Continuous Administration of Angiostatin Inhibits Accelerated Growth of Colorectal Liver Metastases after Partial Hepatectomy


Departments of Surgery [T. A. D., I. H. M. B. R., E. D. R., T. J. M. V. v. V.] and Internal Medicine [T. A. D., M. F. B. G. G., E. E. V.], Laboratory of Medical Oncology, University Medical Center Utrecht, 3584 CX Utrecht, the Netherlands

ABSTRACT

Human plasminogen-derived angiostatin is one of the most potent antiangiogenic agents currently known. However, it is unclear whether angiostatin is also effective against accelerated tumor growth induced by local up-regulation of growth factors, including angiogenesis stimulators, such as in regenerating liver. Prior to addressing this question, we tested, in mice, whether continuous administration of angiostatin could improve its biological effects. This assumption was based on the relatively short half-life of angiostatin in mice, as well as on the theoretical necessity to suppress tumor-induced angiogenesis continually. The findings presented here clearly indicate continuous administration to be superior to the conventional twice-daily bolus injections. Using the maximally effective regimen of 100 mg/kg/day via s.c. pump infusion, we found angiostatin to not only suppress s.c. primary tumors but also to significantly inhibit the outgrowth of colorectal hepatic metastases in resting liver and even to inhibit accelerated tumor growth in regenerating liver after 70% partial hepatectomy. In conclusion, angiostatin could play an important role in patients subjected to partial hepatectomy to prevent outgrowth of residual micrometastases, provided it is administered continuously to obtain maximal biological effects.

INTRODUCTION

Antiangiogenic treatment has become one of the most exciting new developments in anticancer therapy. Among the most potent antiangiogenic substances is angiostatin, a proteolytic cleavage product of plasminogen (1). Angiostatin was discovered in mice bearing Lewis lung carcinoma as an endogenous peptide, generated by the primary tumor, and capable of suppressing outgrowth of metastatic tumor cells beyond microscopic dimensions. In contrast, endogenously formed angiostatin was unable to inhibit propagation of the primary malignancy. This phenomenon was attributed to local production of proangiogenic factors by the tumor, which outweighed the antiangiogenic regulators such as angiostatin, shifting the balance between the two forces toward angiogenesis (i.e., the angiogenic switch; Ref. 2). In contrast, exogenous human plasminogen-derived angiostatin has been shown to effectively suppress growth of s.c. primary tumor lesions in mice, regardless of tumor cell type (3).

In analogy herewith, liver regeneration after extensive hepatic resection is accompanied by a similar peak in local growth factor production, including proangiogenic factors, eliciting proliferative responses along a variety of auto- and paracrine pathways (4–9). As a consequence, it has been suggested that residual “dormant” micrometastases in the liver remnant might also display stimulated growth after major hepatic resection. Recent experimental data appear to support this concept (10–12). The role of angiogenesis under these circumstances is unknown. One could speculate that antiangiogenic treatment to prevent outgrowth of liver metastases might be problematic because of the overwhelming number of secondary tumor cell deposits. These deposits each produce their own proangiogenic microenvironment in a host organ where angiogenesis dependency has been demonstrated to differ from that in s.c. deposits (13). Moreover, the local secretion of large amounts of growth factors after partial hepatectomy may present additional loss of effectiveness of angiogenesis inhibitors. On the other hand, there is sufficient experimental evidence that antiangiogenic treatment using exogenously administered, plasminogen-derived human angiostatin effectively inhibits outgrowth of pulmonary metastases (3). In addition, angiostatin causes regression of existing macroscopic solid tumors, supporting our hypothesis that angiostatin does inhibit accelerated metastatic outgrowth in regenerating liver (3, 14, 15).

To test this hypothesis, optimal administration of angiostatin is a prerequisite. Hitherto, most animal studies on angiostatin have used a twice-daily s.c. treatment schedule. The half-life of human angiostatin in mice is around 4–6 h (1). This implies that twice-daily injections do not allow for continuous suppression of angiogenesis. Accordingly, we have previously demonstrated that twice-daily bolus injections with IFN-γ at a sublethal concentration only partially suppressed angiogenesis, whereas continuous infusion of IFN-γ at half the daily dose of the bolus injections completely inhibited angiogenesis (16). The idea that antitumoral effects could be improved by continuous administration of an antiangiogenic peptide (TNP-470), instead of intermittent administration, was also supported by Yamaoko et al. (17).

The present study was undertaken to define the optimal route of administration of angiostatin and to evaluate the antitumoral efficacy of such treatment in experimental colorectal hepatic metastases in resting liver, as well as in liver regeneration after major partial hepatectomy. We show that continuous administration of angiostatin significantly improves its antiangiogenic and antitumoral effects when compared with twice-daily s.c. bolus injections. Furthermore, continuous administration of angiostatin was able to suppress accelerated growth of colorectal liver metastases in a regenerating liver.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The mouse colon adenocarcinoma cell line C-26 was maintained as a monolayer culture in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 10% CO₂ environment. Early passage cells (<15 times) were used. Confluent cultures were harvested by brief trypsinization (0.05 trypsin in 0.02% EDTA), washed three times with PBS, and resuspended to a final concentration of 10⁶ cells/ml (s.c. tumor) and 10⁷ cells/ml (liver metastases) in PBS. The presence of single-cell suspension was confirmed by phase contrast microscopy, and cell viability was determined by trypan blue staining.
ANGIOSTATIN INHIBITS ACCELERATED TUMOR GROWTH

Antiangiogenic Effects of Continuous Administration

Angiostatin Production

Human angiostatin was generated as described by O’Reilly et al. (1) using minor modifications. Briefly, recovered outdated human plasma was diluted 2:1 with PBS, supplemented with 3 mM EDTA, warmed up (37°C), and filtered (0.1 μm). The plasma was then applied to a lysine-Sepharose column (Phar- macia, Uppsala, Sweden) at room temperature. After washing the column with 0.5 M phosphate buffer, plasminogen was eluted with 0.2 M prewarmed (37°C) e-amino-n-caproic acid at pH 7.4. SDS-PAGE of the eluate revealed one band of apparent Mr 92,000 corresponding to plasminogen. The eluate was dialyzed against demi-water (MWCO: 6–8000 Spectra/Por; >4 × 10⁴ dilution; 4°C), followed by proteolytic digestion (12 h at 37°C; 120 rpm) with porcine pancreatic elastase (Calbiochem, San Diego, CA) in a concentration of 0.8 unit/mg plasminogen using a shaker (24 h at 37°C; 120 rpm). Next, the solution was applied to a lysine-Sepharose column that had been equilibrated with a salt solution pH 7.4 (0.5 M NaCl, 0.2 M e-ACA, 0.03 M NaH₂PO₄, 0.02 NaN₃, and 0.1% Triton X-100). Furthermore, the column was re-equilibrated with a 30 mM phosphate buffer at pH 7.4. Finally, angiostatin was eluted with 0.2 mM e-ACA and dialyzed against demi-water (MWCO: 6–8000 Spectra/Por; >4 × 10⁴ dilution). SDS-PAGE revealed three distinct bands of approximately Mr 40,000, Mr 42,000, and Mr 45,000, resembling the triplet first described by O’Reilly et al. (1). After freeze-drying, angiostatin was dissolved in PBS (175 mg/ml) and stored at −80°C.

Animals

Male Balb/c mice, 8–10 weeks of age, were used in all experiments and were purchased from the General Animal Laboratory, University Medical Center Utrecht. Animals were maintained under specific pathogen-free conditions, food and water ad libitum, and kept on a 12-h light/12-h dark cycle. Experiments were performed according to the guidelines of the Utrecht Animal Experimental Committee, University Medical Center Utrecht.

Corneal Neovascularization Assay

Antiangiogenic effects and dose dependency of angiostatin were tested in cornea neovascularization assay as described previously, using minor modifications (18). Briefly, mice (n = 8 eyes/group) were anesthetized using a mixture of Hypnorm (0.3 mg/mouse i.p.; Janssen-Cilag, Brussels, Belgium) and Dormicur (12.5 mg/mouse i.p.; Roche, Brussels, Belgium). In addition, both corneas were anesthetized locally with 4 mg/ml oxybuprocaine (0.4%). Corneal micropockets were created with surgical blade number 10 and a pair of microsurgical tweezers, followed by implantation usually a −100 ng basic fibroblast growth factor (Life Technologies, Inc., Rockville, MD) containing sucrose aluminum sulphate coated pellet placed with Hydron (IFN Sciences, New Brunswick, NJ). To prevent corneal infection, Aknemycin (Merck-Belgolabo NV, Overijse, Belgium) was applied to the cornea. The corneas of mice were examined daily by microscope. The surface area of newly formed blood vessels was determined using a formula (0.2 × π × maximal vessel length × clock hours) described by Kenyon et al. (19). The experiment was ended when in untreated animals the newly formed blood vessels had reached the pellet (6 days after implantation of pellets).

Tumor Models

s.c. Tumor. Mice (n = 7/group) received a s.c. injection of 10⁷ C-26 cells in 100 μl of PBS. Tumor diameters were determined daily by caliper, and tumor volume was calculated by the formula: width² × length × 0.52. The experiment was ended after 14 days when the tumors of control mice reached a volume that started to affect the quality of life.

Liver Metastases. Liver metastases were induced as follows. After anesthesia (n = 7/group), a transverse incision in the left flank was performed, exposing the spleen, followed by intrasplenic injection of 10⁷ C-26 tumor cells in 100 μl of PBS using a 27-gauge needle (20). A visible “paling” of the spleen and the lack of bleeding were the criteria for a successful inoculation. Five min later, the spleen was removed to prevent growth of tumor cells in the spleen. In this model, metastases are confined to the liver (data not shown). Seven and 14 days after tumor inoculation, mice were sacrificed, and wet liver weight was measured. Then, left lateral and median liver lobes were frozen in liquid nitrogen and stored at −80°C until determination of the hepatic replacement area.

Accelerated Intrahepatic Tumor Growth.

Five min after intrasplenic injection of 10⁷ C-26 tumor cells, the spleen was resected; 5 min later, the left lateral and caudal liver lobes were ligated and resected, resulting in a partial hepatectomy of 70%. Tumor growth was evaluated on days 7 and 14. Mice were randomized into four groups: tumor growth in resting liver (n = 12); tumor growth in resting liver + angiostatin treatment (n = 11); tumor growth in regenerating liver (n = 6); and tumor growth in regenerating liver + angiostatin treatment (n = 6).

Intrahepatic tumor angiogenesis was evaluated by sectioning 10 standard- ized cryosections (5 μm) of each liver lobe and reacting with monoclonal antibodies (MEC 13.3) against CD31 and counterstained with hematoxylin (21). Furthermore, in each section the intrahepatic metastases were encircled using a digitalized pen that was connected to a light microscope unit (Axiostkop; Zeiss, Oberkochen, Germany) and video camera (Sony CCD/RGB color video camera: Hamburg, Germany). In this way, microscopic images were transferred into a computer frame store. Calculation of the hepatic replacement area, i.e., the ratio of tumor volume/total liver volume (%), was calculated using software of Videoplan 2.2 (Kontron Elektronik GmbH, Eching, Germany).

Treatment Schedules

All animals, except hepeatectomized mice, were treated with angiostatin twice-daily (bolus group) or continuously (pump group). Bolus injections were given as s.c. injections (twice-daily) with 100 μl of angiostatin (12.5 mg/ml PBS) or 100 μl of PBS (control). Continuous administration was performed by loading a mini-osmotic Alzet pump [Alza, Palo Alto, CA; type 2001 (7 days) or type 2002 (14 days)] with 200 μl angiostatin (type 2001, 87.5 mg/ml PBS; type 2002, 175 mg/ml PBS). The pump was implanted s.c. in the dorsal skin fold. In mice bearing s.c. tumors, absence of contact between tumor deposit and pump was assured by implanting the pump in the contralateral side. Animals received s.c. an initial bolus injection of 2.5 mg angiostatin in 100 μl PBS. In view of the results on continuous administration, subsequent experiments on accelerated tumor growth in regenerating liver were performed using continuous administration only.

Antiangiogenic effects and dose response were tested in the cornea neovascularization assay for 6 days using three daily doses (1, 10, and 100 mg/kg; n = 8/group). The Alzet 2001 pump was used. In all tumor models, a cumulative daily dose of 100 mg/kg was used. Furthermore, in all of the pump groups in the tumor models, the Alzet 2002 type was used.

Statistical Analysis

Data were expressed as mean ± SE unless mentioned otherwise in the text. The significance of differences in corneal angiogenesis response, s.c. tumor growth (volume), and liver metastases (wet liver weight and hepatic replacement area) among groups was determined by the unpaired Students t test. P < 0.05 was considered to be statistically significant.

RESULTS

Dose-dependent Inhibition Affects Treatment Schedule

After 6 days, new blood vessels from the limbal plexus had reached the pellet in all control mice. Both treatment groups consistently showed positive angiogenic responses, but these were only a fraction of the response of control mice (Fig. 1). In the bolus group, inhibition was dose dependent: twice-daily injection with 1, 10, and 100 mg/kg/day resulted in 14.02, 33.38, and 72.10% inhibition, respectively. Furthermore, continuous administration of angiostatin resulted in a dose-dependent inhibition, which was significantly greater than bolus injection at all dose levels: 22.71, 67.07, and 92.68% inhibition. No corneal edema was seen. Histological examination revealed no signs of inflammation.

Antitumoral Effects Improved by Continuous Administration of Angiostatin

Of the three daily doses tested in the cornea neovascularization assay, 100 mg/kg revealed maximal angiogenesis inhibition. There-
ANGIOSTATIN INHIBITS ACCELERATED TUMOR GROWTH

Accelerated Tumor Growth in Regenerating Liver

with hepatic replacement areas of 12
cmaximal inhibition of tumor growth was observed in the pump group,

On POD7, the hepatic replacement area had more than doubled: 53

Fig. 1. Evaluation of antiangiogenic effects of human angiostatin in the cornea neovascularization assay. Measurements were taken at 6 days after implantation. Each group (column) represents eight mice. ■ control group. All dose levels differ significantly from the control group. At a dose level of 1 mg/kg/day, the difference between the bolus and pump group was not significant (P = 0.1778). At the dose levels of 10 and 100 mg/kg/day, the difference between the bolus and pump groups was significant (P < 0.001 and P = 0.001, respectively). Bars, SE.

tumor volume was 919 ± 94 mm³. Near total suppression of tumor growth was observed in mice that received angiostatin continuously (tumor volume = 104 ± 16 mm³).

Liver Metastases. Mean wet liver weight (g) of normal mice was 1.35 ± 0.07 g. Seven and 14 days after tumor inoculation in control mice, wet liver weight had increased to 2.29 ± 0.22 g and 3.78 ± 0.16 g, respectively. The wet liver weight in the bolus group was 2.16 ± 0.03 g (POD;3 not significant versus control) and 2.98 ± 0.06 g (POD;14; P < 0.05 versus control). Tumor growth was inhibited significantly in the pump group, as judged by wet liver weight of 1.77 ± 0.12 g on POD7 (P < 0.05 versus control) and 2.24 ± 0.13 g on POD14 (P < 0.01 versus control). The difference between both treatment groups was significant at both time points. Similar results were obtained using the hepatic replacement area (%) as parameter for intrahepatic tumor growth (Fig. 3). In control animals, the respective hepatic replacement areas were 59.3 ± 7.5% and 97 ± 2% on POD7 and POD14. In the bolus group, they were 43.3 ± 8.1% (POD7) and 81 ± 10.1% (POD14), respectively, whereas maximal inhibition of tumor growth was observed in the pump group, with hepatic replacement areas of 12 ± 4.6% on POD7 and 43 ± 5.3% on POD14.

Accelerated Tumor Growth in Regenerating Liver
and Angiostatin

All mice recovered well from liver resection. When compared with intrahepatic tumor growth in nonresected, nonregenerating liver, acceleration of growth of colorectal hepatic metastases was observed in the remnant liver after 70% partial hepatectomy (Fig. 4). On POD7, the hepatic replacement area of control mice carrying colorectal liver metastases was 24.6 ± 4.1%, whereas in the hepatectomized group, the hepatic replacement area had more than doubled: 53 ± 3.2% (Fig.

5). This discrepancy between nonhepatectomized and hepatectomized groups had disappeared on POD14 (64 ± 7.1% and 68 ± 3.7%, respectively). Accelerated tumor growth in regenerating liver was inhibited significantly by angiostatin. On POD7, the hepatic replacement area of nontreated hepatectomized mice and angiostatin-treated hepatectomized mice was 53 ± 3 and 20.8 ± 1.4%, respectively. Seven days later, on POD14, the hepatic replacement area in angiostatin-treated mice was 34 ± 1%, i.e., approximately half that in non-treated control mice (68 ± 3.7%).

DISCUSSION

In the present study, we demonstrated that continuous administration significantly improves both inhibition of angiogenesis and tumor growth as compared with bolus injections of angiostatin. This was shown not only for primary s.c. tumors but also for metastatic outgrowth using a highly aggressive cell line, which in normal circumstances leads to murine death within 15 ± 2 days after inoculation. Furthermore, using the same route of administration and a daily dose

Fig. 3. Influence of method of administration on the antitumoral effects of human angiostatin in mice bearing s.c. tumors. ●, PBS control (n = 6); ■, pump (n = 7; angiostatin, 100 mg/kg/day); ■, twice-daily (n = 7; angiostatin 100 mg/kg/day). Significantly different from control value: *, P < 0.001. Bars, SE.

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of 100 mg/kg body weight, accelerated outgrowth of colorectal hepatic metastases after 70% partial hepatectomy was inhibited as well.

As reported by others, we confirmed that performing a partial hepatectomy leads to accelerated intrahepatic tumor growth (22, 23). Because angiostatin strongly inhibited liver metastases in resting liver, we expected that angiostatin could suppress the accelerated outgrowth of liver metastases in regenerating liver as well. However, we did not expect that antitumoral effects of angiostatin would be as strong as the inhibition of liver metastases in a resting liver. Surely, in a regenerating liver, both remnant liver and colorectal liver metastases contribute to local expression of growth factors, whereas in a resting liver, only the tumor contributes to up-regulation of local growth factors. This is supported by others; vascular endothelial growth factor, one of the major proangiogenic factors, is up-regulated in colorectal hepatic metastases in resting (24, 25) and in remnant hepatocytes in regenerating liver (26). On POD7, no significant differences could be observed between inhibition of tumor growth of colorectal metastases in resting (58% inhibition) and regenerating liver (60% inhibition). But on POD14, inhibition of tumor growth of metastases was significantly stronger in the resting liver (63% inhibition) compared with the regenerating liver (49% inhibition; \( P < 0.05 \)). This might be explained by the concept that compared with tumor growth in a resting liver, in a regenerating liver the local imbalance of growth factors may be shifted more to the side of the proangiogenic factors. One way to overcome this problem could be to further increase the total daily dose of angiostatin. Furthermore, the effect of angiostatin, or any antiangiogenic treatment, on liver regeneration after partial hepatectomy deserves attention. In preliminary experiments, we have found that angiostatin, in addition to an antitumor effect, reduces the rate of physiological liver regeneration.\(^4\)

It is important to note that antitumor activity of angiostatin was measured differently in the s.c. and liver metastases model; liver metastases were measured by surface area (mm\(^2\)), whereas sc. tumor growth was evaluated using tumor volume (mm\(^3\)). It is a mathematical fact that any difference in tumor growth between control and test groups will be more strongly exhibited using volume then area. Therefore, it appears that angiostatin is less effective in the liver metastases in comparison with s.c. tumors. The sc. injection of tumor cells in the s.c. dorsal skinfold results in a localized tumor deposit containing 10\(^6\) tumor cells, whereas in the liver model, an intrasplenic injection with 10\(^5\) tumor cells results in a large number of intrahepatic tumor deposits. The liver metastases may all grow to 1–2 mm\(^2\) without neovascularization. This results in a substantial tumor growth not affected by antiangiogenesis treatment. However, all described models demonstrate that continuous administration compared with twice-daily injections is crucial for inhibiting angiogenesis and tumor growth.

The role of antiangiogenic treatment for colorectal hepatic micrometastases after partial hepatectomy is unclear. In agreement to our results, Tanaka et al. (27) reported previously that the angiogenesis inhibitor TNP-470 inhibited growth of colorectal liver metastases in rabbits. Our findings are relevant to the future use of antiangiogenic agents in cancer patients. In this study, we focused on colorectal hepatic metastases. The optimal clinical use of angiogenesis inhibitors may be in the adjuvant setting (28). Patients at risk for recurrence, either after complete surgical resection of the primary tumor/hepatic metastases or after successful chemotherapy or radiation therapy, could be candidates for antiangiogenic treatment in an attempt to impose dormancy on residual clinically undetectable micrometastases. Continuous suppression appears to be crucial in preventing the outgrowth of tumors. This may be done by continuous administration of

these agents by ambulatory infusion pumps, by modulating angiogenic drugs to increase their plasma half-lives, or by new approaches such as gene therapy.

In conclusion, plasminogen-derived human angiostatin is a very potent antiangiogenic and antitumoral agent. Its biological effects can be improved remarkably by continuous administration instead of only twice-daily. Furthermore, angiostatin might play an important role as an adjuvant antitumoral agent in patients subjected to partial hepatectomy for colorectal hepatic metastases.

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