CD44 Expression Is Aberrant in Benign Schwann Cell Tumors Possessing Mutations in the Neurofibromatosis Type 2, but not Type 1, Gene

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ABSTRACT

Atypical expression of CD44 splice variants has been implicated in the progression of numerous tumors. This abnormal CD44 expression is presumed to result from gene alterations that cause tumorigenic transformation. Two tumor types that have been linked to specific gene alterations are schwannomas, which have mutations in the neurofibromatosis (NF) type 2 (NF2) gene, and neurofibromas, which characteristically possess NF type 1 (NF1) gene mutations. We examined CD44 expression in normal sciatic nerves, in schwannomas with confirmed NF2 mutations, and in neurofibromas and malignant peripheral nerve sheath tumor tissue and cell lines from NF1 patients. Compared to normal nerves, schwannomas express higher total levels of CD44 and additional splice variants, whereas CD44 expression in neurofibromas is unaltered. Malignant peripheral nerve sheath tumor tissue and cell lines express the CD44v6 epitope, which is not expressed by normal Schwann cells or by other Schwann cell tumors. These data indicate that altered CD44 expression correlates strictly with mutations in the NF2 but not NF1 gene and suggest that CD44v6 might be a marker for the malignant transformation of Schwann cells.

INTRODUCTION

Schwann cells are a neural crest-derived population of supportive cells enfolding all axons within the peripheral nervous system (1, 2). Tumors that contain Schwann cells can be divided into two major groups on the basis of their histology. The first group, schwannomas, account for approximately 8% of all primary intracranial neoplasms and are composed entirely of Schwann-like cells (3). These benign tumors commonly arise on the vestibular branch of the eighth cranial nerve but also occur on other nerve roots (3, 4). The onset of schwannomas has been linked to mutations in the NF3 type 2 (NF2) gene located on chromosome 22 (5, 6). The second group of Schwann cell tumors, neurofibromas, contains numerous cell types in addition to Schwann cells, including perineurial fibroblasts, neurons, vascular elements, and mast cells (7). These tumors are a characteristic feature of NF type 1 (NF1), which has an incidence of 1 in 3500 live births (7). The NF1 gene is located on chromosome 17 and encodes a protein, neurofibromin, which functions as a Ras-GAP (8–10). In rare instances, tumors from NF1 patients undergo malignant transformation (7), resulting in MPNSTs.

Previous investigations have indicated that schwannomas express members of the CD44 family of transmembrane glycoproteins (11–14). These studies did not demonstrate, however, whether this expression differed from normal nerve tissue. CD44 proteins are the products of a single gene and result from the alternative splicing of up to 10 variant exons, referred to as v1–v10, and extensive posttranslational modifications (15). CD44 proteins therefore have a wide range of molecular weights, from approximately Mr 85,000 for the “standard” form (CD44s), which lacks variant exon-encoded sequences, to more than Mr 250,000 for isoforms containing all of the variant products. CD44s has a virtually ubiquitous pattern of expression and has been implicated in lymphocyte homing and activation, as well as in cellular adhesion (16). Alternatively spliced variant isoforms (CD44v), however, are only expressed in certain cells and tumors of epithelial origin.

Normal functions have not yet been defined for most CD44 splice variants. Two such variants (CD44v4–v7 and CD44v6–v7), however, can confer metastatic behavior to a benign rat pancreatic carcinoma cell line (17, 18). CD44s has no effect in this regard, indicating that some CD44 variants could have roles in tumor progression. This notion is supported by numerous studies of human cancers that have demonstrated that CD44 variant expression correlates with the onset of metastasis and poor prognosis (16, 19–21). Such abnormal CD44 expression could be due either to alterations in splicing or to highly up-regulated promoter activity, in which case all CD44 isoforms transcribed by a given cell would be expressed at high levels, including those normally in low abundance. Either of these mechanisms presumably can be influenced by mutations in proto-oncogenes or tumor suppressor genes that cause cellular transformation (22, 23).

If CD44 expression is perturbed in Schwann cell tumors, then it might contribute to Schwann cell tumor progression in a manner similar to its role in other tumors. Furthermore, aberrant expression of CD44 proteins by these tumors would suggest that mutations resulting in Schwann cell tumor formation may influence CD44 expression. We have examined the cells expressing CD44 in neurofibromas from NF1 patients and in schwannomas with confirmed NF2 mutations. We have determined which CD44 splice variants are expressed by these tumors and compared this expression to that of the cells comprising normal human sciatic nerves. Furthermore, we have examined cell lines derived from MPNSTs of NF1 patients and determined whether altering their Ras activity, which is abnormally high compared to non-NF1 cells, influences CD44 expression and splicing.

MATERIALS AND METHODS

Antibodies. The antihuman CD44 antibodies VFF11 (specific for the CD44v4–v7 epitope), VFF8 (CD44v5), VFF18 and VFF7 (CD44v6), VFF9 (CD44v7), VFF17 (CD44v7 and -v8), and VFF16 (CD44v10) were obtained from Bender and Co. (Vienna, Austria) and have been described previously (21). The antihuman CD44 antibody Hermes-3, which recognizes the NH2-terminus of CD44 proteins, was the generous gift of Dr. Sirpa Jalkanen (Turku, Finland). Antibodies FW11.10 (CD44v4), FW11.9 (CD44v6), and FW11.24 (CD44v9; Ref. 24) were prepared from hybridomas obtained from the European Collection of Cell Cultures (Salisbury, Wilt, United Kingdom). The S-100 protein and CNPase antibodies were both purchased from Sigma Chemical Co. (Taufkirchen, Germany).
Cells were then pelleted by centrifugation and resuspended in ice-cold FACS buffer (PBS + 3% FBS) and incubated for 1 h at 4°C. Cells were pelleted and resuspended in冰-cold FACS buffer for 30 mm, washed, and then incubated with phycoerythrin-conjugated goat antimouse IgG (Jackson Laboratories, West Grove, PA), followed by incubation in streptavidin peroxidase and 3,3-amino-9-ethyl carbazole protection with fluorescence-labeled secondary antibodies, sections were incubated in streptavidin peroxidase and 3,3-amino-9-ethyl carbazole

**Immunohistochemistry.** Sections of frozen tissues (7 μm) were fixed in ice-cold methanol for 5 min and then washed twice in PBS. Following preincubation with 10% normal goat serum in PBS, sections were incubated for 1 h in 0.5–10 μg/ml of mouse antihuman CD44 antibodies in PBS + 1% BSA. Sections were then washed three times in PBS. For visualization with the DAKO StreptABCComplex kit (DAKO Corp., Santa Barbara, CA), endogenous peroxidases were blocked by incubation with 0.3% H2O2 in methanol. Sections were then washed and incubated with goat antiamouse IgG secondary biotinylated antibody for 30 min (Jackson Laboratories, West Grove, PA), followed by incubation in streptavidin peroxidase and 3,3-amino-9-ethyl carbazole (Sigma) as described in the manufacturer’s instructions. Sections were then counterstained with hematoxylin and mounted with glycerol-gelatin. For detection with fluorescence-labeled secondary antibodies, sections were incubated for 30 min with either FITC- or tetramethylrhodamine isothiocyanate-conjugated secondary antibodies (Jackson Laboratories) and then washed five times in PBS and mounted for double-labeling experiments, both primary antibodies were added simultaneously, and then, after washing, both secondary antibodies were added. Negative controls included staining with no primary antibody, and, in the case of double-labeling experiments, switching secondary antibodies to confirm their specificity. Stained tissues were examined using a Zeiss Axioscope equipped with epifluorescence.

**Western Blot Analysis.** Frozen tissues were diced into small pieces and then homogenized in ice-cold 50 mm Tris-HCl (pH 7.4) containing 150 mm NaCl, 0.5% NP40, 1 mm phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin (Boehringer Mannheim, Mannheim, Germany). Lysates were clarified by centrifugation at 14,000 x g at 4°C for 30 min. The protein concentrations of the supernatants were then determined using a Bradford assay (Bio-Rad, Richmond, CA). Lysates were mixed with Laemmli’s sample buffer (29) containing 100 mm DTT and then heated to 100°C for 4 min. Equal amounts of protein were loaded into each lane of a 7% SDS-polyacrylamide gel. Proteins were electrophoretically onto an Immobilon membrane (Millipore Corp., Bedford, MA) and were blocked subsequently in 5% dry milk in PBS plus 0.25% Tween 20. For detection of total CD44 proteins, membranes were next incubated with 2 μg/ml Hermes-3 for 1 h at room temperature, washed, and then incubated for 30 min with goat antiamouse IgG conjugated to horseradish peroxidase (Jackson Laboratories). CD44 proteins were visualized using an enhanced chemiluminescence system (Amersham, Braunschweig, Germany).

**Flow Cytometry.** Live Schwann cell and MPNST cultures were brought into single-cell suspension by incubation in Ca2+/Mg2+-free PBS containing 5 mm EDTA for 10 min at room temperature, followed by repeated trituration. Cells were then pelleted by centrifugation and resuspended in ice-cold FACS buffer (PBS + 3% FBS) and incubated for 1 h at 4°C. Cells were pelleted again, resuspended in 1 μg/ml either mouse IgG or one of the primary antihuman CD44 antibodies in FACS buffer for 30 min, washed, and then incubated with phycoerythrin-conjugated goat antiamouse IgG (Jackson Laboratories) for an additional 30 min. Cells were then washed four times, and immunofluorescence was analyzed using a FACS-Star Plus Cell Sorter system (Becton Dickinson, Mountain View, CA).

**Northern Blot Analysis.** Polyadenylated RNA was isolated from cells as described previously (17). Approximately 2 μg of the RNA was electrophoresed through a 1.2% agarose gel containing formaldehyde, blotted onto a Hybond-N nylon filter (Amersham) in 20× SSC (0.15 m NaCl, 0.015 m Na2-citrate·2H2O, pH 7.0), and then baked at 80°C for 1 h. Following 2 h of prehybridization, filters were hybridized successively overnight with GAPDH, v-fos (provided by Peter Angel, Heidelberg, Germany), and CD44s probes that were labeled using a Prime It II random primer labeling kit (Stratagene, La Jolla, CA) at 65°C using the method of Church and Gilbert (30). Following each hybridization, filters were washed under stringent conditions (once in 1× SSC, 0.1% SDS at 65°C for 30 min and then twice in 1× SSC, 0.1% SDS at 65°C for 30 min) and then exposed to X-ray film at −80°C for 2–48 h. Filters were stripped at 100°C for 30 min and checked via autoradiography for remaining signals before rehybridization.

**RT-PCR and Southern Blot Analysis.** There was sufficient tissue in 13 of the 21 schwannomas to prepare RNA for RT-PCR analysis. Approximately 5 μg of total RNA from each of these tumors were isolated and reverse transcribed as described previously (17). First-strand cDNA (5 μl) was amplified by Taq polymerase (Amersham) in 50 μl using buffer conditions described by the manufacturer. For GAPDH PCR, oligonucleotides homologous to positions 8–29 and 362–339 of the published cDNA sequence (31) were used. After 20 rounds of amplification (95°C for 1 min, 62°C for 1 min, and 72°C for 1.5 min), 10 μl of the reaction were resolved on a 1.4% agarose gel and then transferred to Hybond-N nylon filters for Southern blot analysis with a GAPDH probe. For amplification of variant CD44 transcripts, primers homologous to positions 513–540 (in the 5' nonvariant region) and 900–922 (in the 3' nonvariant region) of the published human CD44s sequence (32) were used. After 35 cycles of amplification (94°C for 1 min, 62°C for 1 min, and 72°C for 2.5 min), 10 μl of the product reaction were resolved as above and transferred to nylon filters. These filters were then probed with variant CD44 exon-specific probes as described previously (33). Probes were synthesized by PCR using variant CD44 primers homologous to the following positions (5'/3'): 24–53/81–110 (v3), 128–154/213–239 (v4), 243–271/327–356 (v5), 357–383/389–418 (v6), 425–456/482 (v7), 489–515/585–614 (v8), 621–647/692–718 (v9), 722–750/779–808 (v9), and 812–838/907–1013 (v10; see Ref. 33). Before hybridizing with another probe, the filters were boiled in a 0.5% SDS solution and checked via autoradiography for the absence of remaining signal.

**RESULTS**

**Schwann Cells from Normal Human Peripheral Nerves Express Predominantly CD44s.** Peripheral nerve tissue is composed of multiple cell types, including Schwann cells, perineurial and epineurial fibroblasts, endothelial cells, and axons (Fig. 1A). A number of previous studies have indicated that cells from human nerves express CD44 (34, 35). These studies did not, however, precisely identify which cell types are CD44 positive nor the particular splice variants that are expressed by these cells. To determine which cells in human peripheral nerve express CD44, snap-frozen sciatic nerve tissue was sectioned and examined via double-labeling immunohistochemistry using Hermes-3, an antibody that recognizes all forms of human CD44 and that is highly specific for CD44 proteins (36, 37) and using antibodies against either S-100 protein or CNPase, which recognize Schwann cells in both normal and tumor tissues (38, 39). As shown in Fig. 1, D–G, all of the CD44-positive cells were also S-100 protein positive. Numerous S-100-positive cells, however, lacked CD44 immunoreactivity (Fig. 1, D–G). Similar results were obtained using the CNPase antibody (data not shown), indicating that all of the CD44-expressing cells in axon bundles are Schwann cells. There was some cellular staining in the perineurium (Fig. 1H), supporting previous suggestions that perineurial fibroblasts also express CD44. Antibody specificity was confirmed using isotype control primary antibodies (Fig. 1, B and C).

We next determined which splice variants of CD44 are expressed...
Fig. 1. Coexpression of CD44 and S-100 protein in a section of normal human sciatic nerve tissue. A, light photomicrograph of a nerve cross-section, showing a single axon bundle surrounded by perineurium. Myelinated axons appear as donut-shaped structures within the bundle. B, isotype control from a parallel section of the nerve in A (rabbit IgG). C, isotype control, same section as in B (mouse IgG). D–H, fluorescence photomicrographs of the same section as in A, showing S-100 protein (green) immunoreactive cells (D), CD44 (red) immunoreactive cells (E), and cells immunoreactive for both S-100 protein and CD44 (yellow; F and G). Note that axons are negative for both proteins. At high magnification (G), it is clear that all CD44-positive cells also express S-100. H, fluorescence photomicrograph showing CD44-positive cells in the perineurium.
by human peripheral nerve tissue. Using a panel of antihuman variant epitope-specific CD44 antibodies, CD44v3, -v4, -v5, and an epitope at the border between CD44v7 and -v8 were detected within the axon bundles of each of the three nerve fragments examined (Table 1). This expression was very weak compared to sections of skin or of Schwann cell tumors (see below). The epitope encoding exon v6 could not be detected using three separate antibodies. Compared to the expression in axon bundles, only CD44v3, -v4, and -v5 could be detected in perineurium at very low levels (Table 2). This finding indicates that CD44 proteins are differentially expressed in a cell type-specific manner within nerve tissue. To determine the relative amounts of different CD44 isoforms expressed by peripheral nerves, the CD44 proteins expressed in normal nerve tissue were examined via Western blot analysis using the Hermes-3 antibody (Fig. 3). We found that normal nerve samples expressed a strong, broad band between Mr 85,000 and 95,000, typical of glycosylated CD44s and a second molecular weight bands not observed in normal nerves, likely representing the border between CD44v7 and -v8 were detected within the axon bundles of each of the three nerve fragments examined (Table 1). This expression was very weak compared to sections of skin or of Schwann cell tumors (see below). The epitope encoding exon v6 could not be detected using three separate antibodies. Compared to the expression in axon bundles, only CD44v3, -v4, and -v5 could be detected in perineurium at very low levels (Table 2). This finding indicates that CD44 proteins are differentially expressed in a cell type-specific manner within nerve tissue. To determine the relative amounts of different CD44 isoforms expressed by peripheral nerves, the CD44 proteins expressed in normal nerve tissue were examined via Western blot analysis using the Hermes-3 antibody (Fig. 3). We found that normal nerve samples expressed a strong, broad band between Mr 85,000 and 95,000, typical of glycosylated CD44s and a second band at approximately Mr 60,000, likely representing underglycosylated CD44, as described previously (18, 40). Higher molecular weight proteins (e.g., between Mr 95,000 and 250,000) indicative of CD44 splice variants were not detected. These data suggest that, although certain splice variants of CD44 are present in normal nerve, they are only weakly expressed.

Schwannomas Express High Levels of CD44s and Aberrant CD44 Splice Variants. Previous investigations have demonstrated that schwannomas express at least some CD44 splice variants (11–14). However, these studies did not compare this expression with that of normal nerve tissue. We found via immunohistochemistry that nearly all of the cells in 21 schwannomas expressed high levels of CD44 compared to normal nerve tissue. All of these CD44-positive cells were S-100-protein positive (data not shown), confirming that these tumors are composed almost entirely of Schwann-like cells. In addition to the splice variants expressed by normal Schwann cells, 10 of these schwannomas expressed detectable levels of the CD44v9 epitope, and all of them expressed CD44v10 (Fig. 2A; Table 1). None of these tumors expressed the v6 epitope (Fig. 2B; Table 1). To compare overall levels of CD44 expression in schwannomas and normal nerve tissue, we analyzed lysates from four schwannomas and three samples of normal sciatic nerve by Western blot analysis. Schwannomas are composed almost entirely of Schwann-like cells, whereas the contribution by Schwann cells to peripheral nerve is smaller (30–40% less, depending on the sample). We found, however, that schwannomas typically expressed three to four times more total CD44 protein than normal nerve tissue (Fig. 3), indicating that CD44 expression is elevated in the Schwann-like cells of these tumors. As with the normal nerve tissue, schwannomas expressed a broad band between Mr 85,000 and 95,000, typical of glycosylated CD44s, and a range of under-glycosylated CD44 proteins between Mr 55,000 and 70,000. Schwannomas also expressed very weak higher molecular weight bands not observed in normal nerves, likely representing proteins encoded by CD44 splice variants (18, 40).

To confirm that schwannomas express CD44 splice variant transcripts encoding the variant epitopes described above, we examined CD44 expression in 13 tumors via RT-PCR analysis. Total RNA was reverse transcribed, and the resulting cDNAs were amplified using CD44 primers in the 5' and 3' nonvariant coding regions. The PCR products were then analyzed via Southern blotting using variant exon-specific probes. We found that most of these tumors expressed a variety of CD44 transcripts containing different combinations of each of the variant sequences (Fig. 4). The CD44s sequence (predicted size, 409 bp) was not detected by any of the variant exon-specific probes, confirming the high stringency of the hybridizations. One transcript that was detected consistently by the CD44v3 probe was slightly above 700 bp and likely represents CD44v3—v5 (predicted size, 741 bp). This band was also detected with CD44v4 and -v5 probes (data not shown). Another band often observed using the CD44v10 probe migrated near 680 bp and likely represents a splice variant containing only v10 (predicted size, 610 bp). This band was not detected using any of the other probes. The presence of this transcript is consistent with our immunohistochemical findings using a CD44v10-specific antibody. Although the CD44v6 epitope could not be detected in any of the schwannomas via immunohistochemistry, some of these tumors expressed CD44 transcripts containing exon v6. This expression, however, was generally weak compared to that of other variants, and it is unclear whether these transcripts are actually translated (see “Discussion”).

### Table 1 CD44 variant epitopes expressed by normal human sciatic nerves, schwannomas, and neurofibromas

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CD44 ABODY (epitope)</th>
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<tr>
<td>Normal sciatic nerves (Schwann cells only)</td>
<td>VFF11 (v3)</td>
</tr>
<tr>
<td>Schwannomas (NF2 mutations confirmed)</td>
<td>FW11.10 (v4)</td>
</tr>
<tr>
<td>Schwannomas (NF2 mutations not determined)</td>
<td>VFF8 (v5)</td>
</tr>
<tr>
<td>Neurofibromas (from NF1 patients)</td>
<td>VFF8 (v5)</td>
</tr>
<tr>
<td>MNPST5* (from NF1 patients)</td>
<td>VFF17 (v7/v8)</td>
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Data are presented as the number of positive samples and, in parentheses, the percentage of positive samples.

<table>
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<tr>
<th>CD44 EXPRESSION IN NF</th>
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<tr>
<td>Patient(v3)(v4)(v5)(v6)(v7/8)(v9)(v9/v10)(pan)</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>3</td>
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* - no detectable staining; +, weak staining; ++, moderate staining; ++++, intense staining. Note that additional antibodies for the v6 epitope (VFF7 and FW11.9) were also used and yielded identical results.

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of the variant epitopes) from these tumors for all of the antibodies tested, the tumor cells from these samples only expressed the same CD44 variants that are expressed by normal nerve (Fig. 2, C—F; Table 1). As with schwannomas and normal nerve tissue, the CD44v6 epitope could not be detected using three separate antibodies. The level of CD44 staining was comparable to that of normal nerves (i.e., weak compared to skin), suggesting that total CD44 expression is not elevated in neurofibroma tissue.

MPNST Cell Lines from NF1 Patients Express Additional CD44 Variants Compared to Normal Cultured Human Schwann Cells. Malignant Schwann cell tumors (MPNSTs) are extremely rare. In NF1 patients, however, these malignancies occur at a higher incidence compared to the unaffected population and represent a significant contribution to NF1 patient morbidity (7). Because the expression of certain CD44 splice variants correlates with malignant
CD44 EXPRESSION IN NF

Transformation in other tumors, we compared the expression of CD44 proteins in primary cultures of normal human Schwann cells and two MPNST cell lines derived from NF1 patients (28). Both of the MPNST cell lines expressed splice variants of CD44, including variants with the v6 and v10 epitopes (Figs. 5A and 6A). These splice variants were not expressed by normal Schwann cells in vitro (Fig. 6A). Furthermore, total CD44 and CD44 variant expression was significantly higher in the MPNST cells compared to normal Schwann cells (Figs. 5B and 6A). To verify that this aberrant CD44 expression also occurs in situ, we examined tissue sections from a MPNST via immunohistochemistry, as above. This tumor expressed weak levels of the CD44v6 epitope (Table 1).

 Altering Ras Activity in MPNST Cell Lines Does Not Affect Their CD44 Expression. Because neurofibromas and MPNSTs both possess mutations in the NF1 gene, our finding that CD44 expression is unaltered in neurofibromas suggests that the abnormal CD44 expression in MPNSTs is not due to their NF1 mutations. However, the NF1 gene product, neurofibromin, is a functional Ras-GAP-related

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**Fig. 3.** Western blot analysis of CD44 expression by normal peripheral nerves (N1, N2, and N3) and by schwannomas. Equal amounts of total protein from four schwannomas and three samples of normal sciatic nerve were analyzed for CD44 protein expression using the Hermes-3 antibody. Note that schwannomas express higher overall levels of CD44 proteins and very weak additional molecular weight species.

**Fig. 4.** RT-PCR analysis of CD44 variant expression in 13 schwannomas. CD44 cDNA transcripts were amplified with primers in the 5' and 3' nonvariant coding region of human CD44. Filters were then probed with radioactively labeled exon-specific probes for each CD44 splice variant with similar specific activities and then exposed for 16 h and examined. Using these primers, a full-length CD44 product containing all of the variant exons would have a predicted size of 1398 bp, whereas the size of a product lacking any variants would be 409 bp. Results from CD44v3, v5, v9, and v10 are shown, as are results from a GAPDH-PCR used to confirm the abundance of the cDNA. Samples from four patients (patients 6, 9, 11, and 12) expressed low signals due to poor-quality RNA. Patients 1–4, 7, 9, and 11–13 have confirmed NF2 mutations. Patient numbers correspond to the patient numbers in Fig. 3.
The two MPNST cell lines analyzed above, for example, each have higher than normal levels of GTP-bound (active) Ras cells, and two p120AP-transfected clones (GAPC 5.2 and 4.4). Note that CD44 expression is elevated in MPNST cells compared to normal Schwann cells and that reducing ras activity in the MPNST cell lines could reverse the high CD44 expression by these cells. Therefore, we tested whether reverting high Ras activity in the MPNST cells is not involved in their anomalous CD44 expression. Interestingly, at least some cells transfected with c-Ha-ras express significantly higher levels of CD44 proteins and new splice variants compared to their wild-type counterparts (22). We therefore tested whether reverting high Ras activity in the MPNST cell lines could reverse the high CD44 expression by these cells. We examined two clones of one of the MPNST cell lines from above (ST8814) that had been stably transfected with the catalytic domain of p120GAP ("GAP-C"). Compared to untransfected cells, these clones have altered morphology, grow more slowly in agar, and have significantly lower constitutive Ras activity (28). However, we found that these clones expressed the same levels of total CD44 proteins and individual splice variants that were expressed by the parental ST8814 cells (Figs. 5B and 6, A and B). To verify that the lowered Ras activity in these cells was sufficient to influence the expression of other Ras-induced genes, we examined the expression of fos transcripts in these cells via Northern blot analysis. Signals were quantified by scanning densitometry and then normalized to GAPDH. We found that fos expression was reduced approximately 3-fold in the GAP-C 4.4 clone and 8-fold in the GAP-C 5.2 clone. CD44 expression, however, was essentially unchanged (1.1-fold higher in clone 4.4 and 1.4-fold lower in clone 5.2). These data suggest that the altered Ras activity in MPNST cells is not involved in their anomalous CD44 expression.

**DISCUSSION**

Although the molecular events mediating tumor progression remain poorly defined, it is clear that cell surface proteins, including adhesion molecules, contribute significantly to this process. Members of the CD44 family of transmembrane glycoproteins are among such molecules. In several experimental systems, CD44 has been shown to play a pivotal role in promoting tumor growth and metastasis (16–18). Clinical investigations of a number of different human cancers have also indicated a correlation between the expression of certain CD44 variants and tumor behavior (16, 19–21, 33). Here, we find that the occurrence of mutations in the NF2 gene correlates strictly with increased CD44 expression and the appearance of atypical CD44 variant proteins in schwannomas. This finding is consistent with the possibility that the NF2 gene product, merlin, can either directly or indirectly influence CD44 expression and possibly splicing. Because CD44 variants have previously been implicated in the formation of various other tumors, it is intriguing to speculate that CD44 proteins may play a similar role in Schwann cell tumor progression.

Interestingly, the normal nerve tissue, neurofibromas, and schwannomas examined in this study did not express the CD44v6 epitope. Although this finding was confirmed with three separate antibodies, protein, and cells with NF1 mutations have high Ras activity (28, 41, 42). The two MPNST cell lines analyzed above, for example, each have higher than normal levels of GTP-bound (active) Ras (28). Interestingly, at least some cells transfected with c-Ha-ras express significantly higher levels of CD44 proteins and new splice variants compared to their wild-type counterparts (22). We therefore tested whether reverting high Ras activity in the MPNST cell lines could reverse the high CD44 expression by these cells. We examined two clones of one of the MPNST cell lines from above (ST8814) that had been stably transfected with the catalytic domain of p120GAP ("GAP-C"). Compared to untransfected cells, these clones have altered morphology, grow more slowly in agar, and have significantly lower constitutive Ras activity (28). However, we found that these clones expressed the same levels of total CD44 proteins and individual splice variants that were expressed by the parental ST8814 cells (Figs. 5B and 6, A and B). To verify that the lowered Ras activity in these cells was sufficient to influence the expression of other Ras-induced genes, we examined the expression of fos transcripts in these cells via Northern blot analysis. Signals were quantified by scanning densitometry and then normalized to GAPDH. We found that fos expression was reduced approximately 3-fold in the GAP-C 4.4 clone and 8-fold in the GAP-C 5.2 clone. CD44 expression, however, was essentially unchanged (1.1-fold higher in clone 4.4 and 1.4-fold lower in clone 5.2). These data suggest that the altered Ras activity in MPNST cells is not involved in their anomalous CD44 expression.

**Fig. 5.** FACS analysis of CD44 expression in ST8814 cells. A. cells were serum starved for 24 h and then labeled with variant specific antibodies. Results from labelings with VFF18 (CD44v6) and VFF16 (CD44v10) are shown. Note that high levels of each variant are expressed by these cells. Similar results were obtained when analyzing NF90-8 cells. B. analysis of Hermes-3 (total CD44) immunoreactivity in normal Schwann cells, ST8814 cells, and two p120GAP-transfected clones (GAPC 5.2 and 4.4). Note that CD44 expression is elevated in MPNST cells compared to normal Schwann cells and that reducing ras activity in the ST8814 cells has no significant influence on CD44 expression.

**Fig. 6.** Analysis of CD44 expression in normal cultured human Schwann cells and ST8814 MPNST cells overexpressing the catalytic domain of p120GAP. A. Western blot analysis of total CD44 expression by normal cultured Schwann cells. MPNST cells (ST8814 and NF90-8), and GAPC 5.2 cells using the Hermes-3 antibody. Note that Schwann cells express lower levels of CD44 proteins than MPNST cells and express no detectable higher molecular weight splice variants. Note also that the GAPC5.2 cells express the same level and pattern of CD44 proteins as the parental ST8814 cells. B. Northern blot analysis of CD44 and fos expression by the ST8814 cells and the GAPC clones. Lane 1, ST8814 cells; Lane 2, clone GAPC 4.4; Lane 3, clone GAPC 5.2. Note that, although the levels of fos are reduced in the GAPC clones compared to the ST8814 cells, CD44 transcript levels are not significantly different.
and RT-PCR data were reported previously in a study of normal and neoplastic breast tissues (33). It is possible that the v6 epitope is expressed in these tumors (and in normal nerve tissue) but is somehow masked. We had previously found very weak v6 immunoreactivity in two of five schwannomas (11). However, only a small number of cells in these samples were positive, and the identity of these cells was not confirmed. Because RT-PCR is a sensitive enough technique to detect RNA species that are expressed by small numbers of cells, it is more likely that contaminating cell types (i.e., from blood or surrounding tissues) contributed the weak v6 signals observed in Fig. 4. Activated T lymphocytes, for example, are known to express the v6 epitope (43). Furthermore, some of these v6-containing transcripts may be very unstable and might not be translated. Although increases in such transcripts may reflect a general altered state of transcription in tumor cells, their expression is not consistent and is, therefore, less informative than that of the translated species.

In contrast to schwannomas, the neurofibromas examined in this study, which likely possessed mutations in the NfI gene, appeared to have unaltered CD44 expression compared to normal nerves. Cells from patients with NfI mutations have elevated Ras activity (28, 39, 43). Furthermore, some of these v6-containing transcripts may be less informative than that of the translated species.

compared to cultures of normal human Schwann cells, these MPNST cell lines from NfI patients following reversal of high Ras activity with p120GAP. Because NFI mutations alone are not sufficient to cause malignant transformation, it is likely that MPNSTs result from additional mutations in cells that already possess altered neurofibromin expression. Such mutations are likely to be responsible for the high CD44 expression in the MPNST cell lines examined in this study. The identification of these mutations will better our understanding of the mechanisms by which CD44 promoter activity and possibly splicing are regulated in Schwann cells.

Compared to cultures of normal human Schwann cells, these MPNST cell lines expressed significantly higher levels of CD44 and CD44 splice variants, including the epitope encoded by exon v6. This epitope was not expressed in detectable levels by normal cultured Schwann cells, verifying that CD44v6 expression is not simply a feature of Schwann cells in vivo. This finding is consistent with our previous data from malignant rat schwannomas (11) and our present observations of MPNST tissue and suggest that the CD44v6 epitope might be a marker for malignant transformation in Schwann cells. If CD44v6 is reliable in this regard, then its detection in Schwann cell tumors could provide a means to diagnose the onset of MPNSTs earlier than is presently possible, reducing the morbidity associated with these tumors.

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REFERENCES


CD44 Expression Is Aberrant in Benign Schwann Cell Tumors Possessing Mutations in the Neurofibromatosis Type 2, but not Type 1, Gene


Cancer Res 1997;57:4889-4897.

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