Effect of Endostatin on Spontaneous Tumorigenesis of Mammary Adenocarcinomas in a Transgenic Mouse Model

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Abstract

A transgenic mouse model was used to evaluate the effect of endostatin treatment on spontaneous tumorigenesis. In this model system, female mice develop multiple mammary adenocarcinomas and male mice develop prostate cancer. Female mice treated with mouse endostatin during a 12–15-week period showed delayed tumor development by 4–6 weeks and significantly decreased tumor burden. Furthermore, endostatin treatment reduced the number of malignant lesions per mouse. In a separate set of experiments, male mice treated with endostatin showed a survival advantage, and their life spans were prolonged by 10.5 weeks over control animals. These data demonstrate that mouse endostatin is effective in delaying spontaneous tumor development and growth.

Introduction

Neovascularization is one of the important steps involved in tumor growth and metastasis. At least three different mechanisms are recognized in the angiogenesis of tumors. These include: (a) vascular sprouting; (b) recruitment of circulating endothelial progenitors; and (c) cooption (1). Cancer cells actively participate in creating an angiogenic microenvironment, which provides a survival advantage. Extensive angiogenesis is, therefore, linked to aggressive tumor growth and poor prognosis. Consequently, methods to inhibit the angiogenic process provide a unique opportunity to arrest tumor growth, either alone or in combination with chemotherapy and radiation. A number of endogenous antiangiogenic molecules have been identified recently. These include angiotatin, endostatin, antithrombin fragment, and canstatin (2–4). In addition to proteolytic fragmentation. A number of endogenous antiangiogenic molecules have been identified recently. These include angiotatin, endostatin, antithrombin fragment, and canstatin (2–4). In addition to proteolytic fragmentation, thrombospondin, retinal epithelium-derived factor, interleukin 12 are also shown to be potent antiangiogenic molecules (5, 6). Treatment with angiostatic molecules such as endostatin leads to regression of established tumors in certain model systems (3). Studies on the effect of angiogenic therapy on spontaneously arising tumors are, however, sparse. Bergers et al. showed recently that endostatin and angiotatin treatment of RIP-Tag mice could delay pancreatic tumorigenesis and inhibit tumor growth (7). In the present study, we investigated the effect of murine endostatin on spontaneous growth of mammary adenocarcinomas in a transgenic model system developed by Maroulakou et al. (8). In this model system, the SV40 early region transforming sequence was cloned under the regulatory control of a rat prostatic steroid binding protein [C3(1)] promoter. SV40 Tag functionally inactivates p53 and Rb through the direct binding to these proteins (9) and appears to interfere with cell cycle regulation, as often occurs in human cancer. Female transgenic animals develop mammary adenocarcinomas over a predictable time course, whereas male transgenic mice develop prostate adenocarcinomas. Although TAg is not an etiological agent for human mammary and prostate carcinomas, the genetically engineered mouse model is very useful in evaluating potential therapeutic agents in preventive and interventional settings. Using the C3(1)/TAg transgenic model, we investigated the effect of endostatin on tumor incidence, growth, and survival. Endostatin treatment initiated before the development of gross tumor lesions delayed the onset of mammary adenocarcinoma formation. Mice treated with endostatin showed reduced tumor burden and number of lesions. In male mice, endostatin treatment prolonged survival time.

Materials and Methods

C3(1)/SV40 TAg Transgenic Mice. Phenotypes of male and female C3(1)/TAg transgenic mice have been described previously (8, 10–12). Heterozygous TAg transgenic mice were maintained by breeding with FVB/N mice. All manipulations of mice were performed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, 1985).

Purification of Recombinant Mouse Endostatin. Mouse endostatin has been cloned and expressed in Pichia pastoris by Dhanabal et al. (13). A selected Pichia clone was cultivated in a 10-liter fermentor (BioFlow 3000, New Brunswick, NJ) and induced to express endostatin by methanol feed (14). Supernatants from fermentation runs were first concentrated by ultrafiltration and then dialyzed against 10 mM Tris-HCl buffer (pH 7.6) and 0.5 mM PMSF. Further purification was carried out by heparin affinity column. The heparin column was equilibrated with 10 mM Tris-HCl buffer (pH 7.6) and 0.5 mM PMSF. Samples were applied to the column at a flow rate of 1.0 ml/min using a fast protein liquid chromatography (Amersham Pharmacia Biotech, Piscataway, NJ). After thorough washing to remove unbound proteins, bound proteins were eluted with a continuous gradient of 0–1 M NaCl in 10 mM Tris-HCl (pH 7.6) and 0.5 mM PMSF. Further purification was carried out by heparin affinity column. The heparin column was equilibrated with 10 mM Tris-HCl buffer (pH 7.6) and 0.5 mM PMSF. Samples were applied to the column at a flow rate of 1.0 ml/min using a fast protein liquid chromatography (Amersham Pharmacia Biotech, Piscataway, NJ). After thorough washing to remove unbound proteins, bound proteins were eluted with a continuous gradient of 0–1 M NaCl in 10 mM Tris-HCl (pH 7.6) and 0.5 mM PMSF. Endostatin eluted at ~0.5 M NaCl. Purified endostatin was analyzed on an SDS-PAGE (12% acrylamide gel) under nonreducing conditions. Routinely, the samples were subjected to NH3 terminus microsequencing (10 cycles) and mass spectrometry. Purified materials were dialyzed against PBS [137 mM NaCl, 8.1 mM Na2HPO4, 2.68 mM KCl, and 1.47 mM KH2PO4 (pH 7.3)] and stored in aliquots at −70°C.

Treatment of Female Transgenic Mice. Female C3(1)/TAg transgenic mice develop mammary intraepithelial neoplasia originating in ducts and terminal ductal lobular units by 3 months of age. Mammary intraepithelial neoplasia lesions (~5–10 months of age. By 6 months of age, all of the female mice die because of universal development of multifocal mammary adenocarcinomas with occasional evidence of metastatic involvement to the lung (8, 16). We tested the efficacy of mouse endostatin beginning at 12 weeks of age by daily administration for a period of 3 weeks. Tumor growth was monitored by periodic

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The abbreviations used are: TAg, SV40 large T antigen; PMSF, phenylmethylsulfonyl fluoride.
EFFECT OF ENDOSTATIN ON TRANSGENIC MICE

Fig. 1. Inhibition of mammary adenocarcinoma development by endostatin. Female C3(1)/T_{AG} transgenic mice were treated with 20 mg/kg of soluble mouse endostatin for 3 weeks beginning at 12 weeks of age. All injections were given s.c. Tumor development represents the percentage of mice showing palpable tumors. ---, control (n = 14); ----, endostatin-treated group (n = 5). P = 0.037.

caliper measurements, and the number of tumor nodules was also counted. Tumor volume was calculated by the following formula: tumor volume (mm$^3$) = $(a \times b^2)/2$, where $a$ is length in mm and $b$ is width in mm. Statistical significance between control and treated groups was determined by Student’s $t$ test.

Treatment of Male Transgenic Mice. In male C3(1)/T_{AG} transgenic mice, hyperplastic changes in the epithelium of the dorsal/ventral regions of the prostate usually occur as early as 3 months of age. Adenomas develop in about one-third of animals between 6 and 8 months of age. About 40% of male mice develop invasive prostate adenocarcinomas by 9 months of age (8, 10). For male mice, treatment started at 22 weeks of age, ~7 weeks before the appearance of visible tumors. Mouse endostatin expressed in yeast was s.c. injected at a dose of 20 mg/kg/day for 30 days. Injections were given s.c. at the neck, and the survival of mice was monitored.

Results and Discussion

A number of antiangiogenic inhibitors are currently being studied for their efficacy to inhibit tumor growth, either alone or in combination with chemo/radiotherapy. Recombinant endostatin is expressed in bacteria as an insoluble protein. When the insoluble form was administered into C57/Bl6/J mice transplanted with Lewis lung carcinomas, T241 fibrosarcomas, or B16F10 melanomas, tumor regression was observed (3). Repeated cycles of endostatin treatment led to tumor dormancy and a complete cure in mice (17). Endostatin has been expressed in soluble form in yeast (13). The soluble protein was found to inhibit tumor growth in a number of transplanted tumor models (13, 14, 18). In the present study using a transgenic animal model system, we investigated the effect of endostatin on spontaneous formation of mammary adenocarcinomas in female mice and on survival of male mice prone to develop prostate cancer.

Treatment of Female C3(1)/T_{AG} Transgenic Mice by Mouse Endostatin. Female mice were treated at a dose of 20 mg/kg/day, started at 12 weeks of age and continued up to 15 weeks of age. Palpable tumors begin to arise at about 14 weeks of age in these mice. Daily endostatin administration for 3 weeks significantly delayed the appearance of tumors. For example, 50% of the control mice showed visible tumors by 16.7 ± 0.70 weeks of age. However, the endostatin-treated group showed tumors in 50% of animals about 22.0 ± 2.58 weeks of age ($P = 0.037$). In the control group, 100% of mice developed mammary adenocarcinomas by week 21.3. The endostatin-treated group showed delayed appearance of tumors although 100% of the animals developed malignant lesions by 28.6 weeks of age (Fig. 1), well after the termination of endostatin treatment. Because female transgenic mice develop multiple mammary tumor nodules, tumor burden per mouse and number of tumor lesions were determined after endostatin treatment. These data are summarized in Figs. 2 and 3. At week 18, ~3 weeks after the termination of treatment, tumor burden (mean) of the control group was 387 mm$^3$, whereas tumors in the endostatin-treated group were barely detectable (4.8 mm$^3$; Fig. 2). At week 23, ~8 weeks after the termination of treatment, tumor burden of control mice reached a value of 2794 mm$^3$. At this time point, the endostatin-treated group showed a tumor burden of 278 mm$^3$, 10-fold reduction in tumor burden. In addition to a decrease in tumor burden, endostatin treatment also significantly altered the number of tumor nodules per mouse. Fig. 3 shows a representative group of female mice from control (A) and endostatin-treated group (B) at ~23 weeks of age. Throughout the observation period, endostatin-treated mice showed a lower number of tumor nodules. At the end of the experiment, the control group of mice had an average of 7.8 lesions, but the endostatin treatment group showed a mean of 3.3 nodules/mouse (Fig. 3C). These data demonstrate that endostatin treatment during the early phase of spontaneous tumorigenesis delayed tumor development and significantly reduced tumor burden.

In an earlier study, Bergers et al. reported that Fe-endostatin fusion protein was effective against pancreatic islet cell carcinoma in RIP1-Tag2 transgenic mice (7). Endostatin treatment inhibited angiogenesis and tumor growth more in the prevention and the intervention stage than in the regression stage. Fe fusion was used to improve the pharmacokinetics of endostatin. Bioavailability and serum half-life was suggested to be critical in determining the efficacy of endostatin treatment. For example, bacterially expressed endostatin, when given as a suspension, was highly effective against established tumors and induced regression (3). A recent study using a rat endostatin preparation was administered as a suspension, which inhibited carcinoembryonic-induced mammary carcinomas in rats (19). Using endostatin as a precipitate is believed to result in slow release in vivo. Furthermore, daily injections of insoluble preparation will result in a progressive accumulation of endostatin during treatment. Another strategy is to administer endostatin twice daily (split dose), which can improve antitumor activity. In the case of angiostatin, administration two times and three times per day showed better tumor growth inhibition than a once-a-day schedule (20). In a preliminary experiment, radioiodinated endostatin was used to determine the clearance rate in mice. These studies showed that >50% of injected endostatin is rapidly cleared from circulation with an α phase of about 5 min (data not shown). Consequently, a slow-release formulation will improve the efficacy of endostatin therapy significantly.

![Fig. 2. Inhibition of mammary adenocarcinoma growth by endostatin treatment. Tumor burden of individual nodules of female C3(1)/T_{AG} transgenic mice was determined by caliper measurements. □, control; ●, endostatin. Tumor burden represents cumulative value from all of the tumor nodules from individual mice. Data are expressed as means of tumor burden; bars, SE. Statistical significance was determined using Student’s $t$ test. *, $P < 0.05$.](cancerres.aacrjournals.org)
Treatment of Male C3(1)/T<sub>AG</sub> Transgenic Mice by Mouse Endostatin. In a separate experiment, the efficacy of endostatin on the survival of male C3(1)/T<sub>AG</sub> mice was determined. The male mice are prone to develop prostate cancer as well as proliferative lesions in other genitourinary organs (12) and glandular tissues (21). Data in Fig. 4 show the survival of male mice. Mean survival of the control group of mice treated with PBS from weeks 22 to 25 was 35 weeks. Endostatin treatment (20 mg/kg) during the same period prolonged their survival time for an additional 74 days (survival time, 45.6 weeks). Increased survival by endostatin treatment is statistically significant (P < 0.0045).

In the present study, endostatin was injected from 5 months of age, and at this time point, high-grade prostatic intraepithelial neoplasia is expected to occur in these mice. Additional studies will determine the effect of endostatin treatment on the histopathological progression of prostate lesions and other glandular lesions in male mice.

References
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