MBP-1 Inhibits Breast Cancer Growth and Metastasis in Immunocompetent Mice

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Abstract
Breast cancer is the leading cause of cancer death among women. We have shown previously an antiproliferative effect of MBP-1 on several human cancer cells. In this study, we have examined the potential of MBP-1 as a gene therapeutic candidate in regression of breast cancer growth and metastasis in an immunocompetent mouse model. For this, we have used a mouse breast cancer cell line (EO771) and syngeneic C57BL/6 mice. EO771 cells were implanted into the mammary fat pad of C57BL/6 mice. Replication-deficient recombinant adenovirus expressing MBP-1 was administered intratumorally to determine gene therapeutic potential. The results showed a significant reduction of primary and distant (lung) tumor growth. Animals exhibited prolonged survival on treatment with MBP-1 compared with the control group (dl312). Subsequent studies suggested that MBP-1 inhibits matrix metalloproteinase expression in human breast cancer cells. Cells transduced with MBP-1 displayed inhibition of migration in a wound-healing assay. The conditioned medium from MBP-1–transduced cells blocked in vitro tube formation assay and inhibited expression of several angiogenic molecules. Taken together, our study shows that MBP-1 acts as a double-edged sword by inhibiting primary and metastatic tumor growth and modulating matrix metalloproteinase expression with a therapeutic potential against breast cancer progression. [Cancer Res 2009;69(24):OF1–6]

Introduction
Breast cancer is the most frequent neoplasm in women from western countries. The etiology of breast cancer involves a complex interplay of genetic, hormonal, and probably dietary factors. Loss of genetic material, identified by loss of heterozygosity, is the most frequent genetic alteration in breast cancer (1, 2). For prognostic evaluation of breast cancer, the most important information is size of the primary tumor, involvement of regional lymph nodes, and occurrence of distant metastasis. Histopathologic and biochemical markers are important tools for identifying aggressive breast cancers with poor prognosis and for predicting response to treatment (3, 4). However, the genes and pathways associated with these markers are not sufficiently known and the processes that lead to their clinical manifestation are not fully understood. There have been significant advances in breast cancer treatment, which have improved patient survival and quality of life. However, women continue to die from the disease and additional treatment strategies are essential.

We identified a c-myc promoter binding protein, MBP-1, from a human cervical carcinoma (HeLa) cell expression library (5). In vitro transient transfection assay suggested that MBP-1 negatively regulates both human and mouse c-myc promoter activity (5–7). We have shown that MBP-1 is ubiquitously expressed in different human tissues and located at human chromosome 1p35-pter (8, 9). Sequence analysis suggests that MBP-1 has a high homology with ENO1 cDNA (5), designated as human enolase cDNA, encoding a protein of 49 kDa (10). However, the enolase enzymatic activity was not shown from the ENO1 cDNA. MBP-1 has also been suggested as an alternative product of ENO1 gene (7, 11), although the function of full-length ENO1 gene product remains unknown.

Matrix metalloproteinases (MMP) are proteolytic enzymes and regulate various cell behaviors with relevance to cancer biology (12). The MMPs are synthesized as inactive zymogens (pro-MMPs). The expression and activity of MMPs are increased in almost every type of human cancer, including breast cancer, and correlate with advanced tumor stage, increased invasion, metastasis, and shortened survival. MMPs are not upregulated by gene amplification or activating mutations. The increased MMP expression in tumors is probably due to transcriptional changes rather than genetic alterations. This might be the result of activation of oncogenes or loss of tumor suppressors; for example, MMP-2, MMP-9, or MMP-14 is upregulated through combined activation of the transcription factors PEA3, Ets-1, c-Jun, β-catenin, LEF-1, MEK5, and Notch1, and the transcription of MMP-1 and MMP-13 is repressed by the tumor suppressor p53 (12–14). MMP-2, MMP-9, and MMP-14 also directly regulate angiogenesis. Tumor angiogenesis and growth is reduced in MMP-2–deficient mice compared with wild-type mice (12).

Clinical applications involving gene therapy require specific delivery of therapeutic genes to the tumor site to maximize therapy and minimize potential side effects. We have shown that intratumor injection of MBP-1 inhibits prostate and lung tumor growth in xenograft nude mice and induces cell death in several cancer cells without affecting the normal cell growth (15–17). Ubiquitous antitumor activities were also observed with other growth suppressors. Transduction of MDA-7/interleukin-24 by means of a replication-incompetent adenovirus displayed broad-spectrum antitumor activity against breast, lung, and gliomas (18). Proteins involved in the cell death process may also have a role in the regulation of metastasis (19). Notch1 has been indicated in both antitumor and antimetastatic activities in animal models (14). MDA-7/interleukin-24 is one of the broadest-acting, cancer-specific, and apoptosis-inducing cytokine genes, which also displays antiangiogenic, radiosensitizing, and antimetastatic activities (20, 21). In this report, we have shown that MBP-1 regresses primary and metastatic breast tumor growth in immunocompetent mouse model. We have also shown that MBP-1 modulates MMP expression and inhibits in vitro angiogenesis.
Materials and Methods

Cell culture. Breast cancer cell lines (MCF-7 and MDA-MB-231) were procured from the American Type Culture Collection and maintained in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified CO2 incubator. The C57BL/6-derived breast cancer cell line EO771 was obtained from Dr. Rong Xiang (Scripps Institute) and maintained in DMEM containing 20% fetal bovine serum. Human coronary microvascular endothelial cells (HCMEC) were obtained from Lonza and maintained in EC medium.

Tumorigenicity assay. Female C57BL/6 mice (5-7 weeks old) were obtained from the National Cancer Institute. EO771 cells were grown, harvested, washed, and resuspended in DMEM. mice were injected s.c. into the mammary fat pad with ~5.0 x 10^6 cells (0.1 mL). When tumor volume reached a mean volume of 200 mm^3, mice were randomized into two groups, and gene transfer treatment was started. A dose of ~2 x 10^9 virus particles of dl312 or AdMBP-1 was administered intratumorally each day for a total of 5 consecutive days. Tumor growth was monitored twice a week using a slide caliper and their volume was calculated as described previously (16). All mice were sacrificed when tumor growth reached ~2,200 mm^3 using the highest standards for animal care in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and approval of the Saint Louis University Animal Care Committee.

Statistical analysis. Two-tailed Student’s t test was used for statistical analysis as described previously (16).

Western blot analysis. Breast cancer cells were transduced with AdMBP-1 or dl312 control virus, and cell lysates were prepared after 72 h post-infection in 2× SDS sample buffer. Cell lysates were analyzed for Western blot analysis. MMP-2 was detected by immunoblotting the membrane using a specific antibody (Santa Cruz Biotechnology) followed by enhanced chemiluminescence (Amersham Biosciences). The blot was reprobed with actin to compare protein load in each lane.

In vitro reporter assay. Full-length human MMP-2 promoter with luciferase reporter gene was obtained from Tika Benveniste (University of Alabama at Birmingham), and full-length MMP-9 promoter was obtained from Doug Boyd (M. D. Anderson Cancer Center). Cells were cotransfected with MMP-2 or MMP-9 promoter with luciferase gene and increasing doses of cytomegalovirus MBP-1 plasmid DNAs. Luciferase activity was measured 48 h post transfection as described previously (15).

Gelatin zymography. Breast cancer cells were transduced with control dl312 or AdMBP-1 for ~36 h. Cell culture supernatants were replaced with serum medium and incubated for additional 24 h. Cell supernatants were denatured in the absence of β-mercaptoethanol and electrophoresed in a 10% SDS-polyacrylamide gel containing 0.1% gelatin. Following electrophoresis, the gel was washed three times with double-distilled water for SDS removal and dialyzed twice with gentle shaking at room temperature for 30 min using renaturing buffer (Invitrogen). The gel was placed in activation buffer (Invitrogen) and incubated at 37°C with gentle rocking overnight (16-20 h). Coomassie blue staining was done with gentle shaking for 1 h at room temperature. The gel was destained with double-distilled water until white bands were observed within a dark blue background.

Tube formation assay. Matrigel (BD Biosciences) was thawed at 4°C for overnight, and each well of prechilled 24-well plates was coated with 100 μL. Matrigel and incubated at 37°C for 45 min. HCMECs (50,000) were mixed with conditioned medium (collected from cells transduced with either control virus or AdMBP-1) and overlaid onto the Matrigel. After 6 to 16 h of incubation at 37°C, 5% CO2, endothelial cell tube formation was assessed with Olympus inverted microscope. Tubular structures were quantified by manual counting of low-power fields (×25), and inhibition percentage was expressed using untreated wells as 100% (P < 0.001, Student’s t test).

Human angiogenesis antibody array. The expression profile of angiogenesis-related proteins was detected and analyzed using human angiogenesis array kit (ARY007; R&D Systems). This array contains duplicate spots of 55 angiogenesis-related proteins. Briefly, the membrane containing immobilized angiogenesis-related antibodies was blocked with bovine serum albumin for 1 h on a rocking platform at room temperature. Membrane was then incubated with culture supernatants from dl312 or AdMBP-1-transduced MDA-MB-231 cells along with Detection Antibody Cocktail overnight at 2°C to 8°C on a rocking platform. The membrane was incubated with streptavidin-horseradish peroxidase conjugate followed by chemiluminescent detection reagent. The membrane was scanned and pixel density was presented by quantifying the mean spot densities from two experiments.

Results

Therapeutic use of AdMBP-1 against breast cancer growth in immunocompetent mice. Because MBP-1 expression in human breast cancer cells resulted in induction of cell death (15), we

Figure 1. AdMBP-1–mediated inhibition of tumorigenicity ex vivo. A, EO771 cells were transduced with AdMBP-1 or control dl312 virus for 16 h. Cells were washed, prepared for single-cell suspension, and implanted into mammary fat pad of immunocompetent C57BL/6 mice. Tumor growth was monitored twice a week. Bars, SE. B, EO771 cells were transduced with control dl312 or AdMBP-1 for 36 h. Cell lysates were subjected to Western blot analysis for MBP-1 expression. The blot was reprobed with actin antibody to compare the protein load. C, EO771 cell lysates transduced with control dl312 or AdMBP-1 were subjected to Western blot analysis using a specific antibody to PARP or caspase-3. On treatment with AdMBP-1, PARP was cleaved to an 86 KDa signature peptide (antibody used here recognized the cleaved product). The antibody used for caspase-3 in this experiment only recognized procaspase-3 form. The blot was reprobed with an antibody to actin for comparison of equal protein load.
examined whether MBP-1 could be used as a gene therapeutic intervention against breast cancer in a preclinical mouse model study. For this, we chose to use an immunocompetent mouse model. EO771 tumors, implanted s.c. into mammary fat pad of female mice, are immunosuppressive and highly aggressive, invading locally into dermal layers and the peritoneum as well as distantly to the lung and have characteristics that closely mirror those of the human disease (22). Initially, EO771 cells were infected for 16 h with either control adenovirus (dl312) or AdMBP-1 at an MOI of 100. After dislodging cells, the cell suspensions in culture medium were pipetted to obtain single-cell suspensions and counted for cell viability by trypan blue exclusion. Cells were resuspended in sterile serum-free DMEM at 5 × 10^5 per 100 μL. Orthotopic injection in mammary fat pad was done as described previously (23). Two weeks after injection, breast tumors appeared in the control mice (dl312 adenovirus-infected cells), and tumor volume reached ∼2.2 cm^3 after 3 weeks of implantation, and then mice were sacrificed. We did not observe any palpable tumors at this point in our experimental group. However, two of six mice in the experimental group developed tumor at day 25 (Fig. 1A), and the experiment was terminated on day 33. We have examined the MBP-1 expression level following transduction to AdMBP-1 into EO771 cells and observed higher MBP-1 expression (Fig. 1B). Subsequent experiments revealed that transduction of MBP-1 in EO771 cells displayed caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage (Fig. 1C). These data are in agreement with previous results observed in MCF-7 cells following MBP-1 transduction (15). Together, these results clearly suggested an inhibitory role of MBP-1 on EO771 cells in tumor formation.

Next, we examined the therapeutic efficacy of MBP-1. For this, EO771 cells were implanted orthotopically in the mammary fat pad of female mice. When the tumor volume reached an average of 200 mm³, the mice were randomized into two groups for administration of dl312 or AdMBP-1. A dose of ∼5 × 10^9 virus particles of dl312 (control virus) or AdMBP-1 was administered intratumorally each day for five consecutive daily injections. A significant reduction in tumor growth was observed following administration of AdMBP-1 (Fig. 2; Table 1). In contrast, tumors in the control group receiving dl312 control virus grew aggressively and the mice were sacrificed because of large tumor volume (>2,200 mm³) within a couple of weeks following treatment. Interestingly, 60% of the mice lived tumor-free until the day of sacrifice (day 41) following gene therapeutic intervention with AdMBP-1. Some of the tumors displayed a significant reduction of tumor growth until day 25 and then relapsed, although the tumor growth remained very slow until the observed period of time. Intratumor injection of AdMBP-1 significantly prolonged animal survival from 27 to 41 days (P < 0.001; Fig. 2B). Five of six mice in our control group displayed lung metastasis within 3 weeks of injection of tumor cells into the mammary pad. In fact, several spots were visualized macroscopically. Neoplastic cells invaded into the dermal layer in two mice in the control group. On the other hand, very small or no metastasis in lungs was seen in most of the AdMBP-1–treated mice. Two mice in the experimental group displayed small lung metastases.
Representative photomicrographs of lung tissue sections from control (a) and experimental (b) mice are shown in Fig. 2C. These results suggested that MBP-1 could be an effective therapeutic intervention strategy against breast cancer growth.

**Introduction of MBP-1 into breast cancer cells inhibits MMP-2 expression.** Tumors express high levels of MMPs that degrade tissue matrix and facilitate tumor as well as endothelial cell invasion and migration (24). Because we have observed the inhibition of lung metastasis by MBP-1 in a mouse model, we examined whether MMP-2 is regulated by MBP-1. For this, gelatin zymography was done to detect MMP-2 protease activity from MCF-7 cells transduced with control adenovirus (dl312) or AdMBP-1. As expected, MCF-7 cells highly expressed MMP-2 (Fig. 3A). Inhibition of MMP-2 expression was observed in AdMBP-1–transduced MCF-7 cells, suggesting that, in the presence of MBP-1, MMP-2 expression was inhibited. We further performed Western blot analysis for MMP-2 expression of breast cancer cells transduced with control virus or AdMBP-1. Our results suggested that MMP-2 expression is inhibited in MCF-7 cells following MBP-1 transduction (Fig. 3B). Similarly, we have observed MMP-9 inhibition in conditioned medium of AdMBP-1–transduced MDA-MB-231 cells (data not shown). We did not observe MMP-2 expression in EO771 cells using several commercially available antibodies. It is possible that EO771 cells express other MMPs to promote migration and need to identify from future studies.

Because MBP-1 acts as a transcriptional repressor, we examined whether MMP-2 is transcriptionally regulated by MBP-1. MCF-7 or MDA-MB-231 cells were transfected with full-length MMP-2 promoter with luciferase reporter construct and different doses of MBP-1. Luciferase activity was measured 48 h posttransfection. The results suggested that MBP-1 downregulates full-length MMP-2 promoter activity in a dose-dependent manner (Fig. 3C). Similarly, MBP-1 transcriptionally inhibited MMP-9 promoter activity (data not shown).

**MBP-1 inhibits capillary-like tube structure network.** Tumor cells can induce the formation of new blood vessels by secreting endothelial cell–specific growth factors. We performed a tube formation assay to determine whether conditioned medium from MDA-MB-231 cells transduced with or without MBP-1 generates tube formation of HCECs. Conditioned medium from control adenovirus (dl312)–transduced MDA-MB-231 cells were able to induce tube formation of HCECs within 18 h of incubation (Fig. 4A, a an b) as seen earlier (25, 26). On the other hand, conditioned medium from AdMBP-1–transduced MDA-MB-231 cells significantly reduced tube formation (Fig. 4A, c and d). Capillary-like tube structures were scored from control (dl312) and experimental (MBP-1) adenovirus-transduced cells, and results are summarized in Fig. 4B. Our results show that exogenous expression of MBP-1 blocks tube formation in endothelial cells, suggesting an inhibition of *in vitro* angiogenesis.

**Wound-healing migration assay.** We further determined the ability of MBP-1 to inhibit migration of breast cancer cells using an *in vitro* wound-healing motility assay (27). MDA-MB-231 cells were transduced with control dl312 virus or AdMBP-1. After 24 h, the plates were scratched with a thin disposable tip to generate a wound in the cell monolayer. The cells were incubated for additional 24 h and photographed. AdMBP-1 transduction inhibited a substantial migration of the cells into the denuded area (Fig. 4C) in contrast to control dl312 virus-transduced cells.

**Angiogenesis antibody array.** To determine specific molecules modulated by MBP-1 for inhibition of angiogenesis, we performed an antibody array using conditioned medium from breast cancer cells transduced with control dl312 virus or AdMBP-1. Several angiogenesis-related proteins were modulated following introduction of MBP-1. Endothelin-1 (ET-1), angiogenin (ANG), interleukin-8 (IL-8), MMP-9, placental growth factor (PIGF) and vascular endothelial growth factor (VEGF) were significantly inhibited following transduction of MBP-1 in MDA-MB-231 cells (Fig. 5). Future studies will be initiated to elucidate an in-depth function of angiogenesis-related molecules modulated by MBP-1.

**Discussion**

Development of therapeutic strategies using negative regulatory or killer genes has been shown to be effective against many cancers. In this study, a strong antitumor effect of MBP-1 in breast cancer growth was observed when administered intratumorally in immunocompetent mice. Therapeutic administration of MBP-1...
against breast tumors in C57BL/6 mice significantly increased life expectancy. Although we have observed recurrence of tumor growth in ∼30% mice 25 days after AdMBP-1 administration, tumor volume never reached the size of control mice at the time of sacrifice. In fact, ∼60% of the MBP-1-treated mice lived tumor-free until the date of sacrifice. Therefore, this study suggests that MBP-1 could be a useful therapeutic intervention against breast tumor growth. More importantly, we have observed the inhibition of breast cancer metastasis into lungs following MBP-1 introduction. Introduction of MBP-1 into several cancer cells inhibits cell growth and induces cell death (15). Similar results were observed with p53 or MDA-7/interleukin-24. The p53 tumor suppressor gene is thought to be central in protecting against development of many cancer types, and p53 gene therapy is becoming established as a useful strategy in many cancers adjunct to conventional treatments (28). MDA-7/interleukin-24 displayed cancer-specific, apoptosis-inducing properties and ubiquitous antitumor activities (18).

MMPs are proteolytic enzymes that regulate cancer cell growth, differentiation, apoptosis, migration and invasion, and regulation of tumor angiogenesis and immune surveillance (12). During metastasis, cancer cells must cross several extracellular matrix barriers. First, they cross the epithelial basement membrane and invade the surrounding stroma and then enter blood vessels or lymphatics, extravasate, and establish new proliferating colonies (29). In experimental metastasis assays, the number of colonies formed in the lungs of mice is reduced by downregulation of MMP-9 in cancer cells and is also reduced in the MMP-2 and MMP-9 null mice compared with wild-type mice. In our studies, we have observed that MMP-2 and MMP-9 expression is inhibited by MBP-1. Human MMP-2 promoter sequences possess several transcriptional factor binding sites, including Ets (30). MBP-1 cooperates with Ets for modulation of Bcl-x promoter activity (15). Therefore, it is conceivable that MBP-1 represses MMP-2 at the transcriptional level by cooperating with Ets-1. However, other transcription factors such as Notch1 and Mek5 may also be involved in the regulation of MMPs. Recent studies have shown that MBP-1 interacts with these proteins (16,31), and future studies will be initiated to further delineate MBP-1–mediated MMP-2/9 regulation.

MMP-2 and MMP-9 are known to play important roles in the proteolysis of extracellular matrix, which allows endothelial cell migration toward the angiogenic stimuli to form new blood vessels and nurture tumor cells to grow. Our results showed that transduction of MBP-1 inhibits in vitro angiogenesis. We have also observed that several angiogenic factors such as endothelin-1, angiogenin, fibroblast growth factor-1, insulin-like growth factor binding protein-1, vascular endothelial growth factor, and MMP-9 are inhibited following introduction of MBP-1. In summary, our preclinical study suggests that therapeutic administration of MBP-1 against breast tumors in C57BL/6 mice significantly increased life expectancy and inhibit primary breast tumor growth and lung metastasis. Thus, MBP-1 acts as a double-edged sword by inhibiting cancer growth and metastasis. Future studies will attempt to investigate a more generalized role of MBP-1 as a cancer therapy.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
References

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