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Expression of a Decorin-Like Molecule in Human Melanoma

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Decorin, a member of the family of small leucin-rich proteoglycans, has originally been described as a secreted proteoglycan component of the connective tissues, and has been implicated in the negative regulation of cell proliferation directly or via interactions with TGF-β. It was reported to be generally absent from tumor cells. Here we show that human melanoma cell lines express a decorin-like molecule. We detected decorin mRNA by RT-PCR in 7 out 7 human melanoma lines characterized by various metastatic potential. Using polyclonal antiserum against the core protein of decorin, the typical 80-120 kD glycanated form as well as a high molecular weight aberrant version (200-210 kD) of decorin were demonstrated by Western blot technique in the culture supernatants as well as in lysates of human melanoma cells. Finally, decorin epitope was also demonstrated immunohistochemically in human melanoma xenografts, as well as in tumor cells of surgically resected melanomas but not in melanocytes of nevi. The expression of this aberrant decorin did not inhibit the *in vitro* or *in vivo* growth of human melanoma cells, and it was independent of their metastatic potential. Human melanoma cell lines expressing aberrant decorin retained sensitivity to the antiproliferative and gelatinase-stimulatory effects of exogenous TGF-β. (Pathology Oncology Research Vol 7, No 4, 260–266, 2001)

Keywords: decorin, human melanoma, mRNA, protein, TGF-beta

Introduction

Decorin[1] belongs to the family of small leucin-rich proteoglycans (SLRPs)[2] containing predominantly chondroitin sulfates or dermatan sulfate. It is involved in the regulation of various cellular functions such as proliferation, adhesion and migration. Decorin can bind extracellular matrix components including fibronectin, thrombospondin and several types of collagen, and regulate collagen fibrillogenesis.[2] It was shown to interact with TGF-β via its core protein, and has been suggested to function as a "natural inhibitor" of this cytokine.[3, 4] However, other observations indicate that interactions of decorin with TGF-β do not necessarily block (or in some cases, may even enhance) the cytokine activity.[5, 6] Decorin has also been implicated in the direct control of cell proliferation. The upregulation of decorin gene expression was associated with growth arrest of several cell types.[7, 8] In gene transfer experiments decorin transfection of CHO cells or human tumor cell lines caused suppression of *in vitro* growth and tumorigenicity, associated with upregulation of $p21^{Cip1/WAF1}$ protein.[9, 10, 11] Decorin has originally been described as a secreted PG expressed by a wide variety of tissues of mesenchymal origin including tumoral stroma[12] but not by transformed cells.[2]

Melanoma expresses two known transmembrane CSPGs, the human homologue of the rat NG2 (melanoma-associated CSPG, MCSP) and CD44, both involved in cell-matrix interactions.[13,14] Previous studies on human melanoma cell lines of various experimental metastatic potentials indicated

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Abbreviations: CS, chondroitin sulfate; PG, proteoglycan; TGF, transforming growth factor

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that these cells express a significant amount of CSPG[15] and a proportion of it was a yet uncharacterized low molecular weight secreted PG species.[16] The first suggestion of a possible decorin expression in human melanoma came from our studies detecting decorin-related antigen epitope in melanoma cell lines.[17] In the present study we demonstrated the presence of this PG in several human melanoma cell lines, at mRNA and protein level as well. The expression of this decorin-like molecule did not correlate with the growth and metastatic potential of the tumor cells, or with their sensitivity to exogenous TGF-β. Immunohistochemistry however detected decorin antigen in human malignant melanoma but not in benign melanocytic tumor samples, suggesting a transformation-related expression pattern.

Materials and Methods

Cell lines and culture conditions

The A2058 melanoma cell line was provided by LA Liotta (NCI, Bethesda, MD). The HT168 and HT168-M1 lines are derivatives of A2058.[18] The HT199 line was developed in our institute.[19] M24met was kindly provided by B.M. Mueller (Scripps Research Institute, La Jolla, CA). The WM983B and WM35 cell lines were gifts from M. Herlyn (Wistar Institute, Philadelphia, PA). The HT58 human non-Hodgkin's lymphoma line was provided by L. Kopper (Semmelweis University, Budapest). HepG2 hepatocellular carcinoma is an ATCC cell line. Cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum (Sigma, St. Louis, MD), 2 mM glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 μg/ml gentamicin sulfate (all from Gibco BRL, Life Technologies, Paisley, Scotland), at 37°C in a 5% CO_2 atmosphere.

Cell proliferation assay

4 x 10^3 melanoma cells were allowed to adhere to flat-bottomed 96-well tissue culture plates overnight in serum-containing medium, then the medium was replaced with serum-free medium and TGF-β (R&D Systems, Minneapolis, MN; 0.1, 1.0, 10 ng/ml) was added. At the end of the 96-h incubation period, a colorimetric assay (MTT test) was performed as described earlier.[20]

Determination of collagenase activity

4 x 10^3 cells, plated in flat-bottomed 96-well tissue culture plates were treated with TGF-β (0.2, 1.0, 5.0 ng/ml) for 48 h in serum-free media. The conditioned media were centrifuged and the supernatants were frozen until use for zymography. The measurements of type IV collagenase activity were performed as described.[20] Briefly, samples were applied on an 8% SDS-PAGE (Serva Feinbiochemica, Heidelberg, Germany) gel containing 0.3 mg/ml gelatin (Sigma). After electrophoresis the gels were incubated overnight at 37°C, and stained with Coomassie Brilliant Blue (Serva), followed by destaining in methanol/acetic acid. Enzyme activities were quantitated by Eagle Eye II video densitometer (Stratagene GmbH, Heidelberg, Germany).

RNA isolation and cDNA synthesis

Total RNA was isolated from 10^7 cells by homogenization in guanidium isothiocyanate/2-mercaptoethanol followed by phenol extraction and ethanol precipitation. 200 ng of total RNAs were used for cDNA synthesis with 0.25 μg random hexamers (Promega, Madison, WI), 0.3 mM dNTP (Promega) and 200 U MMLV reverse transcriptase (Gibco BRL) for 45 min at 42°C in a total volume of 20 μl.

PCR primers and conditions

From each cDNA products 4-4 μl was amplified with 4 different primer pairs selected to cover almost the entire coding region of decorin gene *(Figure 1).* The oligonucleotide primer pairs used were: 1F: GGC AAA TTC CCG GAT TAA – 1R: TTG TGT CAG GGG GAA GAT; 2F: GAT GAG GCT TCT GGG ATA – 2R: CAT CTG GTT CAG TCC ATT; 3F: AAT GGA CTG AAC CAG ATG – 3R: GGA GCT TCA CTT GGA CAA; 4F: GGA GCT TCA CTT GGA CAA – 4R: CAT TGC AGT TAG GTT TCC. An additional aliquot was used for β-actin amplification (primers: forward: GTG GGG CGC CCC AGG CAC CA – reverse: CTC CTT AAT GTC ACG CAC GAT TTC). PCR amplifications were performed in 50 μl reactions, containing 3.5 mM (1F-1R and 4F-4R) or 2.5 mM (2F-2R, 3F-3R and β-actin) $MgCl_2$, 0.2 mM dNTP (Promega), 2 U Taq polimerase (Boehringer

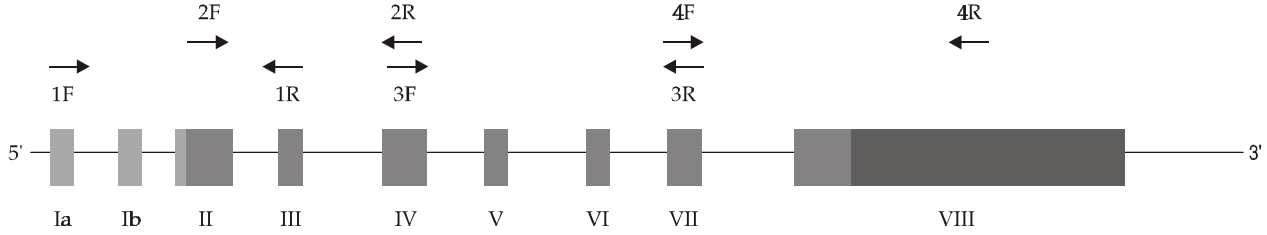

Figure 1. The intron-exon organization of decorin gene,[27] and the primer pairs used.

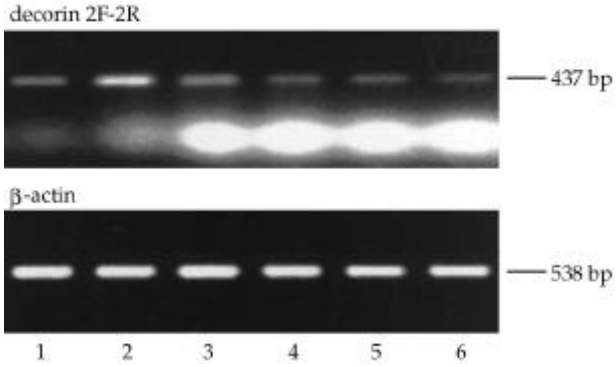

Figure 2. Expression of decorin mRNA in melanoma cell lines. RT-PCR analysis with decorin-specific (2F-2R, upper panel) and β-actin-specific primers (control, lower panel). Lane 1, WM35; lane 2, HT199; lane 3, HT168-M1; lane 4, HT168; lane 5, WM983B; lane 6, M24met.

Mannheim, Indianapolis, IN), and 0.5 μM of the forward and the corresponding reverse primers. Reactions were performed in a PTC-100 thermocycler (MJ Research Inc., Watertown, MA) for 35 cycles (94°C – 1 min, 60°C – 30 sec, 72°C – 90 sec). cDNA from cirrhotic liver and from HT58 human lymphoma cells served as positive and negative control, respectively.

Southern blot analysis of PCR products

PCR products were separated on a 1.5% agarose gel, and transferred to Hybond-N+ membrane (Amersham, Little Chalfont, UK). Southern blots were hybridized with [^{32}P]dCTP-labelled decorin probe: human PG40 cDNA[1], a kind gift from E. Ruoslahti (Burnham Inst., La Jolla, CA), labelled with random priming kit (Boehringer Mannheim, specific activity >10^9 cpm/mg DNA). Hybridization was carried out at 65°C for 2 h in Quick Hyb solution (Stratagene) containing 10^6 cpm/ml radioactivity, then the filters were washed and exposed to Kodak X-Omat films.

Western blot analysis

Samples prepared from lysates of cultured cells, from 100x concentrated serum-free culture supernatants or isolated PGs, containing equal amounts of protein, were size fractionated by SDS-PAGE (8%) and transferred to nitrocellulose or PVDF membranes (Bio-Rad, Munich, Germany). Nonspecific binding sites were blocked by non-fat milk (for cell lysates and supernatants). The immune reaction was performed using polyclonal anti-human decorin antibody (PG40; Telios, La Jolla, CA; 1:500), or nonimmune rabbit serum as negative control, and the binding of the secondary biotinylated donkey anti-rabbit Ig (Amersham; 1:1500) was detected using streptavidin-peroxidase (Amersham; 1:3000) and DAB. HT58 lymphoma cells were used as negative control.

Flow cytometry

Cells were fixed in methanol for 10 min, and nonspecific protein binding sites were blocked with 1% BSA in PBS. Cells were then incubated with PG40 antibody (1:100, 1 h), followed by incubation with FITC-conjugated donkey anti-rabbit IgG (Amersham; 1:100, 1 h), and analysed for fluorescence with FACStar flow cytometer (Becton Dickinson, Sunnyvale, CA). Nonimmune rabbit serum was used for negative control. Cells were considered positive when fluorescence intensity was above that of 90% of control cells.

Immunohistochemistry of melanoma xenografts

Formaldehyde-fixed, paraffin-embedded tissue sections from HT168-M1 melanoma liver metastases (in SCID mouse) were deparaffinized. After antigen retrieval by proteinase K digestion (75 μg/ml, 37°C, 10 min), the slides were incubated with PG40 antibody (1:3000), and a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and biotinylated tyramine amplification were used to detect the antigen-antibody reaction. The negative control was prepared by omitting the primary antibody.

Immunohistochemistry of human skin melanoma samples

Representative samples from 8 consecutive, surgically removed pigment cell tumors (diagnosis: malignant melanoma – 5 cases, and intradermal nevus – 3 cases,

Table 1. **Expression of aberrant decorin at mRNA and protein level by human melanoma cell lines**

Cell line	*mRNA expression RT-PCR – primers:*				*Protein expression*		
					Western blot		*FCM*
	1F-1R	*2F-2R*	*3F-3R*	*4F-4R*	*cells*	*supernat.*	*(% of cells)*
A2058	+	n.d.	+	+	+	n.d.	63
HT168	n.d.	+	n.d.	n.d.	+	+	76
HT168-M1	(+)	+	+	+	+	+	70
HT199	+	+	+	+	+	+	75
M24met	(+)	+	+	(+)	+	+	37
WM983B	+	+	+	(+)	+	+	78
WM35	(+)	+	+	+	+	+	71

+: strong expression; (+): weak expression (detected only after hybridization with labelled decorin probe); FCM: flow cytometry; n.d.: not determined

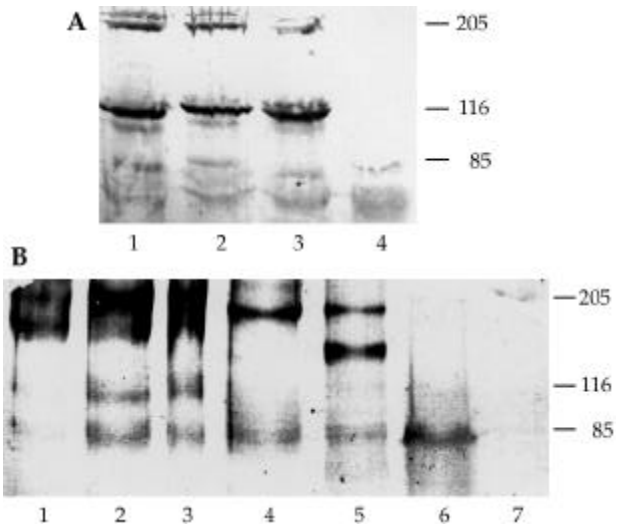

Figure 3. Western blot analysis of aberrant decorin in melanoma cell lysates (a) and cell culture supernatants (b). a) Lane 1, HT168-M1; lane 2, HT199; lane 3, M24met; lane 4, negative control (FCS). b) Lane 1, HT168-M1; lane 2, HT168; lane 3, HT199; lane 4, M24met; lane 5, WM983B; lane 6, WM35; lane 7, HT58 (negative control).

Department of Dermatology, Semmelweis University) were taken with the patients' informed consent, snap-frozen using isopenthane and liquid nitrogen. Methanol-fixed frozen sections were blocked with non-immune goat serum (1:2 in PBS, 30 min) and incubated with PG40 antibody (1:50, 1 h), then biotin-conjugated goat anti-rabbit IgG was applied (Amersham, 1:200, 1 h), followed by Streptavidin-Texas Red (Amersham, 1:100, 30 min). In the negative controls the primary antibody was omitted but nuclear counterstaining with Hoecht's dye was performed. Sections were viewed in an Olympus (Tokyo, Japan) Vanox epifluorescent microscope.

Tumor cell injections

Single-cell suspensions, containing 10^6 viable cells were inoculated intrasplenically in SCID mice or immunosuppressed CBA mice, in a volume of 0.05 ml.[18,19] Recipients were killed when they became moribund or 10 weeks after tumor cell implantation.

Results

By RT-PCR using four primer pairs amplifying different regions of human decorin cDNA *(Figure 1),* we detected expression of decorin mRNA in 7 out of 7 melanoma cell lines studied (*Figure 2, Table 1).* The identity of the PCR products was ascertained by hybridization with [^{32}P]dCTP-labelled decorin cDNA (not shown). Following this, the expression of decorin was studied at protein level, by the Western blot technique using a polyclonal antiserum against the core protein. The antigen was detected both in the cell lysates and in culture supernatants of all the melanoma lines *(Figure 3, Table 1)*. However, besides the typical smear of 80-120 kD, characteristic for glycanated decorin, higher molecular weight bands also appeared at 200-210 kD. Flow cytometric analysis of the expression of decorin antigen at population level indicated that (with the exception of M24met, which contained a smaller decorin-positive subpopulation) all the melanoma cell lines expressed decorin antigen in the majority of the cell population (63-78%; *Table 1*).

The expression of human decorin antigen was also demonstrated on paraffin-embedded primary (data not shown) and metastatic liver tumors of human melanoma xenograft using immunohistochemistry (*Figure 4a,b*). Decorin antigen was localized pericellularly and to the interstitial matrix of melanomas suggesting that it is expressed *in vivo,* similarly to the melanoma cell lines. Following this surgically removed melanocytic tumor samples were analyzed by immunohistochemistry. None of the studied three benign melanocytic tumors (common nevi) exhibited positive labeling for the decorin-related epitope *(Figure 4c),* but five out of five frozen human skin melanoma samples showed strong positive labeling in the cytoplasm of the majority of tumor cells *(Figure 4d),* beside the expected stromal signal (data not shown). On the other hand, cells of the covering epithelia including normal melanocytes did not show reactivity with the anti-decorin antibody used (not shown).

The melanoma cell lines showed comparable growth characteristics *in vitro* (not shown). With the exception of the weakly tumorigenic and nonmetastatic WM35 line, all the melanoma lines formed tumors when injected subcutaneously[18, 22, 23] or intrasplenically in immune deficient mice, while the liver colonization capacity varied among the cell lines *(Table 2).* There was no correlation between the expression of decorin antigen and a reduced growth rate, tumorigenicity or metastatic potential.

Table 2. **Tumorigenicity and metastatic potential of melanoma cell lines after intrasplenic injection[a]**

Cell line	*Tumorigenicity (spleen)*	*Metastatic potential (liver)*
A2058	+	high
HT168	+	medium
HT168-M1	+	high
HT199	+	high
M24met	+	low
WM983B	+	low
WM35	+/–	non-met

[a]Injection of 10^6 cells in the spleen of immunocompromised mice

Since decorin is known to be able to bind TGF-β and it was suggested to function as an inhibitor of this cytokine, we studied the effect of TGF-β on these aberrant decorin-expressing melanoma cells. *Figure 5* indicates that the majority of the cell lines retained sensitivity to the proliferation-inhibitory effect of TGF-β to a certain extent and this effect was independent of the expression level of the aberrant decorin. Furthermore, in three out of four cell lines studied (HT168-M1, M24met, WM983B, WM35) TGF-β stimulated the secretion of MMP-2 or MMP-9 type IV collagenases *(Figure 6).*

Discussion

The NG2-homologue MCSP and CD44 are the two transmembrane PGs expressed regularly by melanoma cells, where MCSP expression seems to be tumor-type specific.[13,14] However, secreted CSPGs have not been found to be expressed by melanoma cells before. There are two subclasses of secretory CSPGs, SLRPs and modular multidomain CSPGs, which play an important role in organizing pericellular matrices. Screening of human melanoma cell lines for the expression of proteoglycan antigen epitopes revealed the presence of aggrecan and decorin epitopes in melanoma cells, suggesting that some of the secretory CSPGs might also be present.[17] Our present analysis provided evidence for the expression of the decorin gene both at mRNA and protein level in seven out of seven human melanoma cell lines and five out of five human skin melanoma tumor samples. According to our previous data, human melanoma cell lines contain hardly any DS,[16,24] therefore, the GAG chain in "melanoma-decorin" is most probably CS. Previous analysis of decorin expression in human skin did not detect the presence of the core protein either in epithelial cells or in melanocytes.[25] This finding was supported by the immunofluorescence analysis described in this study, showing the expression of a decorin-like molecule in human melanoma samples, while benign tumors of melanocytes and nevi remained negative. On the other hand, the level of the expression of this gene in the melanoma cell

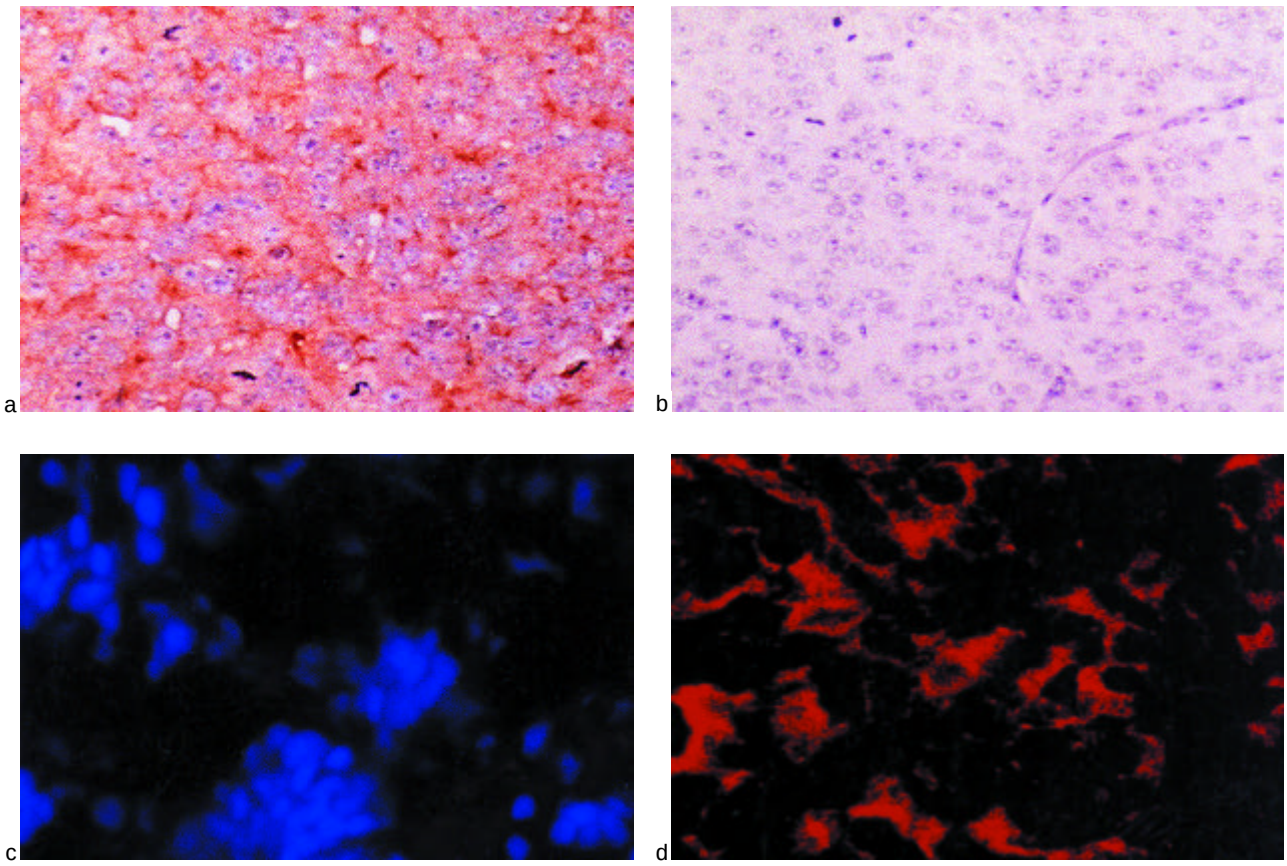

Figure 4. a,b) Immunohistochemistry of decorin antigen in human melanoma xenograft tumor (HT168-M1 melanoma liver metastasis in SCID mice). a) Note the intense reaction (DAB) in the pericellular and extracellular tumor matrix. b) Negative control sample prepared by omitting the primary antibody. c,d) Dual channel immunofluorescence of decorin epitopes in skin sample of benign and malignant human melanocytic tumors. c) Example of benign melanocytic tumor (nevus): note the absence of cytoplasmic labeling. Nuclear staining (blue) identifies melanocytes. d) Decorin epitopes are present in the cytoplasm of melanoma cells (red fluorescence) in a malignant melanoma.

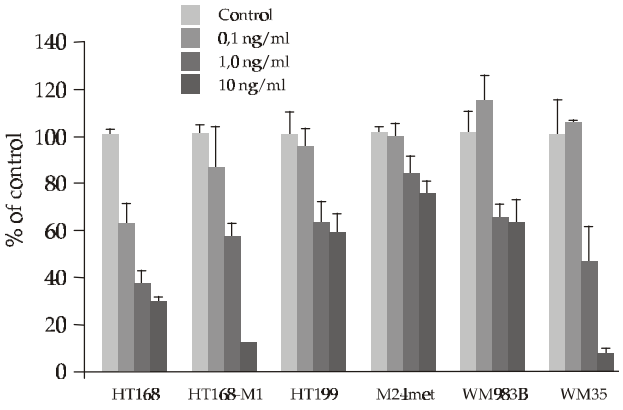

Figure 5. Effect of TGF-β on the proliferation of melanoma cells (96 h, MTT test). Data are given as % of control (mean +/– SD of 3-5 parallel samples; results of a representative experiment of 2 to 5 separate experiments).

lines was independent of their metastatic potential. These observations suggest that the expression of the decorin-like molecule is associated with malignant transformation but not to progression of melanoma.

The constitutive expression of this aberrant decorin does not influence cell proliferation, tumorigenicity or metastatic potential of the human melanoma lines, nor their sensitivity to the biological effects of TGF-β. A potential explanation for the lack of effect of this decorin-like molecule on melanoma cell proliferation is degradation by MMPs.[26] Alternatively, our observations may suggest that the aberrant decorin in human melanoma is different from the wild type form. Further studies are required to determine the precise alterations of the melanoma-decorin gene and its protein product. The Western blot experiments showed that the aberrant decorin produced by the melanoma cells is not secreted completely. The intracellular fraction seemed to be under- or unglycanated, while the glycanation of the secreted molecule varied. The presence of 200-210 kDa glycanated bands vs. the average ~80-120 kDa size found in other tissues further suggests the presence of a different protein compared to the wild-type decorin core.

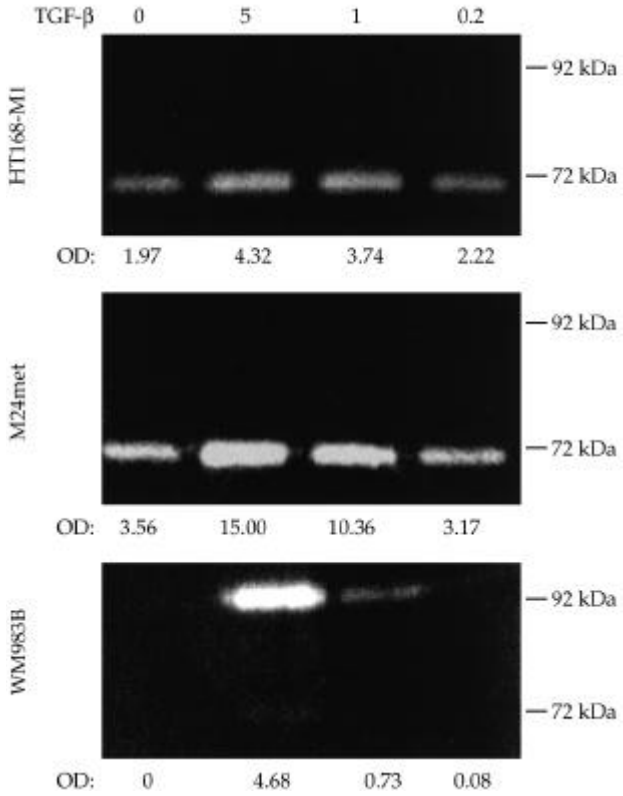

Figure 6. Effect of TGF-β treatment (48 h) on collagenase production by melanoma cells. Samples of supernatants were subjected to gelatin zymography, and the density of the resulting bands was quantitated (OD, optical density).

Expression of aberrant proteins in malignant tumors is a common feature and is due to the genetic instability of malignant cells. The presence of some of these proteins in tumor cells has no functional significance – as it was demonstrated in our study for "melanoma-decorin" – but could be exploited for the differentiation of benign lesions from their malignant counterparts. The differential diagnosis of malignant melanoma and its predecessor, the so-called dysplastic nevus is an extremely difficult task for surgical pathologists. The neoexpression of two CSPG species, NG2/MCSP[13] and the aberrant decorin (this study), in transformed melanocytes seems to be a new molecular marker of human melanoma which could be developed for the diagnosis of this disease.

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