

A Human Homologue of the Rat Metastasis-associated Variant of CD44 Is Expressed in Colorectal Carcinomas and Adenomatous Polyps

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Abstract. A recently described splice variant of CD44 expressed in metastasizing cell lines of rat tumors has been shown to confer metastatic potential to a non-metastasizing rat pancreatic carcinoma cell line and to non-metastasizing sarcoma cells. Homologues of this variant as well as several other CD44 splice variants are also expressed at the RNA level in human carcinoma cell lines from lung, breast, and colon, and in immortalized keratinocytes.

Using antibodies raised against a bacterial fusion protein encoded by variant CD44 sequences, we studied the expression of variant CD44 glycoproteins in normal human tissues and in colorectal neoplasia. Expression of CD44 variant proteins in normal human tissues was readily found on several epithelial tissues including the squamous epithelia of the epidermis, tonsils, and pharynx, and the glandular epithelium of the pancreatic ducts, but was largely absent from other epithelia

and from most non-epithelial cells and tissues. In human colorectal neoplasia CD44 variant proteins, including homologues of those which confer metastatic ability to rat tumors, were found on all invasive carcinomas and carcinoma metastases. Interestingly, focal expression was also observed in adenomatous polyps, expression being related to areas of dysplasia. The distribution of the CD44 variants in human tissues suggests that they play a role in a few restricted differentiation pathways and that in colorectal tumors one of these pathways has been reactivated. The finding that metastasis-related variants are already expressed at a relatively early stage in colorectal carcinogenesis and tumor progression, i.e., in adenomatous polyps, suggests the existence of a yet unknown selective advantage linked to CD44 variant expression. The continued expression in metastases would be compatible with a role in the metastatic process.

TUMOR metastasis is the principal cause of death for cancer patients. A subset of parental tumor cells acquire metastatic properties, presumably through a series of genetic alterations (Nicolson 1987; Hart et al., 1989). As a result of this process of tumor progression, carcinoma cells detach from the primary tumor, penetrate the basement membrane into the connective tissue and invade adjacent structures including lymph and blood vessels. The tumor cells are subsequently transported to sites of metastatic outgrowth via lymph or blood. This dissemination process obviously requires a complex series of interactions of tumor cells with extracellular matrix components and with other cells probably involving adhesion receptors, proteolytic enzymes, growth factors, and growth factor receptors.

In an analysis of the metastatic properties of rat carcinoma cells, antibodies were raised that recognize antigens exclusively expressed in the metastasizing clonal variants of these tumors (Matzku et al., 1989). Using one of these antibodies, we have isolated cDNA sequences that encode splice variants of CD44 (Günthert et al., 1991; Rudy, W., M. Hofmann, R. Schwartz-Albiez, M. Zöller, K.-H. Heider, H. Ponta, and P. Herrlich, manuscript submitted for publication). The metas-

tasis-associated epitope is part of an enlarged extracellular region that is not present in standard CD44. Several metastasizing rat tumor cell clones express a heterogeneous set of splice variants. Coinjection of variant-specific mAb with the metastasizing cells provoked retardation and even complete block of metastatic spread in vivo (Reber et al., 1990; Seiter, S., R. Arch, D. Komitowski, M. Hofmann, H. Ponta, P. Herrlich, S. Matzku, and M. Zöller, manuscript submitted for publication). Moreover, overexpression of either one of two of the metastasis-specific variants in non-metastasizing tumor cell lines conferred metastatic abilities on them (Günthert et al., 1991; Rudy, W., M. Hofmann, R. Schwartz-Albiez, M. Zöller, K.-H. Heider, H. Ponta, and P. Herrlich, manuscript submitted for publication). Hence, these splice variants of CD44 are causally involved in the metastatic process. Homologues of the rat CD44 splice variants are also expressed at the RNA level in human carcinoma lines from lung, breast, and colon (Hofmann et al., 1991). Using antibodies raised against a fusion protein that carries sequences of the human variant domains, we have now studied the expression of CD44 variant glycoproteins in human colorectal neoplasia and normal human tissues. While normal colon

epithelium is largely negative, all metastases were positive. The expression is acquired early in tumor progression during the adenomatous polyp stage. According to PCR data, one specific CD44 variant is expressed predominantly. The data establish the variant CD44 exon sequences as tumor progression marker in human colorectal cancer.

Materials and Methods

Cell Culture

The two cell lines Colo 205 and Colo 320 were received from the American Type Culture Collection (Rockville, MD); all other cell lines and their culture conditions have been described previously (Hofmann et al., 1991).

Cloning of pGEX Fusion Proteins

The whole variant region of the HPKII-type CD44v (Hofmann et al., 1991; and see Fig. 1) was cloned from cDNA via PCR amplification. The two PCR primers (5'-CAGGCTGGGAGCCAAATGAAGAAAATG-3' and 5'-TGA-TAAGGAACGATTGACATTAGAGTTGGA-3') correspond to positions 25 to 52 and positions 1013 to 984, respectively, of the LCLC97 variant region as described in Hofmann et al. (1991). In addition they carry an EcoRI recognition site at the 5' end that was used to clone the PCR product directly into the pGEX-2T vector (Smith and Johnson, 1988). The resulting construct (pGEX CD44v HPKII = v3 to v10) codes for a fusion protein of ~70 kD.

To obtain subclones of the variant region that could later be used for affinity purification, fragments comprising DI (v3) and DIII (v6, v7) (see Fig. 1) were cloned using appropriate restriction sites. Fusion protein DI contains the CD44 sequence from position 744 (Stamenkovic et al., 1989) to position 142 of variant CD44 (Hofmann et al., 1991), and fusion protein DIII the variant sequence from positions 378 to 638 (Hofmann et al., 1991). Both fragments were cloned into the pGEX vector system.

Immunization and Purification of Antisera

New Zealand white rabbits were immunized according to standard procedures. 200 µg of affinity purified fusion protein derived from pGEX CD44v HPKII (v3 to v10) was injected s.c. together with 400 µl Freund's incomplete adjuvant four times within 9 wk.

To remove antibodies directed against the glutathione transferase part of the fusion protein, the pGEX-2T glutathione transferase fragment was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ), according to the manufacturers instructions. The serum was applied to the column three consecutive times, the flow through was collected and loaded onto affinity columns to which either CD44v HPKII (v3 to v10), DI (v3), or DIII (v6, v7) was coupled. After washing the columns with coupling buffer (0.1 M NaHCO₃, pH 8, 0.5 M NaCl), specific antibodies were eluted with 100 mM glycine, pH 2.5, and the relative antibody concentration was measured in an ELISA assay. For the ELISA the fusion protein

CD44v HPKII (v3 to v10) was coated onto a 96-well microtiter plate in excess. The wells were then incubated with several antibody dilutions for 2 h, washed with PBS, and incubated with alkaline phosphatase-coupled anti-rabbit IgG. The conversion of *p*-nitrophenyl-phosphate was measured at 405 nm in an ELISA reader. For Western blot analysis and immunohistochemistry equal relative concentrations of each antibody were used.

mAbs

The mAb NKI-P1 is directed against the standard form of CD44 (Pals et al., 1989a). The mAb pAb1801 directed against p53 was purchased from Cambridge Research Biochemicals Ltd., Northwich, UK.

Western Blot Analysis

Cells were lysed by sonication and boiling in SDS gel sample buffer (Laemmli, 1970). Frozen tumor material was pulverized under liquid nitrogen, taken up in gel sample buffer and sonicated. Proteins were electrophoretically resolved on 6% reducing SDS polyacrylamide (Laemmli, 1970). Bacterial fusion proteins were taken up in gel sample buffer, boiled, and resolved on 10% SDS polyacrylamide. Proteins were transferred to polyvinylidene difluoride membranes (Millipore Continental Water Systems, Bedford, MA) using a transblot apparatus (Bio-Rad Laboratories, Cambridge, MA). Nonspecific interactions were blocked with PBS containing 10% dry milk. Then the membranes were incubated at room temperature with the polyclonal antibodies, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Amersham Corp., Arlington Heights, IL), for 1-h each. After each individual antibody incubation, the membranes were washed with PBS containing 0.3% Tween 20 (Sigma Chemical Co.). Signals were developed with the enhanced chemoluminescent system (Amersham Corp.).

Tissues

Normal and pathological tissues were selected from the files of the Department of Pathology, Academic Medical Center, University of Amsterdam, The Netherlands, and tested for expression of CD44, variant CD44, and p53, employing the mono- and polyclonal antibodies described above. All normal tissues were obtained as a corollary of standard surgical procedures. Colorectal adenocarcinomas (*n* = 17), adenomatous polyps (*n* = 17) and colorectal carcinoma metastases (*n* = 8) were obtained from a total of 33 patients. Of these, six patients (four carcinomas and six polyps) suffered from familial polyposis coli, other cases being sporadic. Of the primary carcinomas two were Dukes' A, six were Dukes' B, five were Dukes' C, and four were Dukes' D (Dukes, 1932). Histologically, the degree of dysplasia in the polyps ranged from mild to severe, while the histological grade of the tumors ranged from well differentiated to poorly differentiated.

Immunohistochemistry

Frozen sections were fixed in methanol for 10 min, washed in PBS, and preincubated with normal goat serum (10% in PBS). After three washes in PBS the sections were incubated with the primary antibody (in PBS/1%

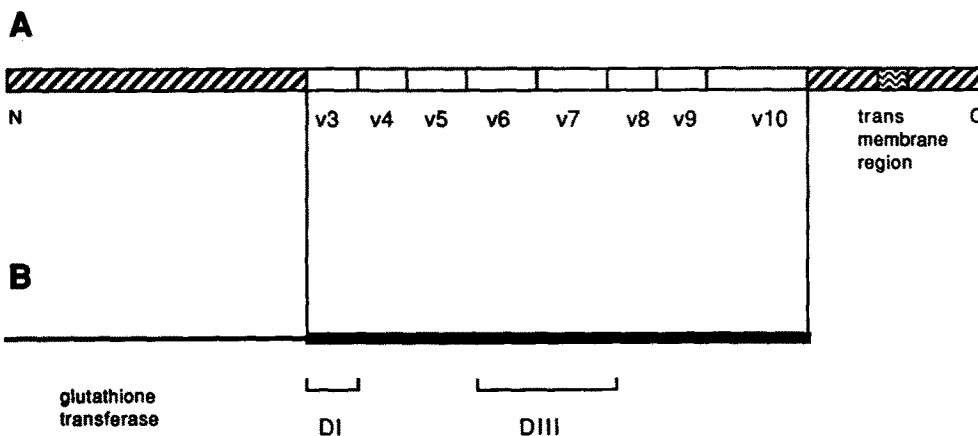


Figure 1. (A) Schematic representation of HPKII-type CD44v. Boxes v3 to v10 indicate exons that are not spliced out in the immortalized keratinocytes (HPKII). Hatched boxes indicate standard type CD44 sequences. (B) Schematic representation of bacterially expressed fusion protein encoded by pGEX CD44v HPKII (v3 to v10). Location of the subcloned regions DI (v3) and DIII (v3, v7) is indicated.

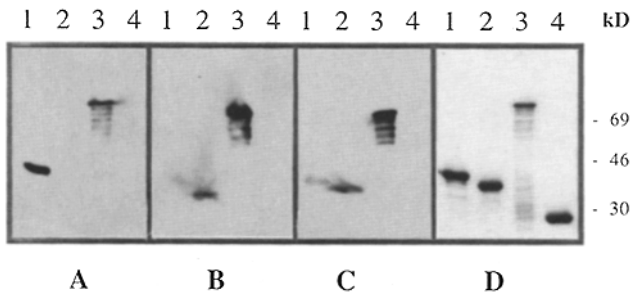


Figure 2. Western blot analysis of CD44v-encoding bacterial fusion proteins with different polyclonal CD44v-specific antibodies. Replica gel resolutions (10% SDS polyacrylamide) are shown all of which carry the following order of the fusion proteins: DI (lane 1), DIII (lane 2), CD44v (v3 to v10) (lane 3), and glutathione transferase (lane 4). The figure shows the reaction of the fusion proteins with anti-DI (A), anti-DIII (B), and anti-CD44v (v3 to v10) (C). In D the Coomassie-stained SDS gel is shown. All antibodies used had been affinity-purified as described in Materials and Methods. Note that the fusion proteins migrate abnormally in the SDS gel (the DI peptide is 33 kD in size but runs at an apparent molecular weight of 42 kD).

BSA) for 1 h. Before incubating with the secondary biotinylated antibody for 30 min (anti-mouse and anti-rabbit F(ab)₂; Dako Corp., Santa Barbara, CA) endogenous peroxidases were blocked by 0.3% H₂O₂ in methanol. As an enzyme for color development horseradish-peroxidase was used, which had been coupled to the biotine via a streptavidine-biotin-peroxidase complex (Dako Corp.). After incubation for 30 min the sections were incubated in 3,3-amino-9-ethyl carbazole (Sigma Chemical Co.) for 5 to 10 min and the reaction was stopped in H₂O. The cells were counterstained with hematoxyline, mounted with glycerine-gelatine and were then ready for microscopy.

Results

Polyclonal Antibodies Directed against the Variant Portion of the Human CD44 Glycoprotein

To obtain antibodies directed against the variant portion of the human CD44 molecule, we immunized rabbits with a fu-

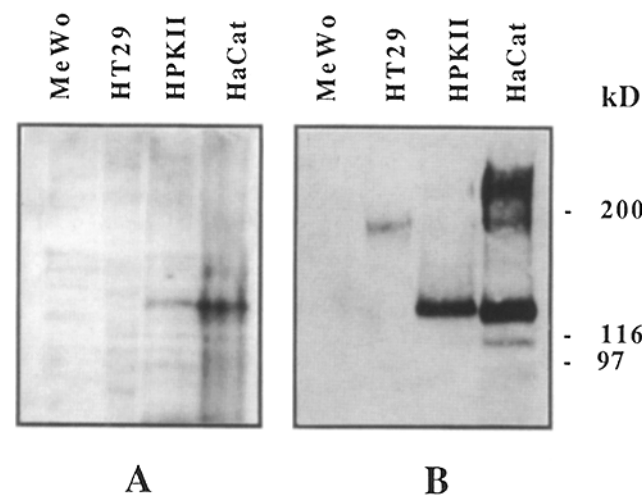


Figure 3. Western blot analysis of several human cell lines with polyclonal CD44v-specific antibodies. (A) Reaction with anti-DIII. (B) Reaction with anti-CD44v (v3 to v10). Cell lysates were resolved on 6% SDS polyacrylamide.

Table I. Expression of Variant CD44 in Normal Human Tissues

Tissues	Antibody specificity			
	CD44s (NKI-P1)	CD44v (v3-v10)	DIII (v6,v7)	DI (v3)
Lymphohematopoietic				
lymphocytes	+++	(+)	-	-
granulocytes	+++	-	-	-
monocytes/macrophages	+++	(+)	-	-
erythrocytes	+++	-	-	-
thrombocytes	-	-	-	-
Epithelial tissues				
keratinocytes	+++	+++	++	++
mucosa of tonsils and pharynx	+++	+++	++	+
pancreas				
ducts	+++	+++	++	+
acini	-	-	-	-
thyroid	+++	+++	++	-
liver				
hepatocytes	-	-	-	-
bile ducts	-	-	-	-
bronchus	+++	+++	++	-
kidney tubules	-	-	-	-
small intestine				
crypts	+	(+)	(+)	-
villi	-	-	-	-
large intestine				
crypt base	+	(+)	(+)	-
crypts	-	-	-	-
Other				
fibroblasts	+++	-	-	-
endothelium	+	-	-	-
smooth muscle	+++	-	-	-
skeletal muscle	-	-	-	-
brain				
neurons	-	-	-	-
astrocytes	+++	-	-	-
microglia	+++	+	+	-

(+), very weak/equivocal; +, weak; ++, moderate; +++, strong

sion protein composed of variant exon-encoded sequences v3 to v10 of human CD44 and the bacterial glutathione-S-transferase (Fig. 1; and see Materials and Methods). Immunoreactive rabbit antiserum was purified stepwise by affinity chromatography (Material and Methods). In Western blots, fusion proteins with all exon sequences found in keratinocytes (v3 to v10), as well as proteins composed of only exon v3 sequence (DI) and of exons v6, v7 (DIII), respectively, were detected by the antibodies (Fig. 2 C, lanes 1-3). Antibodies with specificity against the v3 sequence and the v6/v7 sequence, respectively, were purified using the appropriate affinity columns (Materials and Methods). Their specificity is demonstrated by Western blot analysis (Fig. 2, A and B, lanes 1-3).

To prove that the antibodies react also with variant CD44 protein expressed on cells, we prepared cell extracts from several human cell lines and subjected these to Western blot analysis. The cells were chosen on the basis of previous Northern blotting data (Hofmann et al., 1991). The immortalized keratinocyte cell lines HaCat and HPKII expressed a CD44v-RNA species containing sequences complementary to exons v3 to v10 (Hofmann et al., 1991). The polyclonal rabbit antiserum affinity-purified on CD44v v3 to v10

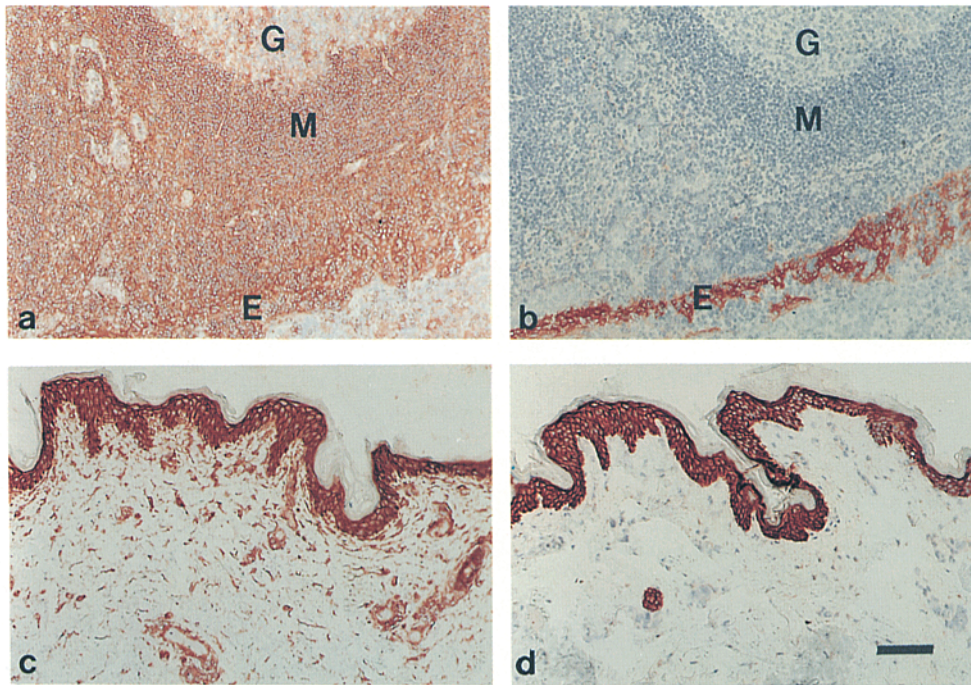


Figure 4. Expression of variant CD44 on normal tonsil and skin: (a and b) serial sections of tonsil, stained with mAb NKI-P1 against the standard part of CD44 (a), and with antibodies against variant CD44 (v3-v10) (b). GC, germinal center; M, mantle zone; E, epithelium. (c and d) Serial sections of skin, stained with mAb NKI-P1 against standard part of CD44 (c), and with antibodies against variant CD44 DIII (v6/7) (d). While the epitope on the standard part of CD44 detected by NKI-P1 is expressed on a variety of cell types including epithelium, lymphocytes, fibroblasts and blood vessels, expression of variant CD44 is limited to epithelium. Counterstain hematoxyline. Bar, 100 μ m.

stained proteins from HaCat cell extracts with apparent molecular weights of 110, 130, 180, and 230 kD, and a major protein of \sim 130 kD from HPKII-extracts (Fig. 3 B). By immune precipitation with the polyclonal antiserum and subsequent Western analysis with a mAb directed against CD44s, identity of the proteins with CD44 variants was confirmed (not shown). The colon carcinoma cell line HT29 expressed one major RNA species that hybridized with exons v8 to v10 (Hofmann et al., 1991). The polyclonal antibody stained a single protein band with the apparent molecular weight of 180 kD in the Western blot analysis (Fig. 3 B). This could be taken as an indication that the polyclonal serum reacts also with sequences derived from exons v8 to v10. The CD44v-negative cell line MeWo (Hofmann et al., 1991) did not react with the antisera. Glutathione transferase specific antibodies that were purified similarly to CD44v antibodies from the same rabbit antiserum showed no staining with either protein extract tested (not shown). The anti-DIII (v6 and v7) antibodies only recognized a protein of 130 kD in both HPKII and HaCat cells (Fig. 3 A). Possibly this (these) epitope(s) is (are) masked on the larger proteins. With anti-DIII there was no reaction with HT29 or MeWo cell extracts.

Expression of CD44 and CD44 Variant Proteins on Human Cells and Tissues

The cell and tissue distribution of CD44 and CD44 variant proteins was assessed in immunohistochemical studies using mAb NKI-P1 against the standard part of CD44 and the antibodies against the variant portion of CD44. It should be noted that NKI-P1 also recognizes the variants of CD44 as they contain the complete standard sequences, and that immunostaining indicates accessibility of epitopes, not necessarily synthesis or absence of the protein. In accordance with previous observations (Pals et al., 1989a), CD44 as detected by mAb NKI-P1 was found to be expressed on a variety of normal cells and tissues (Table I; and Fig. 4). In the lympho-

hematopoietic system high level expression of the NKI-P1 epitope was observed on B and T lymphocytes (with the exception of germinal center B cells and cortical thymocytes), on macrophages, including alveolar macrophages of the lung and Kupffer cells of the liver, on dendritic cells, and on granulocytes and erythrocytes. In epithelial tissues, the squamous epithelia of the epidermis, tonsils and pharynx, and the glandular epithelia of the salivary glands, intralobular pancreatic ducts and of the thyroid stained positive, whereas hepatocytes, bile duct epithelium and kidney tubules did not express CD44. In the epithelia of the small and large intestine weak staining was observed at the base of the crypts. Furthermore, we observed CD44 expression in fibroblasts, endothelial cells, and smooth muscle cells in many tissues. In brain, astrocytes and microglia were positive. Similar tissue distribution of CD44 has been found in the rat (Wirth, K., R. Arch, C. Somasundaram, M. Hofmann, P. Herrlich, S. Matzku, and M. Zöller, manuscript submitted for publication).

Compared to the wide tissue distribution of standard CD44 as detected by NKI-P1, the expression of epitopes of the splice variant domains was much more restricted. Antibodies raised against the fusion protein containing all variant domains from v3 to v10, as well as those against v6/v7 (DIII)

Table II. Expression of CD44 and Variant CD44 in Colorectal Neoplasia

	Antibody specificity (No +/No. tested)			
	CD44 (NKI-P1)	CD44v (v3-v10)	DIII (v6, v7)	D1 (v3)
Adenomatous polyps	17/17*	16/17*	14/17*	0/17
Adenocarcinoma	17/17	17/17	16/17	0/17
Metastasis	8/8	8/8	8/8	0/8

* Focal expression in areas showing moderate or severe dysplasia.

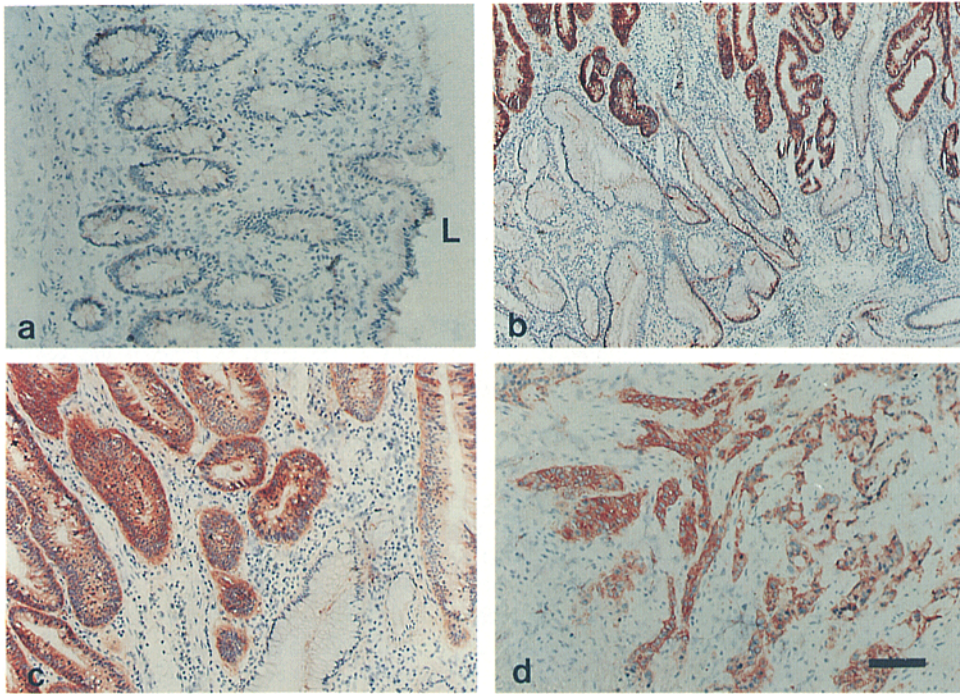


Figure 5. Expression of variant CD44 in colorectal neoplasia. (a) Normal colon mucosa stained for CD44 (v3-v10); no unequivocal staining is observed. (b) Expression of variant CD44 (v3-v10) in an adenomatous polyp. Expression is clearly related to the parts of the tumor with advanced dysplasia. (c) Detail of b. (d) Expression of variant CD44 DIII (v6 and v7) in an invasive colorectal carcinoma. (b) or (a, c, and d), counter-stain hematoxyline. Bars, (b) 400 μ m; and (a, c, and d) 100 μ m.

strongly stained epidermal keratinocytes, tonsillar and pharyngeal epithelium, bronchus and thyroid gland (Table I, and Fig. 4). High level expression was also observed in pancreatic epithelium, which seems at variance with previous studies on rat pancreas (Wirth, K., R. Arch, C. Somasundaram, M. Hofmann, P. Herrlich, S. Matzku, and M. Zöller, manuscript submitted for publication). At the base of intestinal crypts very weak signals were found. Microglia cells in the brain were slightly positive. Expression of epitope DI (v3) was limited to epidermal keratinocytes, the mucosa of tonsils and pharynx, and pancreatic ducts. CD44 variant epitopes were not found on numerous other tissues including all mesenchymal cells (fibroblasts, smooth, and striated muscle endothelium). CD44v expression thus is limited to few differentiation pathways. We note, however, that expression of an epitope does not imply that the CD44 variants expressed are identical.

Expression of CD44 and CD44 Variant in Adenomatous Polyps and Colorectal Carcinomas

As mentioned in the previous section, specimens of histologically normal colon mucosa showed weak expression of standard CD44 on epithelial cells localized at the base of the crypts, while staining with antibodies to CD44v (v3 to v10) or DIII (v6 and v7) was either very weak or undetectable. In marked contrast to this restricted expression of CD44 (standard) in the normal colon, all invasive colon carcinomas and carcinoma metastases expressed CD44 at moderate to high levels. These tumors were stained by NKI-P1, CD44v (v3 to v10) and v6/v7-specific antibodies. In most carcinomas, the majority of tumor cells were positive for the NKI-P1 and v3 to v10 epitopes, but considerable intratumor heterogeneity in staining intensity was generally observed. Importantly, areas positive for v3 to v10 antibodies were present in all carcinomas, and all (but one) showed staining with DIII (v6, v7)-specific antibodies (Table II; and see Fig. 5).

Five of the colorectal carcinomas that were positive for CD44v expression upon histological staining with antibodies were examined by Western blot analysis (Fig. 6, lanes 6-10). Arrows indicate prominent bands that react specifically with antibodies against CD44v (v3 to v10). The unspecific band at \sim 120 kD will be discussed below. The different tumor samples show pronounced differences in specific staining intensity. These differences are in agreement with those found by immunohistochemical surface staining, both with respect to number of positive cells and intensity of CD44v expression, e.g., in tumors examined in Fig. 6, lanes 9 and 10, 90% of cells were strongly positive for CD44v expression, whereas in those used in lane 6 and 8 only 30 to 50% of the cells were CD44v positive and

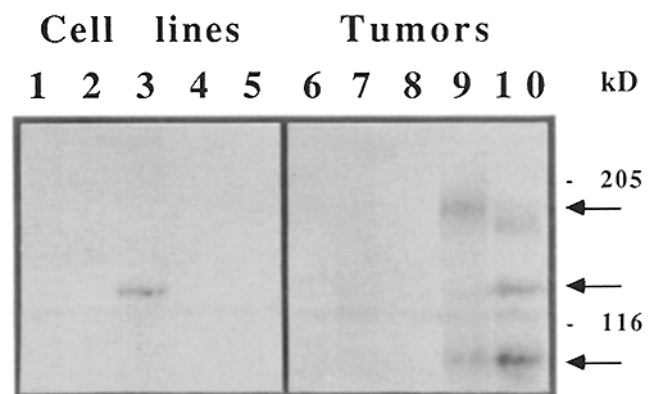


Figure 6. Western blot analysis of several human cell lines and colorectal carcinomas with polyclonal CD44v (v3 to v10) specific antibodies. In lane 1 to 4 extracts from the following colorectal carcinoma cell lines were used: SW 480 (1), SW 620 (2), Colo 205 (3), and Colo 320 (4). The extract used for lane 5, was obtained from the melanoma cell line MeWo. In lanes 6 to 10 extracts obtained from five different colorectal carcinomas were applied. Proteins were resolved on 6% SDS polyacrylamide.

showed weak to moderate staining. Interestingly cell lines derived from colorectal carcinomas differ in their expression of CD44v (Fig. 6, lanes 1–4). One cell line apparently expresses proteins similar to those found in colorectal carcinomas (lane 3), the others are negative for CD44v. The two cell lines used in lanes 1 and 2 (SW480 and SW620) had been examined for RNA expression previously and were also negative for CD44v expression in this test (Hofmann et al., 1991). The cell line MeWo (Fig. 6, lane 5) was used as negative control (see also Fig. 3) to identify unspecific staining.

For one carcinoma (Dukes C) and two lymph node metastases it was possible to elucidate the structure of the variant portion of the CD44v-RNA expressed. RNA prepared from these samples was subjected to reverse transcription-PCR-amplification using primers corresponding to sequences within either the v6 or v8 region and the 3' or the 5' standard region. The data are compatible with expression of a single major RNA species comprising sequences v6 to v10 but lacking v3 to v5 (data not shown).

Interestingly, CD44 was also expressed on adenomatous polyps which represent precursor lesions of colon carcinoma. In addition to staining with NKI-P1, these adenomas showed focal staining with CD44v v3 to v10-specific as well as DIII (v6 and v7)-specific antibodies. Expression of these variants in the polyps was clearly associated with areas of either moderate or severe dysplasia, and hence with morphological evidence of tumor progression within the adenomas.

We observed no correlation between staining for CD44 v3-v10 or DIII (v6 and v7) and overexpression of p53 as detected by mAb pAb1801. Both colocalization and differential localization were observed in adenomas and carcinomas which showed an overall p53 positivity of 50 and 18%, respectively.

Discussion

Expression of CD44 determinants has recently been linked to tumor dissemination in several systems including human non-Hodgkin lymphomas (Pals et al., 1989b; Horst et al., 1990; Jalkanen et al., 1990) and rat adenocarcinomas (Günthert et al., 1991). In the latter system, a new variant of the CD44 molecule generated by alternative splicing was shown to be causally involved in metastasis formation. Using antibodies raised against fusion proteins of human homologues of the variant portion of CD44, we now demonstrate expression of variants of the CD44 glycoprotein family in several normal human tissues. Furthermore, we show that CD44 variant sequences, which in the rat confer metastatic behavior, are overexpressed in human colorectal carcinomas and carcinoma metastases, and, to a lesser extent, in adenomatous polyps.

While biochemical studies indicated the presence of heterogeneous sets of CD44 glycoproteins on many cell types (Jalkanen et al., 1988; Pals et al., 1989a; Picker et al., 1989), and several CD44 RNA splice variants have recently been identified (Stamenkovic et al., 1991; Günthert et al., 1991; Hofmann et al., 1991; Dougherty et al., 1991; Brown et al., 1991; Shtivelman and Bishop, 1991), our present study is the first to document differential expression at the protein level of variant domains in humans. In comparison with the abundance of standard CD44, these variants show a very restricted distribution in normal human tissues. Thus,

whereas many epithelial as well as non-epithelial cells express high levels of standard CD44, variant CD44 glycoproteins are found, at detectable levels, at only a subset of epithelial cells (Table I).

The causal role of variant CD44 glycoproteins in the lymphatic spread of carcinomas in the rat prompted us to study expression of CD44 variants in spontaneous human tumors. For this study we chose colorectal neoplasia, since in this tumor type individual stages of the multi-stage carcinogenesis process can be morphologically distinguished. From normal mucosa through local hyperplasia and various adenoma stages to carcinoma, morphological phenotypes have been related to genetic changes (reviewed by Fearon and Vogelstein, 1990). Here we observe that variant CD44 glycoproteins, which in the rat are associated with metastatic spread, are strongly overexpressed in colorectal neoplasia. Thus, whereas in the normal colon CD44 variant epitope expression is very weak and restricted to epithelial cells at the base of the crypts, a moderate to strong diffuse expression was found in most of the invasive carcinomas and carcinoma metastases tested. (Table II; and Fig. 5). In adenomatous polyps, focal expression of variant CD44 epitopes was found (Fig. 5). This overexpression was clearly related to the degree of dysplasia within the adenomas and hence to progression within these limits.

The progressive accumulation of genetic changes in multi-step carcinogenesis is thought to be caused by mutation and clonal selection. Changes at chromosomes 5q (putative gene: APC and MMC), 12p (RAS), 17p (p53), and 18q (DCC) (reviewed by Fearon and Vogelstein, 1990) appear to produce growth advantages. DCC interestingly codes for a putative adhesion protein (Fearon et al., 1990). Loss of adhesive functions and gain of new adhesive functions are likely to be important in the development of metastatic cancer. The CD44 variants could be such new adhesion proteins. Overexpression in colorectal epithelial cells could lead to altered adhesion and to growth-promoting signals associated with adhesion. If we assume, as is supported by the data obtained in rats, that the CD44 variants are part of the mechanism of metastasis formation, it is likely that CD44v expression is not the only property required. All relevant features need to be assembled to cause metastatic spread but the order of their acquisition may not matter. Further, metastatic properties may develop independently of cellular transformation. Metastasis formation depends, however, on the growth potential established by transformation. In colorectal cancer, the acquisition of CD44v seems to be an early event which precedes overexpression of p53. We should, however, be cautious as the possibility exists that the variant proteins expressed on polyps and those on metastases are not identical.

Interestingly, colon carcinoma cell lines differ dramatically with respect to CD44 variant expression. Only one out of five expresses proteins that migrate similarly in SDS-gel electrophoresis to those expressed in tumors. Another colon carcinoma cell line, HT29, carries a prominent CD44 variant with only the exons v8 to v10 (Stamenkovic et al., 1991; Hofmann et al., 1991) whereas three others were negative for CD44v. Primary colon carcinomas and metastases synthesize variants that contain exons v6 and v7 according to staining with the affinity-purified antibody. PCR analysis of three samples examined indicate the presence of v6 through v10 sequences in CD44v.

The expression pattern of CD44 as found in our present study, suggests a role for variant CD44 glycoproteins in certain normal epithelial differentiation pathways, while apparent deregulation and overexpression in colorectal neoplasia is consistent with a possible role in metastatic behavior, analogous to that found in the rat (Günthert et al., 1991). It will be important to determine whether this overexpression in colon tumors is regulatory or is mutationally fixed. Clearly, overexpression can serve as a tumor progression marker in human cancer. In this context, it is of interest that the genetic control of susceptibility to colon carcinoma in mice has been linked to the chromosomal region of the CD44 gene (Moen et al., 1992).

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