Expression of CD44, particularly of certain splice variants, has been linked to tumor progression and metastasis formation in a number of different animal and human cancers. Because human cutaneous melanoma is among the most aggressive human cancers, we explored expression of CD44 isoforms (CD44v) in lesions of melanocytic tumor progression. In addition, by RT-PCR and FACS analysis we assessed CD44v RNA species and cell surface expression of CD44v in cultured melanocytes isolated from human foreskin and in a panel of 2 non-, 2 sporadically and 2 highly metastatic human melanoma cell lines. We observed that all melanocytic lesions examined showed strong uniform expression of standard CD44 (CD44s) epitopes. We did not detect CD44v6 expression in the melanocytic lesions. However, CD44 isoforms containing v5 or v10 were differentially expressed. V5 was expressed in 16%; 0%, 20%, 67% and 58% of common nevi, atypical nevi, early primary melanomas (≤1.5 mm), advanced primary melanomas (≥1.5 mm) and metastases, respectively, and hence was related to tumor progression. In contrast, CD44v10 was expressed in all common nevi, whereas part of the atypical nevi and most primary melanomas (≤1.5 mm), advanced primary melanomas (≥1.5 mm) and metastases lacked v10. CD44v RNA patterns were closely similar in cultured melanocytes and all melanoma cell lines. Melanocytes expressed high levels of CD44s but no CD44v, whereas all melanoma cell lines expressed CD44v10 at the surface. Interestingly, expression of v5 was strongly increased in the highly metastatic cell lines. Our results suggest a role for CD44 variant domains, particularly v5 and v10, in human melanocytic tumor progression.

**MATERIAL AND METHODS**

**Antibodies**

MAbs directed against the variant portions of CD44 were VFF4 anti-CD44v6 (IgG2b); VFF7 and VFF18 anti-CD44v6 (both IgG1); VFF8 anti-CD44v5 (IgG1) and VFF14 and VFF16 anti-CD44v10 (both IgG1) (Wielenga et al., 1993; Koopman et al., 1993). The VFF series of antibodies were kindly provided by Drs. E. Patzelt and G. Adolf, Bender-Wien, Vienna, Austria. The MAb against an epitope on the constant portion of the extracellular domain of CD44 was NKI-P1 (Pals et al., 1989). Specificity of the MAbs is schematically shown in Figure 1.

**Tissues**

Human melanocytic lesions were selected from the files of the Department of Pathology, University Hospital, Nijmegen, The Netherlands, and the Department of Dermatology, University Hospital, Würzburg, Germany, and tested for expression of CD44s and CD44v by Drs. E. Patzelt and G. Adolf, Bender-Wien, Vienna, Austria. The MAb against an epitope on the constant portion of the extracellular domain of CD44 was NKI-P1 (Pals et al., 1989). Specificity of the MAbs is schematically shown in Figure 1.

Immunohistochemistry

Immunoperoxidase staining was performed as described previously (Danen et al., 1993). Briefly, 4 μm cryostat sections were fixed in acetone for 10 min, washed in PBS and incubated with the primary antibody for 1 hr. Bound MAbs were visualized using the peroxidase-based Vectastain elite ABC Kit (Vector, Burlingame, CA) and amino-ethyl-carbazole. Lesions were designated "positive" when they were estimated to show staining in more than 10% of the melanocytic cells.

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CD44 VARIANTS IN HUMAN MELANOMA

183

FIGURE 1 – (a) Schematic representation of the CD44 gene. Open boxes indicate exons that are spliced out of the “standard” type of CD44. TM, transmembrane region. (b) Schematic representation of the CD44 protein with location of the epitopes which are recognized by the MAb NKI-P1, VFF4, VFF7, VFF8, VFF14, VFF16 and VFF18. Anti-variant antibodies were raised against a bacterially expressed fusion protein encoded by pGEX CD44v HPKII (v3 to v10). Dark area, “standard CD44”; v1–v10, domains encoded by variant exons.

Cell lines and cell cultures

The human melanoma cell lines 530 and IF6 (non-metastatic upon s.c. injection in nude mice), M14 and MEL57 (sporadically metastatic upon s.c. injection in nude mice) and MV3 and BLM (frequently metastatic upon s.c. injection in nude mice; Danen et al., 1993) were studied for expression of CD44 variants. The cell lines were grown in DMEM (GIBCO BRL, Grand Island, NY) supplemented with 1 mM glutamine, 10% (v/v) heat-inactivated FCS (Hyclone, Logan, UT) and antibiotics. Primary melanocytes were isolated from human foreskin and cultured in Ham’s F10 (Flow, Irvine, UK) supplemented with 2% Ultroser-G synthetic serum (GIBCO), glutamate, penicillin, streptomycin, 0.1 mM IBMX (Sigma, St. Louis, MO) and 16 mM phorbol 12-myristate 13 acetate (PMA; Sigma) for a maximum of 5 passages, as described previously (Danen et al., 1993).

Flow cytometry

Cells were sequentially incubated in PBS containing 1% BSA and 0.02% sodium azide with appropriate dilutions of the various antibodies, followed by incubation with biotin-conjugated rabbit–anti-mouse Ig and phycoerythrin-labeled streptavidin (Dakopatts, Glostrup, Denmark). Fluorescence was measured by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Reverse transcriptase polymerase chain reaction

Cells (5 to 10 × 10^6) were harvested, washed and lysed by the addition of 0.2 ml of RNAzol (Campro Scientific, Veenedaal, The Netherlands) per 10^6 cells. Total RNA was extracted by adding 0.2 ml chloroform per 2 ml of cell lysate. After centrifugation at 12,000 g for 15 min (4°C) the aqueous phase was transferred to a fresh tube and RNA was precipitated using isopropanol at −20°C for 45 min. Isolated RNA was stored at −80°C in 70% ethanol until use; 2 μg aliquots of total RNA were used to synthesize cDNA using an oligo dT primer with DNA reverse transcriptase, according to the protocol provided with the GeneAmp RNA-PCR kit (Perkin Elmer Cetus, Norwalk, CT). Amplification of CDNA was performed using CD44 5’ constant and CD44 3’ constant primers corresponding to positions 513–540 and 900–922 of the standard CD44 sequence described by Scream et al. (1992). Amplification was performed in 35 cycles (30 sec, 94°C; 1 min, 55°C and 2 min, 72°C) in a microwave-based Amplitube DNA incubator (Kreatech, Amsterdam, The Netherlands). PCR products were analyzed on a 2% agarose gel (Brunschwig, Amsterdam, The Netherlands) containing ethidium bromide, subsequently transferred to nylon filters (Gene Screene Plus, NEN, Boston, MA) and hybridized with a 5’ constant CD44-specific oligonucleotide probe (position 513–540; CA GAC CTG CCC A AT GCC TTT GAT G G A CC) or exon v5 (position 1164–1184; AT GGC ACC ACT GCT TAT GAA G), exon v6 (position 1322–1346; TGG TTT GGC AAC AGA TGG CAT GAG G), exon v7 (position 1443–1464; CA AGC CCA GAG AGT TCC TG) and exon v10 (position 1799–1817; CT CAT TAC CCA CAC ACG AA) specific oligonucleotide probes labeled with gamma(32P)P (Amersham, Den Bosch, The Netherlands) using polynucleotide kinase (Boehringer Mannheim, Germany).

RESULTS

Expression of CD44 variants in common and atypical nevocellular nevi and malignant melanomas

Immunohistochemical studies demonstrated differential expression of the various CD44 epitopes in melanocytic lesions (Figs. 2, 3). In all types of lesion, there was a strong homogenous expression of CD44 proteins, determined by the pan-CD44 MAb NKI-P1 directed against an epitope on the NH2 terminal constant part of CD44. By contrast, expression of epitopes encoded by v6 was not observed with any of the 3 MAb used, although normal epidermal keratinocytes present in all tissue sections strongly stained (not shown).

Interestingly, there was a marked difference in the expression of v5 and v10 in the different types of melanocytic lesion. In the lesions that were positive for v5 or v10, a heterogeneous staining pattern was observed with 25–100% positive melanocytic cells. V5 was detected in only a low percentage of the common and atypical nevi and early primary melanomas, i.e., in 3/19 (16%), 0/9 (0%) and 2/10 (20%), respectively (Figs. 2, 3). However, the majority of the advanced primary melanomas (6/9, 67%) and melanoma metastases (11/19, 58%) expressed CD44v5 (Figs. 2, 3). Hence, over-expression of v5-containing CD44 isoforms is strongly related to tumor progression (χ^2 trend 1 d.f. = 12.82, p = 0.0005).
Figure 2 – Expression of CD44v on human melanocytic lesions. n, nevus cells; m, melanoma cells; s, stroma. (a) Common melanocytic nevus strongly positive for CD44s (NKI-P1). (b and c) Comparable region of the same lesion as in (a) negative for CD44v5 (VFF8) (b) and positive for CD44v10 (VFF14) (c). (d) Atypical nevus strongly positive for CD44s (NKI-P1). (e and f) Comparable region of the same lesion as in (d) negative for CD44v5 (VFF8) (e) and positive for CD44v10 (VFF14) (f). (g and h) Early primary melanoma strongly positive for CD44s (NKI-P1) (g) and negative for CD44v5 (VFF8) (h). (i) Advanced primary melanoma strongly positive for CD44s (NKI-P1). (j and k) Comparable region from the same lesion as in (i) positive for CD44v5 (VFF8) (j) and negative for CD44v10 (VFF14) (k). (l) Melanoma metastasis strongly positive for CD44s (NKI-P1). (m and n) Comparable region from the same lesion as in (l) positive for CD44v5 whereas the stroma cells are negative (VFF8) (m) and negative for CD44v10 (VFF14) (n). Bar: 20 μm.
V10-encoded epitopes were also differentially expressed in the various types of melanocytic lesion. The pattern of expression of v10, however, was strikingly different from that of v5. V10 was detected in 100% of common nevi (19/19), in 67% of atypical nevi (6/9), in 10% of early primary melanomas (1/10), in 11% of advanced primary melanomas (1/9) and in 32% of the melanoma metastases (6/19) (Figs. 2, 3). Hence, malignant transformation of melanocytes is often associated with a loss of exon v10 expression ($\chi^2$ trend 1 d.f. = 9.25; $p = 0.026$).

Expression of CD44 isoforms on cultured melanocytes and melanoma cell lines

We next assessed the expression of CD44 isoforms on cultured normal melanocytes isolated from human foreskin as well as on a panel of human melanoma cell lines that are non- (IF6, 530), sporadically (M14, MEL57) and highly (BLM, MV3) metastatic after s.c. inoculation into nude mice. Cultured normal melanocytes were found to express high levels of standard CD44, with a mean relative fluorescence intensity of approximately 5 x 10^3 (not shown). Epitopes encoded by v5, v6 and v10 were hardly or not detectable on melanocytes (Fig. 4).

The human melanoma cell lines also showed a strong expression of standard CD44 epitopes (not shown). This expression was independent of the metastatic phenotype of the cell lines. Interestingly, however, expression of v5- and v6-containing CD44 splice variants was variable, and expression of v6 and particularly v5 related to the metastatic potential of the cell lines (Fig. 4). Thus expression CD44v6 in the highly metastatic cell lines was much higher than in the non-metastatic cell lines. High expression of CD44v6 was also observed in one of the sporadically metastatic lines (i.e., MEL57). Furthermore, CD44v5 was weakly expressed on the non- and sporadically metastatic cell lines, but expression on the highly metastatic cell lines was 5-10 fold higher. All melanoma cell lines weakly expressed CD44v10. The difference in culture conditions of melanoma cell lines and primary cultured melanocytes did not influence the CD44 expression profile on melanocytes and melanoma cell lines (data not shown).

RT-PCR analysis demonstrates multiple CD44 splice variants

To gain insight into the diversity and structure of the CD44 splice variants expressed in human melanocytes and melanoma cell lines, RNA prepared from these cells was subjected to RT-PCR amplification using primers corresponding to sequences of the 5' and the 3' standard constant region of CD44. With these primers a major PCR product of approximately 450 bp was obtained from the normal melanocytes as well as from the melanoma cell lines (Fig. 5a). The size of this product corresponds to that expected for the standard CD44 message. In addition, up to 6 minor bands of approximately 530, 580, 615, 660, 850 and 1,100 bp were obtained from the melanoma cell lines. Human cultured melanocytes gave rise to 5 minor bands of 530, 580, 660, 850 and 1100 bp (Fig. 5a).

Hybridization of the PCR products of melanocytes and melanoma cell lines with specific oligonucleotide probes (Fig. 5b-f) showed that all contained one to several splice variants with v6, v7 and v10. Except for M14, all melanoma cell lines contained splice variants with v5, whereas melanocytes did not hybridize with v5-specific probes. In general, the patterns obtained with the different cell lines were very similar.

**DISCUSSION**

Adhesion molecules mediating cell–cell or cell–matrix interactions are involved in several steps of the metastatic cascade. In malignant melanoma increased expression of integrins $\alpha$2$\beta$1 (Bröcker et al., 1985), $\alpha$3$\beta$1 (Natali et al., 1993), $\alpha$5$\beta$1 (Dancen et al., 1994) and $\alpha$v$\beta$3 (Seftor et al., 1993) and of ICAM1 (Johnson et al., 1989) and decreased expression of integrin $\alpha$6$\beta$1 (Natali et al., 1991) have been reported to correlate with tumor progression. Furthermore, functional studies implicate integrin $\alpha$5$\beta$1 and $\alpha$v$\beta$3 in melanoma cell proliferation and invasion (Seftor et al., 1993).

Several studies show a correlation between expression of members of the CD44 family of adhesion molecules and tumor progression in human non-Hodgkin's lymphoma (Horst et al., 1991) and carcinoma (Wielenga et al., 1993). For human melanoma, a correlation has been reported in sublines from a single cell line between high CD44 expression and metastatic property, but in a large panel of different human melanoma cell lines no such correlation was confirmed (East et al., 1993).

In addition, CD44 is strongly expressed in all stages of human melanocytic tumor progression *in vivo* (Moretti et al., 1993). Our finding of comparable levels of CD44 on the surface of normal human melanocytes and melanoma cell lines with different metastatic capacities confirms earlier findings that CD44 expression does not correlate with the metastatic capacity of melanoma cells (East et al., 1993). Moreover, in line with previous reports (Moretti et al., 1993) we also find that CD44 is strongly expressed in all stages of melanocytic tumor progression *in vivo*. Hence, expression of CD44s appears to be independent of the presence of a malignant or metastatic phenotype. However, functional studies have provided evidence for a role for CD44 in human melanoma cell motility and invasion (Faassen et al., 1992), and we have found that a combination of high production of hyaluronate and expression of "active" CD44, mediating adhesion to hyaluronate, correlates with the metastatic capacity of human melanoma cell line (data not shown).

Recently, no evidence exists for the presence of CD44 splice variants in human melanoma. Therefore, in the present...
study we have investigated expression of CD44 variants (i) in lesions representing various stages of human melanocytic tumor progression \textit{in vivo} and (ii) in a panel of human melanoma cell lines with different metastatic potential after s.c. inoculation into nude mice.

In contrast to CD44s, CD44 isoforms are differentially expressed. Expression of CD44 isoforms containing v5 is not found on normal human melanocytes, but v5 is expressed on human melanoma cell lines and is strongly enhanced in the highly metastatic cell lines. In addition, v5 is expressed in a very low percentage of benign melanocytic lesions and early primary melanomas, whereas it is expressed in the majority of advanced primary melanomas and melanoma metastases. Thus enhanced expression of CD44v5 is related to high metastatic potential of human melanoma cell lines and correlates with human melanocytic tumor progression \textit{in vivo}. Interestingly, CD44v5 is also induced in human colorectal tumor progression (Wielenga \textit{et al.}, 1993). Similar to this type of cancer, where v5 is already expressed in early stages of adenoma, v5 can also be detected in a few benign melanocytic nevi. However, in contrast to colorectal tumor progression, the transition from early primary melanoma to the highly aggressive stage of advanced primary melanoma is attended by a marked increase of the percentage of v5-positive lesions. Gastric diffuse-type adenocarcinomas and their metastases also express CD44v5 and are devoid of v6 as well (Heider \textit{et al.}, 1993).

CD44v6-containing isoforms have been related to tumor progression in human colorectal carcinomas and malignant lymphomas (Wielenga \textit{et al.}, 1993; Koopman \textit{et al.}, 1993). Moreover, v6-containing isoforms have been shown to play a causal role in metastasis of rat pancreatic carcinoma cells (Günthert \textit{et al.}, 1991; Seiter \textit{et al.}, 1993). However, by immunohistochemical staining of malignant melanomas and melanoma metastases, we do not detect any expression of CD44v6. This lack of CD44v6 expression contrasts with the results obtained in the 6 human melanoma cell lines, which all express v6. We have no explanation for the discrepancy in CD44v6 expression between cultured cell lines and fresh human malignant melanomas. Expression of v6 on the melanoma cell lines may be induced by the \textit{in vitro} culture conditions. Alternatively, the v6 epitope might be masked by ligand binding or alterations in the conformation of the CD44 molecule \textit{in situ}.

In complete contrast to expression of v5, we find that expression of CD44v10 is inversely correlated to melanocytic tumor progression \textit{in vivo}. Most metastases lack expression of this variant, and in line with this finding, a very low surface expression of CD44v10 is found on all melanoma cell lines tested, which were originally generated from metastases.

At the RNA level, the patterns of CD44 splice variant expression are closely similar in the various cell lines. This
finding is remarkable since the cell lines studied are mutually unrelated. Hence, the splicing patterns observed do not represent idiosyncratic changes of an individual clone but may represent a tissue- or tumor-type-specific quality.

The functional consequence of insertion of various exons into the CD44 molecule remains unknown. It may be speculated that the ligand-binding specificity is altered. The 90 kDa CD44s isoform (CD44H) expressed on most cells binds hyaluronate (Stamenkovic et al., 1991). For hyaluronate binding of CD44E, an isoform that contains a 132 amino acid-inserted domain, available data are conflicting (Stamenkovic et al., 1991; He et al., 1992). All of our cell lines, including melanocytes, express the 90 kDa CD44s molecule, and CD44-mediated attachment to hyaluronate observed for melanocytes 1F6, Mel57, BLM and MV3 (data not shown) is probably via this isoform. Therefore, in this system we cannot look into the role of the splice variants in attachment to hyaluronate. Further studies are needed to provide more insight in this matter.

Taken together, we observed that an increase of CD44v5 and a decrease of CD44v10 expression is related to melanocytic tumor progression in vivo and that enhanced expression of CD44v5 is related to the metastatic capacity of melanoma cell lines. Our findings suggest a role for CD44v5 and CD44v10 in human melanocytic tumor progression and melanoma metastasis.

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