Liposome-delivered Angiostatin Strongly Inhibits Tumor Growth and Metastatization in a Transgenic Model of Spontaneous Breast Cancer

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ABSTRACT

The possibility to inhibit tumor growth by interfering with the formation of new vessels, which most neoplasias depend on, has recently raised considerable interest. An angiogenic switch, in which proliferating cells acquire the ability to direct new vessel formation, is thought to be an early step in the natural history of solid tumors. Using a transgenic model of breast cancer, which shows many similarities to its human counterpart, including ability to metastasize, we targeted angiostatin production to an early stage of tumor formation. Liposome-delivered angiostatin considerably delayed primary tumor growth and, more importantly, inhibited the appearance of lung metastases. These findings can be relevant to the design of therapeutic intervention in humans.

INTRODUCTION

Tumors are dependent on blood supply to express their full malignant potential (1–4). Although this need has long been recognized, the possibility of treating malignancies by acting on their vasculature has gained popularity only since the discovery of angiogenesis inhibitors, which dramatically affect cancer growth in experimental murine systems. Highly promising results on experimental tumors in rodents with purified angiostatin and endothastin injection, alone or in combination with traditional means, have been reported (5–8). The establishment of a “dormancy status,” which in some cases persists even after the therapy has been discontinued, has been obtained with both gene products (6, 7), and this, together with the absence of drug resistance (9), raised the possibility that the antiangiogenic effects could be useful in the treatment of human tumors.

Most of these results have been obtained on transplantable tumors, i.e., tumoral cell lines cultured in vitro and transplanted in immunocompromised or syngenic mice. Although this approach has greatly benefited basic investigation in tumor biology, it is now being recognized that it is artificial to a great extent and does not recapitulate what really happens in humans (4, 10–12). However, the relevance of the obtained results for the treatment of human spontaneous tumors, which arise from a single cell that must adapt to its microenvironment, compete with similar adjacent cells, escape immune surveillance (if any), grow to a sizeable mass, acquire further genetic changes, and give rise to distant metastases, is unknown. Moreover, to evaluate the effect of the transferred gene, clones of these cells engineered to express a predetermined gene product are often used, and the effect on their growth compared to that of the parental, unmodified cell line, is investigated. This is not representative of what can be done at present in humans, where only a portion of the tumoral cells can be transfected or transduced with the expression vector and in which transgene expression is often shut off with unknown mechanisms. Therefore, it appears that to obtain results that can be transferable to the human situation, the choice of the animal model used is crucial.

Transgenic mice can represent an alternative to transplantable tumors for the evaluation of novel therapeutic strategies. However, thus far, the advantages offered by transgenic mice in this regard have only been exploited by Hanahan’s and Folkman’s groups, who after pioneering the use of this technology for the study of the early step of tumor pathogenesis (13, 14), subsequently reported detailed studies on the angiogenesis step involved in the growth of a few tumors and on gene therapy trials targeted to tumor vasculature in these mice (4, 15). These groups have very recently reported the first study investigating endogenous angiogenesis inhibitors for tumor therapy in transgenic mice (16).

With all these considerations in mind, we have investigated the effect of liposome-mediated angiostatin administration on a transgenic model, which we have been using for the last few years for gene therapy approach evaluation. This model, based on MMTV-neu transgenic mice bearing an activated rat neu oncogene under the control of the regulatory sequences contained in the long terminal repeats of the MMTV (17), was chosen for its features that make it relatively similar to its human counterpart (10, 18–22). Female mice develop multiple mammary tumors in 100% of cases, in a very predictable manner, which makes them very useful as providers of homogeneous neoplastic material. Tumors are symmetrical, giving the possibility of comparing the treated tumors on one side to the untreated tumors on the contralateral side, and they have been grown for many generations without modification of tumor growth kinetics, allowing us to meaningfully compare mice treated over the years with different approaches. In this way, the early steps of tumor development can be investigated thoroughly and the stage at which to start the treatment can be easily determined even at the preclinical level. These tumors represent neoplasias arising relatively slowly in an immunocompetent host and involve an oncogene that is known to also play a role in human breast cancer (albeit with an amplification/overexpression mechanism, as opposed to the activating point mutation present in these mice). In addition, these tumors give rise to distant metastases in the 4th or 5th month of age, which are easily detected by histopathological examination.

Exploiting the reproducibility of tumor insurgence in our MMTV-neu model, we targeted liposome-delivered angiostatin to mammary tumors before they become clinically detectable, i.e., at a stage in which this switch is likely to occur. The response obtained with such a protocol was impressive and clearly superior to other approaches previously tested on the same model.
MATERIALS AND METHODS

Mice. The production and screening of MMTV-neu transgenic mice as well as the histological features of the tumors have been previously described (10, 19).

DNA Construct. Angiostatin cDNA was obtained by RT-PCR on RNA extracted from liver of a CD-1 mouse using the following primers: H3KangF, 5’-CAAAGCTTCCACATGGACCATAAAGGAATTAACG3’; and R1HAanrB, 5’-CGAATTTCTAAGCAGTGATCTGGAGAGCTCGAT-TGGTATGTTGGGCAATTTCCCAACACCT3’. After the denaturation step for 3’ at 94°C, PCR was carried out for 35 cycles at 94°C for 20’ (denaturation step), 58°C for 30’ (annealing), and 72°C for 20’ (extension). The product was cloned in TA cloning and checked for the correct sequence.

Angiostatin cDNA was digested with HindIII/EcoRI and cloned in the HindIII/EcoRI site of MMTV Aneu Neo (20) after the removal of the Aneu sequences contained in an internal HindIII/EcoRI segment. The clone A3/3 was selected, characterized, and used for all of the experiments.

In Vivo Treatments. A3/3 construct DNA was prepared and added to the DOSPER liposomal transfection agent (Roche Diagnostics SpA, Monza, Italy) according to manufacturer’s instructions, as previously described (20). Fifty μl of the DOSPER/A3/3 mixture, containing 2 μg of DNA, were injected into the mammary glands of 2-month-old MMTV-neu female mice. In the first set of experiments, both the right and left breasts were injected every 15 days until the 5th month of age. In a second group of mice, only the breasts of the right side were injected monthly, whereas those of the left side were not injected and used as an internal control. At the end of the 5th month, the mice were sacrificed, and tumors were removed, weighed, and subjected to molecular and histopathological analyses.

Statistical Analysis. Local and systemic effect of angiostatin treatment were tested through repeated measures ANOVA. In this model, we defined two factors, side and breast, representing the repeated measures to assess within-subject (local) effect, and a third factor, angiostatin treatment, to evaluate between-subject (systemic) effect. Statistical analysis of vessel density was performed with the Student t test. The statistical software we used was SPSS for Windows 95.

Analysis of Angiostatin DNA and RNA. DNA and RNA were extracted from tumors inoculated or not with A3/3 DNA, and RNAs were retrotranscribed as previously described (19). PCR was performed with the following primers: Ang338F, 5’-GGAACCATGCTCCAGCAAGA and Ang1097R, 5’-GGAACCTGAGGAATCTGACTGG, which amplify a fragment of 759 bp.

Histopathological Analysis. At necropsy, all tissues were examined grossly for lesions. Mammary tumors, lungs, liver, kidneys, and spleen were fixed in 10% neutral buffered formalin. After fixation, lungs were sectioned in 2-mm thick slices and processed as a whole, while single, transverse sections of every mammary tumor and other organs were studied. All tissues were dehydrated in graded ethanol, embedded in paraffin, sectioned at 5 μm, and stained with H&E according to standard procedures. Metastases were evaluated in histological sections of the lungs, with careful examination of all lung sections: they were counted and scored as small (<10 cells), intermediate (10–100 cells), and large (>100 cells).

Vessel Density Evaluation. Mammary tumors were snap-frozen on liquid nitrogen and sectioned at 6 μm with a cryostat. Sections were mounted on poly-L-lysine-coated slides, air-dried at room temperature for 24 h, and fixed at −20°C for 10 min in aceton. Endogenous peroxidase activity was blocked by incubating the slides in 0.5% H2O2 and 0.1% sodium azide in Tris buffer (pH 7.6) for 20 min at room temperature. The slides were washed in Tris buffer for 5 min and then exposed for 30 min to 2% normal rabbit serum. Without washing, the first antibody, rat antimouse CD-31 (Mec13.3), a specific marker of endothelial cells (23), was applied overnight in a humidity chamber at 4°C (dilution 1:50). The slides were incubated with the second antibody, biotinylated rabbit antirat immunoglobulin G (Vector Laboratories, Burlingame, CA). The slides were then incubated in avidin-biotin peroxidase complex according to Hsu et al. (Ref. 24; Vectastain elite, Vector Laboratories, Burlingame, CA). The reaction was revealed by incubating the sections with 0.05% diaminobenzidine and 0.01% H2O2 substrate for 1 min. The slides were washed for 5 min in tap water, counterstained with hematoxylin, and mounted. Control slides were prepared by replacing the primary antibody with normal rat serum.

Vascularization was evaluated in the area of tumor containing the most capillaries and small venules by light microscopy. The areas of highest vascularization were found by scanning the tumor sections at low power. In the selected area, vessels were counted on three ×100 fields (1 mm²), and the mean was calculated (25, 26).

RESULTS

Liposome-mediated Angiostatin Administration in MMTV-neu Mice. Liposome-complexed angiostatin cDNA under the control of the MMTV promoter (see “Materials and Methods”) was injected into the mammary tumors of transgenic mice, while control transgenic mice were inoculated with empty liposomes. An additional set of five mice were injected with liposomes carrying a grossly deranged endostatin cDNA. Injections were started at 2 months of age, an age at which mammary tumors are not yet clinically detectable, although histological investigation reproducibly shows the presence of a mammary adenocarcinoma. Two groups of MMTV-neu mice were treated and compared with eight controls. The first group (five mice) was treated every 2 weeks with injections of the DNA-liposome complex in all of the breasts of both sides, while the second group (six mice) was treated monthly with injection in the breasts of the right side. All of the 19 mice were sacrificed at 5 months of age when MMTV-neu mice consistently showed large tumors and become recumbent, and a complete necropsy was performed.

As explained before (10, 19), this protocol allows the evaluation of both local and systemic effect (if any) because the comparison of treated tumors of treated mice against un.injected (contralateral) tumors of treated mice and tumors of untreated mice gives an estimate of the effect exerted at the local level. In addition, if a systemic effect is also present, the weight of the untreated tumors of treated animals should be less than that of tumors of control (untreated) mice. In the extreme situation in which the locally administrated agent is active independently of the site of administration, there should be no difference among the treated tumors and the contralateral ones. Finally, the effect on metastatic dissemination, either direct or indirect, can be evaluated by careful examination of lungs from treated and control mice.

PCR performed on tumors of treated mice showed that at the moment of sacrifice, the lipofected angiostatin vector DNA was present in injected but not in uninjectected samples (Fig. 1A, Lanes 3–6 and 8). RT-PCR analysis showed that angiostatin expression was still detectable in injected tumors (Fig. 1B, Lane 8), whereas no expression was detected in uninjectected tumors from treated mice (Fig. 1B, Lane 7) and in 10 tumors from MMTV-neu untreated mice (data not shown), suggesting that these tumors do not have basal expression of this inhibitory molecule.

A total of 110 mammary tumors were weighed and compared to the 80 tumors arising in the controls, and the results are summarized in Table 1. Statistical analysis demonstrates that the weight of tumors from both groups of angiostatin-treated mice was significantly different from that of control mice injected either with empty liposomes (P < 0.0001; see also Fig. 2) or with liposomes carrying grossly deranged endostatin (P < 0.0001). The difference was also highly significant when treated (right side) or untreated tumors (left side) from treated mice were separately compared to tumors that had arisen in control mice (P < 0.0001). On the contrary, when treated tumors were compared to untreated tumors in both groups of angiostatin-treated mice, the difference did not reach statistical significance. Likewise, no difference was present between the tumors of the two angiostatin-treated groups. No difference was found between the two

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different control groups (empty liposomes and deranged endostatin/liposomes). Taking all these findings together, we can conclude that angiostatin is able to slow tumor growth \textit{in vivo} by acting mainly with a systemic effect.

In additional experiments, four mice were treated every 2 weeks with injections of liposome-cDNA mixtures in tumors on both sides, starting at 2.5 months of age, when the neoplasias start to be clinically detectable. Two of these mice died abruptly at 4.5 months of age and could not be evaluated for technical reasons. The tumors in the remaining two mice had an average tumor weight (0.60 ± 0.05 g), which is somewhere between that of the treated and that of the control mice, suggesting that a delay in starting the treatment could negatively affect the outcome of the therapy.

Comparison with Other Gene Therapy-based Approaches. The reproducibility of tumor growth in control mice is high, as demonstrated by a statistical analysis of the controls performed in previous experiments. When the tumor weights of the eight controls used in this series were compared to those of eight controls used in previous experiments sacrificed at the same age, there was no significant difference. This is in agreement with the reproducibility of tumor kinetics in MMTV-neu mice and suggests that a comparison between the results obtained in the present study and those achieved with previous treatments can be significant. In the past few years, we have tested various gene therapy approaches on our model: retrovirally delivered herpes simplex virus thymidine kinase gene (8 mice, sacrificed at 4 months of age), retrovirally delivered IL-4 (13 mice evaluated at 4 months and 11 mice evaluated at 5 months), and retrovirally delivered connexin 43 (4 mice evaluated at 4 months of age). These findings are summarized in Table 2. In all these cases, tumors were individually weighed, with a total of 360 tumors in the treated groups. A total of 25 control mice (250 tumors) were also killed at 4 months of age, whereas 9 mice (90 tumors) were sacrificed at 3 months (see Table 2). When the results obtained with liposome-delivered angiostatin were compared to those found with all these approaches as well as with the 16 pooled control mice sacrificed at 5 months, it was clear that the weights of both treated and untreated tumors in angiostatin-treated mice were significantly lower than those of treated and untreated tumors from any of these treatments, with the exception of control mice at 3 months (Table 2). This suggests that angiostatin treatment delays tumor expansion for at least 2 months, a period which is more than one third of the life of MMTV-neu mice.

Table 1 Statistical analysis of angiostatin treatment effect on breast tumor growth in MMTV-neu mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean tumor weight (g)</th>
<th>SE</th>
<th>No. of mice</th>
<th>No. of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>L: 1.15 ± 0.12</td>
<td>8</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 0.99 ± 0.12</td>
<td>8</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Angiostatin (monolateral)</td>
<td>L: 0.17 ± 0.07</td>
<td>6</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 0.10 ± 0.03</td>
<td>6</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Angiostatin (bilateral)</td>
<td>L: 0.13 ± 0.03</td>
<td>5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: 0.16 ± 0.05</td>
<td>5</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

* L, left side; R, right side.

b \( P < 0.0001 \) (see text).

Fig. 1. A, angiostatin expression vector DNA amplification from injected and un.injected breast tumors from two treated mice (6060 and 6076) sacrificed at 5 months of age. Lane 1, 1-kb marker; Lane 2, left-side (untreated) tumor from mouse 6060; Lanes 3–6, four different right-side (treated) tumors from mouse 6060; Lane 7, left-side tumor from mouse 6076; Lane 8, right-side treated tumor from mouse 6076; Lanes 9–10, positive and negative controls, respectively; Lane 11, marker VIII. B, RT-PCR analysis of recombinant angiostatin expression in breast tumors from the treated 6076 mouse. Lane 1, 1-kb marker; Lanes 2 and 3, analysis of HPRT expression in retrotranscribed RNAs from left- and right-side tumors, respectively; Lane 4, negative control; Lanes 5–6, angiostatin expression in nonretrotranscribed RNA from left- and right-side tumors, respectively; Lanes 7–8, angiostatin expression on retrotranscribed RNA from left- and right-side tumors, respectively; Lanes 9–10, positive and negative controls, respectively; Lane 11, marker VIII.

Fig. 2. Comparison between a control (6133) and a monolaterally angiostatin-treated (6126) transgenic mouse. Top, 5-month-old mice were sacrificed and photographed. Bottom, tumors resected at necropsy are shown according to their natural position.
were separated from one another by thin fibrous trabeculae (Fig. 3). In situ carcinomas and enlarged, and solid lobular masses tended to coalesce and form in shape and size. Mitoses were a common finding. The interlobular stroma and fat were infiltrated and replaced by the neoplastic growth. Most of the solid masses of tumor cells displayed some evidence of tubular morphology. The largest solid areas of tumor often became necrotic. The neoplastic cells were usually uniform in shape and size. Mitoses were a common finding.

Vessel density was investigated in tumors from treated and control mice. Treated tumors showed a lower number of vessels than the controls. Reduction of tumor vascularization is evident in angiostatin-treated mice (mean vessel count, 62 ± 4.2) in comparison to the untreated controls (mean vessel count, 122 ± 2.36; P < 0.001). In Fig. 3, c and d, representative fields from a treated and a control tumor are reported.

**Histopathological Analysis.** The main histological features of primary mammary tumors and their metastatic behavior related to the age in female mice MMTV-neu are schematically reported in Table 3. One of the first changes observed in mammary tumors arising in MMTV-neu transgenic mice was the filling of the alveoli and ductules by proliferating cells (carcinoma in situ). The entire lobule proliferated and enlarged, and solid lobular masses tended to coalesce and were separated from one another by thin fibrous trabeculae (Fig. 3a). The interlobular stroma and fat were infiltrated and replaced by the neoplastic growth. Most of the solid masses of tumor cells displayed some evidence of tubular morphology. The largest solid areas of tumor often became necrotic. The neoplastic cells were usually uniform in shape and size. Mitoses were a common finding.

Vessel density was investigated in tumors from treated and control mice. Treated tumors showed a lower number of vessels than the controls. Reduction of tumor vascularization is evident in angiostatin-treated mice (mean vessel count, 62 ± 4.2) in comparison to the untreated controls (mean vessel count, 122 ± 2.36; P < 0.001). In Fig. 3, c and d, representative fields from a treated and a control tumor are reported.

**Angiostatin Delivery in MMTV-neu Mice Inhibits Metastatization.** Lung metastases are a consistent feature of the MMTV-neu system (an example is given in Fig. 3b). To determine whether the delay in tumor weight also reflects on the ability of tumoral cells to spread to distant sites, we performed a careful examination of lungs from MMTV-neu transgenic mice. Vessel density was quantified by counting vessels from three fields in each tumor section (immunoperoxidase for CD-31, hematoxylin counterstain, ×100).

**DISCUSSION**

The present investigation extends previous studies on the potential usefulness of the angiogenic inhibitor angiostatin in cancer therapy. Firstly, our transgenic model is based on spontaneously arising tumors and, for the reasons listed in the “Introduction,” it is clearly more similar to its human counterpart than the artificial systems using transplantaible cell lines which, with the single exception of the RIP-Tag mouse (16), have been used thus far. Therefore, the present report suggests that angiostatin can be effective also on spontaneous arising tumors in which an oncogene known to play a role in human neoplasias is involved, although it must be pointed out that tumors arising in MMTV-neu mice do not completely match human breast carcinomas. In our mice, neu oncogene is mutated in the germ-line examined at 5 months of age. (see Table 3). Taken together, these results show that angiostatin delivered with this protocol delays both primary tumor growth and metastatic dissemination.

**Table 2 Comparison between angiostatin treatment and other gene therapy-based approaches**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Three months&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Four months&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Five months&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mean tumor weight (g) SE</td>
<td>n&lt;sup&gt;d&lt;/sup&gt;</td>
<td>n&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>L</td>
<td>0.12 0.02 9 45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0.13 0.02 9 45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiostatin&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
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</table>

<sup>a</sup> Age at which mice were sacrificed and tumors weighed.

<sup>b</sup> n, number of mice; n', number of tumors; L, left side; R, right side.

<sup>c</sup> Data from all the angiostatin-treated mice are pooled.

<sup>d</sup> P < 0.0001 when compared to all the other groups, except 3-month-old controls (see text).

**Fig. 3. Results of histological and immunohistochemical analysis.** a, mammary tumors in a 2-month-old MMTV-neu transgenic mouse. Lobular, coalescing, tubular-to-solid adenoacarcinoma is recognizable in the s.c. fat (H&E, ×100). b, metastatic nodule in the lungs from a 5-month-old MMTV-neu transgenic mouse (H&E, ×200). c and d, representative tumor vasculature from control (c) and angiostatin-treated (d) 5-month-old MMTV-neu transgenic mice. Vessel density was quantified by counting vessels from three fields in each tumor section (immunoperoxidase for CD-31, hematoxylin counterstain, ×100).

**Table 3 Histopathological analysis of primary tumors and lung metastases at different ages**

<table>
<thead>
<tr>
<th>Age</th>
<th>Prevalent tumor morphology</th>
<th>Lung metastases/mice</th>
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<tbody>
<tr>
<td>1 mo</td>
<td>Carcinoma in situ</td>
<td>0/4</td>
</tr>
<tr>
<td>2 mo</td>
<td>Lobular, coalescing, tubular-to-solid AC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/3</td>
</tr>
<tr>
<td>3 mo</td>
<td>Solid AC with necrosis</td>
<td>0/3</td>
</tr>
<tr>
<td>4 mo</td>
<td>Solid AC with necrosis</td>
<td>3/9</td>
</tr>
<tr>
<td>5 mo</td>
<td>Solid AC with necrosis</td>
<td>12/16</td>
</tr>
<tr>
<td>5 mo (controls)</td>
<td>Solid AC with necrosis</td>
<td>12/16</td>
</tr>
<tr>
<td>5 mo (IL-4)</td>
<td>Solid AC with necrosis</td>
<td>0/11</td>
</tr>
</tbody>
</table>

<sup>a</sup> AC, adenocarcinoma.
and is active in all mammary cells, giving rise to multicentric neoplasias arising in all mammary glands.

Secondly, our system allows the evaluation in a natural setting of the effect of angiogenesis on the metastasization process, which is the leading cause of mortality in human tumoral pathology. When a careful analysis was performed on treated and untreated mice for the presence of pulmonary metastases, 75% of untreated mice had detectable metastases, whereas none of 11 treated mice showed any. This is a very promising finding, which strengthens the putative clinical usefulness of angiostatin treatment in humans. In our model, the inhibition on metastatic growth could be exerted directly at the level of lung microenvironment or could be a consequence of the effect exerted at the level of the primary tumor. We favor the second hypothesis; as in the former case, we would expect small tumor cell clusters to be present in the lungs because it is generally believed that seeding of small metastatic foci does not need new vessel formation. In this context, although the fact that metastatic cells are not usually shed from a primary tumor until this has become neovascularized has long been recognized (1, 2, 27), the present data are the first formal proof of this phenomenon in a quasi-natural setting. Although we were unable to formally distinguish between these two possibilities, which are not mutually exclusive, it is interesting that a relationship between the degree of vessel density and metastatic status has been described in human breast carcinoma patients (25, 28).

Thirdly, whereas most previously reported studies with angiostatin and endostatin made use of purified protein, the findings presented here have been obtained with a gene transfer approach. Cao et al. (29), using cell lines transfected with angiostatin cDNA, demonstrated that the protein can be released in vivo by engineered cells and viral-delivered angiostatin cDNA has been tested in experimental models based on transplantable cell lines (30, 31). Very recently, endostatin and/or angiostatin cDNA have been shown to be effective against transplantable tumors (32, 33), and our preliminary results showed that i.p. administration of liposome-complexed angiostatin could slow tumor growth also in MMTV-neu mice.5 Gene transfer approaches have the disadvantage that the amount of protein delivered cannot be exactly quantified. On the other hand, the production and purification of these two inhibitory proteins has not yet been fully standardized (34); in addition, the availability of purified products could represent a bottleneck in human cancer treatment. These problems could be overcome by gene transfer-based approaches such as the one described here. Liposomes are a vehicle easily prepared, have already been used in gene therapy clinical trials as well as in cosmetics, show little toxicity when administered locally or even parenterally, can be repeatedly administrated, do not raise immunological responses, do not show any danger of recombination with endogenous viral sequences and, in the long run, can be manipulated also by physicians in small peripheral hospitals (35). The latter consideration could be relevant, should the angiogenesis inhibition approach become a widely used tool for cancer therapy.

We must emphasize that we did not provide a formal proof that angiostatin delivered by liposomes acted only through an antiangiogenic effect. However, our results are compatible with such kind of action because it has been shown that similarly engineered vectors act in this way and that angiostatin has little, if any, effect on the growth rate of tumor cell lines (29, 33). Our results are in agreement with those obtained recently with parenterally delivered angiostatin in a SV40 T-antigen-mediated transgenic model of islet cell carcinoma, in which angiostatin was effective mainly when used in the early stages of tumor development (16). In our study, in which angiostatin was delivered before the appearance of clinically detectable lumps, a delay of at least 2 months in tumor growth and metastasis appearance has been obtained, which represents more than one-third of the life of untreated MMTV-neu mice. In addition, our experiments on mice in which injections were started at 2.5 months of age showed a reduced therapeutic effect. Taken together, these results suggest that angiostatin administration could be useful as an adjuvant treatment after surgery, whereas in large established tumors, protocols based on synthetic or natural inhibitory products combined to conventional treatments are needed (8, 36). In this regard, our model could be a useful tool to test all of these combinations.

ACKNOWLEDGMENTS

The technical assistance of Remo Di Bacco, Dario Strina, and Lucia Susani is acknowledged. We thank Prof. R. Dulbecco for encouragement and Victoria Stames for typing the manuscript.

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