

Carboxyl-Terminal Repressor Domain of MBP-1 is Sufficient for Regression of Prostate Tumor Growth in Nude Mice

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Abstract

Prostate cancer is the most frequently diagnosed cancer in men and the second leading cause of male cancer death in the United States. Early detection and improved procedures for surgical intervention and radiation therapy have reduced the fatalities; however, there is no effective cure for men with advanced disease and additional therapy is urgently needed. We have previously shown that MBP-1 acts as a general transcriptional repressor and exerts an antiproliferative effect on several human cancer cells. MBP-1 possesses two repressor domains, located at the amino and carboxyl termini. In this study, we have examined the potential of the repressor domains of MBP-1 as a gene therapeutic candidate in regression of prostate tumor growth. Our results suggested that replication-deficient adenovirus-mediated delivery of amino-terminal (MBP-AR) or carboxyl-terminal (MBP-CR) repressor domain of MBP-1 exerted an antiproliferative effect, like the full-length MBP-1, and induced caspase-independent apoptosis in prostate cancer cells. Next, we investigated the therapeutic effectiveness of MBP-1 repressor domain on prostate tumors. When tested in human tumor xenografts in nude mice, MBP-CR suppressed prostate tumor growth more effectively than full-length MBP-1, whereas MBP-AR delayed prostate tumor growth. Together, these results suggested that MBP-CR expression has an antiproliferative effect in human prostate cancer cells, being more effective than the full-length MBP-1 in preventing tumor growth. (Cancer Res 2005; 65(3): 718-21)

Introduction

Prostate cancer is the second leading cause of cancer death among males in the United States. Although survival rates are good for prostate cancer on early diagnosis, and many patients in this group can be cured by radical prostatectomy or radiation therapy, a significant number will develop local recurrence within the prostate, and some ultimately will develop disseminated disease (1). In 2003, an estimated 220,900 American men were diagnosed with prostate cancer, and ~28,900 men died from the disease (2). Therefore, more effective therapies that can cure localized tumors and prevent their metastasis are urgently needed. Considerable interest has been shown in the discovery of cellular genes, which seem to be antiproliferative in gene therapy studies (3). We have identified a novel cellular gene MBP-1 from a human cervical carcinoma cell expression library (4). 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 (4-7). Subsequent studies suggested that MBP-1 acts as a

general transcriptional repressor (8). Structure/function analysis of MBP-1 mutants revealed that the transcriptional repressor domains are located in the amino-terminal (MBP-AR) and carboxyl-terminal (MBP-CR) regions. Ectopic expression of MBP-1 induces cell death in murine fibroblasts (6) and regresses human breast tumor growth in nude mice (9). In this study, we have examined the potential of MBP-1 repressor domain as a gene therapeutic candidate in regression of prostate tumor growth.

Materials and Methods

Cell Lines. Androgen-independent prostate cancer cell lines, DU145 and PC3, were purchased from 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.

Generation of MBP-AR or MBP-CR in Replication-Deficient Recombinant Adenovirus. The cDNA encoding the amino acid residues 1 to 95 (MBP-AR) or the amino acid residues 190 to 338 (MBP-CR) fused to Flag-epitope were cloned into replication-deficient adenovirus as described previously (10). The expression of MBP-AR or MBP-CR in the recombinant adenovirus was verified by Western blot analysis using an antibody to Flag. Full-length MBP-1 expressing recombinant adenovirus (AdMBP-1) and Adβ-gal were also used in this study (11). All recombinant adenovirus constructs were purified using CsCl density gradient (11).

Cell Proliferation Assay. PC3 cells were infected with Adβ-gal (control virus), AdMBP-1, AdMBP-AR, or AdMBP-CR. Cell viability was determined by trypan blue exclusion at 0, 48, 72, and 120 hours following transduction.

DNA Fragmentation. PC3 cells were infected with the Adβ-gal, AdMBP-1, AdMBP-AR, or AdMBP-CR at 100 multiplicity of infection. After 72 hours of infection, cells were treated with lysis buffer [10 mmol Tris (pH 8.0), 1 mmol EDTA, 100 mmol NaCl, 1.0% SDS, and 200 µg/mL proteinase K]. Genomic DNA was isolated and analyzed on a 1.4% agarose gel by electrophoresis as described previously (6).

PARP Cleavage and Activation of Caspases. PC3 cells were transduced with Adβ-gal, AdMBP-1, AdMBP-AR, or AdMBP-CR for 72 hours. Cell lysates were subjected to Western blot analysis using a rabbit polyclonal antibody to PARP (Alexis, San Diego, CA) or antibodies to procaspase-9 and procaspase-3 (PharMingen, San Diego, CA).

Tumorigenicity Assay. Male BALB/c athymic nude mice (5-7 weeks old) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). In the first set of experiments, prostate cancer cells were transduced with Adβ-gal, AdMBP-1, AdMBP-AR, or AdMBP-CR. After 48 hours of infection, cells were trypsinized, washed and the cell suspension was injected s.c. in the flank region of nude mice. Tumor growth was monitored weekly using a slide caliper. In the second set of experiments, prostate cancer cells were harvested, washed with PBS and resuspended in DMEM containing 40% matrigel (BD Biosciences, Bedford, MA). Male nude mice were injected s.c. into each posterior flank region with 4.0×10^6 cells (0.1 mL amount). Tumors were allowed to grow and gene transfer treatment was started when they reached a mean volume of 88 mm³. A dose of $\sim 5 \times 10^9$ virus particles of Adβ-gal, AdMBP-1 or AdMBP-CR was given intratumorally (i.t.) every 48 hours for a total of three injections. Another group of mice received saline and were used as controls. Regression of tumor growth was monitored weekly using a slide caliper and their volume was calculated by employing the following formula: $[\text{length} \times (\text{width})^2] / 2$. All mice were sacrificed when tumor growth reached $\sim 1,500$ mm³ using the highest

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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* tests was used.

Results and Discussion

Generation of Recombinant Adenovirus Expressing Repressor Domains of MBP-1. We have shown previously that MBP-1 possesses two independent, separable transcriptional repressor domains located at the NH₂- and COOH-termini (8). To investigate whether the repressor domains of MBP-1 exert antiproliferative activity, we generated recombinant adenovirus (Fig. 1A) expressing the amino-terminal repressor domain (AdMBP-AR) or carboxyl-terminal repressor domain (AdMBP-CR). To examine the expression of AdMBP-AR or AdMBP-CR, PC3 cells were infected and cell lysates were prepared after 72 hours of infection and analyzed by Western blot using an antibody to Flag. Results showed expression of both the deletion mutants of MBP-1 in PC3 cells on transduction of AdMBP-AR and AdMBP-CR (data not shown).

Antiproliferative Effect of MBP-AR or MBP-CR on Prostate Cancer Cells. To examine whether expression of MBP-AR or MBP-CR has an effect on prostate cancer cell growth, PC3 cells were transduced with either Adβ-gal, AdMBP-AR, or AdMBP-CR. Cell viability was determined by trypan blue exclusion at various time periods (0, 48, 72, and 120 hours). AdMBP-1 was used in parallel for comparison. Our results showed a significant reduction in cell viability between 48 and 72 hours following transduction with AdMBP-AR or AdMBP-CR when compared with that of Adβ-gal (Fig. 1B). Similar results were obtained when DU145 cells were transduced with these recombinant adenoviruses. These results suggested that both MBP-AR and MBP-CR exert antiproliferative effects, like full-length MBP-1, on prostate cancer cell growth *in vitro*.

MBP-AR or MBP-CR Induces Apoptosis in Prostate Cancer Cells. We next determined whether the MBP-AR- or MBP-CR-mediated inhibition of prostate cancer cell growth is due to induction of apoptosis. For this, we examined intranucleosomal DNA fragmentation in cells infected with either Adβ-gal, AdMBP-AR, or AdMBP-CR. AdMBP-1 was used in parallel for comparison. DNA was isolated from cells after 72 hours of infection and analyzed by agarose gel electrophoresis. We have observed the intranucleosomal DNA fragmentation in cells transduced with full-length MBP-1, MBP-AR, or MBP-CR (Fig. 2A). However, cells transduced with Adβ-gal did not exhibit detectable DNA fragmentation. Thus, both the repressor domains of MBP-1 induce apoptosis in prostate cancer cells.

To further investigate the effect of MBP-1 repressor domain on the apoptotic signaling pathway, PARP cleavage was examined in the lysates of prostate cancer cells (PC3) transduced with recombinant adenoviruses for 72 hours by Western blot analysis using a specific antibody (Fig. 2B). Cells transduced with AdMBP-AR or AdMBP-CR, as well as AdMBP-1, displayed a cleavage of ~116 kDa native PARP to its ~86 kDa signature polypeptide. Cells transduced with Adβ-gal for 72 hours or left untreated did not display any detectable amount of ~86 kDa PARP cleavage product. Therefore, these results suggested that MBP-1-mediated apoptosis involves PARP cleavage. We further analyzed the activation of procaspase-9 and procaspase-3 from AdMBP-1 transduced cells by Western blot using specific antibodies, and did not observe activation. Treatment of cells with a pan-caspase inhibitor,

zVAD-fmk, could not protect from MBP-1-induced apoptosis or PARP cleavage (Fig. 2B, lane 6), suggesting that MBP-1-induced apoptosis in PC3 cells may occur independent of caspase activation. Translocation of apoptosis-inducing factor from mitochondria into the nucleus also induces apoptosis without activation of caspases (12). Here, we did not observe translocation of apoptosis-inducing factor from mitochondria into the nucleus of prostate cancer cells expressing MBP-1 or its repressor domain (data not shown), suggesting that MBP-1-mediated apoptosis does not involve this pathway. Our results also suggested that apoptosis in PC3 cells mediated by MBP-1 repressor domains follow different pathways as compared with HeLa or MCF-7 cells (11). Further in-depth mechanism of MBP-1-mediated apoptosis in prostate cancer (PC3) cells remains to be elucidated.

Effect of MBP-AR and MBP-CR on Prostate Tumor Growth *In vivo*. To investigate whether MBP-AR or MBP-CR could regress prostate tumor growth, DU145 and PC3 cells were transduced with Adβ-gal, AdMBP-AR, and AdMBP-CR, or left untreated. Full-length MBP-1 (AdMBP-1) was used in parallel for comparison. After 48 hours of infection, cells were harvested, washed and injected s.c. into the flanks of nude mice. None of the athymic nude mice given an injection of MBP-CR or full-length MBP-1-transduced cells

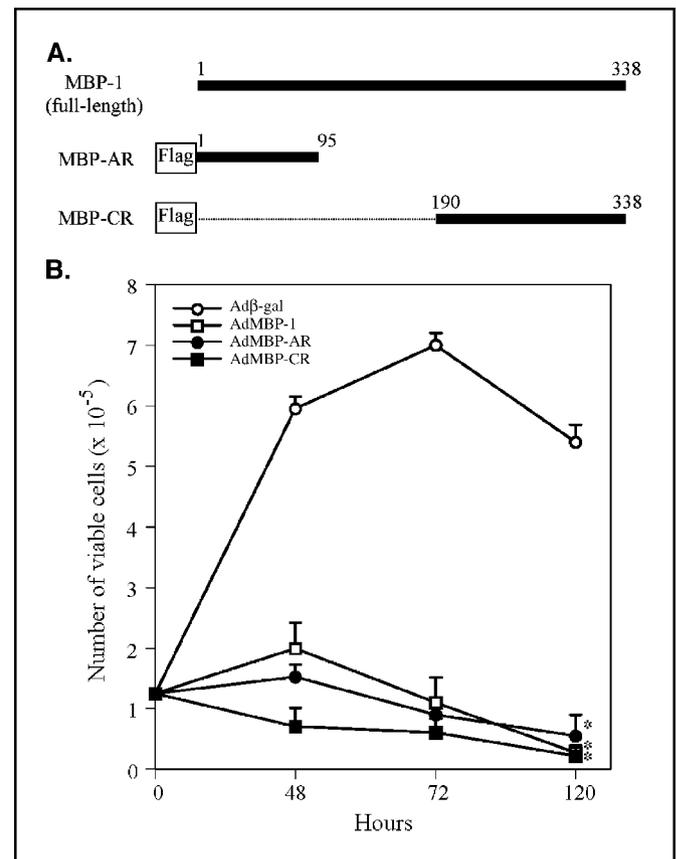


Figure 1. Repressor domains of MBP-1 (MBP-AR and MBP-CR) exert antiproliferative activity on prostate cancer cell growth. **A**, schematic presentation of the MBP-1 repressor domains MBP-AR and MBP-CR showing amino acid positions. Flag epitope was fused to the amino terminus of the repressor domains and cloned in a replication-deficient recombinant adenovirus vector. **B**, expression of MBP-AR or MBP-CR inhibits cell proliferation. Prostate cancer cells were transduced with either Adβ-gal, AdMBP-1, AdMBP-AR, or AdMBP-CR and cell viability was determined by trypan blue exclusion at various time points (0, 48, 72, and 120 hours). Each experiment was repeated using two different preparations of virus stock, *, *P* < 0.001 compared with Adβ-gal-infected cells.

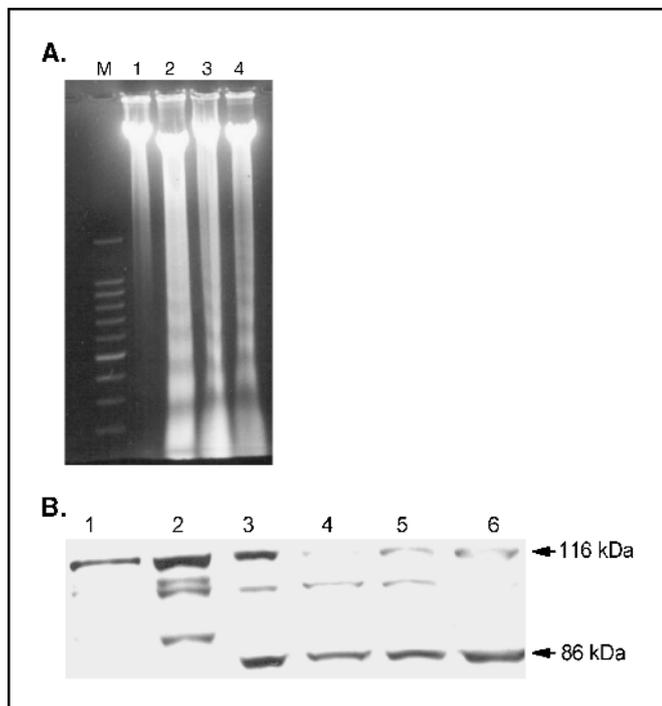


Figure 2. Expression of MBP-AR or MBP-CR induces apoptosis. *A*, cellular DNA was isolated from PC3 cells infected with either Ad β -gal control virus (lane 1), AdMBP-1 (lane 2), AdMBP-AR (lane 3), or AdMBP-CR (lane 4) for 72 hours and intranucleosomal DNA fragmentation was analyzed by agarose gel electrophoresis. The migration of 100 bp standard DNA ladder is shown (lane M). *B*, MBP-AR, MBP-CR, or full-length MBP-1-mediated apoptosis involves PARP cleavage. Lysates were prepared from PC3 cells transduced with Ad β -gal (lane 2), AdMBP-1 (lane 3), AdMBP-AR (lane 4), AdMBP-CR (lane 5) for 72 hours or left untreated (lane 1), and subjected to Western blot analysis using a specific antibody to PARP. Upon transduction of MBP-1, MBP-AR, or MBP-CR, PARP was cleaved to an 86-kDa signature peptide. Treatment of cells with a pan-caspase inhibitor, zVAD-fmk, did not protect against MBP-1-induced PARP cleavage (lane 6).

showed tumor formation during the observation period (Table 1). However, Ad β -gal-transduced control cells and uninfected cells implanted in nude mice displayed tumor growth and were sacrificed at day 19 (DU145) or day 44 (PC3) after injection for large tumor burden. Expression of MBP-AR delayed tumor growth

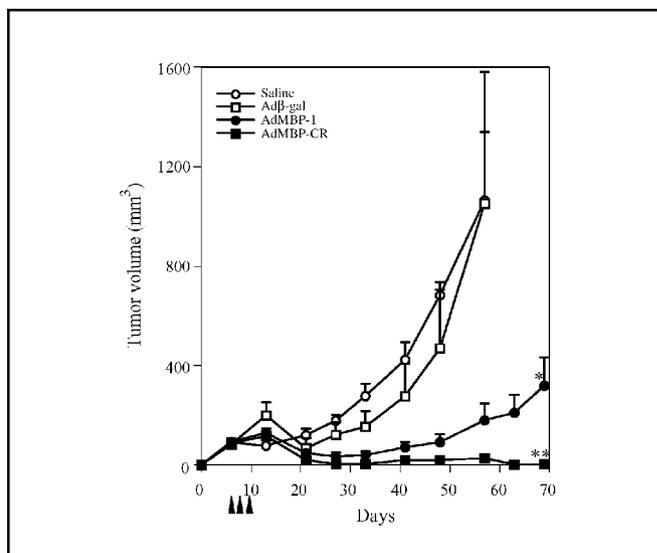


Figure 3. Evaluation of AdMBP-CR and AdMBP-1-mediated gene transfer in human prostate tumor xenografts. PC3 cells were implanted s.c. into the flanks of male nude mice. Tumor bearing mice were randomized into different treatment groups for i.t. administration of saline, Ad β -gal, AdMBP-1, or AdMBP-CR, when the tumor size reached an average volume of 88 mm³. A total of three i.t. injections were applied for each construct at every 48-hour interval. Points, mean volume of tumor growth monitored weekly; bars, SE; arrowheads, day of injections; *, significant difference ($P < 0.001$) from the control groups; **, significant difference ($P < 0.0001$) from the control groups.

and then continued to grow until the mice were sacrificed. These results suggested that the MBP-CR and full-length MBP-1 are able to inhibit *in vivo* prostate tumor growth.

Next, we examined whether the MBP-CR or full-length MBP-1 has a therapeutic effect in regression of prostate tumor growth. For this, $\sim 4 \times 10^6$ cells mixed with 40% matrigel were injected at each site in the flank region of male nude mice. When the tumor volume reached an average volume of 88 mm³, the mice were randomized into different treatment groups for administration of recombinant adenovirus constructs. A dose of $\sim 5 \times 10^9$ virus particles of Ad β -gal, AdMBP-CR, or AdMBP-1 was given i.t. every 48 hours for a total of three injections. Another group of mice bearing prostate tumors were injected with saline at the same interval and used as control.

Table 1. Antitumor effect of MBP-CR in prostate cancer cells implanted in athymic nude mice

Xenograft model	Treatment group	Tumorigenicity	Latency (d)	Day of sacrifice*
DU145	Uninfected	4/4	7	19
	Ad β -gal	4/4	7	19
	AdMBP-1	0/4	NA [†]	43
	AdMBP-AR	3/4	26	43
	AdMBP-CR	0/4	NA	43
PC3	Uninfected	4/4	19	44
	Ad β -gal	3/4	19	44
	AdMBP-1	0/4	NA	55
	AdMBP-AR	2/4	29	55
	AdMBP-CR	0/4	NA	55

NOTE: Cells were infected with recombinant adenovirus constructs or left untreated and implanted into nude mice 48 hours post-infection.

*Mice were sacrificed because of large tumor burden.

[†]NA, not applicable.

Because we observed the delayed tumor growth from AdMBP-AR-infected prostate cancer cells, we did not include AdMBP-AR in this part of our study. The tumor volume from the control groups (treated with saline or Ad β -gal) steadily increased with time and mice were sacrificed at day 57 because of tumor burden (Fig. 3). Administration of AdMBP-CR reduced the tumor size significantly as early as day 11 following treatment, and prostate tumor growth did not increase over time ($P < 0.0001$ between MBP-CR and saline, $P < 0.0001$ between AdMBP-CR and Ad β -gal). In fact, all tumors disappeared as observed following AdMBP-CR administration. Administration of full-length MBP-1 also reduced tumor size. However, a slow growth was observed after 47 days of treatment and continued to grow until day 69 when all the mice were sacrificed ($P < 0.001$ between MBP-1 and saline, $P < 0.001$ between AdMBP-1 and Ad β -gal). Together, these results suggested that administration of AdMBP-CR or full-length MBP-1 significantly reduced prostate tumor growth and the MBP-CR possesses a stronger antitumor activity.

In summary, we have shown that the repressor domains of MBP-1 exert an antiproliferative effect on prostate cancer cell growth by inducing apoptosis. MBP-1-mediated apoptosis in prostate cancer cells involves PARP cleavage without activation of caspases. In a prostate tumor xenograft model, MBP-CR displayed a stronger antitumor activity as compared with MBP-AR. Furthermore, i.t. administration of MBP-CR was more pronounced against prostate tumor growth as compared with the full-length MBP-1. Earlier studies have shown that administration of recombinant adenovirus encoding the ligand-binding ectodomain of the vascular endothelial growth factor receptor 2 fused to an

Fc domain reduced prostate tumor growth by 66% for orthotopic LNCaP tumors and by 42% for spontaneous tumors in TRAMP mice (13). Introduction of the posttranslationally phosphorylated mutant Bik into prostate tumors enhanced antitumor activity as compared with the wild-type (14). Treatment of human non-small cell lung carcinoma and bladder carcinoma cells by a recombinant adenovirus vector expressing the NH₂-terminal truncated retinoblastoma protein (pRB94) completely suppressed the tumorigenicity in nude mice (15). Although adenovirus-mediated gene transfer of both full-length retinoblastoma and pRB94 induce human pancreatic tumor regression in a mouse xenograft, the pRB94 was evidently more potent than the full-length retinoblastoma (16). In conclusion, we have used an established animal model for testing the therapeutic potential of MBP-1 and its repressor domains. The COOH-terminal repressor domain of MBP-1 was proven to have a strong antitumor activity and could be useful in gene therapy against prostate cancer growth. Therefore, it may be possible to design a small molecule to mimic the structure of this peptide by constructing a fusion protein or using a synthetic peptide for specific targeting of prostate cancer cells.

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