Adenovirus-mediated Endostatin Delivery Results in Inhibition of Mammary Gland Tumor Growth in C3(1)/SV40 T-Antigen Transgenic Mice

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Abstract

We demonstrate the efficacy of systemic administration of a replication-defective adenovirus expressing endostatin (Ad-mEndo) administered during the preinvasive stage of mammary tumor development in C3(1)/T antigen transgenic mice. Mean serum levels of endostatin increased about 8-fold above that of controls and resulted in a significant decrease in tumor growth and an increase in survival. The inhibitory effect of endostatin occurred during or after the progression to invasive carcinoma. Reduced levels of vascular endothelial growth factor mRNA were found in association with high levels of endostatin. Our results demonstrate that the adenoviral induction of high levels of circulating endostatin significantly inhibits mammary tumor growth during the period when the "angiogenic switch" occurs.

Introduction

Tumor vascularization is a necessary process for the growth of solid tumors (1, 2). Tumor angiogenesis occurs as a consequence of enhanced expression of proangiogenic factors relative to antiangiogenic factors in the tumor microenvironment (2). This transition has been designated the "angiogenic switch" (2). A large variety of growth factors modulate tumor angiogenesis through endothelial cell proliferation, migration, and formation of new blood vessels (3). The increased expression of the proangiogenic factor VEGF is often observed in association with the angiogenic switch (2, 3). Antiangiogenic therapy has emerged as a means to prevent angiogenesis in primary or metastatic tumors, leading to tumor regression and "dormancy" (2). Endostatin and angiotatin have been identified as compounds with antiangiogenic properties that inhibit tumor growth (1, 2, 4, 5). Endostatin, a 20,000 fragment derived from the COOH-terminal domain of collagen XVIII, has been tested in some animal models of cancer (1, 4, 6–10). Despite its strong antiangiogenic properties, endostatin exhibits minimal detectable toxicity (1).

Gene therapy approaches offer the ability to induce the expression of exogenous compounds, including antiangiogenic factors such as endostatin, to reduce or eliminate tumors (6–10). However, such therapy has not been previously tested in transgenic animal models with intact immune systems where tumors develop spontaneously.

The C3(1)/Tag transgenic mouse model of mammary cancer has been shown to recapitulate several important histopathological and molecular alterations that occur in human breast cancer (11). Lesion progression in this model follows a very predictable time course with low-grade MIN lesions developing at about 8 weeks of age, progressing to high-grade MIN (similar to human ductal carcinoma in situ) by 12–14 weeks of age, leading to invasive carcinomas at about 15–16 weeks of age (11). The expression of Tag in this model results in the functional inactivation of the tumor suppressor genes Rb and p53, which is often lost or mutated in human breast cancer (11). Alterations in the expression of cyclins and cyclin-dependent kinases in this model are similar to those reported for human breast cancer (11, 12).

We have demonstrated previously that the C3(1)/Tag is a suitable preclinical model for testing certain chemopreventive or therapeutic compounds, including recombinant endostatin (13). In this study, we demonstrate that systemic adenoviral delivery of endostatin inhibits the angiogenic switch, delays mammary tumor onset, and decreases tumor burden in C3(1)/Tag mice with high levels of circulating endostatin.

Materials and Methods

Adenoviral Vector. The adenoviral vector used in this study (Ad-mEndo) has been previously characterized and tested for its in vitro and in vivo efficacy (6). Mouse endostatin cDNA was obtained by RT-PCR from liver extracts and inserted, together with the 18-amino acid E3/19K signal sequence, into the adenoviral shuttle plasmid pAdCMV. The resulting plasmid was recombined with 5 E1A/B-deleted Ad and used to infect 293 cells. The virus was amplified in 293 cells and titered using a standard plaque-formation assay. Control virus was similarly produced lacking the endostatin cDNA.

Animals and Treatment Schedule. All manipulations of mice were carried out in accordance with the guidelines of the Animal Care and Use Committee and with the procedure outlined in the guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, 1985). Thirty-six heterozygous C3(1)/Tag transgenic females in the FVB/N background were randomly divided into three groups: (a) experimental group (n = 12), animals treated with adenovirus containing the endostatin gene (Ad-mEndo); (b) control group 1 (n = 12), animals injected with the empty adenoviral vector (Ad-emptyV); and (c) control group 2 (n = 12), animals injected with PBS.

Mice received tail vein injections of 10^9 plaque-forming units of Ad-mEndo, Ad-emptyV, or PBS at 12 weeks of age and again at 13 weeks of age. Blood samples were taken from the saphenous vein 4 days after both injections to measure serum levels of circulating endostatin. Previous experiments have demonstrated that the expression peak for this adenoviral vector occurs 4 days after the i.v. injection (6). Because repeated blood extractions can alter endostatin levels, serum samples were taken only on these particular days.

Five mice from each group were sacrificed at 14 weeks of age, and mammary glands, liver, and blood samples were taken for analyses. Body weight and tumor volume were measured weekly in the remaining animals as described previously (14). Animals were euthanized when tumors reached 1.5 cm or when animals appeared sickly.

Evaluation of Endostatin Levels in Serum and Mammary Glands. Mammary glands were homogenized in radioimmunoprecipitation assay buffer containing protease inhibitors (1 × PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 μg/ml phenylmethylsulfonyl fluoride), maintained at 4°C for 45 min, and cleared by centrifugation. Protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Endostatin levels in serum and mammary gland samples were determined by competitive enzyme immunoassay (EIA; Cytimmune Sciences, College Park, Maryland). Mean serum levels of endostatin increased about 8-fold above that of controls and resulted in a significant decrease in tumor growth and an increase in survival. The inhibitory effect of endostatin occurred during or after the progression to invasive carcinoma. Reduced levels of vascular endothelial growth factor mRNA were found in association with high levels of endostatin. Our results demonstrate that the adenoviral induction of high levels of circulating endostatin significantly inhibits mammary tumor growth during the period when the "angiogenic switch" occurs.

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; MIN, mammary intraepithelial neoplasia; RT-PCR, reverse transcription-PCR; Tag, T-antigen.

Park, MD) according to the manufacturer’s instructions. All determinations were performed in duplicate wells using a Multiskan MCC/340 plate reader (Titertek, Huntville, AL).

Histopathology and Quantification of Mammary Gland Alterations. Mammary glands and liver were fixed immediately in fresh 4% paraformaldehyde overnight, embedded in paraffin, cut (4-μm thick), and stained with H&E. MIN lesions were quantified as described previously (15). Five mice per experimental group were analyzed.

In Situ Hybridization. The plasmid pBS-VEGF, kindly provided by Dr. D. Hanahan, was used to generate antisense and sense probes. 35S-CTP-labeled probes were generated by run-off transcription with T3 or T7 polymerase. In situ hybridization was performed as described previously (16). The developed slides were visualized in both bright field and dark field using a Zeiss Axoplan microscope.

RNA Extraction and Quantitative Real-time RT-PCR. Total RNA was extracted from frozen tissues using the RNeasy mini kit (Qiagen). The quality of the RNA was assessed by running aliquots on agarose gels. Six μg of RNA were reverse-transcribed into cDNA with the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA), as per the manufacturer’s protocol.

PCR primers for real-time RT-PCR of CD-31 (PECAM), VEGF164, and 28S rRNA were designed using Vector NTI software. The specificity of each primer was checked by performing a BLAST search using the National Center for Biotechnology Information tools. The sequences of the primers were as follows (5’-3’): (a) VEGF164 sense primer, ACAGGACAAAGCCA-GAAAAAACAC; (b) VEGF164 antisense primer, GTTTAATCAAAGCTGC-CTCGCCT; (c) CD-31 sense primer, GAGCCCAATCACGTTTCAGTTT; (d) CD-31 antisense primer, TCTCTTGCTTCTTGCTAGCT; (e) 28S rRNA sense primer, GGCTGGTCAAATCCACATCAA; and (f) 28S rRNA antisense primer, AGTTCTTTCAACTTTCCCT.

To verify the specificity of each primer set, the melting temperature curves of the amplicons generated from the PCR reaction were analyzed. Gel analyses were performed as well to show single PCR products. Quantitative analysis of gene expression was done with the SYBR Green master mix kit (Applied Biosystems). A Bio-Rad I-Cycler IQ Real-time detection system was used for PCR amplification and analysis. The levels of CD-31 and VEGF164 expression were normalized to that of 28S rRNA. Results were presented as relative units of expression with respect to 28S rRNA levels. Samples were analyzed in triplicates.

Statistical Analysis. The two-sided Student’s t test was used to evaluate whether significant differences existed between the mean values of the groups analyzed. The Mann-Whitney test was used in the case of tumor volumes. The log-rank test was used to determine differences in survival curves. Pearson’s correlation was used to analyze whether serum endostatin levels correlated with tumor volume, survival, and expression levels of VEGF mRNA in the mammary glands.

Results and Discussion

Antiangiogenic therapy for cancer has received wide attention in recent years because of its potential effectiveness against multiple types of tumors (1, 2, 4, 5). Endostatin, a COOH-terminal fragment of collagens XVIII, has previously been shown to be one of the most effective antitumor compounds by inhibiting angiogenesis in a variety of cancers (1, 2, 4, 5). However, high doses of recombinant endostatin are required to achieve tumor regression when delivered by i.p. or s.c. injection (13). This study is the first report to demonstrate inhibition of tumor development by adenoviral delivery of endostatin in a transgenic mouse model of cancer.

Although administration of either Ad-emptyV or Ad-mEndo did not result in weight loss or signs of distress, a mild liver toxicity was observed in both groups consisting of moderate hepatocytomegaly and inflammation. Therefore, toxicity was not due to endostatin, but to the adenoviral vector. These types of lesions have been described previously after the administration of adenoviral gene therapy vectors (17).

An increase in VEGF expression is a common molecular event that occurs during the angiogenic switch (2, 3). The elevation of VEGF levels promotes migration, sprouting, and blood vessel formation within tumors, with a subsequent increase in vascularization (3). We have demonstrated by in situ hybridization and by ELISA assay that the transition from MIN lesions to invasive carcinoma is accompanied by a significant increase in the levels of VEGF mRNA (Fig. 1). Mammary glands from normal FVB/N female mice had a mean value of 82 ± 12.1 pg VEGF/mg total protein, which was not statistically different from the mean value of 93 ± 15 pg VEGF/mg total protein from transgenic mice with MIN lesions at 14 weeks of age. However, mammary glands from 18-week-old mice with invasive carcinomas had a mean value of 790 ± 100 pg VEGF/mg total protein, an almost 10-fold increase compared with the mice with MIN lesions. These
controls (were modestly increased in the Ad-mEndo group compared with after the first injection. However, the average levels of endostatin circulating endostatin, compared with the values measured 4 days second set of serum samples (collected at day 11 of treatment) had a reached levels over 1300 ng/ml (1321.9 and 1475.7 ng/ml). The therapy achieved serum levels of mEndo-treated mice. Four of 12 animals that received Ad-mEndo ng/ml for Ad-emptyV-treated mice, and 678/H11006 of endostatin were 90 treatment (Fig. 2). Four days after the first injection, the mean values days after the second injection (day 11), which was given on day 7 of Serum samples were analyzed 4 days after the first injection and 4 injections of Ad-mEndo in 12-week-old mice (day 1 of treatment). results show that the transition from preinvasive to invasive carcinoma is accompanied in this model by an increase in VEGF mRNA and protein levels associated with the angiogenic switch.

High systemic levels of endostatin were achieved after tail vein injections of Ad-mEndo in 12-week-old mice (day 1 of treatment). Serum samples were analyzed 4 days after the first injection and 4 days after the second injection (day 11), which was given on day 7 of treatment (Fig. 2). Four days after the first injection, the mean values of endostatin were 90 ± 7 ng/ml for PBS-treated mice, 99 ± 4.5 ng/ml for Ad-emptyV-treated mice, and 678 ± 130 ng/ml for Ad-mEndo-treated mice. Four of 12 animals that received Ad-mEndo therapy achieved serum levels of >800 ng/ml endostatin. Two mice reached levels over 1300 ng/ml (1321.9 and 1475.7 ng/ml). The second set of serum samples (collected at day 11 of treatment) had a significantly lower average level (an approximately 80% decrease) of circulating endostatin, compared with the values measured 4 days after the first injection. However, the average levels of endostatin were modestly increased in the Ad-mEndo group compared with controls (P < 0.01). The mean endostatin values were 66.6 ± 6 ng/ml for PBS-treated mice, 144 ± 15.2 ng/ml for Ad-emptyV-treated mice, and 272 ± 21 ng/ml for Ad-mEndo-treated mice. This result demonstrated that the second injection of Ad-mEndo was unable to maintain or increase the circulating levels of endostatin. Previous reports have shown that repeated injections of adenoviral vectors induce an immune response, thus limiting the effectiveness of sustained therapy (18). Mice given Ad-mEndo had circulating levels of endostatin about 130 ng/ml higher than those of mice given Ad-emptyV on day 11. The Ad-emptyV, however, elicited a mild but significant increase in endostatin levels compared with the PBS-treated mice (P < 0.01). We speculate that this increase may be the result of endostatin released from injured hepatocytes, which are rich in collagen XVIII/endostatin (19).

The levels of endostatin achieved in our study are consistent with earlier reports. Most previous studies using adenoviral gene therapy have reported slightly higher (1770 ng/ml in nude mice; Ref. 6) or lower (857 ng/ml in 129/J mice and 178 ng/ml in BALB/c mice; Ref. 8) peak levels of circulating endostatin after injection of 108 plaque-forming units, compared with our results. Differences in endostatin levels are most likely due to the type of adenoviral vectors used as well as variations between the strains of mice (8).

When delivered by tail vein, adenovirus has a preferential tropism for hepatocytes (20). We found no detectable endostatin levels in the mammary glands of mice treated with Ad-mEndo or controls. This result revealed that either mammary glands were not infected by the adenovirus or endostatin gene expression by the viral vector was not detectable in the mammary glands. Therefore, the inhibitory effect on tumor development appears to be due to the circulating levels of endostatin produced systemically. Tumor volume was significantly reduced in mice injected with Ad-mEndo, compared with controls (Fig. 3). By 20–21 weeks of age, the mice treated with Ad-mEndo had an approximately 50% reduction in the average tumor burden, compared with the control animals. An inverse correlation between endostatin levels and tumor volume was found in the group of mice that received Ad-mEndo (Fig. 4A). Animals with the highest levels of endostatin had the lowest tumor burden (Fig. 4A). For instance, at week 21 of age, the average cumulative tumor volume for the three mice with the highest levels of endostatin was 493 ± 123 mm³, whereas the average for mice with lower endostatin levels was 1020 ± 130 mm³. Tumor multiplicity was not significantly different between the three groups in the study. Survival was increased in mice with the highest levels of endostatin. A statistically significant positive correlation (P = 0.04) was found between levels of endostatin and survival (Fig. 4B) in the group of mice treated with Ad-mEndo. Although tumor volume was reduced in endostatin-treated mice, all of the animals eventually had to be sacrificed due to the presence of large tumors. The extent of tumor reduction that we report in this study is similar to that described for adenoviral gene therapy in xenograft models (6–10). Our previous study on tumor development in C3(1)/

![Fig. 2. Serum levels of endostatin in control animals (injected with PBS or Ad-emptyV) or animals injected with Ad-mEndo. A. 4 days after the first injection, levels of endostatin in the Ad-mEndo-treated group are modestly higher than those in control mice.](image)

![Fig. 3. Cumulative tumor volume in C3(1)/Tag mice treated with PBS, Ad-emptyV, or Ad-mEndo. Tumor volumes in Ad-mEndo-treated mice are decreased with respect to controls and reach statistical significance at 20 and 21 weeks of age (*, P < 0.05).](image)
Tag mice using a 3-week course of recombinant endostatin resulted in an approximately 9-fold decrease in tumor burden at week 21 in treated mice compared with controls (13). In the present work, levels of circulating endostatin were transiently increased in Ad-mEndo-treated mice for about 2 weeks. Other studies using adenoviral gene therapy have reported a similar duration of elevated endostatin levels (6–8, 10).

To determine the effect of endostatin on the development of preinvasive MIN lesions in C3(1)/Tag mice 2 weeks after the initial treatment (14 weeks of age), 5 mice/group were euthanized, and mammary glands were examined. Histopathological analysis revealed that the three study groups had similar numbers and types of mammary gland lesions, consisting of low-grade and high-grade MIN. VEGF and CD-31 mRNA levels in the mammary glands were quantified by real-time RT-PCR in animals sacrificed at 14 weeks of age. Three of the animals sacrificed at this age from the Ad-mEndo group had high levels of circulating endostatin (1475, 736, and 660 ng/ml), whereas two mice had low endostatin levels (167 and 130 ng/ml). VEGF mRNA levels in the mammary glands were significantly decreased in the three 14-week-old mice with high levels of endostatin (P < 0.05) compared with the controls (Fig. 4C). An inverse correlation was observed between circulating endostatin levels and VEGF mRNA levels in the mammary glands in the five animals in the Ad-mEdno group (r² = 0.6, Fig. 4D). This correlation was not statistically significant, probably due to the limited number of animals analyzed. Levels of the endothelial marker CD-31 were slightly reduced in the three Ad-mEndo-treated animals, which had high levels of endostatin compared with controls, but those differences did not reach statistical significance (data not shown).

Our results demonstrate that elevation of systemic levels of endostatin is associated with a significant decrease in levels of the proangiogenic factor VEGF in the preinvasive mammary lesions. The reduction in VEGF levels likely contributes to the inhibition of the angiogenic switch, resulting in a delay in tumor angiogenesis and tumor growth. The elevation of endostatin levels, even for a relatively short period of time during the transition phase from MIN to invasive carcinoma, is able to significantly inhibit tumor growth but does not have a lasting effect. Although the effect of endostatin is transient due to the limitations of the adenoviral system used, it seems possible that sustained treatment with endostatin could significantly prolong the inhibition of tumor growth.

In conclusion, we have demonstrated that the systemic administration of an adenoviral vector expressing endostatin during the transition from preinvasive mammary lesions to invasive carcinomas significantly inhibits tumor growth in association with reduced VEGF mRNA levels. These results suggest that endostatin can retard the angiogenic switch in the C3(1)/Tag mammary gland lesions and inhibit tumor formation. Because the currently available adenoviral vectors are not able to maintain sustained levels of circulating endostatin, improved gene therapy delivery systems that provide prolonged production of endostatin may be quite useful in inhibiting human mammary tumor progression at the ductal carcinoma in situ stage, perhaps in combination with other modalities.

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References


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