Bone Morphogenetic Protein 7 Inhibits Tumor Growth of Human Uveal Melanoma In Vivo

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PURPOSE. Bone morphogenetic protein-7 (BMP7), a member of the TGF-β superfamily, is essential for early ocular morphogenesis, and lack of BMP7 causes epithelial development disturbances in the eye. In the present study, the association of tumorigenicity and malignant behavior of human uveal melanoma with BMP7 expression and the possibility that overexpression of BMP7 in uveal melanoma affects intraocular tumor growth in vivo were investigated.

METHODS. To establish the role of BMP7 in uveal melanoma progression, the human OCM-1 cell line was stably transfected to overexpress BMP7 (OCM-1 FRT/BMP7) using targeted homologous recombination.

RESULTS. Transcriptional profiling revealed low or no detectable expression of BMP7 in primary tumor tissue of patients with uveal melanoma. In line with these clinical observations, BMP7 mRNA levels were low or not detectable in cultured human uveal melanoma cell lines, when compared with normal cultured melanocytes. Inoculation of OCM-1 FRT/BMP7 cells into the anterior chamber of the eye of nude mice inhibited tumor progression significantly, compared with progression in the control cell line (no BMP7 expression).

CONCLUSIONS. Collectively, the data provide novel evidence that decreased BMP7 expression contributes to progression of uveal melanoma. Furthermore, BMP7 may represent a novel therapeutic molecule for repression of tumor growth in vivo melanoma. (Invest Ophtalmol Vis Sci. 2007;48:4882–4889) DOI:10.1167/iovs.07-05050

Uveal melanoma is the most common primary malignant intraocular tumor in the adult, with a varying annual incidence of 6 to 12 per million in Caucasians. It develops in one of the most capillary-rich tissues of the body and has a 5-year survival of 6 to 12 per million in Caucasians. It develops in one of the most capillary-rich tissues of the body and has a 5-year survival of 6 to 12 per million in Caucasians. Knowledge of the nature of growth and metastatic behavior in uveal melanoma is essential for the development of new treatment strategies, especially with regard to improvement of survival.

The 35-kDa homodimeric protein bone morphogenetic protein (BMP)7 is a member of the TGF-β superfamily. Knockout studies have shown that BMP7 (and BMP4) is essential for early morphogenesis of the eye and kidney. BMP7 knockout mice revealed deficient ocular growth due to severe disturbances in epithelial development. Recent evidence suggests that BMP7 plays a role in a functional system in the eye, modulating and balancing the expression of ECM proteins (collagen IV, laminin, fibronectin) by meshwork cells of the trabecular system. Disturbances of this balance may result in primary open-angle glaucoma (POAG). In addition to ocular development, BMP7 is a prerequisite for induction of condensation and epithelialization of metanephric mesenchyme in the kidney. In mesangial cells of the kidney, BMP7 counteracts TGF-β induced fibrosis, reversing the process of chronic renal injury and maintaining an epithelial phenotype. Moreover, BMP7 experimental therapy halts progression and reverses the effects of chronic progressive kidney disease. For instance, BMP7 counteracts the increased expression of several extracellular matrix (ECM) proteins and connective tissue growth factor (CTGF) in chronic renal fibrosis.

This study was designed to investigate whether tumorigenicity and invasive growth behavior are associated with modulated BMP7 expression in primary uveal melanoma and in human uveal melanoma cancer cell lines. Our results suggest that BMP7 inhibits growth of human uveal melanoma xenografted into the eye. BMP7 protein may therefore represent a novel therapeutic molecule for repression of tumor growth of uveal melanoma.

MATERIAL AND METHODS

Uveal Melanoma Cell Line and Uveal Melanocytes

Seven cell lines (92-1; Mel-202, -285, and -290; and OCM-1, -3, and -8) were obtained from primary uveal melanomas. Cell lines OMM-1, 3 and -1.5 were obtained from liver metastases. Cell line 92-1 and normal uveal melanocytes were established in our laboratory. Cell lines OCM-3, OCM-8, Mel-202, OMM-1.5, 92-1, Mel-285, OMM-1.3, and Mel-290 were cultured in RPMI 1640 (Invitrogen-Gibco, Grand Island, NY), 10% heat-inactivated fetal calf serum (FCS), Hyclone, Logan, UT), and 100 IU/ml penicillin (Invitrogen-Gibco). Cell lines OCM-1 and OMM-1 were grown as monolayers in 10 ml/dish Dulbecco’s modified Eagle’s medium (Invitrogen-Gibco) supplemented with 10% FCS (Hyclone), 100 IU/ml penicillin (Invitrogen-Gibco), and 100 µg/ml streptomycin (Invitrogen-Gibco). Normal melanocytes were grown as a monolayer 10 ml/dish Ham’s/F12 (Invitrogen-Gibco) medium as described by Hu et al. All cells were incubated at 37°C in a humidified atmosphere with 5% CO2.


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Primary Uveal Melanoma Tumors
Twenty-eight enucleated eyes containing uveal melanoma were included in this study, as described previously.16 The protocol conformed to the requirements of the Declaration of Helsinki. Two melanomas were treated because of incomplete tumor regression after combined treatment with ruthenium-106 plaque radiotherapy and transpupillary thermotherapy. The remaining 26 eyes were enucleated primarily because of large uveal melanoma. The median age of the patients, 14 women and 14 men, at time of enucleation was 61.5 years (range, 42–84 years). The maximum follow-up was 115 months. Tumor diameter varied from 7 to 18 mm (mean, 12.5 mm; SD 2.9). After histopathological analysis, 10 tumors were classified as epithelioid, 12 as spindle cell, and 6 as mixed histology. There were 24 choroidal tumors, 2 ciliary body tumors, and 2 ciliary body melanomas with iris involvement. Tumor fragments were snap frozen in nitrogen and stored at −80°C or paraffin embedded for immunohistochemistry and real-time PCR, respectively.

Establishment of Stable Transfectants Expressing the BMP7 Gene
For the establishment of an OCM-1 cell line with BMP7 overexpression, we used the OCM-1 FRT cell line.17 The BMP7 coding sequence (1 µg of construct CMV-BMP7 FRT (BMP7 cDNA)) was integrated by targeted homologous recombination using the single FRT site, as described previously.17 Stable transfectants were selected with hygromycin (400 µg/mL; Invitrogen, Carlsbad, CA).

Enzyme-Linked Immunosorbent Assay for Human BMP7
Levels of human BMP7 were measured with a commercially available specific ELISA kit, with the sandwich enzyme immunoassay technique (R&D Systems, Abingdon, UK). A normalized number of cells in conditioned medium were analyzed by ELISA for BMP7 production after 1, 3, and 8 days of culture. Fresh culture medium was used as the negative control. All samples were preformed in duplicate.

Cell Proliferation Assay
OCM-1 FRT and OCM-1 FRT/BMP7 cells were cultured for 1 and 4 days after normalization to 2000 cells/200 µL. An MTS assay was performed according to the manufacturer’s protocol (Promega, Leiden, The Netherlands). All experiments were performed in six times.

Isolation of Cellular RNA and Real-Time Polymerase Chain Reaction
RNA was isolated from cells in culture and tissue from experimentally induced tumors, as described earlier.17,18 RNA from 28 human melanoma tissue samples was isolated with a kit (RNeasy Mini Kit; Qiagen, Venlo, The Netherlands) and proteinase K (20 mg/mL; Qiagen). Reverse transcription was performed with random primers in the presence of an RNase inhibitor (Roche Diagnostics, Rotkreuz, Switzerland). Quantitative real-time PCR (qPCR) was performed with commercially obtained exon-specific primers for BMP7, vimentin, and β-actin (primer catalog numbers: Hs 002333477 m1 [BMP7], Hs 00185584 m1 [vimentin], and Hs 99999903 m1 [β-actin]; Applied Biosystems [ABI], Rotkreuz, Switzerland) on a sequence-detection system (Prism 7700; ABI). Values were normalized with the housekeeping gene β-actin, according to the comparative method of Livak and Schmittgen.19 All experiments were performed in triplicate.

Transient Transfections and Transcription Reporter Assays
OCM-1 FRT and OCM-1 FRT/BMP7 cells were seeded at a density of 7500 cells/cm² in DMEM with 10% FCS in 24-well plates. On subsequent days, the cells were transiently transfected with 1 µg of the indicated constructs (described later), with transfection reagent (FuGENE 6; Roche, Mannheim, Germany) according to the manufacturer’s protocol. To correct for transfection efficacy, 100 ng of Renilla luciferase (pRL-CMV or pRL-CAGCS; Promega) was cotransfected. On day 3, cells were serum starved for 24 hours before stimulation with TGF-β and/or BMP7 for a duration of 30 hours. On day 5, luciferase activities were quantified (Dual Luciferase Assay; Promega).20 Firefly luciferase activity was corrected for Renilla luciferase activity. The experiments were performed in triplicate and repeated at least twice. Results are expressed as the mean ± SEM.

Luciferase Reporter Gene Constructs
For intracellular signaling of TGF-β the CAGA-luciferase construct (kindly provided by Peter ten Dijke, Dept. Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands), consisting of 12 Smad3/Smad4 binding sequences (CAGA boxes), and the luciferase-coding sequences was used. The CAGA boxes confer TGF-β stimulation to a heterologous promoter reporter construct, whose activity depends on binding of activated Smad3/Smad4 transcription factor complexes.20 The BRE-luciferase construct (also kindly provided by Peter ten Dijke), that is based on the mouse Id1 promoter, was used to study the presence and functionality of BMP-receptors.21

Mouse Model of Human Uveal Melanoma in the Anterior Chamber of the Eye
Female BALBc nu/nu mice were purchased from Charles River (Charles River, Maastricht, The Netherlands). They were housed in ventilated cages in sterile conditions according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Sterile food and water were provided ad libitum. Mice were 8 weeks old at the time of the intraocular injection of tumor cells.

To analyze the effect of BMP7 in vivo, we used the model for induction of tumor growth in the anterior chamber of the eye.17 Tumor cells were orthotopically inoculated into the anterior chamber of mouse eyes (only right eyes, each group n = 10) as described earlier by Niederkorn et al.22 With a glass needle, OCM-1 FRT cells or OCM-1 FRT/BMP7 cells at a concentration of 10³/µL were injected into the anterior chamber of the eye of 8-week-old female BALBc nu/nu mice. After 3 weeks, all eyes were enucleated and used for histomorphometric analyses and for immunohistochemistry.

Immunohistochemistry
After enucleation, the eyes were immediately fixed in 5 mL of 4% paraformaldehyde (Lommers Pharma, Oss, The Netherlands). After 24 hours, the eyes were dehydrated for 2 hours in 70% ethanol, 1 hour in 90%, and 30 minutes in 99% and processed for paraffin embedding. Hematoxylin-cosin (H&E) and periodic acid-Schiff (PAS) staining were performed on 5-µm serial sections of tumor eyes. Histomorphometric measurements of tumor burden were performed on central sections through the tumor (largest tumor area). Total tumor area (expressed in square millimeters), as an estimate of total tumor burden, was measured by image analysis (NIH-Image 1.62b; available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).23 Antibodies against human BMP7 (rabbit polyclonal a-BMP7, 285-bab, generated by Vakic Vaccine; 10 µg/mL) and vimentin (Abcam Ltd., Cambridge, UK) were used for immunohistochemistry on serial sections of tumors eyes, OCM-1 FRT/BMP7 cells, and OCM-1 Wt cells (cultured on chamber slides). Polyclonal BMP7 antibody was used in a dilution of 1:100 and vimentin in a dilution of 1:50. For the BMP7 staining, medullary rays of the kidney were used as a positive and glomeruli as negative control.24,25

Sections were incubated with primary antibodies overnight, followed by incubations with biotin-labeled rabbit-anti-mouse IgG and a preformed complex of biotin-labeled horseradish peroxidase and streptavidin (Dako, Glostrup, Denmark). Immune complexes were visualized with 0.05% diaminobenzidine and 0.0015% hydrogen peroxide. Slides were counterstained in Mayer’s hematoxylin (Merck, Amsterdam, The Netherlands). OCM-1 FRT/BMP7 cells and OCM-1 FRT cells cultured on chamber slides were also stained with vimentin antibody.
The level of vimentin staining was quantified by two independent observers (scale 0–3: 0, absent; 1, low; 2, moderate; and 3, high).

**Statistical Analysis**

Statistical analysis was performed with ANOVA (SPSS for Windows, ver. 13.0; SPSS, Chicago, IL). Results are expressed as the mean ± SEM of at least two experiments, unless otherwise indicated. \( P < 0.05 \) was considered significant.

**RESULTS**

**BMP7 Expression Primary Uveal Melanoma Tissue and In Vitro**

Real-time PCR analyses revealed that BMP7 expression was low or undetectable in most of the tested uveal melanoma tumor tissues (in 26 of the 28 patients tested; Fig. 1A). In line with
from two single experiments in duplicate.

BMP7 expression in all retina layers, with high levels of expression being presented in the inner and outer nuclear layers. BMP7 was expressed in the nerve fiber layer, inner nuclear layer, outer segments, and external limiting membrane of the retina, with diffuse staining of the choroid (Fig. 1C). BMP7 immunolocalization was not detected in 93% of the tested tumors in the primary uveal melanomas (Figs. 1E, 1F). Our observations, in line with several studies, showed BMP7 expression in all retina layers, with high levels of expression being presented in the inner and outer nuclear layers. BMP7 was expressed in the nerve fiber layer, inner nuclear layer, outer segments, and external limiting membrane of the retina, with diffuse staining of the choroid (Fig. 1C). BMP7 immunolocalization was not detected in 93% of the tested tumors in the primary uveal melanomas (Figs. 1E, 1F). The BMP7 immunolocalization data are therefore in full agreement with the mRNA expression profile of the same tumors.

**BMP7 Overexpression in Uveal Melanoma Cell Line OCM-1**

Targeted homologous recombination using the Flp-In system (Invitrogen) was performed to overexpress BMP7 in OCM-1 (OCM-1 FRT/BMP7) human uveal melanoma cells, thus avoiding clonal variability, as described previously by us. Real-time PCR and ELISA analysis revealed the expression of BMP7, both at the transcriptional and protein level in OCM-1 FRT/BMP7 (Figs. 2A, 2B, respectively) as expected, whereas the single FRT host control cell line (OCM-1 FRT) did not express detectable amounts of BMP7 protein (≤60 pg/mL).

Striking differences in morphology and inhibition of proliferation were observed between OCM-1 FRT and OCM-1 FRT/BMP7 in vitro. After 3 days, OCM-1 FRT/BMP7 cells grow in a multilayer, in contrast to OCM-1 FRT control cells (Figs. 3A, 3B). In addition to morphologic differences, a significant change in cell proliferation rate was observed between the OCM-1 FRT control cells and OCM-1 FRT/BMP7 (Fig. 3C).

**FIGURE 2.** BMP7 overexpression in the OCM-1 human uveal melanoma cell line. (A) Relative mRNA expression levels of BMP7 in OCM-1 FRT cells and OCM-1 FRT/BMP7 cells. BMP7 mRNA was expressed at significantly higher levels in OCM-1 FRT/BMP7 cells. Data are the mean ± SD. Results are from two single experiments in triplicate. (B) BMP7 protein expression and secretion into the culture medium by OCM-1 FRT cells and OCM-1 FRT/BMP7 cells (ELISA). Results are analyzed from two single experiments in duplicate.

**FIGURE 3.** Growth and morphologic characteristics of OCM-1 in the absence or presence of BMP7. OCM-1 FRT (A) and OCM-1 FRT/BMP7 (B) cells after 5 days of cell culture. (C) BMP7 expression affected cellular proliferation of OCM-1 cells (n = 6). Vimentin immunolocalization in OCM-1 FRT cells (D, E) and OCM-1 FRT/BMP7 cells (F, G) after 2 days in culture. (H) Quantitation of vimentin expression by immunohistochemistry in OCM-1 FRT and OCM-1 FRT/BMP7 cells (scale 0–3; 0, no expression; 1, low; 2, moderate; and 3, high expression). Original magnification: (A, B) ×16; (D, E) ×20; (F, G) ×100.
Furthermore, BMP7 overexpression coincided with diminished expression of vimentin, which was identified previously as a marker of invasiveness for uveal melanoma. Vimentin expression in OCM-1 FRT/BMP7 cells was mainly localized in the cytoplasmic podosomes of the cells, whereas the control cell line displayed cytoplasmic staining (Figs. 3D–G). Enforced BMP7 expression strongly diminished protein levels of vimentin (Fig. 3H).

Smad-Mediated TGF-β and BMP Signaling in OCM-1 Cells

OCM-1 FRT cells showed significant BRE-luciferase activity, indicating the presence of functioning, activated type I BMP-receptor complexes and active Smad-dependent signaling. In OCM-1 FRT/BMP7 cells, which stably overexpressed BMP7, BRE-luciferase activity was significantly increased, as expected (Figs. 4A, 4B). Next, we tested whether BMP7 expression can antagonize Smad-dependent TGF-β signaling, as recently described by us in breast and prostate cancer. The presence of functionally active TGF-β receptor complexes in the OCM-1 cells was demonstrated by the dose-dependent activation of the CAGA-luciferase reporter, whose activity depends on activated Smad3/4 transcription factor complexes. Addition of BMP7 to TGF-β-stimulated OCM-1 FRT cells did not significantly inhibit TGF-β-driven CAGA-luciferase activity (Fig. 4C), which contrasts with studies performed in breast and prostate cancer.

BMP7 Overexpression and In Vivo Tumor Progression of Human Uveal Melanoma

Three weeks after intraocular inoculation of OCM-1 FRT (control cells) and OCM-1 FRT/BMP7 cells, all mice (n = 20) were killed, and the tumors were enucleated. After 21 days, the control group of mice had macroscopically detectable tumors in the eye. These tumors developed in the anterior chamber with outgrowth into the back of the eye. When BMP7-expressing uveal melanoma cells were used, histomorphometric analyses revealed a significant reduction in tumor burden (Fig. 5A). Furthermore the localization of the tumors differed between the two experimental groups (Figs. 5B–E). BMP7-overexpression...
ing tumors were localized in and around the lens and displayed no signs of invasion into the back of the eye (Figs. 5C, 5E).

DISCUSSION

In this study, we provide novel evidence for the role of BMP7 in uveal melanoma progression. BMP7 was expressed in normal epithelial tissues of the eye, particularly in different retinal cell layers and the iris. Low (or nondetectable) expression of BMP7 was found in nearly all of the primary uveal melanoma tumors, whereas BMP7 expression was observed in normal melanocytes, as expected.24,26

Orthotopic xenografts of BMP7-expressing OCM-1 uveal melanoma cells (vs. control cells) resulted in a significant inhibition of tumor progression. These results suggest that decreased BMP7 expression in normal uveal melanocytes disturbs melanocyte homeostasis and may contribute to tumor progression. In line with these in vivo observations, a marker of the aggressiveness and invasiveness of uveal melanoma, vimentin, was strongly downregulated in BMP7-overexpressing uveal melanoma cells.31,32

In a variety of tissues, BMP7 appears to be a mediator of epithelial homeostasis and a prerequisite for maintenance of differentiated epithelial phenotype.7,8,33–35 Furthermore, in mesangial cells of the kidney, BMP7 counteracts TGF-β induced fibrosis, reversing the process of chronic renal injury and maintaining an epithelial phenotype. Moreover, BMP7 experimental therapy has halted progression and reversed the effects of chronic progressive kidney disease.7,36 BMP7, like other BMPs, binds to the BMPRII present on the cell membrane.
and recruits a type I receptor, BMPRIA (ALK-3) or BMPRIB (ALK-6), forming a complex. BMP receptor type I (ALKs) are transmembrane serine/threonine kinase proteins that self phosphorylate after formation of the BMP-receptor II-receptor I complex and acquire the ability to phosphorylate Smad proteins, a family of TGF-β transducers. Recent evidence from our group in breast and prostate cancer supports the notion that BMP7 inhibits tumor progression and metastasis. Furthermore, BMP7 antagonized the protumorigenic effects of TGF-β on epithelium-to-mesenchyme transition via a Smad-mediated mechanism (BMP7-induced inhibition of CAGA-luciferase and stimulation of E-cadherin expression). In the present study, OCM-1 cells expressed functionally active BMP receptor complexes. The presence of functionally active TGF-β receptor complexes in the OCM-1 cells was demonstrated by the dose-dependent activation of the CAGA-luciferase reporter, whose activity depends on activated Smad3/4 transcription factor complexes. In the presence of TGF-β, exogenous BMP7 addition did not result in a downregulation of CAGA-luciferase reporter activity. In OCM-1 cells, BMP7 therefore does not antagonize TGF-β-induced Smad signaling. Our observations seem contradictory to our recent findings in breast and prostate cancer. It should be noted, however, that BMP7 may also interact with other pathways that are critical in tumor progression and metastasis. In line with the latter studies in carcinomas, in the present study, BMP7 expression was decreased in uveal melanoma when compared with normal melanocytes of the eye. BMP7 expression in breast and prostate cancers is inversely related to tumorigenicity, and these findings are in accordance with the observed low BMP7 expression in human uveal melanoma primary tumors and in human melanoma cell lines. In keeping with our observations in uveal melanoma, stable overexpression of BMP7 in human breast cancer cells by targeted homologous recombination also results in a significant decrease in tumor progression and (bone) metastasis. Although speculative at present, decreased BMP7 expression in uveal melanoma development may contribute to increased invasiveness, as has been suggested for epithelial cancers. Of interest, recent evidence suggests that BMPs can also affect the tumorigenic potential of human tumor-initiating cells with a progenitor/stem cell phenotype. Although much remains to be understood about the complex role of BMP signals in cancer, a decrease in BMP7 expression during uveal melanoma progression may contribute to the acquisition of an invasive phenotype. BMP7 may therefore represent a novel therapeutic agent for repression of tumor growth in uveal melanoma.

Acknowledgments

The authors thank June Kan-Mitchell (University of California, San Diego) for kindly providing the OCM-1 cell line.

References


