Antitumor Activity of the 16-kDa Prolactin Fragment in Prostate Cancer

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ABSTRACT

The 16-kDa prolactin (PRL), derived from the proteolytic cleavage of wild-type 23-kDa PRL, has been shown to have antiangiogenic activity. Such an antiangiogenic activity may have an effect on tumor growth in vivo. Here we examined the effect of 23-kDa and 16-kDa PRL on tumor growth, and the potential of using recombinant 16-kDa human PRL for prostate cancer therapy. The effects of 23-kDa PRL and 16-kDa PRL on the tumorigenicity of prostate cancer cells in vivo were studied. Using an adenovirus transfer vector to achieve high efficiency 23-kDa and 16-kDa PRL transfection in DU145 and PC-3 human prostate carcinoma cell lines, we demonstrated that expression of 16-kDa PRL in the prostate cancer cells markedly reduced their ability to form tumors in a xenograft animal model. These studies established that the 16-kDa PRL has antitumor activity in vivo, presumably as a result of its antiangiogenic effect. Interestingly, 23-kDa PRL showed a weak and transient suppression of prostate tumor growth. The weak antitumor activity of 23-kDa PRL may be because of the production of 16-kDa PRL from 23-kDa PRL by the tumor cells. Thus, the apparent effect of 23-kDa PRL on the growth of DU145 and PC-3 cells in vivo may result from the combined effects of 23-kDa PRL and 16-kDa PRL. These results suggest that the 16-kDa PRL has potential as a treatment agent in prostate cancer.

INTRODUCTION

PRL was originally identified as a lactotrophic hormone secreted by the pituitary gland. PRL has multiple targets. Accumulating evidence suggests that PRL is involved in a diverse array of physiological functions, including reproduction, osmoregulation, and behavioral modification (1, 2). Several investigators have shown that PRL has trophic effects on rat prostate gland (3, 4), and PRL is involved in the normal growth, development, and function of the prostate (5–10).

In addition to the pituitary gland, PRL synthesis has been shown in numerous extra-pituitary tissues (1). Nevalainen et al. (11) showed that PRL is produced locally by secretory epithelia in organ cultures of the human prostate. Using in situ hybridization and immunohistochemistry, Leav et al. (12) demonstrated that in the prostate both PRL receptor message and protein are located predominantly in the epithelial cells of the fetal, neonatal, prepubertal, and adult prostate. These observations suggest that PRL can be produced by the prostate in addition to the pituitary and that the PRL receptor is also present in the prostate. Thus, there probably is an autocrine/paracrine pathway that mediates local PRL effects on the prostate gland, and PRL probably influences the development of the human prostate and contributes to the maintenance of the adult gland. Consistent with the proposed functional roles of PRL in stimulating the growth and differentiation of prostate tissue, the prostate gland is dramatically enlarged in transgenic mice overexpressing the PRL gene (13), whereas the prostate size is significantly smaller in PRL-deficient mice (14).

PRL exists in several forms as a result of post-translational modifications such as glycosylation (15), phosphorylation (17), and proteolysis (18–20). Intact PRL (also called 23-kDa PRL) is additionally proteolyzed into fragments with various sizes. One predominant proteolytic PRL fragment has an apparent molecular weight of 16-kDa (16-kDa PRL) and is produced by the removal of about a quarter of the PRL molecule from the COOH terminus (21). PRL can be proteolyzed in vitro by enzymes from PRL target tissues including the mammary gland (22, 23), liver (23), and prostate (24, 25), and by cathepsin D (21). The 16-kDa PRL is found in the hypothalamus of rats (18) and mice (19), and in the pituitary glands and circulation in humans (20).

Rather than being an inactive breakdown product of PRL, 16-kDa PRL has a potent antiangiogenic effect. Ferrara et al. (26) first reported the antiangiogenic activity of 16-kDa PRL. They showed that rat 16-kDa PRL inhibited, in a dose-dependent manner, both the basal and the bFGF-stimulated growths of cultured bovine brain and adrenal cortex endothelial cells. In contrast, the intact rat 23-kDa PRL had no effect on these cells. Clapp et al. (27) showed that the recombinant 16-kDa human PRL is also a potent antiangiogenic factor. This recombinant PRL has a similar potency as that of the rat 16-kDa PRL, and at nanomolar concentrations, the recombinant 16-kDa PRL inhibited the basal growth of bovine and human vascular endothelial cells in vitro, and the proliferative effects of both bFGF and VEGF on these cells. In vivo, normal development of capillaries in chick embryo chorioallantoic membrane was also inhibited by the recombinant 16-kDa PRL (27). Recently, Duenas et al. (28) showed that the recombinant 16-kDa PRL also inhibited FGF-stimulated cornea vascularization. These studies clearly indicate that 16-kDa PRL has an antiangiogenic activity.

Evidence from the literature suggests that antiangiogenic activity of 16-kDa PRL may be because of its ability to affect several cellular events. D’Angelo et al. (29) demonstrated that the recombinant 16-kDa PRL inhibits bFGF- and VEGF-induced phosphorylation and activation of p42 and p44 mitogen-activated protein kinases in capillary endothelial cells. This inhibitory effect occurs at some step distal to the autophosphorylation of the VEGF receptor, suggesting that the 16-kDa PRL blocks the angiogenic effect of VEGF by interfering with VEGF downstream signal transduction pathways. Lee et al. (30) also showed that the recombinant 16-kDa PRL inhibited the activity of urokinase, which is an essential regulator in the formation of new microvasculature. This inhibition of urokinase was mediated indirectly by an increased expression of plasminogen activator inhibitor 1. Martini et al. (31) demonstrated that the 16-kDa PRL induced endothelial cell apoptosis through rapid activation of caspases 1 and 3. Taken together, these data suggest that the 16-kDa PRL inhibits angiogenesis through a unique signal transduction mechanism.

Whether the antiangiogenic action of 16-kDa PRL has an effect on tumor angiogenesis has not been extensively studied. Angiogenesis is necessary for growth and development of normal tissue; it is also essential for the growth and progression of solid tumors (32). In a variety of neoplasms, including neoplasms of the breast, bladder, and cervix, and cutaneous melanoma, the degree of neovascularization correlates with aggressive behavior (i.e., invasive phenotype; Refs.

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2 The abbreviation used are: PRL, prolactin; 16-kDa prolactin, a 16-kDa fragment of human prolactin; 23-kDa prolactin, the full-length 23-kDa wild-type human prolactin; bFGF, basic fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; m16-kDa PRL, a 16-kDa fragment of human prolactin with mutation of Cys58 to Ser; MVD, microvessel density; MOI, multiplicity of infection; pfu, plaque-forming unit(s); rAd, recombinant adenovirus; VEGF, vascular endothelial growth factor.
33–36). MVD is also associated with prognosis in carcinomas of the breast (37–40), lung (41), and head and neck (42), and in cutaneous melanoma (43). Although prostate cancer is an indolent disease, angiogenesis is an important process that correlates with the stage and virulence of tumor. Bigler et al. (44) demonstrated that the ratio of vessels per unit area in sections of carcinoma compared with that in normal tissue was doubled in 15 radical prostatectomy specimens whose microvessels were quantified with immunohistochemistry using antibodies to factor VIII-related antigen. MVD correlates with the stage of prostate cancer, which is one of the most important prognostic factors (45–46). It also may reflect virulence of prostate cancer. Silberman et al. (47) have shown that MVD, determined by immunostaining sections of tumors from radical prostatectomy specimens for CD31, is predictive of progression after surgery for intermediate-grade tumors. Given the importance of angiogenesis in cancer, it is logical to target inhibition of angiogenesis as a potential therapy for prostate cancer.

In this study, we tested the effect of 23-kDa and 16-kDa PRL on prostate tumor growth, and the potential application of 16-kDa human PRL in prostate cancer therapy. An adenovirus transfer vector was used to achieve high efficiency 23-kDa PRL and 16-kDa PRL transfection in the human prostate carcinoma cell lines DU145 and PC-3 in a xenograft animal model. We demonstrated that expression of 16-kDa PRL, in contrast to that of the intact 23-kDa PRL, in the prostate cancer cells markedly reduced their ability to form tumors. 23-kDa PRL showed a weak and transient suppression of prostate tumor growth. These observations suggest that 23-kDa PRL and its proteolytic product 16-kDa PRL may be part of growth regulatory mechanism in vivo.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Purified 23-kDa PRL. PC-3, DU145, and 293 cells were purchased from American Type Culture Collection (Manassas, VA). Monoclonal (E42371M, clone number 164.22.12) and polyclonal antibody (D20910R) against human PRL were purchased from Bioscience International (Sacoo, ME). Rabbit polyclonal antibody against human PRL (NIDDK-anti-hPRL-IC-5) and human pituitary PRL antigen (NIDDK-hPRL-01; AFP-9042-B) were obtained from the NIH (Bethesda, MD).

Construction and Generation of 23-kDa PRL, 16-kDa PRL, and m16-kDa PRL rAd. The cDNA encoding full-length human PRL in plasmid pSK-PRL was constructed as described previously (48). To construct the adenoviral vector containing full-length 23-kDa PRL cDNA, the full-length human PRL cDNA was inserted into the adenovirus shuttle vector pXCMV (49) at the HindIII/NorI sites to generate pXCMV-23-kDa PRL. To construct the cDNA for 16-kDa PRL, the plasmid pSK-PRL was used as the template and amplified with Oligo PRL-For (5’AAGCTTAAACATGAAACTACAAAGGATCCGATGGA3’), including a HindIII restriction enzyme site, the initiation ATG and part of the signal sequence, and Oligo PRL-Rev (5’GCCGCGGTTAGTTGGCTTTCATCATC3’), which contains a NorI restriction enzyme site and nucleotide sequence complementary to amino acids 117–124 with Lys-124 mutated to a stop codon (TAA). The 474-bp PCR product was subcloned into pCRII to generate plasmid pCR-16-kDa PRL, and its sequence was confirmed by DNA sequence analysis. The insert was excised from pCRII-16-kDa PRL by digestion with HindIII and NorI. This cDNA encoding 16-kDa PRL was then inserted into the adenoviral shuttle vector pXCMV (49) at the HindIII/NorI sites to generate pXCMV-16-kDa PRL. The entire 16-kDa PRL sequence in pXCMV-16-kDa PRL was confirmed by sequencing. Because the 16-kDa PRL peptides can form an intermolecular disulfide bond, we performed site-directed mutagenesis of Cys58 to Ser of 16-kDa PRL in a two-step procedure. In the first step, four primers for PCR amplification were used to generate two fragments containing mutation in 16-kDa PRL by using the cDNA encoding intact 23-kDa PRL. The primers used for PCR for fragment one are 5’AAGCTTAAACATGAAACTACAAAGGATCCGATGGA3’ (PRL-For) and 5’AAAGCTTAAACATGAAACTTCTGCGGTGAG3’ (antisense, m16-kDa-oligo1), and for fragment two the primers used are 5’GCCATCAA-CAGAGCCACACCTTGT3’ (sense, m16-kDa-oligo2) and 5’GCCGCGGTTAGTTGGCTTTCATCATC3’ (PRL-Rev). In the second step, the two fragments from the previous PCR were used as templates and amplified with PRL-For and PRL-Rev to generate m16-kDa PRL with mutation of Cys58 to Ser. Construction of m16-kDa PRL into adenoviral shuttle vector pXCMV was carried out as described above.

Adenoviruses Ad-23-kDa PRL, Ad-16-kDa PRL, and Ad-m16-kDa PRL were generated by cotransfecting pXCMV-23-kDa PRL, pXCMV-16-kDa PRL, or pXCMV-m16-kDa PRL with pM17, a vector that contains the adenovirus genome with the E1 gene deleted, into the human embryonic kidney 293 cells by a method published previously (50). Ad-23-kDa PRL, Ad-16-kDa PRL, and Ad-m16-kDa PRL were amplified by infecting 293 cells. The titers of the viral stock, measured in pfu/ml, were determined to be 3 × 10^9, 8.8 × 10^8, and 9.5 × 10^9 pfu/ml for Ad-23-kDa PRL, Ad-16-kDa PRL, and Ad-m16-kDa PRL, respectively.

Analysis of the Structure of the rAd by PCR. Adenoviruses (1 × 10^7 pfu) was used for isolation of adenoviral DNA as described previously (50). The PCR was performed with rAd DNA using the primers XCMV1 and XCMV2 (51), which flank the PRL cDNA sequence, to detect the cDNA insert. Primers XCMV3 and XCMV4 (51) were used to detect the viral genome sequence. PCR products were performed according to published procedures (50).

RNA Analysis. Total cellular RNA was extracted from cells by using RNA-ZolB (Biotechnological Laboratories, Inc., Houston, TX) according to the manufacturer’s instructions. For Northern analysis, 20 μg of RNA was subjected to Northern blot analysis by electrophoresis on a 1% agarose gel containing 0.02% formaldehyde as described by Yang et al. (52), and the blot was hybridized with a random-primed probe generated from 0.7 kb 23-kDa PRL cDNA.

Western Blotting. DU145 or PC-3 cells were infected with Ad-23-kDa PRL, Ad-16-kDa PRL, and Ad-m16-kDa PRL in DMEM-F12 medium containing 5% FCS for 48 h. DU145 cells and PC-3 cells were infected with rAd with an MOI of 10 and 30, respectively, because maximum protein production was achieved without toxicity at these MOIs. The cells were collected at various time points, and the proteins were resolved by SDS-PAGE (4–12%) and transferred to a nitrocellulose membrane. The membrane was exposed to polyclonal rabbit anti-PRL antibody or anti-PRL monoclonal antibody. The secondary antibodies were horseradish peroxidase-conjugated antirabbit or antimouse IgG antibody, and the signals were detected by an enhanced chemiluminescence assay.

Cell Proliferation Assay. DU145 cells or PC-3 cells were plated in six-well culture plates (10^5 cells/well). Cells were infected with Ad-23-kDa PRL, Ad-16-kDa PRL, Ad-m16-kDa PRL, or Ad-Luc (a rAd containing the luciferase gene as a control) at an MOI of 10 (DU145 cells) or 30 (PC-3 cells). Cell numbers were determined at 1, 2, 3, and 4 days after infection using a hemocytometer.

HUVECs (VEC Technologies, Rensselaer, NY) were plated in six-well plates at 10,000 cells/well in DMEM with 10% FCS. Cells were allowed to attach overnight. Then rAd at an MOI of 10 was added to the wells. After 72 h, [H]thymidine (0.6 μCi/well; 25 Ci/mmol; Amersham, Arlington, IL) was added and incubated at 37°C for 4 h. The cells were washed with 5% trichloroacetic acid and solubilized with 0.25 N NaOH. The radioactivity was counted in a scintillation counter. The cell count of HUVECs was also determined. HUVECs were plated in six-well culture plates (20,000 cells/well). Cells were then infected with rAd at an MOI of 10. Cell numbers were determined at 3 days after infection using a hemocytometer.

Measurement of in Vivo Tumor Growth from rAd-Infected DU145 Cells and PC-3 Cells. In the initial experiments, DU145 or PC-3 cells were infected with Ad-16-kDa PRL or Ad-Luc at an MOI of 10 (DU145 cells) or 30 (PC-3 cells) for 48 h, harvested, and resuspended in MEM. DU145 cells (2 × 10^6 cells) or PC-3 cells (1 × 10^6 cells) in a total volume of 100 μl were injected s.c. into the flanks of nude mice (Harlan Sprague Dawley, Indianapolis, IN). Tumor sizes were monitored weekly. Tumor volume was calculated by the following formula: length × width × height / 2 = 0.5236, according to Rockwell et al. (53).

In the second experiments, DU145 cells or PC-3 cells were infected with Ad-23-kDa PRL, Ad-16-kDa PRL, and Ad-Luc as described above. DU145 cells (3 × 10^6 cells) or PC-3 cells (2 × 10^6 cells) in a total volume of 100 μl were injected s.c. into the flanks of nude mice (Charles River Laboratory, Wilmington, MA). Tumor volume was monitored as described above. Animals were killed on day 42. Tumors were removed, fixed in 3.7% formaldehyde overnight, and processed for histopathologic analysis.
16-kDa PRL has antitumor activity, we generated rAd containing cDNA encoding 16-kDa PRL or its related molecules and used it to infect the prostate cancer cells for high-efficiency protein expression. Three rAds were generated for this study. They are Ad-23-kDa PRL, containing full-length human PRL; Ad-16-kDa PRL, containing human PRL gene truncated at Lys 124; and Ad-m16-kDa PRL, containing 16-kDa PRL with Cys58 to Ser mutation (Fig. IA). DNA from rAds was prepared and analyzed by PCR to ensure that no deletion or rearrangement had occurred during viral generation by homologous recombination. PCR of the rAd DNA was performed as described in “Materials and Methods” using primer sets to amplify either the cDNA region or the viral genome adjacent to the recombination site. The resulting PCR products are shown in Fig. 1B. The sizes of all of the PCR products from primers XCMV1 and XCMV2, which are the flanking sequences from the human CMV promoter and SV40 polyadenylation site, matched the predicted sizes of the corresponding DNA fragments. These results indicate that these rAds contain the correct cDNA for PRLs. Furthermore, using primers specific to adenovirus genome (i.e., oligonucleotides XCMV3 and XCMV4), we observed PCR products of 0.86-kb in all three of the rAds tested. These results indicate that these viruses, generated from homologous recombination, have correct DNA arrangement as expected.

Assessment of Transgene Expression. The efficiency of these rAds to produce messages in the target cancer cells was examined by Northern blot analysis. We used two prostate cancer cell lines: DU145, derived from human prostatic carcinoma metastasized to brain (55), and PC-3, an androgen-independent cell line isolated from a prostate cancer patient who had bone metastasis (56). As shown in Fig. 2, no PRL message was detected in DU145 or PC-3 cells, suggesting that the cell lines express no endogenous PRL or express so little that it is undetectable. In both DU145 and PC-3 cells, high amounts of 23-kDa PRL message were already reached at 1 day after infection, and the message levels reached their maximum value 3 days after infection. Significant differences in the transcript levels were observed between cells infected with Ad-23-kDa PRL and Ad-16-kDa PRL (Fig. 2). DU145 or PC-3 cells infected with Ad-23-kDa PRL express abundant 23-kDa PRL message, an amount ~10 times higher than from cells infected with Ad-16-kDa PRL. Ad-m16-kDa PRL infection gave similar results as those of Ad-16-kDa PRL (data not shown). This observation suggests that there are differences in the efficiency of transcription from Ad-23-kDa and Ad-16-kDa PRL.

Proteins Expression. The PRL proteins produced from the DU145 and PC-3 cells after infection with Ad-23-kDa PRL, Ad-16-kDa PRL, and Ad-m16-kDa PRL were examined by Western immunoblot anal-
ysis. In DU145 cells, the production of 23-kDa PRL from Ad-23-kDa PRL was easily detected in the cells (Fig. 3A) and the conditioned medium (data not shown) with anti-PRL antibodies, suggesting that the 23-kDa PRL protein can be produced from the cells efficiently. The 16-kDa PRL proteins were also detected in both cells and medium. Similar to results at the RNA level, the production of 16-kDa PRL protein from Ad-16-kDa and Ad-m16-kDa PRL was much lower than that of 23-kDa PRL. To promote detection and allow comparison of protein products, we loaded for Ad-16-kDa and Ad-m16-kDa PRL about eight times the amount of samples loaded for 23-kDa PRL (Fig. 3). Thus, judging from the slightly weaker signal from 16-kDa PRL compared with that of 23-kDa PRL in the Western blot, the amount of proteins produced from Ad-16-kDa PRL or Ad-m16-kDa PRL is about one-tenth that from Ad-23-kDa PRL. When the DU145 cells were infected with Ad-16-kDa PRL, the protein products were detectable 2 days after infection and slightly decreased with time, suggesting that maximal expression might have been reached within 2 days after infection (Fig. 3A). In the case of Ad-m16-kDa PRL-infected DU145 cells, the protein product peaked 2 days after infection, and declined on days 3 and 4. This phenomenon was reproducible and may be because of the instability of m16-kDa PRL protein in DU145 cells.

PRL has been reported to exist in multiple forms (57). Accordingly, we found that Ad-23-kDa PRL infection produced a major band around 23 kDa, a dimer of 46 kDa, and an antibody-reactive protein of around 80 kDa. We also detected these three major proteins in the 23-kDa human PRL sample purified from human pituitary (NIDDK-hPRL-01; AFP-9042-B; National Institutes of Diabetes, Digestive and Kidney Diseases, NIH; Fig. 3 A and B, Lane 11). These observations suggest that the formation of multiple species with different sizes is an intrinsic property of 23-kDa PRL. Infection of the DU145 cells with Ad-16-kDa PRL produced three PRL antibody reactive-proteins with apparent molecular masses of 16, 18, and 23 kDa, respectively, with the 18 and 16-kDa forms being the major proteins. The Western analysis was performed in the presence of DTT, which should have blocked dimer formation through disulfide-linkage. Indeed, we did not detect a 32 kDa protein, which would have been the 16-kDa PRL dimer. The presence of multiple proteins from Ad-16-kDa PRL may be because of the different post-translational modifications of the 16-kDa PRL. Although m16-kDa PRL differs from 16-kDa PRL by only one amino acid, a substitution of Ser for Cys, an 18- and a 23-kDa protein were detected in cells infected with Ad-m16-kDa PRL. Multiple protein bands from Ad-23-kDa, Ad-16-kDa, and Ad-m16-kDa PRL were detected consistently with several anti-PRL antibodies from several different sources, including polyclonal antibodies from Biodesign and the NIH, and a monoclonal antibody from Biodesign, suggesting that these results are not because of nonspecific cross-reactivity of the antibodies. Thus, the 16-kDa and m16-kDa PRL expressed in DU145 or PC-3 cells may undergo post-translational modification resulting in the detection of multiple protein forms. The types of post-translational modification are not known.

Infection of PC-3 cells with Ad-23-kDa, Ad-16-kDa, or Ad-m16-kDa PRL produced proteins with similar patterns to those of DU145 cells when tested by Western blot (Fig. 3B). Multiple forms of 23-kDa, 16-kDa, and m16-kDa PRL were also detected in the PC-3 cells infected with these rAds suggesting that similar types of post modifications also occurred in PC-3 cells.

**Effect on Prostate Cancer and Endothelial Cell Proliferation.** To determine whether PRL expression affects the proliferation of prostate cancer cells, we infected DU145 cells with Ad-23-kDa PRL, Ad-16-kDa PRL, Ad-m16-kDa PRL, or control adenovirus (Ad-Luc) at an MOI of 10, which is sufficient to completely inhibit DU145 tumor growth in vivo (see below). However, these rAds were ineffective in vitro at this MOI; there were no differences in total cell numbers at any time (Fig. 4A). Similarly, PC-3 cells were infected with these vectors at an MOI of 30, which is optimal for viral infection and has no toxic effect on tumor growth in vivo. Expression of 23-kDa, 16-kDa, or m16-kDa PRL had no effect on the in vitro growth of PC-3 cells (Fig. 4B). These observations suggest that 23-kDa and 16-kDa PRL do not have a direct effect on the growth of prostate cancer cells in vitro. In contrast, expression of 16-kDa PRL or m16-kDa PRL has a direct impact on the growth of endothelial cells. Expression of 16-kDa PRL or m16-kDa PRL in HUVECs inhibited their proliferation by >70% when compared with the control virus- and no virus-treated groups (Fig. 4C). Similar effect on the cell proliferation was also observed by cell count (Fig. 4D). Although Ad-16-kDa PRL and m16-kDa PRL inhibited HUVEC growth, Ad-23-kDa PRL could inhibit only weakly. These data are consistent with previous reports that 16-kDa PRL is antiangiogenic but 23-kDa PRL is not (27).

**Effect of 16-kDa PRL on Prostate Tumor Growth in Vivo.** To examine whether 16-kDa PRL has an antitumor effect, we examined the effect of 16-kDa PRL on the tumorigenicity of DU145 cells in a nude mouse xenograft model. DU145 cells were infected with Ad-16-kDa PRL with an MOI of 10, and the cells were injected s.c. into the flanks of nude mice 2 days later. Expression of 16-kDa PRL inhibited the growth of DU145 cells in vivo, as evidenced by the reduction in tumor incidence and tumor sizes, when compared with
those of the control virus-treated tumor (Fig. 5A). In fact, no tumors developed at the 12 sites injected with 16-kDa PRL-infected DU145 cells, whereas the control virus-treated group developed 11 tumors at the 12 injection sites. The tumor growth rates, calculated by using an exponential curve, were $1.68 \pm 0.29$ mm/day for the control virus-treated tumors but zero for the Ad-16-kDa PRL-treated group.

The effect of 16-kDa PRL on the in vivo growth of PC-3 cells was also tested. As shown in Fig. 6A, 16-kDa PRL suppressed PC-3 tumor growth in vivo. This study shows that the tumor suppressive effect of 16-kDa PRL is not specific to one cell type.

**Comparison of the Effect of 23-kDa PRL, 16-kDa PRL, and m16-kDa PRL on Prostate Tumor Growth in Vivo.** Struman et al. (58), using several measurements of angiogenesis, showed that 23-kDa PRL is angiogenic but 16-kDa PRL is antiangiogenic. Therefore, we compared the effect of 23-kDa PRL and 16-kDa PRL on tumor angiogenesis. In addition, a mutant form of 16-kDa, i.e., m16-kDa PRL, with cys58 mutated to serine was also tested. This mutation avoids possible dimerization or association with other proteins because of the free cysteine in the 16-kDa PRL.

The effect of 23-kDa, 16-kDa, and m16-kDa PRL on prostate tumor growth was examined by infecting DU145 cells with the rAd at an MOI of 10 for 48 h. The cells were then injected s.c. into the flanks of nu/nu mice. As shown in Fig. 5B, both 16-kDa PRL and m16-kDa PRL effectively inhibited the growth of DU145 cells in vivo. Of the 12 sites injected, no tumor growth was observed at 11 sites, and 1 site showed initial growth followed by inhibition (data not shown). This result suggests that both the monomeric (m16-kDa PRL) and natural forms of 16-kDa PRL are effective in tumor suppression. As expected, expression of 23-kDa PRL did not substantially suppress the growth of DU145 cells (Fig. 5B). However, a slight decrease in tumor size was observed (Fig. 5B). When the tumor growth rate was calculated by using an exponential curve, the growth rates of Ad-Luc-treated tumors and Ad-23-kDa PRL-treated tumors differed significantly ($2.11 \pm 0.10$ versus $1.28 \pm 0.10$, respectively; Table 1). These results suggest that 23-kDa PRL, which was suggested to be angiogenic (58), did not stimulate the growth of DU145 tumors in this study but instead, exhibited a weak antitumor activity. There was no significant difference in the histopathology of the control virus- and Ad-23-kDa PRL-treated tumors (data not shown). Neither Ad-16-kDa PRL- nor Ad-m16-kDa PRL-treated groups formed tumors. The MVD of tumors from Ad-23-kDa PRL and Ad-Luc infection were determined. The mean MVD for 23-kDa PRL tumors was $\sim 70\%$ as compared with control Luc tumors. These observations suggest that the transient tumor growth inhibition by 23-kDa PRL is associated with reduced blood vessels.

The effects of 23-kDa, 16-kDa, and m16-kDa PRL on the growth of PC-3 tumors were also examined. Similar to that observed with DU145 cells, the growth of PC-3 cells expressing 16-kDa or m16-kDa PRL was inhibited significantly (Fig. 6B). Expression of 23-kDa PRL in PC-3 cells produced a biphasic growth curve: after initial tumor growth, the tumors stopped growing for 3 weeks and then resumed growth activity 5 weeks after tumor cell inoculation (Fig. 6B). These results indicated that both 16-kDa PRL and m16-kDa PRL has strong antitumor activity on the growth of PC-3 tumors in vivo. In contrast,
23-kDa PRL had weak antitumor activity against PC-3 tumors, as it did against DU145 cells.

**Effect of rAd Administration on DU145 Tumor Growth in vivo.**

The effect of Ad-16-kDa PRL, Ad-m16-kDa PRL, Ad-23-kDa PRL, and Ad-Luc on the growth of established tumors was examined by direct injection of rAd into DU145 tumors in nude mice. Tumors were established in nude mice by the injection of DU145 cells into the flanks; recombinant viruses were injected when the tumors reached \( > 14 \text{ mm}^3 \), and rAds were injected at 2-week intervals. As shown in Fig. 7, administration of Ad-16-kDa PRL and Ad-m16-kDa PRL into the tumors was able to suppress prostate tumor growth, whereas Ad-23-kDa PRL only showed weak antitumor activity.

**Effect of 23-kDa PRL, 16-kDa PRL, and m16-kDa PRL on Tumor MVD.**

The tumor MVD was determined in the established tumors as described above. Tumors were harvested at the end of study. The frozen sections of DU145 tumors were immunostained with anti-mouse CD-31 monoclonal antibody, and the mean MVD of rAd treated tumors were determined. The MVDs were 83% and 54% for Ad-23-kDa PRL and Ad-16-kDa PRL, respectively, as compared with control Ad-Luc treated tumors. These observations suggest that reduced blood vessels may contribute to the decreased tumor growth by 23-kDa PRL and 16-kDa PRL treatment.

**DISCUSSION**

Our studies show that the expression of 16-kDa PRL in prostate cancer cells markedly inhibits prostate tumor growth in vivo, whereas the wild-type 23-kDa PRL does not possess strong antitumor activity. Previous studies have established that 23-kDa PRL and its proteolytic product 16-kDa PRL have different functions with regards to antiangiogenesis (27, 58). Thus, the differential antitumor activity between the 23-kDa and 16-kDa PRL may result from the ability of 16-kDa PRL to inhibit tumor angiogenesis. The presence of 16-kDa PRL that possesses properties different from those of its intact molecule 23-kDa PRL was first reported by Ferrara et al. (26). Subsequently, several research groups have confirmed the antiangiogenic activity of 16-kDa PRL (27–30, 58, 59). However, there was no report on the effect of 16-kDa PRL on tumorm regression. During our studies on the effect of 16-kDa PRL on prostate tumor growth, Bentzen et al. (60) reported that transfection of 16-kDa PRL into human HCT116 colon cancer cells inhibited tumor growth in Rag1 \(-/-\) mice, thus corroborating our result showing the antitumor effect of 16-kDa PRL. These findings suggest that the effect of PRL, which was considered to be growth stimulatory, may be complicated by the presence of its cleavage product, 16-kDa PRL, which is antiangiogenic. Thus, the effect of PRL in various tissues may result from the combined effects of 23-kDa PRL, 16-kDa PRL, and the presence of their respective receptors in these tissues. Such an intricate regulatory process may form the basis of growth control under normal physiological conditions.

It has been reported that 23-kDa PRL stimulates growth and differentiation of prostate tissue (11). Transgenic mice overexpressing the PRL gene develop dramatically enlarged prostate glands (13), whereas prostate size is reduced in PRL-deficient mice (14). In addition, Struman et al. (58) showed that the 23-kDa PRL is angiogenic, whereas 16-kDa PRL is antiangiogenic. These observations suggest that the 23-kDa PRL have growth stimulatory effect under normal physiological conditions. In contrast with previous studies, we found that the growth of prostate tumors was reduced slightly by the expression of 23-kDa PRL. Because tumor cells are highly proliferative, 23-kDa PRL probably cannot additionally increase the growth of DU145 or PC-3 cells. However, the initial decrease in tumor growth in the presence of 23-kDa PRL was unexpected. It is possible that the weak antitumor activity of 23-kDa PRL may be because of the production of a small amount of 16-kDa from 23-kDa PRL by the tumor cells. Although the breakdown product of 23-kDa PRL was not detected, the proteolysis may occur at the cell surface to produce a local concentration sufficient to elicit weak antitumor activity. Thus, the apparent effect of 23-kDa PRL on the growth of DU145 and PC-3 cells in vivo may result from the combined effects of 23-kDa PRL and 16-kDa PRL. Consequently, whether 23-kDa is angiogenic or not cannot be concluded from this study.

Evidence suggests that cleavage of the 23-kDa PRL molecule to 16-kDa fragment may be a normal function of its target tissues and could be of physiological significance. Studies by Baldocchi et al. (21) suggested that the 16-kDa PRL can be produced in vivo from cleavage of the 23-kDa PRL by cathepsin D. Thus, the level of 16-kDa PRL in vivo is determined mainly by the amount of 23-kDa PRL and the presence of cathepsin D localized in close proximity to the 23-kDa PRL. Compton and Witorsch (24, 25), and Wong et al. (22) demonstrated that the prostate and mammary gland contain enzymes that could convert intact rat 23-kDa PRL into the cleaved 16 kDa fragment. In the prostate, Nevalainen et al. (11) reported that PRL is locally produced in human prostate epithelium, and acts as a direct growth and differentiation factor for human prostate. Whether changes in the balance between the production of 16-kDa PRL from 23-kDa PRL contribute to prostate tumorigenesis is not clear.

The amount of 23-kDa PRL produced from the prostate cancer cells from Ad-23-kDa PRL infection is \(~10\) times that of 16-kDa PRL or

<table>
<thead>
<tr>
<th>Cells and vector</th>
<th>Growth rate</th>
<th>SE</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145 cells</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ad-Luc</td>
<td>2.11</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Ad-23-kDa PRL</td>
<td>1.28</td>
<td>0.10</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Ad-16-kDa PRL</td>
<td>0.05</td>
<td>0.03</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Ad-m16-kDa PRL</td>
<td>0.01</td>
<td>0.01</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>PC-3 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad-Luc</td>
<td>1.85</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Ad-23-kDa PRL</td>
<td>1.39</td>
<td>0.05</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Ad-16-kDa PRL</td>
<td>(-0.01^b)</td>
<td>0.02</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>Ad-m16-kDa PRL</td>
<td>(-0.01^b)</td>
<td>0.02</td>
<td>(&lt;0.001)</td>
</tr>
</tbody>
</table>

*The P represents comparison to Ad-Luc group.

Negative growth rate resulted from tumors that initially grew and subsequently regressed.
The administration of antiangiogenic agents was associated with unwanted side effects, degradation of proteins by peptidases present in the serum, and poor target tissue penetration. Local therapy by expression of the 16-kDa fragment PRL in situ can bypass these common challenges of systemic administration of proteins. The prostate is anatomically well suited for local delivery of gene vectors because the entire organ may be accessed easily. By in situ expression of angiogenesis inhibitor using a viral vector system, the common challenges of systemic administration of these proteins may be avoided. Gene therapy using nonviral or viral vectors for clinically localized or recurrent prostate cancer has emerged as a new treatment modality (67). Adenoviral vectors have several advantages over retroviruses for use in prostate cancer. The adeno virus can provide a highly efficient method for gene transfer. The virus has significant tropism for epithelial cells by receptor-mediated endocytosis. In contrast to retrovirus, adenovirus does not rely on cell replication for expression of its genetic material. Therefore, the local expression of the 16-kDa PRL in the prostate can be achieved by using the intracellular machinery of the malignant epithelial cells by gene transfer using adenoviral vector. This study establishes the possibility of applying 16-kDa PRL for prostate cancer therapy. Because of the transient expression of replication-deficient adeno virus-mediated gene transfer, it is likely that multiple injections of rAd will be needed in clinical application. Antitumor effect of angiogenesis inhibitors may be dependent on the timing during tumorogenesis (68) and method of delivery (69). Additional studies on the efficacy of 16-kDa PRL in prostate tumor growth in an orthotopic prostate tumor model and transgenic mouse prostate cancer model will provide important biological information for the appropriate timing and delivery of 16-kDa PRL gene therapy in prostate cancer treatment.

REFERENCES

Antitumor Activity of the 16-kDa Prolactin Fragment in Prostate Cancer


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