Autocrine Stimulation by Osteopontin Contributes to Antiapoptotic Signalling of Melanocytes in Dermal Collagen

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ABSTRACT

The growth of melanocytes, the pigment-producing cells of the skin, normally is restricted to the epidermis. Transformed melanocytes, which have invaded the dermis, however, have gained the ability to grow in this new environment and to counteract apoptosis induced by the dermal connective tissue. The expression of genes contributing to the survival of melanocytes in the dermal environment, therefore, might be involved in melanoma development. Using a differential display approach, we identified osteopontin as such a gene. In melanocytes, expression of the secreted adhesion protein OPN was up-regulated by the melanoma-inducing receptor tyrosine kinase Xmrk as well as by the fibroblast growth factor receptor, which plays a decisive role in human melanoma. Activation of both receptors triggered survival of melanocytes in three-dimensional dermal collagen gels. Competition experiments revealed that the presence of OPN in the medium as a result of receptor signaling was contributing to these effects. Addition of exogenous OPN allowed melanocytes to adhere, spread, and survive in three-dimensional collagen gels, whereas in the absence of OPN, the cells underwent apoptosis. The integrin $\alpha_3\beta_1$, known to be involved in melanoma cell survival and growth was identified as an OPN receptor, which points to an OPN-mediated cross-talk between growth factor receptors and this integrin receptor in melanocytes. In summary, we could show that in melanocytes growth factor receptor-induced secretion of OPN can promote antiapoptotic signaling and mediate appropriate interactions with the extracellular matrix in an autocrine way. Our findings suggest a new role of growth factor receptors of the family of receptor tyrosine kinases in processes associated with melanoma development and progression.

INTRODUCTION

Under physiological conditions, melanocytes are restricted to the stratum basale of the epidermis, where they transport pigment-containing melanosomes to adjacent keratinocytes and exhibit a low mitogenic activity. Transformed melanocytes, however, are able to leave the epidermis, to break through the basal membrane, and to invade the dermis (1). This transition from the so-called RGP to the VGP is a biologically and clinically critical step for the establishment of malignant melanomas that exhibit a metastatic competence (1).

A crucial difference between nontransformed and transformed pigment cells is that normal melanocytes cannot survive for longer periods in the dermal environment but melanoma cells can. The survival and continuous growth of transformed cells in the dermal tissue are important steps in melanoma progression (2). Survival of pigment cells in the dermis seems to be dependent on the presence of cell surface molecules that regulate the contact between the cells and the dermal ECM, i.e., components of the dermal connective tissue such as collagen. A surface molecule, which can mediate the adhesion of melanoma cells to dermal collagen thereby inducing antiapoptotic signaling, is the integrin receptor $\alpha_3\beta_1$ (3, 4). The expression of $\alpha_3\beta_1$ has been directly linked to neoplastic progression and tumorigenicity of melanoma (5, 6). Also, bFGF, a growth factor most uniformly expressed by melanoma cells but not by normal melanocytes (7), is able to counteract apoptosis induced in the presence of type I collagen. Addition of this melanoma-associated factor to melanocytes cultured in three-dimensional collagen gels, which mimic the conditions in the dermis in vitro, prevented apoptosis (8). However, the mechanism by which bFGF exerts its positive effect on melanocyte survival is not clear yet.

The receptor for bFGF is a receptor tyrosine kinase that triggers proliferation in melanocytes as well as in melanoma cells, where it is stimulated in an autocrine way (9, 10). Similarly, the receptor tyrosine kinase Xmrk, the overexpression of which is the initiatory event of the formation of highly invasive melanoma in Xiphophorus (11), induces mitogenic signaling in melanocytes and in melanoma cells (12, 13). Remarkably, activation of the Xmrk receptor does not only trigger proliferation, it also stimulates antiapoptotic signaling. We have established a cell system in which Xmrk-specific antiapoptotic and mitogenic signaling can be dissected (Ba/F3 cells; Ref. 14). Using this cell system, we identified genes that have an expression that is regulated either by mitogenic or by antiapoptotic signaling triggered by the melanoma-inducing receptor.

One of the identified Xmrk target genes, with an expression that is correlated with antiapoptotic signaling in Ba/F3 cells, is opn. Additional investigation in pigment cells revealed that OPN can promote survival of melanocytes in three-dimensional gels of the ECM component collagen. This attribute to OPN a new and relevant role in processes correlating with pigment cell transformation and tumor progression in melanoma development.

MATERIALS AND METHODS

Cell Culture and Long-Term Growth. The IL-3-dependent mouse pro-B cell line Ba/F3 (15) and Ba/F3-derived cell lines were cultured in RPMI 1640, with 5% FCS and 5% of supernatant from X63Ag8-653 BVP m-IL-3-expressing cells (16). Ba/F Hm transfectants were generated by electroporation as described previously (17). Before, stimulation cells were washed twice with PBS and starved for 2–6 h in RPMI 1640. Starved cells were treated with IL-3 (5% supernatant) or EGF (50 ng/ml) for the indicated times and collected by centrifugation. Mouse melanocytes [melan-a (18) and melan-a Hm cells (13)] were cultured in DMEM, 10% FCS in the presence of cholera toxin (12 nM), and TPA (200 nM). Human melanoma cell lines (WM35, A375, Bro, IFB, Mel26, D10) were cultured in RPMI 1640 with 10% FCS and were a kind gift of Dr. C. R. Goding and Dr. J. Becker. The MAP/ERK kinase inhibitor PD98059 (Calbiochem, Eschborn, Germany) or the solvent DMSO alone was added 30 min before stimulation. For long-term growth experiments, cells were seeded at 3 $\times$ 10$^5$ cells/well of a 6-well plate in medium containing the indicated factors. After 4 days, cells were stained with trypan blue and counted.

Differential Display Analysis. For identification of Xmrk target genes, a modified differential display procedure according to Liang et al. (19–21) was performed. Amplifications and subsequent analyses were done in parallel from three independent cell cultures for each condition. DNa-se-digested total RNA.
(2.5 μg) from unstimulated and 2-h EGF-stimulated BaF Hm cells were used for RT using one-base-anchored oligodeoxythymidylate primers. Subsequent PCR amplifications of 1 μl of the RT reaction with the appropriate oligodeoxythymidylate primer in combination with an arbitrary 10-mer primer using [α32P]dCTP were performed. The PCR reactions were separated on a 6% polyacrylamide sequencing gel. Gels were dried and exposed to X-ray films. Differentially displayed bands were excised from the gel, and one-third of a gel slice was directly used for reamplification with the primer combinations of the original differential display PCR. PCR products were cloned, using the Sure-Clone kit (Amersham, United Kingdom) and subsequently sequenced. GCG software was applied to compare sequences to Genbank and European Molecular Biology Laboratory databases. The differential expression of the respective genes was confirmed by Northern blot analysis using probes generated by random priming with [α32P]dCTP. The probes used were a 550-bp opn differential display-PCR-fragment or a mouse cDNA probe, which was kindly provided by Dr. L. W. Fisher (22). A 96-well ELISA plastic plate (Greiner, Frickenhausen, Germany) was then used to amplify the respective integrine genes:

***RT-PCR and Northern Blot Analysis.*** After stimulation and/or inhibitor treatment, RNA was isolated with Trizol reagent (Life Technologies, Inc., Karlsruhe, Germany) following the instructions of the supplier. First-strand cDNA synthesis was performed by using Superscript RT reverse transcriptase (Life Technologies, Inc.), using 1 μg total RNA and random hexanucleotides (0.5 μM) in 20 μl following the suppliers protocol. RT reaction (0.5 μl) was then used to amplify the respective integrin genes: α5, 5'-ATCCGACCGAGCACTGTTTCT-3' and 5'-TCACTTCGACTGCTGTGTCG-3' (30 cycles); and β3, 5'-GAGTCGTCGTGGAGTCG-3' and 5'-GGGACGGCTTCGTTTCT-3' (36 cycles). For Northern blot analysis, total RNA was blotted onto Hybond-N nylon membranes (Amersham, United Kingdom). Prehybridization and hybridization were carried out at 42°C in 50% formamide. Filters were hybridized with a [3P]labeled mouse OPN probe that was generated by random priming with [α32P]dCTP. The probes used were a 550-bp opn differential display-PCR-fragment or a mouse cDNA probe, which was kindly provided by Dr. L. W. Fisher (22).

***Western Blot Analysis.*** After stimulation for the indicated times, cells were rinsed twice with PBS and lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 μg/ml leupeptin, and 10 μg/ml apro tinin for 20 min at 4°C. Proteins were separated by SDS-PAGE and analyzed by Western blotting onto nitrocellulose. Filters were blocked for 10 min with 10 mM Tris-CI (pH 7.9), 0.5% Tween, and 1.5% BSA and were incubated at least for 60 min with the first antibody. Horseradish peroxidase-coupled secondary antibodies were used for nonradioactive detection. Monoclonal anti-phospho MAPK (clone 12D4) was from Nanotools (Teningen, Germany). Anti-ERK-2 (C-14), antimouse OPN (P-18), antihuman OPN (K-20), anti-α5 (Q-20), and anti-β3 (N-20) were from Santa Cruz Biotechnology (Santa Cruz, CA).

***Preparation of Collagen Gels and Analysis of Cell Spreading.*** Collagen gels were prepared using Cellagen solution DMEM (pH 7.4) containing type I collagen (ICN Biomedicals, Eschwege, Germany). Cells were seeded into 24-well plates (4 × 103/well) and suspended in the Cellagen solution. After gelation, either melan-a HER-mrk, CM or FCS (5%) containing medium with the respective factors was added. The concentrations were as follows: 8 nM EGF; 0.3 nM bFGF; 100 nM OPN; and 200 nM TPA. Cell morphology and spreading were examined hourly for the first 12 h, and cells were photographed after 48 h.

***Adhesion Assay.*** The adhesion assay was performed according to Smith et al. (23). A 96-well ELISA plastic plate (Greiner, Frickenhausen, Germany) was coated with OPN (100 μg) or BSA (100 μg) overnight at 4°C. The wells were washed twice with PBS and blocked with PBS/1% BSA for 1 h at 37°C. Melan-a cells were preincubated with either anti-α5 (clone H9.2B8; Pharmingen, BD Biosciences, Germany), anti-β3 (clone 2C9.G2; Pharmingen, BD Biosciences), an unspecific control antibody or without antibody in DMEM/0.1% BSA for 15 min at RT, and 5 × 104 cells/well were allowed to adhere to the substrate at 37°C for 2 h. Nonadhering cells were washed off with PBS, and the adhering cells were fixed in 4% paraformaldehyde/PBS for 1 h at RT and stained with 0.5% toluidine blue in 4% paraformaldehyde for 1 h at RT. After three washes with PBS, the color was solubilized using 1% SDS, and the color intensity was measured with an ELISA reader using a filter of 595 nm.

***Determination of Apoptotic Cells.*** Cells (2 × 106) suspended in Collagen solution were seeded over chamber slides (Nunc), and after gelation, the gels were overlayed with DMEM containing 5% FCS without any additives or supplemented with the indicated factors (concentrations see above). For the blocking experiments, either a neutralizing anti-OPN antibody, kindly provided by Dr. Hsin-Fang Yan-Yen (24), was added to the medium at a concentration of 10 μl of serum/ml or anti-α5 (clone H9.2B8) or anti-β3 (clone 2C9.G2) was added to the preincubated cells in a concentration of 5 μg/ml. As control, antibody anti-MAP kinase phosphatase 1 (Santa Cruz Biotechnology, Heidelberg, Germany) was used at a concentration of 10 μg/ml. After 4 days, the cells were fixed in formaldehyde for at least 30 min and after washing with PBS incubated in 0.1% Triton X-100 for 30 min. Cells were washed again in PBS, and the DNA was stained with 1 μg/ml bisbenzimide ( Hoechst 33342) in PBS. After embedding the fixed cells in mounting medium, they were analyzed using an UV microscope. Condensed and fragmented nuclei were counted in a total of 1000 cells from each sample, and the amount of fragmented nuclei was calculated in percentages.

***DNA Fragmentation.*** Cells (4 × 106) were seeded in Collagen on 10-cm culture dishes and cultured for 4 days under different conditions. Cell-containing gels were washed with PBS, and the cells were scraped off the dish. After centrifugation, collagen-containing cell pellets were lysed in 500 μl lysis buffer (0.2% SDS, 100 mM EDTA, and 200 mM NaCl) complemented with protease K (100 μg/ml) and then incubated at 80°C for 1 h. After phenol/chloroform extraction using SST tubes (BD Biosciences, Heidelberg, Germany), the DNA was recovered by ethanol precipitation. The DNA was dissolved in water, complemented with RNase A (500 μg/ml) and gel-loading buffer, and analyzed on an agarose gel.

***RESULTS.***

Identification of the opn Gene as a Gene Induced by the Xmrk Kinase and Involved in Antiapoptotic Signaling. To identify genes, which are regulated by the Xmrk receptor and involved in proliferation and/or survival, we used the well characterized IL-3-dependent cell line Ba/F3 as a sensitive system to analyze mitogenic as well as antiapoptotic signaling. BaF Hm cells are a Ba/F3 derived cell line, which stably expresses a chimeric and thus regulatable Xmrk receptor (HER-mrk). EGF stimulation of HER-mrk induces activation of the Xmrk kinase domain, and treatment of BaF Hm cells with EGF leads to specific induction of intracellular Xmrk signaling (17).

For a differential display analysis of Xmrk target genes, quiescent BaF Hm cells were treated for 2 h with EGF, and RNAs were analyzed by differential display RT-PCR for differentially expressed genes in EGF-treated and nontreated cells. One of the PCR products was found at particularly high levels after amplification of cDNA from EGF-treated cells. The respective fragment was therefore isolated from the gel, reamplified, cloned, and sequenced. The sequence matched 100% to the mouse opn gene. Differential expression of opn in BaF Hm cells was confirmed by Northern blot analysis. EGF treatment of BaF Hm cells led to a rapid up regulation of OPN, which lasted up to 14 h (Fig. 1A). Also mIL-3-induced opn expression in BaF Hm cells, although the expression level was significantly weaker (Fig. 1A).

To further examine which signaling pathways are involved in the up regulation of the opn gene induced by the HER-mrk receptor, we made use of additional Ba/F3 cell lines expressing different truncated versions of HER-mrk (BaF Hm Δ1006 and BaF Hm Δ1075, Ref. 14). Hm Δ1006 possesses no tyrosine that can be phosphorylated after receptor stimulation, and it is incapable of activating the MAPK pathway (Fig. 1B). Hm Δ1075 possesses a growth factor receptor binding protein 2 binding site and activates MAPK such as the full-length HER-mrk receptor. Activation of MAPK was detectable by its phosphorylation using anti-phospho MAPK (Fig. 1B). The induction of opn expression by Hm Δ1006 was clearly affected (Fig. 1C), and the fact that Hm Δ1075 efficiently up-regulated the opn RNA level (Fig. 1C) points to a role of MAPK in regulation of opn expression by HER-mrk. Interestingly, Hm Δ1006 in contrast to Hm Δ1075 is inefficient in mediating antiapoptotic signaling in Ba/F3 cells (14), confirming an involvement of OPN in cell survival as it was described in this cell system by Lin et al. (24).
ROLE OF OPN IN SURVIVAL OF MELANOCYTES

Xmrk-induced opn Expression in Melanocytes Is Dependent on MAPK Activation. Since activation of the Xmrk receptor induces melanoma formation in fish, we asked whether the Xmrk kinase also induces opn expression in pigment cells and which role osteopontin plays in these cells. Therefore, we analyzed opn expression after stimulation of the chimeric Xmrk receptor in HER-mrk-expressing mouse melanocytes melan-a Hm. These cells proliferate, dedifferentiate, and show a transformed phenotype in response to receptor stimulation (13). Stimulation of melan-a Hm cells with either EGF or with TPA, a general growth factor for melanocytes in vitro, led to strong activation of the MAPKs ERK1 and 2 (Fig. 2A). This activation was almost completely abolished in the presence of the MAPK kinase inhibitor PD98059 (Fig. 2A).

In melan-a Hm cells, stimulation of the HER-mrk receptor resulted in a strong induction of opn expression within the first couple of hours (Fig. 2B). Nearly the same level of induction could be seen when melan-a Hm cells were stimulated with TPA alone confirming, the fact that opn originally has been identified as a TPA-inducible gene (25). Because TPA activates MAPK in melan-a cells (Fig. 2A), MAPK might be involved in up regulation of opn expression in melanocytes. Using PD98059, we blocked MAPK activity during HER-mrk stimulation. In the presence of PD98059, no induction of opn could be detected (Fig. 2B), pointing to a role of MAPK in HER-mrk-induced opn expression in melanocytes.

OPN Protein Expression Is Induced by Xmrk as well as by the FGF Receptor in Melanocytes and Is Constitutively Enhanced in Melanoma Cells. The expression of the OPN in melan-a Hm cells was analyzed after stimulation of the HER-mrk receptor using a polyclonal anti-OPN antibody. OPN was almost completely absent in cells that were factor starved (no TPA, no EGF) for 24 h, pointing to a fast turnover of the protein in these cells. After 2 h of receptor stimulation, OPN was clearly detectable as a Mr 55,000 protein, and this protein expression increased even more during the next 16 h (Fig. 3A).

To investigate whether the induction of OPN in melanocytes is a specific feature of the Xmrk kinase or whether it is a more general characteristic of receptor tyrosine kinases acting in pigment cells, wild-type melan-a cells were treated with bFGF and analyzed for OPN expression. Stimulation with bFGF led to a significant up regulation of OPN within 2–4 h (Fig. 3B), indicating that opn is also a target for the signaling induced by the FGF receptor. However, HER-mrk stimulation appeared to be more effective in inducing OPN expression. In contrast to melanocytes where OPN basal expression was almost not detectable, constitutive-enhanced expression of OPN was found in several different human melanoma cell lines (Fig. 3C). Interestingly, the lowest expression was seen in WM35 cells (Fig. 3C), which are derived from a noninvasive RGP melanoma (26).

OPN Contributes to Adhesion of Melanocytes to Type I Collagen. OPN is known to function both as cytokine as well as cell attachment protein with integrins α3β1, αvβ3, and αvβ5 acting as OPN

Fig. 1. opn is a target gene of the HER-mrk receptor in Ba/F3 cells, and its expression is dependent on MAPK activation. A, Ba/F3 cells were IL-3 deprived and then stimulated with either 8 nM EGF or IL-3 (5% supernatant) for the indicated time points before total RNA was isolated. For Northern blot analysis, 20 μg of total RNA were probed with an opn probe. Equivalent RNA blotting was verified by methylene blue staining (data not shown). B, Ba/F3 cells expressing either the full-length HER-mrk receptor (Hm) or two different COOH-terminal-truncated receptor variants (Hm Δ1075 or Hm Δ1006) were either stimulated with 8 nM EGF for 10 min (+) or left untreated (−). Cellular lysates were analyzed on a Western blot using anti-phospho MAPK and after stripping the membrane using anti-ERK2. C, Ba/F3 Hm Δ1075 and Ba/F Hm Δ1006 cells were IL-3 deprived and then stimulated with 8 nM EGF for the indicated time points before total RNA was isolated and probed with an opn probe on a Northern blot. Equivalent RNA blotting was verified by methylene blue staining (data not shown).

Fig. 2. Opn expression is dependent on MAPK signaling in melanocytes. A, melan-a Hm cells expressing the HER-mrk receptor (melan-a Hm) were either left untreated (−) or stimulated for 10 min with 8 nM EGF or 200 nM TPA in the presence of 40 μM PD98059 or the solvent DMSO. Cellular lysates were analyzed on a Western blot using anti-phospho MAPK and after stripping the membrane using anti-ERK2. B, melan-a Hm cells were treated for the indicated times with either 8 nM EGF, 200 nM TPA, or 8 nM EGF in the presence of 40 μM PD98059 or the solvent DMSO alone before total RNA was analyzed for opn expression on a Northern blot. Equivalent RNA blotting was verified by methylene blue staining (data not shown).
receptors (27). In addition, OPN can bind to the ECM component collagen, including type I collagen (28). Melanocytes, which grow in the epidermis, normally do not get into contact with type I collagen, which is an ubiquitous component of the connective tissue of the dermis. Moreover, melanocytes are not able to adhere properly and spread over a long-term period in three-dimensional type I collagen gels, which simulate the conditions in the dermis in vitro (8). Strikingly, bFGF was found to induce spreading of melanocytes in collagen gels, although the underlying mechanism is not clear yet.

In melan-a cells, OPN expression was substantially increased in response to HER-mrk activation and also by stimulation with bFGF. Because OPN is described to act as a mediator between integrin receptors and collagens (29), we wondered whether it might increase the capacity of melanocytes to adhere to type I collagen and to spread in three-dimensional type I collagen gels. Wild-type melan-a cells were embedded in type I collagen gels, and the cells were cultured in the presence of different factors. Cells in the presence of serum alone and no additional factors initially showed increased spreading during the first 8 h. However, between 30 and 48 h, cell rounding increased, and the cells lost the spread phenotype (Fig. 4A). In contrast, cells cultured in the presence of TPA, which is a general growth factor for melanocytes in vitro, spread much faster, and the spread dendritic phenotype lasted up to 48 h. Also bFGF (0.3 nM), which is described to mediate spreading of human melanocytes in type I collagen gels (8), induced this phenotype in melan-a cells. Strikingly, the addition of 100 nM OPN to the medium also increased the fraction of spread cells in the gel (Fig. 4A).

Because OPN expression is up regulated after stimulation of the HER-mrk receptor in melan-a Hm cells, this secreted protein should be present in supernatants of EGF-stimulated melan-a Hm cells. The fact that melan-a Hm cells were able to spread and grow in the collagen matrix when stimulated with EGF (data not shown) supported this assumption. Therefore, wild-type melan-a cells were cultured in three-dimensional collagen gels in melan-a HER-mrk CM. Significant cell spreading could be observed under these conditions (Fig. 4A), pointing to factors in the HER-mrk CM that enhance interactions of the cells with the collagen matrix. That the observed cell spreading was not due to EGF present in the conditioned medium was seen in wild-type melan-a cells cultured in medium containing 8 nM EGF and FCS (Fig. 4A). These cells showed the same round phenotype as cells cultured in the presence of FCS alone.

Although OPN and HER-mrk CM could prevent cell rounding...
induced by collagen over a long-term period, they did not induce an increase in the cell number. TPA and bFGF, in contrast, very efficiently triggered cell growth in the collagen matrix. To verify that OPN, EGF, and factors in HER-mrk CM do not trigger mitogenic signaling in melan-a cells, the growth response of melan-a cells was assessed by counting the cells after 4 days in medium containing the different factors. TPA, as well as bFGF, clearly induced cell growth and led to a 3–4-fold increase. However, neither OPN, EGF, or factors in HER-mrk CM produced a significant mitogenic effect (Fig. 4B).

**OPN Counteracts Type I Collagen-induced Apoptosis in Melanocytes.** Melan-a cells not only showed inefficient spreading in collagen gels, they also started to apoptose in the absence of any additional factors. Already after 30 h in medium containing only FCS, the first apoptotic cells could be detected, and the number increased dramatically between days 2 and 4. It should be mentioned that this is not a general response of melan-a cells to the absence of defined growth factors since cells cultured under these conditions on conventional culture plates for 6 days, although showing a starved phenotype, do not apoptose (13, 18).

To quantify the antiapoptotic effect of OPN on melanocytes, cells were stained with DAPI after 4 days in collagen (Fig. 5A), and the ratio of intact to fragmented nuclei was determined (Fig. 5B). The effects quantified by DAPI staining were confirmed by analysis of DNA fragmentation (Fig. 5C) clearly showing that apoptosis is induced by type I collagen in melan-a cells.

According to the cellular changes seen by light microscopy, an increased number of intact nuclei was seen in the presence of TPA due to the mitogenic effect (Fig. 5A). Nuclei were also intact in cells cultured in HER-mrk CM, but the total number of nuclei was lower. Confirming the apoptotic phenotype seen in cells cultured in FCS alone, most of the nuclei were fragmented (Fig. 5A), and 80–90% of the cells were apoptotic (Fig. 5B). In contrast, TPA almost completely prevented collagen-induced apoptosis and only 5–10% of the cells showed fragmented nuclei. Also, OPN suppressed cell death in the collagen gel with only 40% of the cells undergoing apoptosis. The conditioned medium HER-mrk CM was almost as efficient as TPA in reducing the number of apoptotic cells (13%). Using a neutralizing antibody to OPN (24), the protective effect of HER-mrk CM was significantly reduced. Approximately 60% of the cells were apoptotic (Fig. 5B), which clearly shows that OPN contributes to the antiapoptotic effect of the HER-mrk CM. Taken together, these data demonstrate that OPN is able to counteract apoptosis induced in melanocytes by collagen.

**Integrin αvβ3 Acts as Receptor for OPN in Melanocytes and Contributes to the Survival Effect Produced by bFGF.** Because it is known that the vitronectin receptor αvβ3 is also a receptor for OPN (27), and αvβ3 has been linked to the tumorigenicity of malignant melanoma (6), we asked whether this integrin might also act as a receptor for OPN in melanocytes.

Using specific primers for the respective integrin subunits, the expression of αv as well as of β3 was readily detectable in melan-a cells by RT-PCR analysis (Fig. 6A). Specific antibodies against αv and β3 identified the respective proteins in melan-a cells (Fig. 6B). Strikingly, αv expression was much higher in melan-a cells than in the human melanoma cell lines IFB and A375. This was not attributable to reduced cross-reactivity of anti-αv with the human protein because the antibody used (Q-20) is directed against the human αv chain.

To further investigate the role of αvβ3 as a potential OPN receptor, we made use of neutralizing antibodies against αv and β3 in an OPN
ROLE OF OPN IN SURVIVAL OF MELANOCYTES

To examine whether α3β1 is involved in the antiapoptotic effect in melan-a cells, the blocking antibodies against α3 and β1 were used to interfere with the OPN interaction in the presence of collagen, and apoptotic cells were identified by DAPI staining as described in Fig. 5B. The presence of the antibodies did not influence apoptosis induced in the absence of any defined factor (Fig. 6D). However, the survival effect brought about by OPN was significantly reduced by addition of anti-α3 with ~80% apoptotic cells compared with 40% in the absence of any antibody (Fig. 6D). Also, anti-β1 had an influence on OPN-mediated cell survival (55% apoptotic cells), although the effect was lower than that of blocking α3 (Fig. 6D).

Because these data showed that OPN-mediated cell survival is correlated with binding to α3β1, and we had found that bFGF induces OPN expression in melan-a cells (Fig. 3B), we wondered whether the OPN/α3β1 interaction also contributes to the survival effect on melanocytes seen and described for bFGF (8). Therefore, cells were cultured in the collagen matrix in bFGF containing medium either in the presence of the α3- and β1-blocking antibodies or in the presence of a control antibody, bFGF alone or in the presence of the control antibody very efficiently suppressed apoptosis with only 5–10% apoptotic cells after 4 days in the collagen gel (Fig. 6E). However, both blocking antibodies, anti-α3 as well as anti-β1, had a significant influence on bFGF-induced cell survival in the collagen matrix, and the number of apoptotic cells increased to ~35 and 20%, respectively (Fig. 6E). This clearly indicated that α3β1 interactions are contributing to the antiapoptotic signaling brought about by bFGF.

OPN Triggers Antiapoptotic Signaling in Human Melanoma Cells. To finally investigate if OPN is also playing a role in survival of human melanoma cells in dermal collagen, the different human melanoma cell lines were cultured in collagen gels, and the influence of constitutive OPN expression was analyzed by using the OPN-
blocking antibody. The various cell lines showed a distinct behavior in the collagen matrix with Bro and Mel26 cells spreading and growing very efficiently during 4 days. Also A375 cells proliferated in the collagen gel but many cells showed a round phenotype. IFB and D10 grew very slowly, and no increase in the cell number of WM35 cells was seen during 4 days. Approximately 10% of WM35 cells were apoptotic when cultured in three-dimensional collagen. None of the other analyzed cell lines showed significant apoptosis either in the absence of any defined factor or in the presence of the control antibody. Addition of the OPN-blocking antibody, however, resulted in an increase in apoptotic cells in all cell lines except WM35 (Fig. 7). The highest increase was seen in Bro cells with 25% of the cells undergoing apoptosis. Mel26, A375, IFB, and D10 showed 9–20% more apoptotic cells when the neutralizing OPN antibody was added (Fig. 7), which points to a contribution of OPN to the survival of transformed pigment cells in dermal collagen.

DISCUSSION

OPN is a secreted, phosphorylated glycoprotein, which contains an arg-gly-asp sequence and has been described to act both as cell attachment as well as survival and growth factor (27).

We have identified OPN as a factor that can transmit survival signals in melanocytes grown in dermal collagen, and its expression is regulated by two receptors (tyrosine kinases that play an important role in pigment cell transformation and melanoma progression. OPN originally was cloned as a tumor promoter-inducible gene, and it is frequently overexpressed in human tumors (27). Its expression is regulated by various factors, including cytokines like IL-3 and granulocyte macrophage colony-stimulating factor in Ba/F3 cells, where it contributes to cellular survival and proliferation induced by these cytokines (24). Also, growth factors (30–32) and oncogenes such as ras can induce OPN expression. Increased expression of OPN is associated with ras-transformation of NIH3T3 cells (33), and its expression is enhanced in mammary tumors of v-Hras transgenic mice (34). This points to a role of MAPK, which acts downstream of ras, in the induction of the OPN promoter. It is also in line with our results showing that the HER-mrk-triggered up regulation of OPN expression was dependent on intact MAPK signaling in both cell lines Ba/F3, as well as melan-a cells. This also might explain the higher level of OPN expression induced by HER-mrk than by the FGF receptor in melan-a cells (Fig. 3) because HER-mrk stimulates strong MAPK activation that lasts several hours (13), whereas bFGF-induced MAPK activation in melanocytes is only transient (35).

Activation of both the HER-mrk as well as the FGF receptor not only induced OPN expression but also enabled murine melanocytes to survive in three-dimensional collagen gels. This could be due to a direct activation of antiapoptotic signaling pathways by the receptors. However, using HER-mrk-conditioned medium and an OPN-neutralizing antibody, we clearly could show that OPN in an indirect way contributes to the survival effect produced by the HER-mrk receptor. For the FGF receptor, it has been described that its stimulation by bFGF induces antiapoptotic signaling in human melanocytes, but the mechanism for this effect has not been elucidated yet (8). Because we found that bFGF stimulation of melanocytes can induce OPN expression, this might point to a contribution of OPN to the survival effect seen by Alanko et al. (8). One function of OPN, once up regulated by either the FGF or the HER-mrk receptor, could be an interaction of the factor with other receptors present on the melanocyte surface. Our studies revealed that in melan-a cells αβ3 can act as such a receptor for OPN and that the OPN/αβ3 interaction is crucial for the mediation of cell survival in collagen. The fact that blocking β3 interactions with a specific antibody did not have an equally strong influence on the OPN-produced antiapoptotic effect suggests that other β-subunits such as β1 or β5 might also participate in binding OPN. Because the ability of melan-a cells to adhere to OPN was reduced to 20% in the presence of the β3 antibody, it nevertheless seems very likely that αβ3 is the main receptor for OPN in this melanocyte cell line.

Most striking, αβ3 is known to play an important role in melanoma cell survival (3), and its expression correlates with melanoma progression from RGP to VGP melanoma and tumorigenicity (5, 6, 36, 37). It should be mentioned, however, that for the effects produced on melanoma cells by αβ3, not only OPN (38) but also denatured (proteolytically-cleaved) collagen was identified as the relevant ligand (3, 4). Thereby, the melanoma cells were able to alter the collagen to promote a stable interaction of αβ3 with the matrix protein. Because melan-a cells do express a functional αβ3 but were not able to spread properly and survive in collagen gels for a long-term period in the absence of defined factors, this particular interaction seems not to occur in untreated melanocytes. Interestingly, it is described that expression of αβ3 in the absence of an appropriate ligand, which is the case for melan-a cells in native collagen, induces apoptosis because of the presence of unligated integrins (39). This integrin-mediated death (39) is described to be biologically and biochemically distinct from anoikis, which is characterized as apoptosis induced by the loss of adhesion, per se (40).

The presence of OPN induced spreading and survival of melan-a cells in collagen gels, and OPN is known to bind collagen (28). This suggests a role for OPN as attachment protein for melanocytes mediating an interaction between collagen and the OPN receptor αβ3. A function of OPN such as an adhesive intermediate (bridging molecule) for cellular attachment to matrix components has also been discussed for vascular smooth muscle cells, where addition of exogenous OPN could restore normal cell adhesion of OPN-deficient cells to collagen (41). On the other hand, recent studies revealed that in OPN-deficient vascular smooth muscle cells, the expression of receptors necessary for adequate adhesion was affected (29). Hence, OPN, instead of acting directly as attachment protein for melanocytes, might also act as an activating ligand for αβ3 thus, stimulating intracellular pathways associated with regulation of adhesion, spreading, or survival.

Just recently it has been reported that in B16 mouse melanoma cells, stimulation of αβ3 by OPN leads to the up regulation of the matrix metalloproteinases MMP-2 and MT1-MMP (38). OPN also enhanced cell migration and ECM invasion of the cells, demonstrating that it can induce all cellular alterations necessary for the most
important step in the establishment of a VGP melanoma from RGP lesions.

The interaction of OPN with $\alpha_5\beta_3$ not only can influence cell attachment, spreading, and migration but also mediates survival signals. Such a survival signaling induced by $\alpha_5\beta_3$ has been described in endothelial cells. Stimulation of $\alpha_5\beta_3$ by OPN thereby resulted in activation of nuclear factor $\kappa B$ through Src- and ras-dependent pathways (42). Activation of the nuclear factor $\kappa B$ with OPN as stimulating ligand for $\alpha_5\beta_3$ was also found in melanoma cells. The antiapoptotic effect of $\alpha_5\beta_3$ in melanoma cells after interaction with denatured collagen, however, was linked to an increase in the Bcl-2: Bax ratio (4), which is known to correlate with cell survival. Whether the interaction of OPN with $\alpha_5\beta_3$ in melanocytes also leads to an induction of bcl-2 expression or triggers other signaling pathways finally leading to survival of the cells in dermal collagen remains to be investigated.

We found that OPN, once expressed after treatment of the cells with growth factors or tumor promoters, can act as a ligand for $\alpha_5\beta_3$ in melanocytes in an autocrine way. Moreover, in several human melano-

malignant melanoma is notorious for its high metastatic potential once the pigment cells are transformed. Since recent data proved that OPN can trigger migration and invasion and enhances tumor growth of melanoma cells (38), production of OPN by melano-

cells might be a crucial step in establishing cells with the potential to give rise to tumorigenic VGP melanoma. Our data demonstrate that growth factor receptors of the family of receptor tyrosine kinases thereby contribute to this step by up regulating $opn$ expression in melanocytes, which points to a new role of these receptors in pro-

cesses associated with melanoma development and progression.

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