Adenovirus-mediated Expression of PML Suppresses Growth and Tumorigenicity of Prostate Cancer Cells

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Abstract

Our previous studies demonstrated that the promyelocytic leukemia gene, PML, encodes a growth and transformation suppressor. Overexpression of PML inhibits cancer cell growth in vitro and in vivo. In this study, we further explored the possibility of applying PML as a potential agent for developing prostate cancer gene therapy using an adenovirus delivery system. We have constructed and produced the recombinant PML-adenovirus, Ad-PML, in which the full-length PML cDNA is driven by the strong cytomegalovirus promoter. In LNCaP, DU145, and PC-3 prostate cancer cell lines, an infection efficiency of 90% can be achieved at a concentration of 2, 10, and 100 multiplicity of infection (MOI), respectively. Western blotting and immunofluorescence staining demonstrated that the Ad-PML-infected cells expressed a high level of PML protein. The protein expression peaked at days 3–4 postinfection, and a detectable level of PML was found at day 18 after viral infection. To test the effect of Ad-PML on the growth of prostate cancer cells, the DU145 and LNCaP cells were infected with 10 and 2 MOI of Ad-PML. We found that the growth rate of the Ad-PML-infected DU145 and LNCaP cells were significantly inhibited. A tumorigenicity test in nude mice showed that the Ad-PML-treated DU145 cells failed to form tumors. Most importantly, direct injection of Ad-PML into DU145-induced tumors was able to repress tumor growth in nude mice by 64%. Taken together, these data indicate that PML is a tumor growth suppressor in prostate cancer and that Ad-PML may be a potential candidate for human prostate cancer therapy.

Introduction

Prostate cancer is the most commonly diagnosed malignancy in American men, with an estimated 317,100 new cases diagnosed in 1996; of this estimate, 41,400 patients are expected to die from this disease (1). Almost 50% of the patients diagnosed with prostate cancer already developed metastatic lesions at initial clinical presentation, and an additional 20% of the patients who initially have local disease will eventually develop metastasis, for which an effective therapeutic regimen is androgen ablation. These tumors eventually relapse and become androgen independent. At this stage, an effective therapeutic regimen is lacking (2).

Throughout the last decade, studies on oncogenes and tumor suppressor genes have provided evidence that cancer is a disease developed through a process of genetic disorders (3–5). Based on this concept, new strategies have been rapidly developed as alternatives to conventional cancer therapy. One of this is cancer gene therapy (6, 7), in which tumor suppressor genes, antisense oligonucleotides to oncogenes, and other related genes are used therapeutically to eradicate cancer cells (2, 8–10).

The PML gene, which encodes a growth and transformation suppressor (11, 12), is disrupted by the 15;17 chromosomal translocation in acute promyelocytic leukemia. The PML gene has been shown to have an altered expression during human oncogenesis (13, 14). Its expression reduced dramatically when breast cancer cells turned invasive, suggesting that PML may be an antioncogene (13). Our previous study demonstrated that PML suppressed transformation of rat embryo fibroblasts by cooperative oncogenes and suppressed growth, clonogenicity and tumorigenicity of NIH3T3 cells induced by activated neu oncogene (11, 12). Overexpression of PML protein by transient transfection induced a sharp reduction in growth rates in vitro and in vivo of human tumor cell lines (13). This observation confirmed our finding that PML is a growth suppressor.

In this study, we further tested whether the PML growth suppressor could be a potential agent for prostate cancer therapy. We constructed a recombinant adenovirus carrying the PML gene, which was controlled by the CMV promoter. Western blot and immunofluorescence staining analysis indicated that Ad-PML mediated a high level of expression of PML protein in LNCaP, DU145, and PC-3 prostate cancer cells. Furthermore, PML overexpression could inhibit cell growth and tumorigenicity. Our results also demonstrated that injection of Ad-PML into the DU145-induced tumors suppressed tumor growth in vivo.

Materials and Methods

Construction of the PML Recombinant Adenovirus. To construct a recombinant adenovirus containing the PML cDNA, the full-length PML cDNA was excised by EcoRI and BglII from pSG5PML (11), blunt ended with Klenow, and ligated into the HindIII site of the shuttle vector, AdE1CMV, as described previously (2), using a HindIII linker. Expression of the PML cDNA was driven by the human CMV promoter, which is linked to the simian virus 40 early polyadenylation signal on the 3' end. This recombinant adenovirus vector is a modification of the plasmid pXCL.1, initially constructed by Graham and Prevec (15). Orientation of the PML cDNA insert was verified by a brief restriction mapping and DNA sequencing. The PML recombinant adenovirus plasmid, pAdPML, and pM17, which carry both adenoviral genome (Ad5) and pBR322 sequences, were cotransfected into the 293 cells using the DOTAP reagent (Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer's protocol. The presence of PML recombinant adenovirus, Ad-PML, was identified, isolated, and purified from a single plaque after re-infecting 293 cells as described previously (2, 8). A small aliquot of the recombinant adenoviral DNA was prepared from the cell culture medium as described previously (16). The presence of PML cDNA in these viral clones
Twenty-four h later, infection was carried out with Ad-PML or Ad-control. Cells were incubated in 0.1% BSA/PBS for 30 min to block nonspecific binding. Cells were washed in PBS and harvested by digestion with 0.05% trypsin and 0.5% EDTA. After washing in PBS containing 0.1% BSA for three times, cells were resuspended in 0.1% Triton X-100. Subsequently, a large quantity of viral stocks was produced as described previously (18).

**Results**

**Generation of the PML Recombinant Adenovirus, Ad-PML.** The PML recombinant adenovirus, Ad-PML, was created as described in “Materials and Methods.” After cotransfection of pAdPML and the recombinant plasmid pJM17 in 293 cells, the presence of Ad-PML was identified and purified. PCR was performed using DNA samples prepared from the cell culture medium to ensure the presence of PML cDNA in the recombinant adenoviruses. As shown in Fig. 1, a predictable 0.58-kb DNA fragment was detected in all four clones (Lanes 1–4). No such band was detected in the negative control (Lane N). This result demonstrated that the recombinant adenoviruses carry the expected PML cDNA sequences. To determine the possibility of contamination of the Ad-PML with the wild-type adenovirus, E1 primer sets were used to amplify the genomic DNA of the nonrecombinant adenovirus. An amplified DNA fragment of 1-kb was detected in the Ad-control DNA but was not detectable in the DNA isolated from all of the Ad-PML clones. This result confirmed that our Ad-PML clones are not contaminated with the wild-type virus.

**Transfection Efficiency and PML Protein Expression in Ad-PML-infected Prostate Cancer Cells.** One of the advantages of adenovirus-mediated gene transfer is its high infectivity in a wide range of host cells (16). To determine the transfection efficiency of adenovirus in prostate cancer cells, LNCaP, DU145, and PC-3 cells were infected with Ad5-CMV-Gal at various MOI for 24 h. The efficiency of viral infection was determined by the expression of PML protein, as determined by Western blotting.

**In Vivo Tumorigenicity Assay.** DU145 cells were seeded directly on 6-well plates, infected either with Ad-PML or Ad-control at a MOI of 10 and continued to be cultured for 24 h. Cells were washed in PBS and harvested by digestion with 0.05% trypsin-EDTA. Cells were immobilized on slides by cytosin at 500 rpm for 5 min and air dried. Cells were then fixed for 20 min with 4% formaldehyde and 10 min with 0.1% Triton X-100. After washing in PBS containing 0.1% BSA for three times, cells were incubated in 0.1% BSA/PBS for 30 min to block nonspecific signals. Immunofluorescence staining was performed using the rabbit anti-PML antiserum raised in rabbits against the GST-PML fusion protein at a dilution of 1:100, as described in our previous report (17).

**In Vivo Inhibition of Tumor Growth.** To address the feasibility of PML gene therapy for established tumors, the efficacy of Ad-PML in inhibiting tumor growth was evaluated in a tumor-bearing nude mouse model using human prostate cancer DU145 cells in a defined pathogen-free environment. Experiments were reviewed and approved by institutional committees for both animal care and use for recombinant DNA research. Briefly, 6 × 10⁶ DU145 cells in 0.5 ml medium were injected s.c. into the right and left flanks of eight 6-week-old male nude mice. The tumors became palpable, the tumor volume was measured, and then 100 μl (5 × 10⁶ pfu) of Ad-PML diluted in buffer were injected at multiple sites around the tumor on the right flanks, and 100 μl (5 × 10⁶ pfu) Ad-control was injected at multiple sites around the tumor on the left flanks. Two weeks later, a second dose of Ad-PML/Ad-control were injected. Changes in tumor volume were measured weekly and calculated. The Student’s t test was used to determine the significance of the effect of Ad-PML on tumor growth.

**Fig. 1. Identification of the PML recombinant adenovirus by PCR.** To confirm the presence of PML cDNA sequences in the recombinant adenovirus, oligonucleotides synthesized as described in “Materials and Methods” were used to amplify a 580-bp PML cDNA fragment by PCR. Lane N, a negative control using DNA isolated from the supernatant of Ad-control infected 293 cells; Lanes 1–4, DNA isolated from the supernatant of four different clones of Ad-PML. Lane C, a positive control using the pSG5-PML plasmid DNA that carries the full-length PML cDNA.
PML ADENOVIRUS IN PROSTATE CANCER THERAPY

Table 1  Infectivity of LNCaP, DU145, and PC-3 prostate cancer cells by Ad5-CMV-Gal

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MOI</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td></td>
<td>34</td>
<td>58</td>
<td>91</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DU145</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>25</td>
<td>54</td>
<td>90</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>27</td>
<td>46</td>
<td>67</td>
<td>93</td>
</tr>
</tbody>
</table>

* ND, not determined.

In Vitro Growth-suppressing Effect of Ad-PML. Our previous report demonstrated that PML is a growth and transformation suppressor (11, 12). Overexpression of PML could suppress cell growth in vitro and in vivo. To analyze the effects of Ad-PML on the growth of prostate cancer cells, we infected DU145 and LNCaP cells with Ad-PML or the control adenovirus (Ad-control) at a virus concentration that achieves 90% infectivity (MOI of 2 for LNCaP and MOI of 10 for DU145 cells). A dramatic increase in expression of PML protein in the Ad-PML-infected cells was confirmed by immunofluorescence staining. Results as shown in Fig. 4 demonstrated that the growth rates of Ad-PML-infected LNCaP cells was completely suppressed at day 4, and a significant number of cell deaths were found at day 6. The growth rate of Ad-PML-infected DU145 cells was also inhibited dramatically at days 4 and 6. No significant changes in growth rate were found in Ad-control-infected cells compared with the negative control. This study indicated that the prostate cancer cell growth was significantly suppressed by overexpression of PML compared to the control.

Fig. 2. Immunofluorescence staining of the PML protein before and after Ad-PML infection of LNCaP, DU145, and PC-3 cells. Immunofluorescence staining and in vivo localization of the PML protein in various cell lines 24 h after infection with Ad-PML was performed as described in “Materials and Methods.” A, C, and E, the normal staining pattern of PODs in LNCaP, DU145, and PC-3 cells, respectively; B, D, and F, the PML staining pattern in LNCaP, DU145, and PC-3 cells 24 h after Ad-PML infection.

\( \beta \)-galactosidase activity. As shown in Table 1, the transfection efficiency of Ad5-CMV-Gal varies significantly in different cell types. A 90% efficiency can be achieved at a MOI of 2 for LNCaP, a MOI of 10 for DU145, and a MOI of 100 for PC-3 cells. LNCaP appears to be most sensitive to adenovirus infection; a 100% infectivity can be achieved by infecting the cells with the virus at a MOI of 5. On the other hand, PC-3 is least sensitive. These results indicated that LNCaP and DU145 cells are more sensitive than PC-3 cells to adenovirus infection. It is also important to note that the Ad-PML-encoded PML protein is capable of organizing normal PODs (Fig. 2).

When DU145 cells were infected with Ad-PML, both Western blotting and immunofluorescence staining demonstrated that PML expression could be detected 24 h after infection. The level of PML protein expression increased proportionally at an increasing Ad-PML concentration. (Fig. 3, A and B). These results indicated that Ad-PML expresses a high level of PML protein in these cells. Results obtained from the time course study indicated that the PML protein expression peaked at days 3–4 postinfection and persisted for at least 2 weeks (Fig. 3C). A detectable level of PML was found 18 days postinfection by Western blotting. This long-lasting expression of PML may provide an advantage for PML as a potential agent for prostate cancer therapy.

Fig. 3. Western blot analysis of PML protein expression in DU145 cells infected with Ad-PML. Cell lysate was prepared from DU145 cells infected with Ad-PML and analyzed by Western blotting using polyclonal antibody against GST-PML fusion protein. A, dosage-dependent expression of PML after infection with various concentrations of Ad-PML. DU145 cells were infected with Ad-PML at various MOI (0, 1, 2.5, 5, and 10). Expression of PML protein was analyzed 24 h postinfection. B, duration of PML expression after Ad-PML infection. DU145 cells were infected with Ad-PML at 5 MOI. The cell culture medium was replaced every 3 days. At the indicated time points (days 1, 2, 3, 4, 6, 10, 14, 18, and 22), cells were collected, and proteins were isolated for Western blot analysis.

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Fig. 4. Ad-PML-mediated inhibition of prostate cancer cell growth. LNCaP and DU145 cells infected with Ad-PML or Ad-control were analyzed for their growth at different time points after infection. The uninfected cells were also analyzed as a negative control. Analysis of the growth rate was performed as described in "Materials and Methods."

### Table 2 Effect of Ad-PML on tumorigenicity of DU145 cells in nude mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of tumors/ No. of mice (%)</th>
<th>Mean volume (mm$^3$ ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>5/5 (100)</td>
<td>170 ± 107</td>
</tr>
<tr>
<td>Ad-control</td>
<td>4/5 (80)</td>
<td>108 ± 45</td>
</tr>
<tr>
<td>Ad-PML$^b$</td>
<td>0/5 (0)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The treated DU145 cells were injected s.c. at 6 × 10$^6$ cells/site.

$^b$ No tumor was observed in these mice for an extended period of 12 weeks. Tumor sizes were determined at the end of a 6-week period.

### Effect of Ad-PML on Tumorigenicity in Nude Mice

To examine whether Ad-PML inhibits tumorigenicity of human prostate cancer cells, nude mice were injected s.c. with DU145 cells infected with Ad-PML and Ad-control. After 6 weeks, all five mice inoculated with uninfected cells formed tumors; cells infected with Ad-control formed tumors in four of the five mice tested. None of the five mice injected with Ad-PML-infected cells formed tumors (Table 2) over an extended period of observation of up to 12 weeks. These results clearly demonstrated that Ad-PML effectively suppress tumorigenicity of DU145 prostate cancer cells.

**In Vivo Tumor-suppressing Effect of Ad-PML in Nude Mice.**

The results presented above indicated that overexpression of PML mediated by Ad-PML in LNCaP and DU145 cells reduces cell growth and inhibits tumorigenicity. To continue to characterize the tumor-suppressing effect of Ad-PML, we tested the therapeutic efficacy of Ad-PML on tumor growth induced by the prostate cancer cell line. Prostate tumor (average volume, 19.1 and 20.3 mm$^3$) induced by DU145 were injected with 5 × 10$^8$ pfu of Ad-PML or Ad-control on days 14 and 28 at multiple sites around the tumors. Tumor volume was measured every week after adenovirus injection. As shown in Table 3, a significant ($P < 0.05$) inhibition of tumor growth was found in tumors injected with Ad-PML when compared to the tumors injected with Ad-control. This result demonstrated that adenovirus is capable of delivering the PML cDNA into tumor cells and that overexpression of PML (confirmed by immunohistochemical staining, data not shown) can inhibit tumor growth in vivo.

### Discussion

It is well known that aberrant expression of normal cellular oncogenes or loss of function of a tumor suppressor as a result of genetic abnormality leads to the development of human cancer. Based on this concept, one of the new direction of cancer therapy is to reestablish the function of a tumor suppressor, to suppress the function of an overexpressed oncoprotein, or to overexpress a growth suppressor gene by novel gene delivery methods. The purpose of these strategies is to reverse the malignant phenotype of the transformed cells and subsequently suppress tumor growth. To deliver a gene into somatic cells with high efficiency, two major genetically engineered vector systems, retrovirus and adenovirus, have been developed and well characterized. The retrovirus-mediated gene transfer system has limited use in prostate cancer gene therapy because of the following reasons (2, 19): (a) infection by retrovirus depends on host cell division, whereas very few mitotic cells can be found in the prostate tumors; (b) the integration of retrovirus into the host genome may cause adverse effects by activation of cellular oncogenes; (c) retrovirus infection is often limited by a certain host range; (d) retrovirus has been associated with many malignancies in both mammals and vertebrates; and (e) in general, the titer of retrovirus is 100- to 1000-fold lower than that of adenovirus.

Along with the advantage of producing high-titer viral stocks, adenovirus allows a large foreign cDNA insert of up to 8 kb (19). Adenovirus infection does not result in an integration of the adenoviral DNA into the host genome. The virus can replicate in an episomal manner without potential genotoxicity. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. Adenovirus produces little morbidity and has not been associated with human malignancies (19). It was reported that adenovirus has a much

### Table 3 Suppression of DU145-induced tumor growth by intratumoral injection of Ad-PML

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average tumor volume (mm$^3$)</th>
<th>Average tumor weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 14</td>
<td>Day 21</td>
</tr>
<tr>
<td>Ad-PML$^a$</td>
<td>19</td>
<td>38</td>
</tr>
<tr>
<td>Ad-control</td>
<td>20</td>
<td>65$^e$</td>
</tr>
</tbody>
</table>

$^a$ An average of tumor volume and weight from eight mice.

$^b$ Athymic mice were inoculated s.c. with 6 × 10$^6$ DU145 cells/site at both right and left flanks of the 6-week-old animals at day 0. Animals were treated with either Ad-PML (5 × 10$^8$ pfu in 100 μl) or control (Ad-control, 5 × 10$^8$ pfu in 100 μl) at day 14 and repeated injection with the same dose of therapeutic and control viruses at day 28. Tumor volumes were assessed by a formula: width × length × height × 0.5236 (27).

$^e$ $P < 0.05$. 

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higher infection efficiency than retrovirus in human prostate cancer cells (20). Results from the current study (Table 1 and Figs. 3 and 4) and others (2, 21, 22) showed that prostate cancer cells are sensitive to adenosine infection. Our study demonstrated that a consistently detectable level of PML protein can be found in the viral-infected cells (Fig. 3), even after 18 days of Ad-PML infection. Compared to the p53-adenovirus infection, a rapid decrease in p53 protein was found after 5 days postinfecion by Ad5CMV-p53 (8). This observation suggests that PML may be a more stable protein with a long half-life compared to other tumor suppressors, e.g., p53, which have a half-life of 1.5 h (23). Indeed, our recent study showed that the half-life of PML is about 6–8 h, which is much longer than that of p53. To achieve therapeutic efficacy, frequent administration of the recombinant p53 adenosivirus is required (24, 25). The stable nature of the PML protein may help to reduce the frequency of in vivo viral administration, which may elicit an adverse immunological response.

Therefore, PML may be a potential agent for prostate cancer therapy. Results observed from in vitro cell growth assay and in vivo tumorigenicity assay (Fig. 4 and Table 2) indicated that overexpression of PML suppressed the growth of prostate cancer cells and inhibited their tumorigenicity in nude mice. This effect is comparable to those observed by using tumor suppressor p53 and C-CAM1 (2, 22). In our previous reports, we showed that PML is a growth and transformation suppressor; it suppresses neo expression and blocks signaling events associated with activated neo (11, 12). Furthermore, our study also demonstrated that the ability of PML to form PODs are essential for the suppression of growth and transformation (17). PML mutants that failed to form PODs in vivo were unable to suppressed transformation of NIH3T3 cells by neo. The growth suppressor function of PML was also confirmed by another group. In that study, they further demonstrated that when breast and colon cancers turned invasive, the expression of PML was lost, suggesting that PML may be an antioncogene involved not only in APL but also in nonhematological oncogenesis (13). Interestingly, Terris et al. (14) reported that PML plays a role in the inflammatory process and in cell growth control. Furthermore, Studler et al. (26) reported recently that PML is a primary target gene of IFN action, and it appears that PML may be directly involved in mediating some of the antiproliferative effects of IFNs. However, the molecular mechanism of growth and tumor suppression induced by overexpression of PML requires further investigation.

Encouraging results were also obtained in studying the effect of Ad-PML on tumorigenicity of prostate cancer cell lines in nude mice (Table 3). Our result showed that direct injection of Ad-PML into the DU-145 cell-induced tumors suppressed their growth by 41–66% compared the control group (P < 0.05). This in vivo study confirmed the in vitro effects of Ad-PML on human prostate cancer cells, suggesting that PML protein mediates a potentially therapeutic tumor-suppressing effect. These results clearly demonstrated that adenovirus-mediated PML expression efficiently suppressed prostate tumor growth.

In conclusion, our findings indicated that overexpression of PML protein using the adenovirus vectors may promote efficient growth inhibition of tumors in vitro and in vivo. The growth- and tumor-suppressing effect of PML and its stable nature indicate that PML may be a potentially important agent for cancer gene therapy. We, therefore, believe that the potential of Ad-PML in suppressing tumorigenicity should be further explored in other tumor models and in clinical applications.

References
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