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Differential Expression of Proteoglycans on the Surface of Human Melanoma Cells Characterized by Altered Experimental Metastatic Potential

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Heparan sulphate (HS) and chondroitin sulphate (CS) proteoglycans (PGs) frequently have opposite biologic functions in cell-matrix adhesion as well as in the regulation of cell proliferation. Data revealed that sulphated glycosaminoglycans (sGAGs) (sugar chains of PGs) are differently expressed in tumor cells characterized by different metastatic potential; the more metastatic cells contain a higher HS/CS ratio. As the proliferative capacity of tumor cells is also frequently altered in parallel with their metastatic potential, it was not clear whether observed PG alterations reflect changes in cell proliferation or metastatic potential. The cell-associated PG expression and sGAG biosynthesis was studied in tumor cells of human melanoma lines characterized by different experimental metastatic potential to the mouse liver but similar** in vitro/in vivo ***proliferation rates. Using antibodies against PGs we found different expression of PG epitopes in melanoma lines, except from the melanoma antigen. Unlike the low CSPG (melCSPG) metastatic melanoma cells, the cell line with high metastatic capacity contained a higher proportion of positive cells for surface-HSPG without the coexpression of certain cartilage-type CSPG epitopes (recognized by MAb HSFPG 529) as well as by an increased pericellular HS/CS ratio due to intracellular accumulation/retention of CS. Immunocytochemistry of adherent cells revealed HSPGs at substrate-attached membrane areas only in cases of highly metastatic melanoma cells. These data further support our view that the absolute or relative dominance of HSPGs over CSPGs at the cell surface of metastatic tumor cells can be considered a marker of a more metastatic phenotype. (Am J Pathol 1992, 141:467–474)

Cell membrane proteoglycans (PGs) are known to be involved in cell-cell and cell-matrix interactions.[1] Some of them are extracellular components like the large PGs aggrecan, perlecan, and versican, or the small PGs decorin and fibromodulin[1–4] belonging to the chondroitin sulphate proteoglycan (CSPG), aggrecan, versican, and decorin, or the heparan sulphate proteoglycan (HSPG), perlecan, family. Interest has been focused on cell membrane-type PGs. Important members of this subgroup are syndecan and betaglycan; both are glycosylated by heparan sulphate (HS) and chondroitin sulphate (CS) chains,[3,4] fibroblast-membrane HSPGs[5] and CSPG associated with MHC-II.[1,3]

PGs were implicated in the regulation of cell growth.[6–8] It was demonstrated that the membrane HSPG, betaglycan, serves as coreceptor for transforming-growth factor-β (TGFβ), providing and/or concentrating TGFβ for the signaling-TGFβ receptor.[9] Another membrane HSPG was shown to serve as a captive device for FGF through its HS side chains, a link essential for FGF effect.[10–12] The sugar chains of HSPGs, HS and heparin, were also shown to have a direct role in the regulation of the proliferation of smooth muscle, endothelial, and tumor cells.[6,13]

Transformed cells are frequently characterized by altered GAG pattern, decreased HS, and increased CS content,[13–15] as well as accumulation of HSPG and HS in the Golgi region.[16] Well-characterized human tumor PGs

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are the melCSPG,[17,18] HSPG of colon carcinoma,[19] and glioma.[20] Meanwhile, the function(s) of tumor cell PGs is not clear. The most important consequence of the malignant transformation is the ability of tumor cells to invade host tissues and form distant colonies, i.e., metastases. Data show that rodent tumors with different metastatic capacity are characterized by altered sGAG pattern; there is frequently an increased HS/CS ratio in more metastatic variants.[21]

We have established and described human melanoma xenograft lines, HT168/HT168-M1, with different liver-colonizing potentials.[21,22] In this model there is no difference in cell proliferation characteristics or expression of melanoma markers.[22] However, using this model this study describes increased HSPG and decreased CSPG epitope expression on the surface of highly metastatic cells. The alterations at the PG level were also reflected in the glycosylation pattern; due to a decreased CS content, the HS/CS ratio increased in the pericellular compartment of highly metastatic cells. *In situ* immunochemistry showed surface and substrate attachment site accumulation of HSPG epitopes only in the case of highly metastatic human melanoma cells.

Materials and Methods

Human Melanoma Xenografts

HT168 human melanoma xenograft was established from human melanoma cell line A2058[21] (provided by L. A. Liotta, NCI, Bethesda, MD). HT168-M1 xenograft was derived from liver metastasis of HT168 tumor.[22] HT168-M1 cells proved to be eight- to tenfold more metastatic than the HT168 cells in liver-colonization assay after intrasplenic injection of 10^6 cells.[22]

In Vitro *Cultures*

HT168 cell line was established from the sc tumor whereas HT168-M1 was from a liver metastasis of the HT168 tumor; both were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Boehringer, Mannheim, Germany) at 37°C in 5% CO_2. The cells were either scraped from the plastic surfaces by rubber policeman or digested at room temperature by 0.2% trypsin for 5 minutes. The human and melanoma character of the tumor cells was tested regularly by chromosomal analysis as well as by measuring the expression of human and melanoma antigens.[22]

Biosynthetic Labeling of GAGs

Cells in stationary growth phase (10^5 cells/cm^2) were incubated with a medium containing 10 μCi/ml ^{3}H-glucosamine (8 mC/mmol, CEA, Saclay, France) for 24 hours at 37°C. The medium was removed, and the cells were washed with 3 × 5 ml phosphate-buffered saline (PBS) and digested by 0.05% trypsin/PBS for 15 minutes at room temperature. The trypsinization was stopped by adding 20 μl FCS. Cells were scraped from the plastic, sedimented at 2000 rpm for 5 minutes at 4°C, and the supernatant, containing the pericellular proteins, was removed. The cell pellet was washed with 2 × 1 ml PBS.

Separation of GAGs

The sugar chains were released from the PG core proteins by reductive alkaline hydrolysis in 0.05 mol/l NaOH in the presence of 1 mol/l $NaBH_4$ at 45°C for 24 hours. The hydrolysates were desalted on Biogel P2 column equilibrated with 10 mmol/l Tris-HCl, pH 8.2. The excluded ^{3}H-macromolecules either were precipitated by cetylpyridinium chloride (CPC) or were submitted to ion-exchange chromatography using DEAE-Trisacryl column (IBF, France), equilibrated with 10 mmol/l Tris-HCl buffer, pH 8.2. Elution was performed with linear NaCl concentration gradient.

Characterization of GAGs

Analytical scale precipitation of ^{3}H-GAGs was carried out according to Wasteson.[23] CS was identified by its sensitivity to chondroitinase AC (Seikagaku-Kogyo, Japan), whereas HS was identified by deaminative cleavage with nitrous acid. The specific degradation was assessed from the elution diagrams of the starting materials and the hydrolysates obtained on a Biogel P6 column using 0.2 mol/l acetic acid-pyridine, pH 5.0.

Anti-PG Antibodies

Anti-CSPG Antibodies

ME.31.3, an IgG1 (provided by Dr. M. Herlyn, Wistar Institute, Philadelphia, PA), was produced against core protein epitope of a 250 kd melanoma associated CSPG antigen.[24]

HFPG-529/4 (IgM) and MK172 (IgG1) were produced against human fetal articular cartilage CSPG recognizing

core protein epitopes at the linkage region of the CS stub.[25,26]

Anti-HSPG Antibodies

FW16 antibody produced against pig skin fibroblast membrane HSPG (mw 60 kd) recognizing a core protein epitope was provided by M. F. Watt (ICRF, London, UK).

BN42 antibody produced against rat liver basement membrane HSPG (J.V. Hascall, NIH, Bethesda, USA) was a gift of B. Nusgens (Liege University, Liege, Belgium).[27]

Flow Cytometry and Indirect Immunofluorescence

For flow cytometry, confluent phase melanoma cells were collected by scraping or by trypsinization, suspended (10^6 cells/sample), and labeled at 4°C (native cells) or after 10 minutes of fixation in methanol (MetOH). For immunofluorescence, cells were grown on glass coverslips at a density of 10^5 cells/cm^2.

Cells in suspension or on coverslips were labeled either at 4°C or room temperature. First, 10% nonimmune goat serum was applied for 10 minutes, then the samples were incubated with the primary antibody (1:100 working dilution for monoclonals and 1:50 for polyclonals) for 30 minutes. After washings in PBS (three times), an appropriate FITC-labeled secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG, Nordic) was applied (working dilution 1:20 in PBS) for another 30 minutes. After another washing in PBS (three times), the unfixed samples were fixed in 1% paraformaldehyde/PBS (10 min), while the fixed ones were kept in PBS at 4°C. Negative controls for flow cytometry or immunofluorescence were exposed to the secondary FITC-labeled antibody alone. Experiments in which the background fluorescence was higher than the autofluorescence of unstained cells were excluded from this study. Cells adherent to coverslips were viewed and photographed in a Vanox-Olympus epiluminescence microscope or in a confocal laser scanning microscope (BRL). Cell suspensions were tested in FACStar flow cytometer (Becton Dickinson, Sunnyvale, CA) by measuring at least 10^4 cells/sample (in triplicate) and the percent of positive cells (the fluorescence of which was higher than the intensity of 90% of the negative control population) was determined.

Results

Flow Cytometric Analysis and Localization of CSPG Epitopes

The surface of HT168 and HT168-M1 cells were equally positive for the melanoma-CSPG antigen (~60%). One of the cartilage CSPG epitopes, detected by MAb MK172, was equally represented, though at a low level, on the surface of the melanoma cells, but another cartilage, CSPG epitope, recognized by MAb HFPG529, was present only on the native low metastatic HT168 cells (Table 1). After MetOH fixation, which allows the detection of intracellular antigens as well, similar CSPG antigen pattern was detected in the two cell populations (Table 1). The lower proportion of positive cells in fixed samples compared with the native ones is probably due to the loss of antigenicity upon fixation. In the adherent cells of both lines, the melanoma CSPG antigen was present at the apical cell surface and in the cytoplasm (Figure 1). In permeabilized adherent cells, MAb HFPG529 bound diffusely to cytoplasmic domains of the low metastatic HT168 cells (Figure 2a) but it did not label the high metastatic variant (Figure 2b). There was a diffuse faint cytoplasmic positivity in a small subpopulation of cells in both tumor cell lines reacted with MAb MK172 antibody (not shown).

Flow Cytometry and Localization of HSPG Epitopes

On the surface of native highly metastatic HT168-M1 cells, the two anti-HSPG antibodies were detected at low frequency (15%) but HT168 cells remained negative (Table 2). The majority of the HSPG epitopes on HT168-M1 cells were masked according to the significantly increased surface labeling after trypsinization (Ta-

Table 1. *Expression of CSPG Antigens in Human Melanoma Cells (Flow Cytometry)*

	Cell surface		Cell surface + cytoplasm	
Antibodies	HT168	HT168-M1	HT168	HT168-M1
ME.31.3	51.5 ± 10.3	69.4 ± 15.4	41.8 ± 11.7	37.2 ± 7.6
HFPG529	47.8 ± 9.2	0.5 ± 0.4	40.6 ± 8.3	0.0 ± 0.0
MK172	20.6 ± 5.2	28.5 ± 6.3	23.6 ± 4.2	22.2 ± 3.3

Data are expressed in % of positive cells. Each point represents mean of three measurements (±SD). Cell surface; immunolabeling of native cells at 4°C. Cell surface + cytoplasm = immunolabeling of MetOH fixed cells.

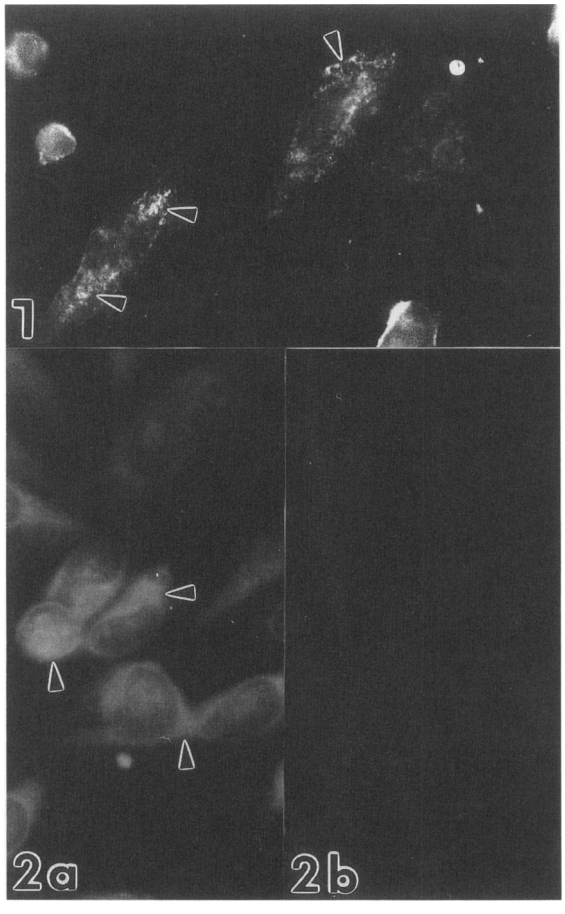

Figure 1. *Immunofluorescent localization of melanoma-CSPG antigen in adherent HT168 cells. Methanol fixation/permeabilization; primary Ab: ME.31.1, secondary antibody: goat anti-mouse IgG-FITC. Note the heterogenous staining of the apical cell surfaces (arrows), ×600.*

Figure 2. *Immunofluorescent localization of cartilage-type CSPG antigen in adherent human melanoma cells. Methodology as in Figure 1, but the primary antibody was MAb HFPG529.* **a**: *In the HT168 line there is a diffuse cytoplasmic staining in some cells (arrows) but negative cells are more frequent, ×600;* **b**: *HT168-M1 cells are uniformly negative, ×600.*

ble 2). One of these antigens, recognized by the polyclonal antibody PAb BN42, was also masked on the surface of a small proportion of HT168 cells (Table 2). After MetOH fixation, both cell lines exhibited cytoplasmic positivity. The PAb FW16-positive cells are dominant over PAb BN42-positive ones in the low metastatic HT168 cell population. In the highly metastatic HT168-M1 cells, the expression of epitopes recognized by PAb FW16 was lower than in HT168 cells, while the HSPG epitope, recognized by PAb BN42, was similarly represented (Table 2).

In adherent cells, PAb FW16 was completely absent from the apical surface of HT168 cells (Figure 3a) but was diffusely present on HT168-M1 cells as discrete domains (Figure 3b). This difference disappeared after permeabilization because both cell types showed positivity with PAb FW16. In the cytoplasm of the adherent low metastatic HT168 cells, the distribution of PAb FW16-recognized antigen was diffuse (Figure 4a). While in HT168-M1 cells, beside the diffuse cytoplasmic localization, these epitopes were concentrated at the focal contact areas at lamellopodia and filopodia (Figure 4b). The difference between the two cell types in the labeling of the ventral membranes with PAb FW16 was further supported by confocal laser scanning microscopic analysis (Figure 5a, b). The staining pattern of PAb BN42 at the cell surface and in the cytoplasm was essentially the same than in the case of PAb FW16 (not shown).

Biochemistry of Cell-associated sGAGs

The incorporation of ^{3}H-glcN into cell-associated sGAGs was predominant in the pericellular (surface) compartment compared with the intracellular one, which represented only one-fifth and one-fourth of the total cellular incorporation in HT168 and HT168-M1 cells, respectively (Table 3). There was no difference in the precursor incorporation into the pericellular sGAGs, but a 50% increase was detected in the incorporation into intracellular sGAGs in HT168-M1 cells compared with HT168 (Table 1).

After β-elimination and ion-exchange chromatography, different sGAG composition was found on the surface of the two melanoma lines. There was no difference in the precursor incorporation into HS but due to the lower level of incorporation into CS in HT168-M1 cells, the HS/CS ratio was three-fold higher on the surface of the high metastatic cells (6:1) than on the low metastatic counterpart (2:1) (Figure 6). Intracellularly, there was no difference between the two cell lines in the precursor incorporation into HS but due to the increased incorporation into

Table 2. *Expressions of HSPG Antigens in Human Melanoma Cells (Flow Cytometry)*

	Cell surface		Cell surface-masked		Cell surface + cytoplasm	
	HT168	HT168-M1	HT168	HT168-M1	HT168	HT168-M1
FW16	0.0 ± 0	13.6 ± 2.9	1.5 ± 0.8	30.3 ± 8.2	73.7 ± 9.2	47.5 ± 6.0
BN42	0.0 ± 0	18.7 ± 5.6	21.5 ± 7.2	71.5 ± 7.0	27.9 ± 5.4	37.2 ± 4.4

Data is expressed in % of positive cells. Each point represents mean of three measurements (±SD). Cell surface = immunolabeling native cells at 4°C. Cell surface-masked = immunolabeling of trypsinized cells at 4°C. Cell surface + cytoplasm = immunolabeling after MetOH fixation.

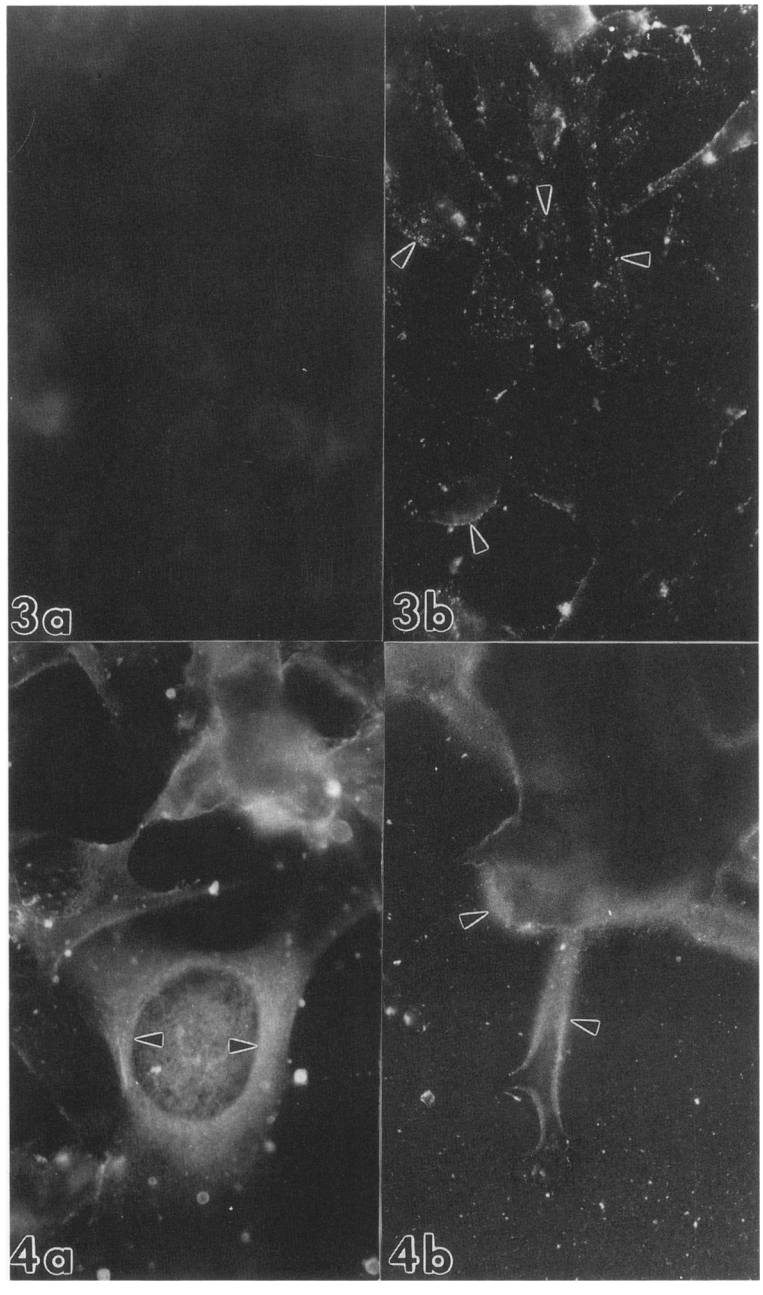

Figure 3. *Immunofluorescent localization of membrane-type HSPG epitopes on the surface of adherent human melanoma cells. Methodology: 4°C labeling with PAb FW16 and goat anti-rabbit IgG-FITC.* **a:** *There is no labeling on the surface of HT168 cells. ×900.* **b:** *Membrane-HSPG epitopes are localized into discrete surface domains (arrows) in HT168-M1 cells. ×900.*

Figure 4. *Immunofluorescent localization of membrane-type HSPG epitopes in human melanoma cells. Methodology: methanol fixation/permeabilization, immunlabeling as in case of Figure 3.* **a:** *There is a diffuse cytoplasmic—mostly perinuclear—staining in HT168 cells (arrow), ×1600.* **b.** *In HT168-M1 cells HSPG epitopes (recognized by PAb FW16) are concentrated at those plasma membrane areas, which are in contact with the solid substrate (arrow), ×1600.*

CS in the highly metastatic cells, the ratio between HS and CS was much lower (around one) in HT168-M1 cells than in the HT168 (around two) (Figure 6). Based on these data, we concluded that the different sGAG composition in the two cell lines is not due to different HS biosynthesis, but rather due to the intracellular accumulation or retention of CS in the highly metastatic cells.

Discussion

The biosynthesis of PGs and their GAG chains is altered during malignant transformation, resulting in a decreased HS sulphation and/or production and increased CS content in the majority of tumor types.[13] The GAG expression is altered in tumor cells during progression and metastatization.[21] In several rodent and in some human metastasis models the increased metastatic capacity is associated with an increased HS/CS ratio in sGAGs.[21] We have studied these phenotypic changes in human melanoma xenografts, HT18/HT168, characterized by different proliferation rate as well as experimental liver metastatic capacity.[21] In this model, we found an increased HS/CS ratio in sGAGs in the more metastatic tumor but the total GAG content was not altered.[21] Due to the fact that be-

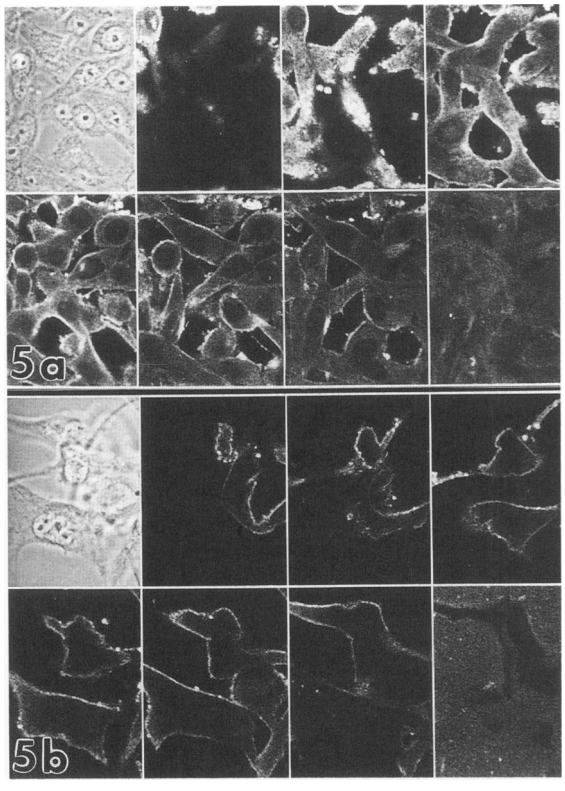

Figure 5. *Confocal laser scanning microscopy of adherent human melanoma cells labeled for intracellular membrane-type HSPG epitopes. Methodology: as in the case of Figure 4. Six serial optical sections (1 μm) were photographed in case of an area.* **Upper left:** *apical membrane;* **lower right:** *substrate attached site.* **a:** *HT168 cells. Note the diffuse cytoplasmic staining (upper center and right) and the lack of labeling at attachment membranes (lower right), ×500.* **b:** *HT168-M1 cells. Note the continuous membrane labeling as well as a significant fluorescence at the membranes attached to substrate (lower right), ×500.*

side the metastatic capacity, the proliferation rate was also different in these melanomas, it was not clear how the difference in sGAG-composition was related to the biological properties. To approach the problem, we have established human melanoma xenograft lines (HT168/HT168-M1) with different liver metastatic capacity but similar *in vivo/in vitro* growth rate.[22] In this model, we found again characteristic differences in cellular PG expression. In the cell-associated sGAG compartment, there was only a slight difference between the two tumor lines, unlike in the relative ratio between HS and CS of the individual fractions (surface or intracellular). The intracellular sGAG was the minority of total cellular sGAGs and the highly metastatic variant, HT168-M1, contained 50% more intracellular sGAG due to the increased CS content than the low metastatic HT168. However, the intracellular HS content was similar in the two cell types. More pronounced differences were found in the pericellular (surface) sGAG composition; the HS/CS ratio increased on highly metastatic melanoma cells due to a decrease in CS content.

Table 3. *Incorporation of ^{3}H-glcN into Cell-associated sGAGs in Melanoma Cells*

sGAG fraction	HT168	HT168-M1
Pericellular	76.83 ± 8.2	81.13 ± 4.5
Intracellular	18.90 ± 1.8	28.00 ± 1.4

Data are expressed as cpm $\times 10^3/10^5$ cells and represent means of three measurements ± SD. Cells were labeled with ^{3}H-glcN, the peri- as well as the intracellular fractions were separated and sGAGs were isolated using β-elimination and ion-exchange chromatography.

In harmony with these observations, flow cytometric measurements revealed characteristic differences between the two melanoma lines in the expression of PG epitopes. A cartilage-CSPG recognizing antibody, MAb HFPG529, did not bind to the highly metastatic melanoma cells unlike its low metastatic counterpart. MAb HFPG529 recognized Ser-Gly-CS-stub epitope on the high molecular weight cartilage CSPG,[26] an epitope that is common in high molecular weight CSPGs.[3] Previous biochemical studies indicated the presence of at least four CSPG species in HT168 cells having approximate molecular weights of 1000, 600, 500, and 400 kd based on the K_{av} values on Sephadex CL 4B.[28] However, only the 1000-kd species was characterized by hialuronic acid (HA) binding capacity.[28] HA-binding characterizes several CSPGs including aggrecan, versican, PG-M, aortic high molecular weight CSPG[2,3], as well as melCSPG.[17,18] Furthermore, there are immunologic similarities between the high molecular weight CSPGs.[3] Interestingly, melanoma CSPG antigen was detected not only in human melanomas but in other tumor cell types as well as in some normal cells including chondrocytes.[29] Decreased expression of a common CSPG motif, Ser-Gly-CS-stub, in the highly metastatic melanoma cells, HT168-M1, may affect the CS-biosynthesis as well. On the other hand, there was no difference between the lines in the expression of melanoma specific CSPG antigen, which supports those previous observations that the expression of this antigen is not linked to the progression.[18] The fact that in highly metastatic melanoma cells the high molecular weight CSPG disappeared[28] when no alteration was found in melCSPG epitope expression suggests that the high molecular weight HA-binding CSPG in HT168 cells is most probably not the melCSPG. Further studies have been undertaken to solve this question.

More characteristic differences were found in basement membrane-type and surface-type HSPG expression in our human melanoma cells. There was an increased surface HSPG epitope expression in the HT168-M1 highly metastatic line; however, the majority of those surface HSPG antigens was masked by trypsin-sensitive domains. Interestingly, there was no difference in the intracellular expression of HSPG antigens between the two melanoma lines. Morphologic study showed that in the

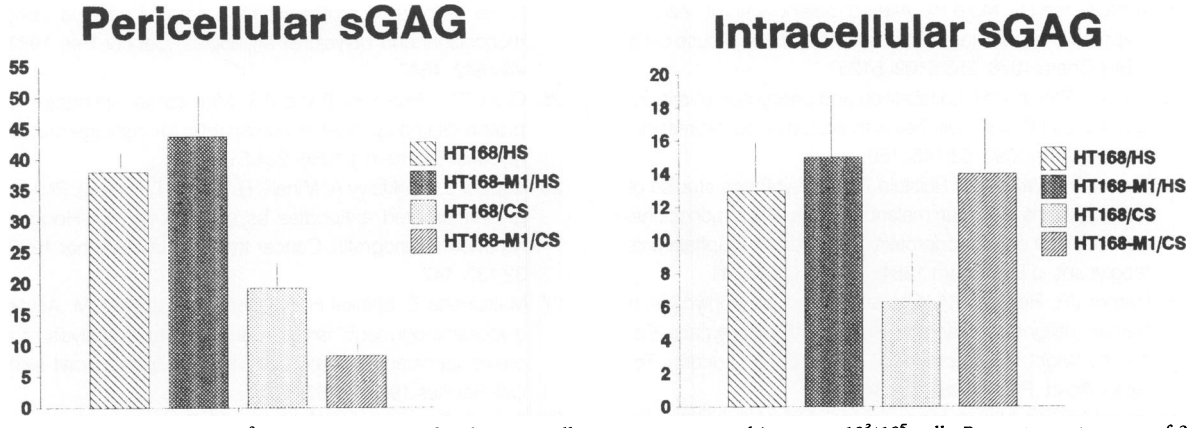

Figure 6. *Incorporation of ^{3}H-glcN into sGAGs of melanoma cells. Data are expressed in cpm $\times 10^3/10^5$ cells. Bars represent means of 3 samples ± SD.*

highly metastatic cells, the surface-type HSPG-antigens recognized by the PAb FW16, was localized not only on the apical plasma membrane but at the substrate attached membrane areas, suggesting that, at least this HSPG, may play a role in attachment. The melCSPG antigen was not present at those areas in the HT168/HT168-M1 model, on the contrary to previous reports in another melanoma line.[30] The A2058 human melanoma cell line, the parent line of the HT168 tumor xenograft, contains at least two different HSPG species,[31,32] a hydrophobic transmembrane molecule with high affinity to thrombospondin and a basement membrane-type HSPG with high affinity to fibronectin.[31] In fact, in HT168/HT168-M1 cells we have shown the presence of two HSPG species characterized by ~200 and ~400 kd[32] and in harmony with this, in the present work, we found binding of an anti-basement membrane (PAb BN42) and anti-plasma membrane (PAb FW16) HSPG antibody to HT168/HT168-M1 cells. The lower level of expression of surface HSPG antigens in low metastatic HT168 cells is due to an intracellular accumulation or retention of these molecules as it was proven by biochemical measurements.[32]

The function(s) of PG molecules in human melanoma cells is still unknown. They might play an important role in matrix adhesion[1,3,4] but according to recent observations they might be involved in the autocrine regulation of the cell proliferation as well.[6] Functions of HSPGs and CSPGs are frequently opposite in adhesion and cell proliferation.[1,3,4,6–8] Our present and previous results[21] suggest that absolute or relative dominance of HSPGs over CSPGs at the surface of human melanoma cells could be considered as a marker of a more metastatic phenotype.

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