Tumor-Suppressive Effects of MBP-1 in Non–Small Cell Lung Cancer Cells

Asish K. Ghosh,¹ Robert Steele,¹ Jan Ryerse,¹ and Ratna B. Ray¹,²

¹Department of Pathology and ²Cancer Center, Saint Louis University, St. Louis, Missouri

Abstract

Lung cancer is the leading cause of cancer death among both men and women. Only ~15% of people diagnosed with non–small cell lung cancer (NSCLC) survive this disease beyond 5 years. Thus, novel therapeutic strategies are urgently needed to improve the clinical management of this devastating disease. We have previously shown the 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 non–small cell lung tumor growth. We have observed that exogenous expression of MBP-1 in NSCLC cells (H1299) induces massive cell death. To determine the gene therapeutic potential of MBP-1, replication-deficient recombinant adenovirus expressing MBP-1 was given intratumorally in human lung cancer xenografts in nude mice. Our results showed a significant regression of lung tumor growth and prolonged survival on treatment with MBP-1 compared with the control groups (saline or dl312). Subsequently, the mechanism of MBP-1–mediated H1299 cell death was investigated. Our results suggested that MBP-1 induced poly(ADP-ribose) polymerase cleavage in H1299 cells; however, treatment with pan-caspase inhibitor did not protect against MBP-1–induced cell death. Cells transduced with MBP-1 displayed early plasma membrane permeability, mitochondrial damage without cytochrome c release, and extensive cytoplasmic vacuolation, yielding a morphotype that is typical of necrosis. Taken together, this study suggests that MBP-1 expression induces a novel form of necrosis-like cell death and MBP-1 could be a potential gene therapeutic candidate against non–small cell lung tumor growth. (Cancer Res 2006; 66(24): 11907-12)

Introduction

Lung cancer is the leading cause of cancer death among both men and women, accounting for ~28% of all cancer deaths. American Cancer Society estimates that there will be ~174,470 new cases of lung cancer in 2006. Lung cancer is the result of a series of genetic and epigenetic alterations in pulmonary epithelial cells (1). There are two major types of lung cancer: small cell lung cancer (SCLC) and non-SCLC (NSCLC). About 85% of lung cancers are NSCLC, and only ~15% of people diagnosed with NSCLC survive this disease after 5 years. Thus, novel therapeutic strategies are urgently needed to improve the clinical management of this devastating disease.

We have identified a novel cellular gene, MBP-1, from a human cervical carcinoma cell expression library (2). MBP-1, an ~37 kDa cellular protein, has multiple functions. MBP-1 binds to the c-myc promoter sequences and transcriptionally represses the c-myc gene (2–4). Subsequent studies suggested that MBP-1 acts as a general transcriptional repressor (5). Structure/function analysis of MBP-1 mutants revealed that the transcriptional repressor domains are located in the NH₂-terminal (MBP-AR) and COOH-terminal (MBP-CR) regions. We have shown that overexpression of MBP-1 gene induces apoptosis in several cancer cells (6, 7). Antitumor activity of MBP-1 was shown on human breast or prostate tumor xenografts in nude mice (6, 8).

Cell death plays an important role in development, tissue homeostasis, and degenerative diseases. The two major forms of cell death are apoptosis and necrosis. Apoptosis is characterized by the ordered cellular degradation of proteins and organelles, maintenance of plasma membrane integrity, and noninflammatory phagocytosis of the intact fragments of the dying cell (9, 10). Necrosis is characterized by cell swelling with plasma membrane rupture, electron-lucent cytoplasm, swelling of cellular organelles, releasing cellular contents into the extracellular environment, and loss of plasma membrane integrity. Biochemical determinants of necrotic cell death are not well understood; however, opening of the mitochondrial permeability transition pore plays a major role in necrosis (11). Recently, it has been suggested that necrotic cell death, like apoptosis, can be a regulated event that contributes to development and to the maintenance of organismal homeostasis (11). Programmed cell necrosis plays a role in several disease processes, such as vascular occlusive disease, neurodegenerative diseases, inflammatory diseases, and cancer (12–14). Because exogenous expression of MBP-1 seems to inhibit proliferation of several diverse cancer cells, we evaluated the therapeutic potential of MBP-1 against lung cancer cells in vivo. We have also examined the underlying mechanism involved in MBP-1–mediated human NSCLC cell death. We have shown that transduction of MBP-1 in H1299 cells induces necrosis-like cell death and regresses tumor growth.

Materials and Methods

Cell culture. NSCLC cell line (H1299) was purchased from the American Type Culture Collection (Manassas, VA) and maintained in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified CO₂ incubator.

Cell proliferation assay. H1299 cells were infected with the replication-deficient control adenovirus (dl312) or AdMBP-1 (7). Cell viability was determined by trypan blue exclusion at days 0, 2, and 4 following transduction.

Tumorigenicity assay. Female BALB/c athymic nude mice (4–5 weeks old) were purchased from Harlan Sprague Dawley (Indianapolis, IN). H1299 cells were grown, harvested with PBS, and resuspended in DMEM. Mice were injected s.c. into each posterior flank region with approximately 2.0 × 10⁶ cells (0.1 ml amount). Tumors were allowed to grow, and gene
transfer treatment was started when they reached a mean volume of 184 mm$^3$. After tumor formation, mice were randomized into three groups. A dose of approximately 5 \times 10^9 virus particles of dl312 or AdMBP-1 was given intratumorally each day for a total of 5 consecutive days. Another group of mice received saline and was used as control group. Tumor growth was monitored twice weekly using a slide caliper, and their volume was calculated as described previously (8). All mice were sacrificed when tumor growth reached \(1,800\) 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.** For statistical analysis, two-tailed Student's t test was used.

**Western blot analysis.** H1299 cells were transduced with AdMBP-1 or dl312 control virus, and cell lysates were prepared after 48 and 96 hours after infection in 2x SDS sample buffer. Poly(ADP-ribose) polymerase (PARP) cleavage was analyzed by Western blot using a rabbit polyclonal antibody to PARP (Alexis Biochemicals, San Diego, CA). Lysates were also prepared from cells following transduction and treatment with the pancaspase inhibitor zVAD-fmk (Santa Cruz Biotechnology, Santa Cruz, CA). To prepare the cytoplasmic fraction of H1299, cells were infected with dl312 or AdMBP-1 for 72 hours. Cytochrome c was detected by immunoblotting the membrane using a specific antibody to cytochrome c (Santa Cruz Biotechnology) followed by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) as described previously (7).

**Immunofluorescence for cytochrome c.** H1299 cells were infected with either dl312 or AdMBP-1 at 500 multiplicities of infection (MOI) for immunofluorescence study. After 72 hours of infection, cells were treated with MitoTracker (Molecular Probes, Eugene, OR) for 30 minutes. After washing, cells were incubated with a mouse monoclonal antibody to cytochrome c (Promega, Madison, WI) for confocal microscopy (Bio-Rad 1024, Hercules, CA) as described previously (7).

**Assessment of permeability transition pore opening by confocal imaging.** H1299 cells were transduced with different doses of AdMBP-1 or dl312 for 24 or 48 hours. Cells were washed with HH buffer [HBSS, 10 mmol/L HEPES (pH 7.2)] before staining with 1 \mu mol/L calcein-AM ester (Molecular Probes) and 5 mmol/L CoCl$_2$ at room temperature for 15 minutes. The CoCl$_2$ was added to quench the cytoplasmic staining so only the fluorescent mitochondria were imaged (15). Cells were washed four times and resuspended in HH buffer before imaging on Bio-Rad 1024 confocal laser microscope. Excitation and emission filters of 488 and 520 nm, respectively, were used for capturing the calcein images.

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**Figure 1.** MBP-1 expression in NSCLC cells inhibits cell proliferation. H1299 cells were transduced with AdMBP-1 or the control virus (dl312), and cell viability was determined by trypan blue exclusion at different time points as indicated. Points, mean of three separate experiments.

**Figure 2.** Evaluation of therapeutic use of AdMBP-1 against NSCLC xenografts in nude mice. A, intratumor administration of AdMBP-1 suppresses tumor growth. H1299 cells were implanted s.c. into flank of female nude mice. Tumor-bearing mice were randomized into three treatment groups for intratumor administration of saline, dl312, or AdMBP-1, respectively, when the tumor size reached an average volume of 184 mm$^3$. A total of five intratumor injections was applied for each group for 5 consecutive days. Mean volume of tumor growth was monitored twice weekly. Bars, SE. B, survival analysis of mice bearing lung tumor (H1299) xenografts following intratumor delivery of saline, dl312, or AdMBP-1.
Electron microscopy. AdMBP-1– or dl312-transduced H1299 cells were pelleted by centrifugation and fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.25) containing 2% sucrose and 2mmol/Lcalciumchloride for 16 hours at 4°C. Cell pellets were washed in 0.1 mol/L sodium cacodylate buffer containing 5% sucrose (this and all subsequent steps up to polymerization were at room temperature) and postfixed in 1% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer containing 2% sucrose for 3 hours. Cell pellets were then washed twice in distilled water, incubated for 1 hour in 2% aqueous uranyl acetate, dehydrated through graded ethanols to 100%, rinsed twice in propylene oxide, and infiltrated with a 1:1 mixture of Polybed resin (Polysciences, Inc., Warrington, PA) and propylene oxide for 3 hours. The cell pellets were then incubated in fresh Polybed resin for 4 to 6 hours, transferred to BEEM capsules filled with fresh resin, and polymerized overnight at 70°C. Thick (0.25 μm) sections were cut with glass knives using a Reichert Ultracut E ultramicrotome (Depew, NY), heat attached to glass slides, and stained with toluidine blue for light microscope evaluation. Thin (50 nm) sections were cut with a diamond knife on a Reichert ultramicrotome, collected on 200 mesh copper grids, poststained with uranyl acetate and lead citrate, and viewed and photographed with a JEOL (Peabody, MA) 100CX transmission electron microscope.

Results

Exogenous expression of MBP-1 in NSCLC cells inhibits cell proliferation. To investigate whether MBP-1 expression negatively regulates NSCLC cell growth, we transduced H1299 cells with replication-deficient adenovirus expressing MBP-1 (AdMBP-1) or control virus (dl312). Viable cell number was counted at 2-day intervals following transduction by trypan blue exclusion (Fig. 1). A significant inhibition of cell proliferation was observed following transduction of H1299 cells with AdMBP-1 compared with the control virus–infected cells. Inhibition of H1299 cell proliferation was also observed in a dose-dependent manner of AdMBP-1 (data not shown). Microscopic observation shows that expression of MBP-1 in H1299 cells resulted in massive cell death, suggesting that MBP-1 negatively regulates H1299 cell proliferation by inducing cell death.

Therapeutic use of AdMBP-1 against NSCLC growth in nude mice. Because MBP-1 expression in H1299 resulted in induction of cell death, we examined whether MBP-1 could be used as a gene therapeutic intervention against NSCLC in a preclinical mouse model study. For this, H1299 cells were implanted s.c. at the flank

![Figure 3. MBP-1–mediated cell death involves PARP cleavage. Lysates were prepared from H1299 cells transduced with dl312 (lanes 1 and 2) or AdMBP-1 (lanes 3 and 4) for 72 hours and subjected to Western blot analysis using a specific antibody to PARP. Lanes 3 and 4, on transduction of MBP-1, PARP was cleaved to an 86-kDa signature peptide. Treatment of cells with the pan-caspase inhibitor zVAD-fmk following transduction with dl312 (lane 2) or AdMBP-1 (lane 4) did not inhibit MBP-1–induced PARP cleavage.](image)

![Figure 4. MBP-1–mediated cell death resembles ultrastructural features of necrotic cell death. A, control virus–infected cells showing intact ultrastructurally normal nuclei (N) and cellular organelles and absence of cytoplasmic vacuolation. B, low-magnification survey electron micrograph illustrating the cellular vacuolation, nuclear densification, nuclear fragmentation, and necrotic cell lysis in AdMBP-1–transduced cells. C, higher-magnification electron micrograph illustrating the swollen, vacuolated, and lipid droplet (L)-filled cytoplasm of an MBP-1–expressing cell. D, a further close analysis showing swollen mitochondria (M) with disintegrated cristae, numerous large and small cytoplasmic vacuoles (V), and a large lipid droplet. Magnifications, ×1,800 (A and B), ×3,500 (C), and ×15,000 (D). Bars, 10 μm (A and B), 5 μm (C), and 1 μm (D).](image)
region of female nude mice. When the tumor volume reached an average of 184 mm³, the mice were randomized into different treatment groups for administration of recombinant adenovirus constructs. A dose of approximately $5 \times 10^8$ virus particles of dl312 or AdMBP-1 was given intratumorally each day by five consecutive injections. Another group of mice bearing xenograft tumors were injected saline and used as controls. A significant reduction in tumor growth was observed following administration of AdMBP-1 (Fig. 2). In contrast, tumors in the control group receiving either saline or dl312 control virus grew aggressively and the mice were sacrificed because of large tumor volume (>1,800 mm³) within a couple of weeks following treatment. Interestingly, 50% of mice lived tumor-free until the day of sacrifice (day 38) following gene therapeutic intervention with AdMBP-1. Some of the tumors ($n = 7$) displayed significant reduction of tumor growth until day 21 and then relapsed, although the tumor growth remained very slow until the observed period (Fig. 2A). As shown in Fig. 2B, intratumor injection of AdMBP-1 significantly prolonged animal survival from 14 to 38 days ($P < 0.0001$). These results suggest that MBP-1 could be an effective therapeutic intervention strategy against NSCLC growth.

**MBP-1–induced cell death involves caspase-independent PARP cleavage.** We next investigated the underlying mechanism of MBP-1–induced H1299 cell death. First, we examined whether MBP-1–induced cell death involves PARP cleavage. H1299 cells were transduced with AdMBP-1 or dl312 control virus for 48 hours. Cell lysates were analyzed for PARP cleavage by Western blot using a specific antibody. Expression of MBP-1 in H1299 cells induces PARP cleavage (~86 kDa), suggesting its involvement in MBP-1–mediated cell death (Fig. 3). We have also examined the status of caspase-9 and caspase-3 on MBP-1–transduced H1299 cells and did not observe activation of caspase-9; however, caspase-3 was activated (data not shown). AdMBP-1–transduced cells treated with the pan-caspase inhibitor zVAD-fmk did not protect from cell death or inhibit PARP cleavage, suggesting that this process may occur independent of caspase activation cascade, although the cause of caspase-3 activation remains unknown. To further investigate whether MBP-1–mediated inhibition of H1299 cell proliferation was due to apoptosis, genomic DNA was isolated from cells infected with AdMBP-1 or dl312. Internucleosomal DNA fragmentation was not detected when analyzed by agarose gel electrophoresis (data not shown). Together, our results indicated that MBP-1–mediated H1299 cell death occurred through non-classic apoptotic pathway.

**MBP-1–expressing cells have ultrastructural features of necrosis.** We next examined the fine ultrastructural features of H1299 cells following transduction with AdMBP-1. At the electron microscope level, the dl312-infected control cells exhibited an intact cellular morphology with ultrastructurally normal nuclei and cellular organelles (Fig. 4A). In contrast, the AdMBP-1–infected cells exhibited either cellular densification with highly disorganized nuclei and numerous cellular vacuoles and lipid droplets or extensive cell lysis with accompanying cellular debris (Fig. 4B and C). The higher magnification electron micrograph of a region in the cytoplasm of an AdMBP-1–transduced H1299 cell shows numerous small and large cytoplasmic vacuoles, swollen and internally disorganized mitochondria, and a large lipid droplet (Fig. 4D). These morphologic observations, along with the total absence of intact cellular blebs or fragmentation (characteristics of apoptosis), are consistent with the idea that MBP-1 causes H1299 cell death by a necrosis-like process.

**MBP-1 induces mitochondrial pore formation.** The intracellular ATP concentration may represent a downstream signal capable of directing cells toward either type of cell death, according to the hypothesis that high energy levels are required for the execution of the apoptotic program, whereas they are dissipated during necrosis (16). Several additional cellular factors, independent of caspase activation, may influence the decision between apoptosis and necrosis, including the duration of mitochondrial membrane pore opening, oxidative stress, and Bcl-2 expression (17). In fact, treatment of H1299 cells with the pan-caspase inhibitor zVAD-fmk did not block MBP-1–induced cell death and PARP cleavage (Fig. 3). The status of the mitochondrial permeability transition pore can be determined with the membrane-permeating fluorescent probe calcine-AM, which freely enters mitochondria but cannot exit except through an open permeability transition pore following processing by cellular esterases. Using CoCl$_2$ quenching of cytosolic fluorescence as described by Bernardi et al. (15), the release of calcine from mitochondria was analyzed by confocal laser microscopy (Fig. 5). Twenty-four hours following AdMBP-1 transduction, H1299 cells lose mitochondrial calcine staining (Fig. 5B and C), indicating opening of the mitochondrial permeability transition pore. In contrast, in control virus–infected cells (Fig. 5A), calcine staining was stronger and confined to the mitochondria. Similar results were obtained when we examined H1299 cells 48 hours following transduction with AdMBP-1 under similar experimental conditions (data not shown).
**MBP-1 Induces Lung Cancer Cell Death**

MBP-1–induced opening of mitochondrial pore does not allow cytochrome c accumulation. To investigate whether opening of the mitochondrial permeability transition pore resulted in accumulation of cytochrome c from mitochondria into the cytosol, H1299 cells were transduced with the control virus (dl312) or AdMBP-1. After 72 hours of transduction, cells were immunostained with an antibody to cytochrome c followed by a secondary antibody to mouse IgG conjugated with Alexa Fluor 568 fluorochrome (Fig. 6a). Images under confocal microscope did not reveal accumulation of cytochrome c in AdMBP-1–expressing cells (Fig. 6a) compared with the control virus–infected cells (Fig. 6a, a).

Subcellular fractionation of H1299 cells infected with AdMBP-1 or dl312 was also done to confirm localization of cytochrome c by Western blot analysis using a specific antibody (Santa Cruz Biotechnology). We have observed that most of the cytochrome c was confined in the mitochondrial fraction from both dl312- and AdMBP-1–infected H1299 cells (Fig. 6b). However, no significant accumulation of cytochrome c was detected in the cytosolic fractions of the control as well as MBP-1–expressing cells, which corroborated with the results from our confocal microscopy. Together, these results suggest that transduction of AdMBP-1 in H1299 cells does not translocate cytochrome c from mitochondria into the cytosol. We have shown previously that transduction of MBP-1 in breast cancer cells induces apoptosis and translocates cytochrome c from mitochondria into cytosol (7). Cancer cells use different signaling pathways for survival as there is aberrant activation or deactivation of various signaling pathways, oncoproteins, and tumor suppressor genes. It is well documented in the literature that cancer cells respond to therapeutic agents differently depending on the cell type, hormonal status, tissue origin, disease stage, and other factors. The distinction of our present results with lung cancer cells compared with previous work is likely to be due to the use of different cell types.

**Discussion**

In this study, we have observed that transduction of MBP-1 induces massive cell death in human NSCLC cells (H1299). Furthermore, a strong antitumor effect of MBP-1 on non–small cell lung tumor growth was observed when given intratumorally. Therapeutic administration of MBP-1 against lung xenografts in nude mice significantly increased life expectancy. Although we have observed recurrence of tumor growth in ~50% nude mice 21 days after the last AdMBP-1 administration, tumor volume never reached to that of control mice at the time of sacrifice. In fact, ~50% of the MBP-1–treated mice lived tumor-free until the date of sacrifice. Therefore, this study suggested that MBP-1 could be a useful therapeutic intervention against non–small cell lung tumor growth. Introduction of MBP-1 into several cancer cells inhibits cell growth and induces cell death (7). Similar results were observed with p53 or Mda7/IL-24. The p53 tumor suppressor gene is thought to be central in protecting the development of cancer, and p53 gene therapy is becoming established as a useful strategy in many cancers adjunct to conventional treatments (18). Mda7/IL-24 displayed cancer-specific apoptosis-inducing properties, and ubiquitous antitumor activities were recently observed (19). Interestingly, MBP-1 has high sequence homology with ENO-1 gene (2, 7). It has been suggested that MBP-1 could be an alternative translated form of α-enolase (20). It is also possible that MBP-1 is an alternative spliced form of ENO-1 gene, and further work is needed to unravel this paradigm.

MBP-1 induces apoptosis in several human cancer cells, including breast and prostate cancers. We next examined the mechanism of MBP-1–mediated NSCLC death. PARP was cleaved to its signature polypeptide of ~86 kDa in H1299 cells on expression of MBP-1.

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Furthermore, treatment of cells with the pan-caspase inhibitor zVAD-fmk did not protect the cells from MBP-1–induced cell death or inhibit PARP cleavage. Interestingly, we have observed the activation of caspase-3 in H1299 cells on 72 hours of MBP-1 transduction without activation of caspase-9 (data not shown) or cytochrome c release. Recently, Pelletier et al. (21) have shown that calcium could activate caspase-3 without involving caspase-9 activation and cytochrome c release, although MBP-1–mediated caspase-3 activation in H1299 cells remains to be elucidated. Apoptosis and necrosis are two forms of cell death with distinct biochemical and morphologic features. We have examined morphologic features of H1299 cells expressing MBP-1 to determine whether MBP-1–mediated NSCLC death was due to induction of apoptosis or necrosis by transmission electron microscopy. We observed that MBP-1 expression causes a necrosis-like cell death in H1299 cells, including cellular densification with highly disorganized nuclei, numerous small and large cytoplasmic vacuoles and lipid droplets, extensive cell lysis with accompanying cellular debris, and numerous swollen and internally disorganized mitochondria. The disorganized mitochondrial structure suggested that MBP-1 expression may induce mitochondrial dysfunction. Using the membrane-permeable fluorescent probe calcine-AM, we observed opening of mitochondrial permeability transition pores on MBP-1 expression in NSCLCs; however, cytochrome c is not released from mitochondria into the cytosol in MBP-1–expressing cells.

The biochemical determinants of necrotic cell death are less well defined, although the mitochondrial permeability transition pore might play a major role. The Bcl-2 family protein BNIP3 induces necrotic cell death independent of caspase activation, cytochrome c release, and translocation of apoptosis-inducing factor from mitochondria (22). Arsenite (As$_2$O$_3$), a clinically efficient agent in anticancer therapy, induces necrotic cell death through a regulated, Bcl-XL–sensitive mitochondrial pathway that is largely caspase independent (23). It has also been reported that PARP cleavage also can cause necrotic cell death (24), although the in-depth mechanism is yet to be elucidated. The physiologic function of the permeability transition pore core complex remains poorly understood; however, as long as the permeability transition pore is open, a cell cannot generate ATP by oxidative phosphorylation (11). Although the opening of the permeability transition pore has been proposed to amplify apoptosis by mediating release of mitochondrial apoptogenic factors, its persistent opening leads to necrotic cell death (25–27). Furthermore, a consequence of persistent permeability transition pore opening is the breakdown of the mitochondrial membrane potential, the inability to synthesize ATP, and, finally, necrotic cell death (28). The opening of the mitochondrial permeability transition pore we observed in the 24- to 48-hour period following MBP-1 expression in H1299 cells may therefore cause induction of necrosis-like cell death.

In summary, we observed that transduction of MBP-1 induces necrosis-like cell death in human NSCLC cells. Our preclinical study suggests that therapeutic administration of MBP-1 against lung xenograft tumors in nude mice significantly increased life expectancy. Therefore, MBP-1 could be a useful therapeutic intervention against non–small cell lung tumor growth.

Acknowledgments

References

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