) Modulation of CD44 variant isoforms on epithelial cells by IFNγ. IFNγ was added to cultures of LCLC97 cells (*top*), HT29 cells (*middle*), and HPKII cells (*bottom*) for 48 h and cells were then stained for CD44s (mAb Hermes-3), CD44-9v (mAb 11.24), and CD44-6v (mAb 11.9) and analyzed on the FACS®. The profile for IFNγ treated cells is indicated in each plot. (*B*) RT-PCR on IFNγ and TNFα stimulated epithelial cells. LCLC97 and RPMI7451 cells were treated with TNFα or IFNγ 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 (Sallusto, 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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