Changes in glycoconjugate production have been reported for tumor cells. In this study, we investigated the glycoconjugate expression pattern in normal human melanocytes and in a panel of 6 human melanoma cell lines with different metastatic capacity after s.c. inoculation into nude mice. Glycoconjugates were labeled in vitro with [35S] sulphate and [3H] glucosamine, purified from cells and culture medium by column chromatography and identified by treatment with specific glycosidases. Characterization of the purified glycoconjugate fractions as well as alcian-blue staining of xenograft lesions revealed that hyaluronic acid (HA) is the main glycoconjugate produced by all cell lines. Highly metastatic cell lines expressed higher levels of HA than melanocytes and than weakly metastatic or non-metastatic cell lines. In addition, a shift in dominance from chondroitin-sulphate proteoglycan to heparan-sulphate proteoglycan was observed with increasing metastatic capacity. We also studied the expression and binding activity of the HA receptor CD44. Immunoprecipitation experiments indicated high CD44 synthesis only in highly metastatic cell lines, but FACS analysis demonstrated approximately the same surface expression in melanocytes as in all cell lines. Adhesion assays to immobilized HA showed that CD44 can be present in an inactive or an active conformation. Our data suggest that a combination of increased HA production and the expression of CD44 on the cell surface may be associated with high metastatic potential of human melanoma cell lines in nude mice.

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The composition of the cell surface and the micro-environment play important roles in tumor progression, especially metastasis formation. To create an environment conducive to tumor-cell invasion, a balance has to be reached between extracellular-matrix (ECM) degradation and production of new ECM components. Local disintegration of the ECM network results in expansion and subsequent hydration of the basic substance. In this environment, glycosaminoglycans bind water accumulating at the tumor invasion zone due to vascular perturbation (Gabbert, 1985). This process is often accompanied and enhanced by the deposition of excessive amounts of hyaluronate (Knudson et al., 1989; Konishi et al., 1990) and/or chondroitin sulphate (Iozzo, 1988; Moczor et al., 1993; Caux et al., 1992).

In tumors, changes in glycoconjugate expression are prominent at the invasion zone: not only the production of excessive quantities of various types of normal glycoconjugates by tumor cells, but also the deposition of chemically altered glycoconjugates, resulting in a pathologic ECM (Pauli and Knudson, 1988). A number of studies have been performed on animal tumor cell lines with different metastatic capacity derived from the same parental line (Kimata et al., 1983; Turley and Tretiak, 1985; Schwartz-Albiez et al., 1988; Pogany et al., 1989; Robertson et al., 1989). Strongly increased expression of HA was found in highly metastatic sub-lines as compared with the weakly metastatic variants (Kimata et al., 1983; Turley and Tretiak, 1985). An important and specific role in tumor-cell invasion was reported for heparan-sulphate proteoglycans (HSPGs) in highly metastatic animal carcinoma and melanoma cell lines (Turley and Tretiak, 1985; Pogany et al., 1989; Robertson et al., 1989) and for chondroitin-sulphate proteoglycans (CSPGs) in a highly metastatic lymphoma cell line (Schwartz-Albiez et al., 1988). Few studies have been published on glycoconjugate expression by human tumor cell lines with different metastatic capacity (Moczor et al., 1993; Caux et al., 1992; Timar et al., 1989). A human melanoma sub-line with 8-to ten-fold capacity to form liver colonies after intrasplenic injection than its parental counterpart was found to have a dominance of HSPG over CSPG at the cell surface (Timar et al., 1992).

Several studies have shown that CD44 can function as a receptor for HA (Aruffo et al., 1990; Thomas et al., 1992). Moreover, CD44 and some isoforms of CD44 are associated with tumor-cell dissemination in some human malignancies (Birch et al., 1991; Horst et al., 1993; Wielenga et al., 1993) and in animal tumor model systems (Güntert et al., 1991). Study of the involvement of CD44 in melanocytic tumor progression has indicated that i.v. inoculation of nude mice with human melanoma-cell variants expressing high levels of CD44 gives rise to more extensive lung colonization than inoculation with cell-line variants expressing low levels of CD44 (Birch et al., 1991). However, the same group was unable to confirm the association after testing a broad spectrum of different melanoma cell lines (East et al., 1993).

The purpose of our study was to investigate whether a correlation exists between the metastatic potential of a panel of human melanoma cell lines after s.c. inoculation into nude mice and their glycoconjugate expression pattern in vitro. Since we found that the level of HA was markedly increased in the frequently metastasizing cell lines, we also studied the expression and functional activity of the hyaluronate receptor CD44, on these cell lines and on cultured normal human melanocytes.

**MATERIAL AND METHODS**

**Cell lines and tissue-culture conditions**

All cell lines were derived from human melanoma metastases, and included 1F6, 550, M14, Mel57, BLM and MV3 (Danen et al., 1993). All cell lines were cultured in Dulbecco's modified Eagle's medium containing penicillin G (50 U/ml) and streptomycin (50 μg/ml), and supplemented with 2 mM glutamine and 10% heat-inactivated FCS. Cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. Isolation and propagation of normal human foreskin melanocytes was performed as described (Smit et al., 1989) in Ham's F10 supplemented with 2% Ultroser-G synthetic serum (GIBCO, Grand Island, NY), glutamate, penicillin, streptomycin, 0.1 mM IBMX (Sigma, St. Louis, MO) and 16 nM phorbol 12-myristate 13-acetate (PMA) (Sigma).

[35S] sulphate and [3H] glucosamine labeling of cells

For radioactive labeling of the glycoconjugates, cells were grown to about 80% confluency in 150-cm² tissue-culture flasks

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and incubated with 20 ml medium containing 20 μCi [35S] sulphate/ml and 10 μCi [3H] glucosamine (Amersham, Houten, The Netherlands) for 24 hr at 37°C. At the end of the labeling, culture medium was removed, centrifuged to remove cell debris and further processed for glycoconjugate analysis. After washing 3 times with PBS, cells were harvested from the bottom of the tissue-culture flask by means of a rubber policeman and stored at -80°C until further analysis.

Isolation of glycoconjugates from cells and culture medium

All extractions and subsequent fractionations were performed in the presence of a mixture of protease inhibitors [1 mM phenylmethylsulphonylfluoride (PMSF)/5 mM benzamidine-HCl/10 mM n-ethylmaleimide/0.1 M 6-aminohexanoic acid/5 mM iodoacetamide/10 mM sodium EDTA].

Labeled human melanoma cells were extracted with 4 M guanidine-HCl/50 mM sodium acetate (pH 5.8) and stirred for 16 hr at 4°C. The residue obtained after centrifugation (20,000g for 30 min at 4°C) was extracted for an additional 16 hr. The combined supernatants were extensively dialyzed at 4°C against 7 M urea/50 mM Tris-HCl (pH 6.8).

Secreted glycoconjugates were isolated from the culture medium. The culture medium was evaporated to dryness and the residue dissolved in 4 ml 7 M urea/50 mM Tris-HCl (pH 6.8). Unincorporated label was removed by gel filtration on a Bio-Gel P2 column (72.0 × 1.6 cm). The peak eluting in the void volume of the column was used for further purification.

Purification of glycoconjugates by Q-Sepharose-HL chromatography

Urea extracts from the melanoma cells or culture medium were chromatographed after centrifugation (100,000g for 30 min at 4°C) and filtration through a 0.2-μm filter on a column (1.6 × 10.0 cm) of Q-Sepharose-HL. The following solvents were used for this FPLC column: A, 7 M urea/50 mM Tris-HCl (pH 6.8); B, 7 M urea/1 M NaCl/50 mM Tris-HCl (pH 6.8). After application of the sample, a linear gradient was used with a flow rate of 2 ml/min. The gradient started at 0% B for 10 min and rose to 100% B at 60 min. The solvent composition was held at 100% B for 10 min, followed by equilibration back to 0% B. Fractions of 1 ml were collected and glycoconjugates were monitored by determination of the radioactivity in the fractions. The glycoconjugate-containing fractions were pooled, dialyzed against distilled water and stored at -20°C.

Identification of glycoconjugates

Glycoconjugates were identified according to established procedures (Van den Heuvel et al., 1989). Enzymes used were: heparitinase (Seikagaku Kogyo, Tokyo, Japan), chondroitinase ABC and (Streptomyces) hyaluronidase (Sigma). The specificity of the glycosidases was first checked on glycosaminoglycan standards. Heparitinase digestion was performed at 43°C for 18 hr in 0.1 M sodium acetate/10 mM calcium acetate (pH 7.0). The enzyme was used at a concentration of 1 U/ml. Chondroitinase ABC digestion was performed at 37°C for 18 hr in 0.1 M Tris-HCl (pH 8.0) at an enzyme

**FIGURE 1** – Histology of BLM xenografts (a, b) and visualization of glycoconjugates in a BLM xenograft (c) and in cultured BLM cells (d). H-E-stained sections of tumor lesions show some compact areas of tumor cells (a), while other parts of the same lesion contain dispersed tumor cells with large intercellular spaces (b). Alcian-blue-positivity suggests the presence of glycoconjugates, mainly in the large intercellular spaces (c). Bar, 50 μm. Similar results were observed with MV3 cells.
concentration of 0.25 U/ml. Hyaluronidase digestion was performed at 37°C for 16 hr in 50 mM sodium phosphate (pH 5.0); the enzyme was used at a concentration of 10 U/ml. Quantitative proportions of glycosaminoglycans were determined by analyzing the radioactivity after BioGel P100 chromatography.

To test for the presence of glycoconjugates in xenograft lesions, formalin-fixed and paraffin-embedded sections from s.c. tumors and from lungs containing metastases were stained using the alcian-blue technique at pH 2.8. To determine the glycoconjugate profile present in alcian-blue-positive xenograft lesion sections from s.c. tumors and from lungs containing metastases were pre-incubated for 1 hr (and overnight) with various glycosidases, as described above, before alcian-blue staining. Parallel sections were treated with HNO₂ for specific chemical degradation of HS.

Analytical procedures
Protein content was determined using the Bio-Rad protein assay using BSA as a standard.

Antibodies
The following MAbs were used: WT31 anti-CD3 (Tax et al., 1983) as a negative control; CD44 MAbs NKI-P2 (Pals et al., 1989); KM201, R7166.7 and 5F12 (Miyake et al., 1990); A1G3 (Liao et al., 1993); A3D8 (Haynes et al., 1983); and 4B4 anti-β1-integrin (Telen et al., 1983).

Immunoprecipitation
Sub-confluent monolayer cell cultures (75 cm²) were labeled overnight at 37°C in methionine-free Eagle's minimum essential medium supplemented with 0.3 mCi [³⁵S] methionine and 10% dialyzed FCS. Before harvesting, cells were washed twice with PBS. NP40-lysis buffer [0.5% NP40, 15 mM NaCl, 10 mM Tris (pH 7.5), 1.0 mM PMSF and 4 μg/ml aprotinin] was then added for 10 min at 4°C, resulting in lysis of the labeled cells. Cell debris were scraped out of the culture flask, repeatedly aspirated into syringes, and forced through needles with decreasing diameters. Absorption to Concanavalin A-Sepharose (Pharmacia, Uppsala, Sweden) was performed to isolate the glycoprotein fraction from the total cell lysate. Finally, immunoprecipitation was performed with NKI-P2 MAb, as described (Danen et al., 1993). Immunoprecipitates were analyzed on a 10% SDS-polyacrylamide gel; gels were dried and exposed to X-ray films (Eastman Kodak, Arnhem, The Netherlands) at ~70°C using intensifying screens. Equal numbers of counts of the glycoprotein fraction were used for immunoprecipitation.

FACS analysis
After short trypsinization of sub-confluent monolayers, cells were incubated for 30 min at 4°C with purified MAbs diluted in PBS containing 0.5% BSA and 0.02% sodium azide. After 3 washes with PBS/BSA/sodium azide, cells were incubated with FITC-conjugated F(ab')₂ fragments of rabbit anti-mouse

**FIGURE 2** – Alcian-blue staining of sections of s.c. lesions of non-metastatic IF6 (a, b) and highly metastatic MV3 (c, d) cells without (a, c) and after (b, d) treatment with chondroitinase ABC. Note alcian-blue positivity in the MV3 tumor (c) and its disappearance after chondroitinase ABC treatment (d). Similar results were found with chondroitinase AC and hyaluronidase. Alcian-blue positivity did not disappear after pre-treatment with heparitinase. No alcian-blue positivity was found in the IF6 tumor (a). Bar, 50 μm. The results with BLM tumors were similar to those with MV3 tumors, while 530, M14 and Mel 57 tumors showed results similar to those with IF6 tumors.
Ig antibodies (Dako, Glostrup, Denmark) and analyzed on an Epics Elite (Coulter, Mijdrecht, The Netherlands).

Adhesion assays

HA isolated from human umbilical cord was purchased from Sigma. Polystyrene microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were pre-coated with 150 μl protamine chloride (200 μg/ml) (KABI, Stockholm, Sweden) for 2 hr at room temperature. After washing with PBS, 100 μl HA solution (200 μg/ml carbonate buffer, pH 9.6) were added and incubated overnight at room temperature. The plates were then washed with PBS. To avoid non-specific binding, plates were subsequently coated for 2 hr at 37°C with 1% (w/v) BSA in PBS and then again washed with PBS.

Labeling of cells and adhesion was performed as described (Danen et al., 1993). In adhesion inhibition assays, cells were incubated with the appropriate MAbs for 30 min at 4°C before seeding into the wells.

RESULTS

In order to study the expression of glycoconjugates in relation to metastatic potential, we used a set of human melanoma cell lines with differing metastatic behavior. This set included 4 non-metastatic or sporadically metastasizing (IF6, 530, M14, Mel57) and 2 frequently metastasizing (BLM, MV3) cell lines after s.c. inoculation into nude mice (Danen et al., 1993). Cultured normal human melanocytes were also included.

Expression and identification of glycoconjugates in tumors

Macroscopic examination of s.c. tumors of the 6 melanoma cell lines demonstrated marked differences in the consistency of the tumors. While the xenografts of the non-metastatic or weakly metastatic cell lines demonstrated a solid aspect, the lesions of the frequently metastatic cell lines showed a mucin-like aspect. Conventionally stained histological sections of the s.c. tumors exhibited exclusively compact areas of tumor cells in IF6, 530, M14 and Mel57 xenograft lesions. In contrast, s.c. tumors and tumor lesions in the lungs of the highly metastatic cell lines BLM and MV3 exhibited, in addition to besides compact areas (Fig. 1a), parts showing dispersed tumor cells with large intercellular spaces (Fig. 1b) containing a mucin-like substance. Alcian-blue staining of sections from lesions of all cell lines showed marked positivity in BLM and MV3 tumors, indicating large quantities of glycoconjugates (Fig. 1c). Very weak positivity, or none, was found in tumors derived from the other melanoma cell lines (not shown). To determine whether the glycoconjugates in BLM and MV3 lesions were produced by the cells of the tumor or by its stromal cells, alcian-blue staining was performed on cells cultured on coverslips. About half of the tumor cells of both cell lines contained markedly positive intracellular vacuoles (Fig. 1d). Separate sections were treated with various glycosidases (chondroitinase AC, chondroitinase ABC, hyaluronidase and heparitinase) to determine the composition of the glycoconjugates in BLM and MV3 xenograft lesions (Fig. 2). Incubation with all enzymes except heparitinase resulted in the disappearance of all or nearly all alcian-blue staining. HN02 treatment, like heparitinase, had no effect. These results strongly suggest that the glycoconju-
Expression and identification of glycoconjugates in vitro

Metabolic labeling with [35S] sulphate and [3H] glucosamine was used to identify the glycoconjugates produced by normal human melanocytes and the different melanoma cell lines in vitro. Purification of the cellular extract on Q-Sepharose-HL resulted for all the cell lines in a 3H peak separated from a 35S peak (Fig. 3), and which eluted as a broad peak between 0 and 0.40 M NaCl from the column and the 35S peak between 0.60 and 0.80 M NaCl.

Chromatography of urea extracts from cellular fractions on Q-Sepharose-HL resulted in elution profiles comparable with those observed for the culture media (Fig. 3), demonstrating that glycoconjugates were secreted in the culture medium.

The composition of the 35S-labeled glycoconjugates, from the cellular fraction as well as from the culture medium, showed differences between the cell lines studied. A clear change from CS to HS dominance was observed in the 35S peak with increasing metastatic capacity of the cell line. This suggests similar changes in the ratio CSPG/HSPG (Table I).

Since the 3H peak contains other products originating from [3H] glucosamine, in addition to HA, this fraction was treated with hyaluronidase to determine the production of HA. As shown in Table I, the highly metastatic cell lines produce by far the most HA, which is in line with the findings in the xenograft lesions.

Expression of CD44

Since the production of HA is clearly elevated in the frequently metastasizing cell lines, and since CD44 may function as a receptor for HA, we were interested in expression of this receptor. To determine the level of biosynthesis of CD44 we performed immunoprecipitation experiments with [35S] methionine-labeled melanocytes and melanoma cells, using MAb NKI-P2, which recognizes an epitope present on all CD44 isoforms. CD44 could be precipitated from all cell lines, including melanocytes, but synthesis was high in BLM and MV3, intermediate in melanocytes, Mel57 and IF6, and very low in 530 and M14 (Fig. 4). In addition, we studied the expression of CD44 by FACS analysis using the same MAb. We found marked CD44 expression on the surface of BLM and MV3 and of the other melanoma cell lines and normal melanocytes (Fig. 5). These data indicate that, in addition to the increased synthesis and secretion of HA, synthesis of the CD44-HA receptor is markedly higher in the highly metastatic cell lines, whereas CD44 surface expression is high on all cell lines tested.

Adhesion to hyaluronic acid

To investigate whether the expression of CD44 was reflected by the capacity to adhere to HA, we performed adhesion assays to immobilized HA. Two melanoma cell lines, 530 and M14, did not adhere to HA, while the other cell lines, including melanocytes, adhered moderately (Mel57) or strongly (melanocytes, IF6, BLM, MV3) (Fig. 6). Pre-treatment of all cell lines with hyaluronidase or chondroitinase did not alter their binding capacity (data not shown).

To demonstrate that binding to HA was CD44-mediated, adhesion inhibition assays were performed with a panel of 6 different CD44 MAb. As a negative control, 4B4, an adhesion-blocking β1-integrin MAb (Danen et al., 1993) was used. As Figure 6 shows, 2 CD44 MAb gave partial (5F12) or almost-complete to complete (R7166.7) inhibition of adhesion. No inhibition of adhesion of melanocytes and all melanoma cell lines was found with CD44 MAb KM201 (data not shown) and with the β1-integrin MAb 4B4, although all cells markedly express β1 integrins on their surface (Danen et al., 1993). From these results we conclude that, while all cells express CD44 on their surface, CD44-mediated adhesion to HA is highly variable.

DISCUSSION

Glycoconjugates are involved in various normal cellular processes, such as cell growth migration and adhesion. They may also be involved in various steps of the metastatic cascade. Transformed cells and tumor cells frequently demonstrate altered glycoconjugate patterns, decreased HS and increased HA and CS expression (Iozzo, 1988). In addition, rodent and human tumor cell lines with different metastatic capacity express altered glycoconjugate patterns (Caux et al., 1992; Moczar et al., 1993), especially increased HS/CS ratios with increased metastatic potential (Timar et al., 1989, 1992).

Since little is known about glycoconjugate expression by human melanoma cell lines in relation to spontaneous metastatic...
sis, we investigated the expression pattern of melanocytes and a panel of 6 human melanoma cell lines with different metastatic behavior after s.c. inoculation into nude mice (Danen et al., 1993). As in other studies using weakly and strongly metastatic mouse-lymphoma cell lines, Lewis lung-tumor cell lines, mouse-melanoma cell lines and various rodent cell lines (Turley and Tretiak, 1985; Schwartz-Albiez et al., 1988; Pogany et al., 1989; Robertson et al., 1989), we found that the production of glycoconjugates is enhanced in the highly invasive and metastatic cell lines. Like Timar et al. (1992), we observed a shift from CSPG to HSPG dominance with increasing metastatic capacity.

Although changes in glycoconjugate synthesis have been found in co-cultures of colon carcinoma and smooth-muscle cells (Iozzo et al., 1989) and in fibroblasts cultured in the presence of tumor-conditioned-medium (Edward et al., 1992), our findings and observations by other groups (Turley and Tretiak, 1985; Kimata et al., 1983) indicate that most glycoconjugates in xenografts are synthesized by melanoma cells, since only a few stromal cells are present in tumor lesions, and melanoma cells show marked production in vitro.

Our finding that the HA receptor, CD44, was expressed on melanocytes and on all melanoma cell lines tested confirms previous findings (Birch et al., 1991; East et al., 1993). The level of cell-surface expression does not correlate with the metastatic propensity of the cell line, since all our cell lines including melanocytes show high expression. A possible explanation for the higher synthesis in the frequently metastasizing cell lines BLM and MV3 may be that CD44, possibly after binding of “endogenous” HA, is shed into the culture medium. This may induce continuous synthesis of CD44, but this is not proven. Furthermore, East et al. (1993) reported that CD44 mRNA levels in their melanoma cell lines did not correlate with the levels of surface expression, suggesting that the control of surface expression is regulated post-transcriptionally. In line with them, we found that melanoma cells express the 90-kDa CD44 form.

Since CD44 is a cell-adhesion molecule which functions in matrix binding and in cell migration and invasion, we studied the functional activity of the receptor by determining its involvement in adhesion of the cells to immobilized HA. In agreement with East et al. (1993), we did not find a correlation between CD44 cell-surface expression and the ability of the cells to adhere to HA. Pre-treatment of the non-adhering or weakly adhering cell lines with hyaluronidase or chondroitinase did not induce or enhance binding to HA, therefore absence of adhesion was not due to binding of “endogenous” HA to the receptor. From these results, it appears likely that CD44 is present in an inactive state on the non-adhering cell lines. Cells that express CD44 do not constitutively bind HA, but binding activity can be induced by antibodies recognizing specific epitopes (Lesley et al., 1993). As activating antibodies were not available to us, we could not prove this supposition. Interestingly, East et al. (1993) reported a reproducible but not significant increase of the binding capacity of CD44-positive human melanoma cells to HA with 2 different CD44 MAb.

The binding of our melanoma cells to HA is indeed CD44-mediated, as was proven by the partial or complete inhibition with anti-CD44 MAb. Regarding the mechanism of inhibition of adhesion by the R7166.7 MAb, we know that this is not due to direct blocking of the HA binding site, since the MAb does not block binding of fluorescein-labeled soluble HA to the cell lines (data not shown). Our interpretation of these data is that binding to immobilized HA might be blocked by MAb R7166.7 by restricting CD44 mobility or distribution. Alternatively, steric hindrance may be involved.

Our results with the KM201 Ab conflict with the report that this antibody should inhibit binding to HA (Thomas et al., 1992). However, we and others (Kincade; Neame and Isacke, personal communication) were unable to demonstrate KM201 binding to human CD44.

In conclusion, we found that (i) melanocytes and non-metastatic or weakly metastatic human melanoma cells secrete lower levels of glycoconjugates than do highly metastatic cell lines; (ii) there is a shift from CSPG to HSPG dominance with
increasing metastatic capacity; (iii) HA is the main glycoconjugate produced by all melanoma cell lines; (iv) the synthesis of HA and its receptor, CD44, are markedly higher in the frequently metastasizing cell lines; and (v) CD44 is strongly expressed on all cell lines but seems to be present in an inactive conformation on some cell lines. Our results confirm the potential role of HA and its receptor in the metastatic process.

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REFERENCES


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