Antitumor Activity of Endostatin against Carcinogen-induced Rat Primary Mammary Tumors

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Abstract

Endostatin, a Mr 20,000 fragment of collagen XVIII, potently inhibits the growth of experimental tumors implanted in mice. Here we report the cloning, expression, and antitumor activity of the rat form of endostatin. When tested on breast carcinomas arising in female virgin rats after intragastric administration of 9,10-dimethyl-1,2-benzanthracene (DMBA), endostatin induced significant inhibition of mammary tumor growth in all of the treated rats during a 4-week treatment period without signs of systemic toxicity. Interestingly, this arrest of tumor growth persisted throughout a four-week off-therapy period. Moreover, endostatin was effective in counteracting the development of multiple primary tumors. These results confirm that rat endostatin is a potent anticancer agent in a carcinogen-induced, spontaneously arising rat breast cancer model. It not only stops the growth of existing tumors but also decreases the incidence of the development of multiple neoplastic lesions.

Introduction

When cells are exposed to carcinogens, oncogenesis can occur, leading to the formation of microscopic foci of proliferating neoplastic cells. The progression of these lesions to larger, invasive tumors requires the formation of new blood vessels (1). This process is regulated by stimulators and inhibitors of angiogenesis (2). In recent years, research has been devoted to characterize the therapeutic potential of angiogenesis inhibitors as antitumor agents. Endostatin, a specific angiogenesis inhibitor produced through enzymatic cleavage of a protein precursor, the multiplexin α1-collagen XVIII, is a potent anticancer agent in animal models. The Mr 20,000 endostatin protein was shown to specifically inhibit the proliferation of endothelial cells and to stop the growth of Lewis lung carcinoma experimental metastases in mice without signs of drug toxicity (3). Furthermore, endostatin suppressed, in a dose-dependent fashion, the growth of a panel of murine primary tumors (3). When therapy was discontinued, tumors regrew at their primary sites. These tumors regressed again at resumption of endostatin treatment. Tumors could be subjected to repeated treatment cycles, without acquired resistance to the therapy (4). Interestingly, after multiple cycles specific for each tumor type, tumors ceased to regrow and remained as dormant microscopic nodules, without further recurrence (4). All of these studies were performed using implanted tumors in mice. Thus, we extended our study to a different animal species and to a model consisting of primary mammary carcinomas arising in rats after administration of DMBA, a potent carcinogen and environmental pollutant. This model of carcinogen-induced predisposition to mammary carcinoma has the advantage of bearing many similarities with human breast cancer. We cloned the rat form of endostatin and demonstrated its ability to inhibit tumor formation in this model.

Materials and Methods

When not specified, reagents were purchased from Sigma-Aldrich (Milan, Italy).

Cloning of Rat Endostatin. Rat liver cDNA (Clontech, Palo Alto, CA) was used as a template for a first round of 25 cycles of PCR amplification with the following primers: GAG GTG CCG GAG GGC TGG CTC ATC TT (forward); and ACT CTA GAG CCT TTT ATT TCT TGA GGA TTA CAT (reverse). The reverse primer was designed to add an XhoI restriction site at the 3’ end of the amplified PCR product, which was digested with XhoI, directionally ligated into the StuI-Xhol sites of a pFastBac 1 vector, and sequenced. This construct was then used as template for a second round of PCR aimed to obtain the exact sequence of rat endostatin. The primers used for this second amplification (forward: CAT ATG CAT ACT CAC GAG CCT TTT CAC; reverse: GCT AGC CAG AGG CCC TAT TTG GAG A) were designed to introduce NdeI and Nhel restriction sites at the 5’ and 3’ extremities of the PCR product, respectively. The PCR fragment was subcloned into the pCR 2.1 vector (Invitrogen, De Schelp, the Netherlands) and sequenced. The rat endostatin cDNA was finally excised from the pCR 2.1 vector by digestion with NdeI-Nhel, and subcloned in-frame into a pET24a-derived bacterial expression plasmid pCTB42#5 (kindly provided by Dr. Thomas Boehm, Children’s Hospital, Boston, MA), between NdeI and Nhel restriction sites. Correct in-frame insertion of rat endostatin cDNA was confirmed by automated DNA sequencing.

Purification of Rat Endostatin. Recombinant rat endostatin was purified from Escherichia coli cultures according to the procedure described by O’Reilly et al. (3). At the end of purification, recombinant endostatin precipitated into a white protein flocculate, which was subsequently concentrated in PBS by centrifugation at a protein concentration of 8 mg/ml, aliquoted, and used for treatment of tumor-bearing rats in its suspension form. The yield of recombinant rat endostatin from E. coli cultures was low (2.6 mg/l), compared with the yield of E. coli recombinant mouse endostatin routinely with murine endostatin (30–40 mg/l). Because of the low yield, a single purification batch was not sufficient for treatment of an individual animal, and several batches had to be randomly pooled. The dose of 20 mg/kg/day was selected on the basis of the maximum antitumor efficacy shown by mouse endostatin on murine tumors (3).

Chemical Carcinogenesis. Fifteen virgin Sprague Dawley rats were used for our experiments (5, 6). At the age of 50 days they were given 20 mg DMBA dissolved at 45°C in 1 ml of corn oil through a stomach catheter. Thereafter, rats were examined to monitor the outgrowth of mammary tumors at weekly intervals for the first 3 weeks, and then twice weekly until the first tumor was detected in each rat. Rats showing signs of persistent toxicity due to DMBA administration (diarrhea, fur ruffling, poor mobility) were excluded from the experiment.

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**Treatment of Rat Mammary Tumors.** At the onset of the first palpable tumors, between 40 and 60 days after DMBA administration, rats were allotted to the endostatin or vehicle groups alternating between the two. When the tumor volume reached 100–200 mm³, the treated group received 20 mg/kg/day rat endostatin in PBS, whereas the control group received equivalent volumes of PBS daily by s.c. injections for a period of 28 days. s.c. injections were given in the flanks of animals alternatively and rotated at different positions. The injection sites were at a distance at least 30 mm from the tumors. The treatment period was followed by an off-therapy observation period of 28 days. Tumors were measured weekly with a caliper throughout both treatment and off-therapy periods; tumor volumes were calculated as previously described (3).

**Results and Discussion**

Rat endostatin sequence was cloned by PCR based on sequence homology between the NC1 region of human and mouse collagen XVIII cDNA (5). Rat endostatin shares a high degree of homology...
with the murine (95% identity) and human (85% identity) proteins (Fig. 1). The antitumor activity of \textit{E. coli}-produced recombinant rat endostatin was tested in a well-established rat mammary tumor model (“Huggins’ model”; Ref. 6). A single intragastric dose of 20 mg of DMBA, administered to immature, virgin rats at 50 days of age, was sufficient to induce the onset within 40–50 days of large, fast-growing tumors, localized in the mammary epithelial area, which have been classified as mammary adenocarcinomas (7). In carcinogen-fed rats, the onset of the first tumor nodule was detectable by palpation as early as 40 days after DMBA administration, followed by other primary tumors appearing within 7–14 days in the mammary area of the rats, reaching the maximum number of five tumors per animal. At the onset of their first palpable tumor (average volume, 100–200 mm\(^3\)), rats were randomly divided in two groups: one group of four rats was treated with 20 mg/kg/day rat recombinant endostatin, and a second group of six control animals was treated with equivalent volumes of PBS.

Endostatin showed a powerful inhibitory activity on mammary cancer growth (Fig. 2A). From the second week of treatment (starting at day 7) up to the end of the off-therapy follow-up period, tumor burdens of endostatin-treated animals remained growth-arrested even in the off-therapy period as indicated by the dotted line. In contrast, two new tumors that developed after treatment stopped grew normally, accounting for the rise of the tumor volume curve during the off-therapy period. In B, pictures show mammary tumors in an endostatin-treated rat (left) and in a control, vehicle-treated animal (right). Both of the pictures were taken at the end of the 28-days treatment cycle.
burden values in the endostatin-treated group were significantly different from those in controls ($P < 0.05$ at day 7; $P < 0.001$ from day 14–56; Student’s $t$ test). At the end of treatment, the ratio between treated tumor volumes and control tumor volumes ($T/C$ ratio) was 0.07. Throughout the whole experiment, no sign of toxicity from endostatin was detected. During the first week of treatment with endostatin, tumors grew very slowly before they arrested by approximately 8 days (Fig. 2A). This pattern is similar to mouse models treated with endostatin (3). Although no data are available about the pharmacokinetics of endostatin, it is possible that slow release of the protein from a s.c. compartment required a long lag time to reach a therapeutic threshold. An alternative hypothesis could be that a significant amount of endostatin must first accumulate in the tumor bed.

Interestingly, endostatin that was administered at the onset of the first palpable tumor was able to counteract the outgrowth of multiple primary tumors in treated rats. Fig. 3 summarizes the number of tumors in treated and control groups during the time course of the experiment. Vehicle-treated control rats developed 3–4 tumors during the treatment period (days 0–28). In contrast, three of four endostatin-treated rats had only one tumor throughout the treatment period (days 0–28). In a fourth rat, a second nodule appeared 3 days after the beginning of endostatin therapy. During the off-therapy period (days 29–56), one tumor underwent complete regression in one rat and one treated tumor regressed completely in another rat. A second tumor module appeared 3 days after the beginning of endostatin therapy in one rat. ***, one new tumor arose in one rat and one treated tumor regressed completely in another rat. ***, one new tumor arose in one rat.

In summary, to our knowledge, this is the first demonstration of antitumor efficacy of endostatin in carcinogen-induced primary tumors. Secondly, it is the first confirmation in a different animal model that the carcinogenic effect of DMBA can be suppressed—although not completely eliminated—by short-term endostatin therapy. Fourthly, the dormant state that occurs when therapy is discontinued seems to be localized to the tumor bed previously exposed to endostatin. In contrast, the fact that new tumors could still arise at remote sites after discontinuation of endostatin therapy suggests that endostatin-induced tumor dormancy may require that therapy be initiated after the angiogenic process has begun. If this latter speculation is valid, it would provide additional evidence that endostatin is acting specifically against growing endothelium.

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References

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