Adenoviral-mediated Expression of MMAC/PTEN Inhibits Proliferation and Metastasis of Human Prostate Cancer Cells

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ABSTRACT

Purpose: The purpose of this study was to determine the effects of adenoviral transgene expression of MMAC/PTEN on the in vitro and in vivo growth and survival of PC3 human prostate cancer cells.

Experimental Design: Adenoviruses expressing MMAC/PTEN or green fluorescent protein as a control were introduced into PC3 cells, and effects on signal transduction pathways and growth of tumors in an orthotopic nude mouse model were determined.

Results: MMAC/PTEN expression in PC3 cells decreased the level of phospho Akt but not that of phospho Mapk or FAK. Expression of MMAC/PTEN inhibited the in vitro growth of PC3 cells primarily by blocking cell cycle progression. Ex vivo introduction of MMAC/PTEN expression did not inhibit the tumorigenicity of orthotopically implanted PC3 cells, but it did significantly reduce tumor size and completely inhibited the formation of metastases. In vivo treatment of pre-established orthotopic PC3 tumors with adenoviral MMAC/PTEN did not significantly reduce local tumor size, but it did diminish metastasis formation.

Conclusions: MMAC/PTEN functionally regulates prostate cancer cell metastatic potential in an in vivo model system and may be an important biological marker and therapeutic target for human prostate cancer.

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths in men in the United States, with a predicted 32,000 patients succumbing to the disease in 2001 (1). Currently, there is no effective treatment for advanced stages of this disease. To improve our understanding of prostate cancer progression, many studies have examined the chromosomal alterations that characterize its different stages (2, 3). Although many alterations have been observed, those involved most frequently include chromosomes 7q, 8p, 10, 13q, and 16q (4–6). Allelic deletions associated with chromosome 10q, specifically the q23–25 region, have been observed to preferentially occur in the advanced stages of disease (7, 8). Reintroduction of this chromosomal region into rat prostate cancer cells significantly inhibited their metastatic capabilities but failed to alter their tumorigenicity (9). Many studies have implicated the tumor suppressor gene, MMAC/PTEN, as critical to the biological effects associated with deletions in (or reintroduction of) chromosome 10. MMAC/PTEN was initially identified because of homozygous deletions of the gene in human glioma and breast cancer cell lines, as well as by its homology to protein phosphatases (10–12). These and subsequent studies demonstrated that deletions and mutations of the gene occur in a wide variety of cancers, including glioma, prostate, breast, melanoma, endometrial, and ovarian (reviewed in Ref. 13). With the exception of endometrial cancer, alterations to MMAC/PTEN are detected almost exclusively in advanced stages of disease. Thus, the loss of MMAC/PTEN function does not appear to be required for tumor initiation in these diseases but instead was a hallmark of tumor progression.

One of the critical functions for the tumor suppressor activity of MMAC/PTEN is its intrinsic lipid phosphatase activity (14), which results in removal of the phosphate moiety at the 3’ position of phosphatidylinositol. By this activity, MMAC/PTEN antagonizes signaling mediated by PI3K (15) and negatively regulates the phosphorylation and activation of a number of important mitogenic and/or prosurvival signaling molecules. Our group and others have shown that enforced MMAC/PTEN expression decreases the phosphorylation and kinase activity of Akt/PKB, a proto-oncogene that is activated by PI3K-mediated signaling events (15, 16). Akt/PKB, a serine-threonine protein kinase, has been shown to be a critical regulator of many different cellular processes, including apoptosis, cell cycle progression, angiogenesis, and metabolism. Other reports (17, 18) have demonstrated that MMAC/PTEN may also dephosphorylate FAK, a protein tyrosine kinase involved in the regulation of cellular adhesion, motility, and survival.

Numerous studies have implicated the loss of MMAC/
PTEN in prostate cancer progression. In the initial identification of the gene, homozygous genetic inactivation of MMAC/PTEN was observed in the LNCaP and PC3 human prostate cancer cell lines, both of which were established from metastatic lesions (10, 11). Multiple cytogenetic studies of prostate tumors detected low rates of mutation and deletion of MMAC/PTEN in organ-confined prostate cancers (19–21). However, ∼60% of metastatic prostate cancers demonstrated loss of heterozygosity for MMAC/PTEN, accompanied by a significant rate of alterations of the second allele (22, 23). In several tumors that retain the MMAC/PTEN gene, decreased expression of the protein may be observed, e.g., Whang et al. (24) demonstrated that MMAC/PTEN mRNA and protein levels were down-regulated in >50% of prostate tumors in which the MMAC/PTEN coding region was not mutated. Furthermore, immunohistochemical analysis of human prostate cancer specimens detected down-regulated expression of MMAC/PTEN protein in a heterogeneous pattern (25). Interestingly, the foci in which MMAC/PTEN expression was lost were generally high-grade lesions, suggesting that these foci might give rise to the aggressive components of these tumors.

In contrast, studies using genetically engineered mice suggest a potential role for MMAC/PTEN at an earlier stage of prostate tumor development. The loss of both alleles of MMAC/PTEN results in embryonic lethality in mice (26–28). MMAC/PTEN p27+/− mice are viable and spontaneously develop lymphoid hyperplasia, several types of tumors, and an autoimmune syndrome (29). The prostates of these mice demonstrate global hyperplasia and frequently develop prostatic intraepithelial hyperplasia, the precursor of invasive prostate cancer. The global dysregulated growth suggests that the loss of a single allele of MMAC/PTEN causes this phenotype (26). Breeding these mice with p27−/− mice, leading to progeny that are genetically MMAC/PTEN p27−/− and p27+/− or p27−/−, resulted in 100% penetrance of frank prostate cancer, including invasive tumors (30). Thus, these knockout studies suggest that MMAC/PTEN is a potent regulator of prostate proliferation and demonstrate in this system a potential role in the earlier stages of prostate cancer tumorigenesis.

To further examine the ability of MMAC/PTEN expression to regulate prostate tumor development and/or progression, we have studied the effects of MMAC/PTEN expression in PC3 human prostate cells. The PC3 cell line was originally derived from a prostate cancer metastasis and represents a model for androgen-insensitive disease. The PC3 cells lack MMAC/PTEN expression because of deletion of both alleles (10, 11) and also lack wild-type p53 function and androgen receptor expression (31). When injected orthotopically into the prostate of nude mice, the PC3 cells grow aggressively locally (32, 33) and readily metastasize to regional lymph nodes (31). In this study, we expressed MMAC/PTEN in PC3 cells via an adenovirus vector Ad-MMAC (15) and examined the effects on cell signaling pathways, growth, and survival of PC3 cells in vitro. Additionally, the effects of MMAC/PTEN expression on the in vivo growth of PC3 cells was tested both by orthotopic implantation of cells infected with Ad-MMAC before their inoculation into nude mice (ex vivo treatment) and by the treatment of pre-established tumors with Ad-MMAC (in vivo treatment). These studies provide further insight into the ability of MMAC/PTEN to regulate specific cell signaling pathways and cellular behaviors in prostate cancer cells and demonstrate for the first time the regulation of in vivo prostate cancer metastatic capability.

MATERIALS AND METHODS

Cell Lines and Adenoviruses. The PC3 cell line was obtained originally from the American Type Culture Collection, Rockville, MD. The cells were maintained in culture in media supplemented with 10% FCS and incubated in 5% CO2/95% air at 37°C. The recombinant adenovirus Ad-MMAC expresses the human wild-type MMAC cDNA under the control of the human cytomegalovirus immediate-early promoter/enhancer (34) and was generously provided by Robert Bookstein (Canjii, Inc., San Diego, CA). Ad-GFP was derived from the same vector as Ad-MMAC; the replication-deficient virus without a transgene (Ad-DE1/Ad5-DE1) has been described previously (35), along with the virus expressing the p53 transgene (36). Adenoviruses were harvested after infection of 293 cells and isolated by cesium chloride gradient, and titer was determined by absorbance. Briefly, viral stock was propagated in 293 cells. Cells were harvested 36–48 h after infection, pelleted, resuspended in PBS, and lysed by three freeze-thaw cycles. Cell debris was removed, and resulting cellular lysates were subjected to double CsCl centrifugation. Concentrated virus was dialyzed, aliquoted, and stored at −80°C. Adenoviral titer represented as optical particles was determined by optical absorbance as described previously (37).

Tumorigenicity Studies. Cells (500,000) were plated on 10-cm tissue culture dishes. Approximately 18 h later, cells were infected with indicated amounts of adenovirus in 4 ml of media. Cells were gently agitated every 15 min for 1 h, then 6 ml of fresh media were added to each plate. Cells were infected with Ad-MMAC, or Ad-GFP at the indicated MOI, or mock infected by incubation with media alone. Twenty-four h after infection, cells were harvested by trypsinization. Cells were counted and resuspended in Ca2+ and Mg2+-free HBSS at 10,000 cells/50 μl. Cells were then implanted in the prostate of nude mice as described previously (33). In brief, nude mice were anesthetized with Nembutal and placed in a supine position. A low-midline incision was made, and the prostate was exposed. Fifty μl of HBSS containing 100,000 cells were injected into a lateral lobe of the prostate. The wound was closed with surgical metal clips. Each experimental condition was performed on groups of eight mice.

Gene Therapy Studies. Subconfluent cultures of PC3 cells were harvested by trypsinization. Cells were counted, then resuspended in Ca2+ and Mg2+-free HBSS at 50,000 cells/50 μl. Cells were then implanted in the prostate of nude mice as described above, and the wound was closed with surgical metal clips. Tumors were treated at 7 and 14 days after implantation. Tumors were exposed by the same procedure, by a low-midline incision, and exposure of the prostate. Tumors were injected with PBS, or 1.5 × 108 plaque-forming units of Ad-GFP or Ad-MMAC, each in a total volume of 20 μl. Tumors and lymph nodes were harvested 14 days after the final gene intratumoral injection. Each experimental condition was performed on groups of eight mice.
Necropsy Procedures and Histological Studies. The mice were euthanized at the times indicated above. Primary tumors in the prostate were excised and weighed. For immunohistochemistry and H&E staining procedures, some tumors were embedded in OCT compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C. Other tumors were formalin fixed and paraffin embedded. Lymph nodes were harvested, and the presence of metastatic disease was determined histologically.

For BrdUrd staining, the mice were injected with 250 μg of BrdUrd. Two h later, the mice were sacrificed, and the prostates were formalin fixed and embedded in paraffin. For staining, tissue sections were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were then treated with 2N HCl for 30 min at 37°C. For CD31 staining, sections of frozen tissues were fixed with cold acetone and transferred to PBS. The slides were then rinsed twice with PBS, and endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 min. The samples were then washed three times with PBS and incubated for 10 min at room temperature with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with 1:100 dilution of monoclonal mouse anti-BrdUrd Ab (Becton Dickinson, Mountain View, CA) or a 1:100 dilution of monoclonal rat anti-CD31 Ab (PharMingen, San Diego, CA). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of peroxidase-conjugated antimouse IgG1 or antirat IgG. The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics, Huntsville, AL). The sections were then washed three times with distilled water and counterstained with Gill’s hematoxylin.

For TUNEL staining, frozen sections were fixed with 4% paraformaldehyde in PBS for 20 min, rinsed twice with PBS, and rinsed with 1% paraformaldehyde and stored in 70% ethanol at −70°C. Other tumors were formalin fixed and embedded in paraffin. For staining, tissue sections were formalin fixed and embedded in paraffin. Lymph nodes were harvested, and the presence of metastatic disease was determined histologically.

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For Western analysis, 250 μg of lysate were incubated with 2 μg of α-FAK monoclonal Ab (Upstate Biotechnology) for 2.5 h at 4°C, with 6 μg of rabbit antimouse IgG Ab for 1 h, then with 35 μl of Pansorbin Cells for 30 min. Complexes were centrifuged for 1 min at 15,000 × g, washed three times with immune complex kinase assay wash buffer, boiled for 5 min in 1× SDS-PAGE buffer, then run on 7.5% gels and transferred to Immobilon-P membranes as described above. Western blotting was performed using antibodies specific for phospho-tyrosine residues (4G10; Upstate Biotechnology) and FAK (Transduction Labs).

Apoptosis and Cell Cycle Analysis. Cells (500,000) were plated on 10-cm tissue culture dishes. Approximately 18 h later, cells were infected with indicated amounts of adenovirus in 4 ml of media. Cells were gently agitated every 15 min for 1 h; then 6 ml of fresh media were added to each plate. Cells were infected with Ad-MMP, Ad-MMAC, or Ad-p53 at the indicated MOI or mock infected with media alone. At the indicated time points, cells were harvested by collecting the supernatant and trypsinizing attached cells. Viable cells were counted by microscopic analysis using a hemocytometer. The significance of differences in cell number was analyzed by unpaired Student’s t test.

Protein Analysis. Cells (500,000) were plated on 10-cm tissue culture dishes. Approximately 18 h later, cells were infected with indicated amounts of adenovirus in 4 ml of media. Cells were gently agitated every 15 min for 1 h; then 6 ml of fresh media were added to each plate. Cells were infected with Ad-GFP, Ad-DE1, Ad-MMAC, or Ad-p53 at the indicated MOI or mock infected with media alone. At the indicated time points, cells were harvested by collecting the supernatant and trypsinizing attached cells. Viable cells were counted by microscopic analysis using a hemocytometer. The significance of differences in cell number was analyzed by unpaired Student’s t test.
apoptosis by TUNEL assay using the Apo-BrdUrd kit (Phoenix Flow Systems, San Diego, CA) or stained solely with propidium iodide (50 μg/ml in PBS). Apoptotic cells were detected and quantitated by flow cytometry. The significance of differences between different treatment groups was analyzed by unpaired Student’s t test.

RESULTS

In Vitro Studies. PC3 cells were chosen for these studies because of their ability to form lymph node metastases after orthotopic implantation into nude mice (33). Before the animal studies, we first examined whether the effects of ectopic expression of PTEN/MMAC were similar to those observed in other studies, we first examined whether the effects of ectopic expression of PTEN/MMAC were similar to those observed in other studies, we first examined whether the effects of ectopic expression of PTEN/MMAC were similar to those observed in other studies.

To further characterize the function of MMAC/PTEN in PC3 cells, the effects of its expression on the phosphorylation status of intermediates of cell signaling pathways were examined by Western blotting. Analysis was performed on cells maintained in FCS-enriched media, as well as in cells that were serum starved or acutely stimulated. For all three tissue culture conditions, MMAC/PTEN expression inhibited phosphorylation of Akt/PKB at its activating residues without diminishing total Akt/PKB protein levels (Fig. 2A). These results are consistent with MMAC/PTEN’s lipid phosphatase activity and are identical to our previous results in both LNCaP prostate cancer cells and human glioma cells (15, 16). The Ad-GFP virus also led to small reductions in phospho Akt expression; however, this reduction was substantially less than that observed with the Ad-MMAC virus. MMAC/PTEN protein expression did not decrease the mitogen-stimulated phosphorylation of p44/42 MAPK, as compared with mock or control adenovirus treatments. This also is consistent with our previous results. The effect of MMAC/PTEN expression on the phosphorylation of FAK was also examined. No decrease was observed in tyrosine phosphorylation, or total protein levels, of FAK in PC3 cells mock treated with media alone (Lane 1) or infected with 25 MOI of Ad-GFP (Lane 2) or Ad-MMAC (Lane 3). Cells were harvested 48 h after indicated treatments. Cell lysates were immuno-precipitated with α-FAK, immunoblotted with α-phosphotyrosine (top), and reprobed with α-FAK (bottom).

The ability of MMAC/PTEN to regulate PC3 cellular growth in vitro was determined by counting viable cell numbers at various time points after infection with control adenoviruses or Ad-MMAC or mock treatment with media alone. Infection with Ad-MMAC decreased the rate of PC3 proliferation at all time points examined (Fig. 3A). Treatment with Ad-MMAC was significantly more growth inhibitory than treatment with a control adenovirus (Fig. 3B; P < 0.05 on day 4, P < 0.01 on day 6). In previous studies, we had observed that MMAC/PTEN expression was more growth inhibitory than p53 expression in

![Fig. 1](image1.png) MMAC/PTEN expression in PC3 cells. Western blotting analysis of PC3 treated with media alone (Lane 1) or infected with 25 MOI Ad-MMAC (Lanes 2–5). Ad-MMAC-infected cells were harvested at 2, 7, 14, and 21 days after infection to determine induction and duration of MMAC/PTEN expression (top). Immunoblotting for actin (bottom) performed to confirm protein loading.

![Fig. 2](image2.png) Effects of ectopic MMAC/PTEN expression in PC3 cells on expression and phosphorylation of signaling intermediates. In A, immunoblotting was performed 48 h after mock infection or infection with 25 MOI of Ad-GFP or Ad-MMAC. Cells were harvested after continuous incubation in complete tissue culture media (Lanes 1–3) or after a 24-h serum starvation with (Lanes 4–6) or without (Lanes 7–9) acute 1% FCS stimulation. Phosphorylation of Akt/PKB and p44/42 MAPK (bottom) was detected by phosphorylation site-specific antibodies. MMAC/PTEN protein expression also shown (middle). B, phosphorylation status of FAK in PC3 cells mock treated with media alone (Lane 1) or infected with 25 MOI of Ad-GFP (Lane 2) or Ad-MMAC (Lane 3). Cells were harvested 48 h after indicated treatments. Cell lysates were immuno-precipitated with α-FAK, immunoblotted with α-phosphotyrosine (top), and reprobed with α-FAK (bottom).
LNCaP prostate cancer cells (16). Therefore, the effect of Ad-MMAC treatment on the growth of PC3 cells was compared with treatment with adenoviral p53 (Ad-p53). In contrast to LNCaP cells, PC3 cells were more sensitive to p53 expression than to ectopic MMAC/PTEN expression. Infection with Ad-p53 resulted in an 85% reduction in viable cell number at day 4 and >90% at day 6 after infection. Both of these effects were significantly greater than was seen with Ad-MMAC ($P < 0.05$ for both days).

To determine the mechanism of MMAC/PTEN-mediated growth inhibition, the effects of these treatments on apoptosis and proliferation were examined by flow cytometry (Fig. 4). Four days after infection with Ad-MMAC, 10% of cells were TUNEL positive, as compared with 5% with control adenovirus, suggesting only a minor increase in MMAC/PTEN-induced apoptosis. In contrast, infection with Ad-p53 produced 52% TUNEL positivity, indicating a significant induction of apoptosis by p53 expression (Fig. 4A). As an independent measure of apoptosis, propidium iodide staining followed by FACS analysis was performed to determine the percentage of cells with sub-G$_0$ DNA content (i.e., cells likely to be undergoing apoptosis). On day 6 after infection, only 7% of Ad-MMAC-infected cells had sub-G$_0$ DNA content, as opposed to 25% of Ad-p53-treated cells (Fig. 4B). Similar differences in relative apoptosis were observed in both assays, suggesting that the growth-inhibitory effect induced by MMAC/PTEN expression cannot be attributed to the induction of apoptosis.

The effect of MMAC/PTEN expression on cellular proliferation was examined by cell cycle analysis (Fig. 4C). Infection
with Ad-MMAC produced an increase in the G1 population of ~20%, with a concurrent decrease in the S phase population versus mock-treated cells. The changes in both the G1 and S phase populations after MMAC/PTEN expression were significant when compared with either mock treatment or infection with a control adenovirus (P < 0.05). This G1 arrest is consistent with experiments examining MMAC/PTEN expression in other cell types (38–42). p53 expression also induced a G1 arrest in the PC3 cells. Similar to the assays of cell growth and apoptosis, the arrest induced by p53 expression was significantly greater than that induced by MMAC/PTEN, as quantified by G1 and S phase cell populations (P < 0.05).

**In Vivo Studies.** Previous studies in a number of human cancer cell types have demonstrated that the orthotopic implantation of cells in nude mice more closely resembles the biological behaviors of these cells in humans, particularly in regards to the development of metastases. This has proven particularly true of human prostate cancer cells, which form primary tumors and metastases with much lower efficiency when implanted ectopically in nude mice.

To assess the ability of MMAC/PTEN expression to regulate PC3 tumorigenicity and in vivo growth, the effects of ex vivo treatment of the cells were examined. PC3 cells were mock infected, or infected with 25 MOI of Ad-GFP or Ad-MMAC, as had been performed for the in vitro studies. After (24 h) these treatments, equal numbers of cells were injected into the proststates of nude male mice. The mice were then sacrificed 21 days after implantation, as was necessitated by the morbidity resulting from the tumors that had formed. The mice were dissected and analyzed for the incidence and size of prostate tumors and for the incidence of metastases to regional lymph nodes. A 100% incidence of prostate tumors was observed for all three treatments, indicating that MMAC/PTEN expression did not inhibit PC3 tumorigenicity. Although MMAC/PTEN expression did not abolish tumor formation, the size of tumors resulting from Ad-MMAC-infected cells was significantly smaller than tumors of the other treatment groups (Fig. 5A). Tumors formed by PC3 cells infected with Ad-MMAC were 77% smaller by mass than tumors formed from mock-infected PC3 cells, whereas Ad-GFP-infected cells were only 25% smaller. In addition to its effect on local tumor growth, MMAC/PTEN expression completely inhibited the development of PC3 lymph node metastases. Although 100% of the mice in the mock treatment group and 80% of the mice in the Ad-GFP treatment group demonstrated enlarged lymph nodes, with the presence of PC3 cells confirmed histologically, none of the mice in the Ad-MMAC treatment group demonstrated lymph node metastases (Fig. 5B). Thus, MMAC/PTEN expression did not inhibit primary tumor formation, but it did reduce primary tumor size and inhibited the development of lymph node metastases.

Immunohistochemical analysis was performed on the harvested tissues to determine the effects of MMAC/PTEN expression on the in vivo behavior of PC3 cells. MMAC/PTEN expression did not affect the morphology of the PC3 cells, or the general architecture of the tumors formed by these cells, as demonstrated by H&E staining (Fig. 6A). These sections also demonstrate the histological evidence of lymph node invasion by mock- and Ad-GFP-infected PC3 cells, whereas the lymph nodes of Ad-MMAC-infected cells demonstrate only lymphoid cells (Fig. 6B). To determine the mechanism of MMAC/PTEN’s inhibitory effect on primary tumor size, the tumors were stained for markers of apoptosis, angiogenesis, and proliferation. TUNEL staining for apoptotic cells did not demonstrate differences between the three treatment groups (Fig. 7A). To assess tumor angiogenesis, the tumors were stained for CD31, a marker of endothelial cells. Again, no significant differences were observed between the different treatments (Fig. 7B). Finally, tumor proliferation was assessed by BrdUrd incorporation. Tumors of both mock- and Ad-GFP-treated PC3 cells showed generalized, strongly positive staining for BrdUrd, indicating that the tumor cells were proliferating globally (Fig. 7C). In contrast, most of the areas of the Ad-MMAC treatment group tumors were negative for BrdUrd incorporation, although there were some positively stained regions around the tumor margin. Thus, ex vivo MMAC/PTEN expression significantly inhibited PC3 cellular proliferation in vivo, without significantly affecting apoptosis or angiogenesis. Western blotting analysis of immunoprecipitated proteins indicated that the tumors of PC3 infected with Ad-MMAC ex vivo did express elevated levels of MMAC/PTEN at the time of tumor harvest (data not shown).

As ex vivo treatment of PC3 cells with Ad-MMAC reduced primary tumor size and inhibited metastasis formation, we next tested the effects of in vivo Ad-MMAC treatment of pre-established PC3 orthotopic tumors. PC3 cells were injected into the prostate of nude mice. The tumors were injected on days 7 and
after implantation with $1.5 \times 10^9$ plaque-forming units of Ad-GFP or Ad-MMAC or an equal volume of PBS. Mice were sacrificed at day 31 after implantation, because of the morbidity of the tumor burden. None of the treatment groups demonstrated tumor regression, as 100% of the mice demonstrated large primary tumors (Fig. 8, A and B) despite the presence of MMAC/PTEN expression in the tumors (Fig. 8C). Treatment with Ad-MMAC resulted in a 35% decrease in primary tumor mass as compared with treatment with PBS ($P < 0.05$; Fig. 5C). However, treatment with Ad-GFP resulted in a similar inhibition of tumor mass ($P = 0.78$ versus Ad-MMAC). This suggested that the observed reduction in tumor size by in vivo Ad-MMAC treatment was largely because of the toxicity of the multiple injections of adenovirus and not because of MMAC/PTEN expression. However, although in vivo treatment with Ad-MMAC did not have a significant effect on primary tumor growth, it markedly inhibited the formation of lymph node metastases (Figs. 5D and 8B). Although the presence of metastases in lymph nodes or at other sites was not examined, any enlarged nodes surrounding the primary tumor were examined for the presence of tumors. Lymph node metastases were grossly detected in 87% of PBS-injected tumors, 63% of Ad-GFP-treated tumors, and 25% of Ad-MMAC-treated tumors. These findings were confirmed by histological analysis. Thus, although multiple lines of evidence support the activity of MMAC/PTEN expression in vivo, the lack of tumor regression and/or reduction in size of tumors cannot be attributed to loss of MMAC/PTEN expression.

DISCUSSION

In the studies reported here, we have examined the impact of adenovirus-mediated MMAC/PTEN expression on the behavior of human prostate cancer cells under both in vitro and in vivo conditions in the human prostate tumor cell line, PC3. The goal of the studies was to determine whether MMAC/PTEN inhibited the development of lymph node metastases in cells implanted orthotopically in the prostate tissue of nude mice. This model more closely mimics the biology of human prostate cancer than s.c. implantation, as previous studies have demonstrated that the tissue microenvironment of the mouse prostate promotes rapid growth of the human prostate cancer cells locally and the development of spontaneous metastases (32, 33).

First, the effects of ectopic expression of MMAC/PTEN on signaling pathways, proliferation, and survival were determined. Our results are consistent with the now well-established model in which MMAC/PTEN regulates the PI3K signaling pathway by decreasing the pools of 3'-phosphorylated phosphatidylinositol (reviewed in Ref. 43). In contrast, no effects of MMAC/PTEN expression on phosphorylation of FAK were observed. These results are similar to those observed in several tumor cell lines in which MMAC/PTEN regulates the PI3K signaling pathway by decreasing the pools of 3'-phosphorylated phosphatidylinositol. Western blotting was performed after immunoprecipitation of tumor cell lysates with MMAC/PTEN-specific antibodies as described in “Materials and Methods.” As is observed in Fig. 8C, primary tumors from Ad-MMAC-infected cells clearly demonstrate the presence of the MMAC/PTEN gene product. Thus, the lack of tumor regression and/or reduction in size of tumors cannot be attributed to loss of MMAC/PTEN expression.
MMAC/PTEN as a phosphatidylinositol phosphatase, the studies presented here do not support the identification of FAK as a substrate of MMAC/PTEN’s putative protein phosphatase activity.

The expression of MMAC/PTEN in PC3 cells resulted in a significant inhibition of growth as compared with mock and control adenovirus treatments. This growth inhibition is not because of increased induction of apoptosis. These biological effects are similar to those we observed previously in experiments using the LNCaP human prostate cancer cell line (16). Although LNCaP cells demonstrate some apoptosis after high levels of MMAC/PTEN expression, quantitatively, the amount of apoptosis induced did not explain the extent of growth inhibition observed. Additionally, overexpression of Bcl-2 protein blocked MMAC/PTEN-induced apoptosis in LNCaP cells but not its growth inhibitory effects. The predominant regulation of prostate proliferation by MMAC/PTEN was also reported in the analyses of hyperplastic prostate tissue of MMAC/PTEN+/− mice and of prostate tumors in MMAC/PTEN+/− Cdkn1b +/− mice. In both of these studies, no changes in apoptotic indices were detected in the MMAC/PTEN+/− prostate tissues, but significant increases in proliferative indices were, as compared with MMAC/PTEN+/+ mice (28).

The tumor suppressor gene p53 is also inactivated frequently in metastatic prostate cancer (2, 44–47) and is currently being used in clinical trials in prostate cancer patients (48). In the studies in LNCaP cells, the expression of MMAC/PTEN was significantly more growth inhibitory than ectopic expression of p53. However, in the studies presented here, p53 expression inhibited the growth and survival of PC3 cells significantly more than the expression of MMAC/PTEN. The extent of growth inhibition induced by p53 observed here is very consistent with earlier published reports (31). The difference in comparative sensitivities to these two treatments between these two prostate cell lines could be because of a number of factors. The most likely explanation for this difference is that LNCaP cells harbor wild-type p53 function, whereas PC3 cells do not. Thus, p53 would be expected to be more growth inhibitory in the latter cells. A similar pattern of differing sensitivity to MMAC/PTEN and p53 has also been observed in human glioma cell lines. We reported previously that the expression of MMAC/PTEN in the

Fig. 7 Effect of ex vivo MMAC/PTEN expression on in vivo apoptosis, angiogenesis, and proliferation of orthotopically implanted PC3 cells. Immunohistochemical analysis of the same primary prostate tumors. A, TUNEL staining for apoptotic cells (top row, ×10 magnification). B, CD31 staining for endothelial cells, indicating vessel formation (middle, ×4 magnification). C, BrdUrd incorporation, indicating cellular proliferation (bottom, ×10 magnification). In each section, positively staining cells appear red. Sections shown are representative of the general appearance of individual tumors in multiple animals in each treatment group.

Fig. 8 PC3 tumors treated in vivo with Ad-GFP or Ad-MMAC. Photographs show representative gross anatomy of mice bearing PC3 tumors that were injected twice with Ad-GFP (A) or Ad-MMAC (B). Green arrows, the prostate tumors; yellow arrows, para-aortic lymph nodes. Prostate tumors treated with Ad-GFP demonstrated large, hardened lymph nodes as seen in A, which histological examination confirmed represented metastases of the primary prostate tumor. Tumors treated with Ad-MMAC showed small, soft lymph nodes that did not contain prostate tumors cell. C, expression of MMAC/PTEN. MMAC/PTEN was immunoprecipitated from 500 µg of lysates from mouse tumors (Ad-MMAC tumor) or cell lines (PC3, PC3 infected with Ad MMAC at 25 MOI, and HT29 cells, a colon tumor cell line expressing MMAC/PTEN). Western blotting was performed to detect MMAC/PTEN as described in “Materials and Methods.”
U251 human glioma cell line, which harbors mutant p53, resulted in a minimal inhibition of growth, whereas p53 expression potently inhibited U251 growth and survival (16, 36). In contrast, the U87 human glioma cell line, which retains wild-type p53 function, is more sensitive to adenosarviral MMAC/PTEN expression than to adenosarviral p53 (34). Paramio et al. (49) have demonstrated that MMAC/PTEN-mediated growth inhibition is not p53 dependent. Although these experiments were not conducted in prostate cells, MMAC/PTEN expression was growth inhibitory to PC3 cells in these experiments, suggesting that MMAC/PTEN-induced growth inhibition in prostate cells also is not p53 dependent. Therefore, there appears to be no direct relationship between the status of p53 mutation and sensitivity to ectopic expression of MMAC/PTEN.

Two previous studies have examined the effects of MMAC/PTEN expression in PC3 cells in vitro. Similar to this report, Sharrard et al. (50) observed an inhibition of PC3 cell growth after MMAC/PTEN expression without an induction of apoptosis. In contrast, Persad et al. (51) reported that the expression of MMAC/PTEN in PC3 cells resulted overwhelmingly in the induction of apoptosis. However, apoptosis was demonstrated only in cells that were maintained in serum-free media for 48 h. Thus, these results do not contradict the results obtained here, as we examined cells maintained in mitogen-enriched tissue culture media. It is clear by the Western blotting presented in this report (Fig. 2) that the mitogenic pathways of PC3 cells are sensitive to the mitogen content of the media, as cells starved for only 18 h demonstrated a decrease in Akt/PKB phosphorylation. Thus, in the studies of Persad et al. (51), the expression of MMAC/PTEN in addition to serum starvation may have inhibited this prosurvival pathway sufficiently to induce the apoptotic cascade.

**In Vivo Studies.** Having established that ectopic expression of MMAC/PTEN in PC3 cells resulted in biological and signaling effects similar to those reported for many tumor cell lines, the main goal of this study was to examine the in vivo function of MMAC/PTEN in human prostate cancer cells. Two different experimental approaches were used. In the first experiments described, MMAC/PTEN expression was introduced ex vivo by treating the cells with Ad-MMAC before implanting the cells in nude mice. This technique has the advantage of allowing uniform gene expression with a single treatment of the cells and involves minimal mechanical toxicity. However, it is a somewhat artificial system to evaluate a gene for potential therapy applications, as treatment of patients will involve expressing the gene in tumor cells already present in the prostate. Therefore, the effects of in vivo treatment of pre-established PC3 tumors with Ad-MMAC were also examined. This allows for a more relevant examination of the potential for clinical gene therapy. However, this technique produces more heterogeneous and limited gene expression in the tumor cells and intrinsically involves more mechanical trauma to the tumor. Thus, the in vivo expression technique may be somewhat limited in accurately representing the true in vivo function of the gene.

The ex vivo expression of MMAC/PTEN in PC3 cells did not inhibit the tumorigenicity of these cells when they were implanted orthotopically. However, ex vivo expression of MMAC/PTEN did significantly reduce local tumor size by inhibiting cellular proliferation, a result that is consistent with the in vitro experiments. Additionally, ex vivo expression of MMAC/PTEN completely inhibited the development of lymph node metastases by PC3 cells. Thus, these experiments suggest that MMAC/PTEN is not a critical regulator of prostate tumor formation but is a critical regulator of aggressive local prostate tumor growth and metastasis. This model is consistent with the pattern of mutations and deletions of MMAC/PTEN that have been detected in human prostate cancer clinical specimens but represents the first in vivo demonstration of this proposed role. However, this finding does contrast with the observation in genetically engineered mice that loss of a single MMAC/PTEN allele promotes prostate hyperplasia and tumor formation (27). Although those murine lesions and tumors may have also included other genetic alterations not recapitulated in the PC3 cell line, it is notable that there is no report of an increased incidence of abnormal prostate proliferation in the familial syndromes that harbor germ-line MMAC/PTEN mutations. Thus, the results in the genetically engineered mice may reflect a different regulation of prostate homeostasis in that species than is functional in the human tissue.

The in vivo treatment of pre-established tumors of PC3 cells with Ad-MMAC did not induce tumor regression but did moderately inhibit localized prostate tumor size. This reduction in tumor size could not be attributed to MMAC/PTEN expression, however, as infection with a control adenovirus resulted in a similar degree of inhibition. Substantial variation in the size of the tumors, particularly in the Ad-MMAC treatment group, indicates that repeated injections into the prostate tumors resulted in nonspecific toxicity. Furthermore, it is expected that not all tumor cells will be infected by this procedure. Thus, in this experimental setting, it is difficult to assess the effect of MMAC/PTEN expression on the local growth of the cancer cells. However, in vivo treatment of these tumors with Ad-MMAC did have an inhibitory effect on the metastatic behavior of these cells. This effect was apparent in the rate of lymph node metastasis, which was confirmed by histological analysis. The potent inhibition of the metastasis of PC3 cells by MMAC/PTEN is consistent with the effects of ex vivo expression and, again, with the detected pattern of genetic inactivation of MMAC/PTEN in clinical specimens.

The inhibition of PC3 prostate cancer cell metastasis after both ex vivo and in vivo expression of MMAC/PTEN is striking. Although these studies did not determine the specific mechanism of this effect, previous studies have demonstrated that MMAC/PTEN can regulate a number of in vitro behaviors characteristic of metastatic cancer cells. These regulated behaviors include anoikis, motility, and invasion (reviewed in Ref. 13). Although MMAC/PTEN expression clearly regulates signaling pathways responsible for the above biological behaviors, it is also possible that MMAC/PTEN’s inhibitory effect on metastasis may be because of the regulation of factors released into the circulation by the tumor cells in the prostate. This hypothesis should also be investigated, as Ad-MMAC treatment of pre-established tumors likely resulted in heterogeneous expression of MMAC/PTEN but still inhibited the formation of metastases.

The effects of adenoviral p53 on the in vivo growth of PC3 cells have also been investigated previously. Two separate reports have demonstrated that the ex vivo expression of p53 in
PC3 cells inhibits the tumorigenicity of these cells (52, 53). More recently, Eastham et al. (31) have reported that a single injection of adenoviral p53 into pre-established orthotopic PC3 tumors resulted in the complete regression of several tumors, a 75% reduction in local tumor size, and a 55% reduction in metastasis formation. Although different numbers of cells and amounts of adenovirus were used, the reported increased in vivo growth-suppressive effects of p53 expression in PC3 cells as compared with MMAC/PTEN agree with the increased inhibitory effect that we observed in vitro. Although it was not determined in vivo, each of these studies demonstrated clear evidence in vitro of a significant induction of apoptosis after p53 expression. Given that MMAC/PTEN-mediated growth inhibition does not seem to be predominantly mediated by apoptosis, a combined gene therapy of MMAC/PTEN and p53 might provide an additive effect.

As yet, few studies have examined the effects of in vivo treatment of pre-established tumors with adenoviral MMAC/PTEN. Sakurada et al. (54) examined the effects of MMAC/PTEN gene therapy on pre-established tumors of human endometrial cancer cells and reported a cytostatic effect. However, the cells were injected s.c. into the mice instead of orthotopically, and the effects of MMAC/PTEN expression on metastasis formation were not reported. Recently, Wen et al. (55) reported the effects of stable ex vivo expression of MMAC/PTEN on the growth of orthotopically implanted U87 human glioma cells. Similar to the results reported here in PC3 cells, MMAC/PTEN expression did not inhibit the tumorigenicity of the U87 cells but did reduce tumor size and prolonged survival.

In summary, the results presented here functionally support the proposed role of MMAC/PTEN as a regulator of prostate cancer progression. Specifically, they have demonstrated that MMAC/PTEN inhibits the metastatic potential of PC3 cells without inhibiting local prostate tumor formation. These studies also suggest that additional investigations are indicated to assess MMAC/PTEN’s potential as a prognostic marker for staging and treatment purposes and also as a potential therapeutic tool.

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