The Designed Angiostatic Peptide Anginex Synergistically Improves Chemotherapy and Antiangiogenesis Therapy with Angiostatin

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ABSTRACT

Recently, we demonstrated that the designed peptide anginex displays potent antiangiogenic activity. The aim of the present study was to investigate anginex treatment as a single-agent therapy and to test its ability to improve conventional chemotherapy and antiangiogenesis therapy. In a human ovarian carcinoma mouse model, anginex inhibited tumor growth by 70%. When anginex was combined with a suboptimal dose of carboplatin, tumors regressed to an impalpable state. Anginex plus angiostatin worked synergistically to inhibit tumor growth. Assessment of microvessel density suggested that the antitumor activity of anginex is mediated by angiogenesis inhibition. In any of the experiments, no sign of anginex-induced toxicity was observed.

INTRODUCTION

Neovascularization, or angiogenesis, is the process of new capillary outgrowth from preexisting blood vessels. Sustained angiogenesis is one of the essential alterations in cell physiology that collectively dictate malignant growth (1). Angiogenesis is required for solid tumors to grow beyond the size of approximately 1–2 mm³. A highly vascularized tumor is associated with poor clinical prognosis, not only because of the potential for exponential tumor growth but also because of the increased access capacity to the capillaries (2), which supposedly facilitates metastasis formation (3). Consequently, methods to inhibit angiogenic sprouting provide a unique opportunity to arrest tumor growth and prevent metastasis, either alone or in combination with conventional therapies. Combination of angiogenic inhibitors with radiation (4, 5), gene therapy (6), or chemotherapy (7) has been shown to be successful. Recently, we reported the design of anginex, a β-sheet-forming peptide 33-mer, with potent in vitro antiangiogenesis activity (8). The aims of the present study were to investigate anginex treatment in vivo as a single-agent therapy and in combination with conventional chemotherapy and the structurally unrelated antiangiogenic angiotatin. Here, we show that although anginex alone significantly inhibits tumor growth, treatment in combination with a suboptimal dose of carboplatin results in tumor regressions to microscopic disease, and treatment in combination with angiostatin demonstrates a synergistic effect at inhibiting tumor growth. The present data warrant further development of anginex for clinical use.

MATERIALS AND METHODS

Reagents. Anginex and control peptide βpep28 were synthesized as described previously (8). βpep28 is 91% homologous and 67% identical peptide compared with anginex but has no antiangiogenic activity (8). Carboplatin (Sigma Diagnostics, St. Louis, MO) was dissolved in PBS (32.5 mg/kg) and administered i.p. once every 3 days. Angiostatin (20 mg/kg) was administered daily s.c. in the neck, as described previously (9).

Culture. MA148, a human epithelial ovarian carcinoma cell line, was cultured on noncoated flasks using 10% fetal bovine serum, 1% penicillin/streptomycin in RPMI 1640. Cultures were split 1:3 every 3 days. Mouse angiostatin (kringle 1–4) was cloned and expressed in Pichia pastoris (10), with culturing, elution, and purification done as described previously (9, 11).

Ovarian Carcinoma Mouse Model. Female athymic nude mice (nu/nu, 5–6 weeks old) were purchased from the National Cancer Institute and allowed to acclimatize for 1 week. Human ovarian MA148 epithelial carcinoma cells were cultured, harvested, and inoculated s.c. into the right flank of the mouse as described previously (9). In the initial experiment, treatment was initiated after randomizing mice and implanting osmotic minipumps (Durect, Cupertino, CA) into the left flank. The pumps had a treatment span of 28 days, which started on the same day as the inoculation of the ovarian carcinoma cells. Subsequently, studies were carried out in a therapeutic intervention model with established tumors to test the capacity of anginex to inhibit tumor growth and to test it in conjunction with angiostatin. In this latter model, treatment was initiated 7 days postinoculation with the MA148 cells. To test the ability of anginex to enhance conventional chemotherapy, carboplatin was used in combination with anginex in the same intervention model.

Tumor volume was determined by measuring the size of the tumors on the flanks of the mice. The diameters of tumors were measured using calipers (Scienceware, Pequannock, NJ), and the volume was calculated using the equation to determine the volume of a sphere: \((a^2 \times b \times \pi)/6\), where \(a\) is the width of the tumor, and \(b\) is the length of the tumor.

Immunohistochemistry. Tumor tissues were embedded in tissue freezing medium (Miles, Inc., Elkart, IN) and snap frozen in liquid nitrogen. Preparation and procedures were done as described previously (12). Samples were subsequently incubated in a 1:50 dilution with phycoerythrin-conjugated monoclonal antibody to mouse CD-31 (platelet/endothelial cell adhesion molecule 1; Pharmingen, San Diego, CA) or a FITC-conjugated PCNA (Ab-1; Oncogene, San Diego, CA) to stain for MVD or proliferation, respectively. After a 1-h incubation at room temperature, slides were washed with PBS and immediately imaged in an Olympus BX-60 fluorescence microscope at ×200 magnification. Sections were also stained for cell death using a TUNEL assay carried out according to the manufacturer’s instructions (in situ cell death detection kit, fluorescein; TUNEL; Roche). Although the TUNEL assay detects apoptosis, it cannot be ruled out that TUNEL will also stain for necrosis, where extensive DNA fragmentation may occur. Digital images were acquired and processed using Adobe Photoshop (Adobe Inc., Mountain View, CA). Vessel density was quantified as described previously (12). Statistical analysis was performed using Student’s t test.

Toxicity Assays. As an indirect measurement of general toxicity, body weights of mice were monitored twice weekly using a digital balance (Ohaus Florham). To determine hematocrit and creatinine levels, blood samples were extracted by tail vein bleedings 1 day after terminating treatment, and blood was collected in heparinized microhematocrit capillary tubes (Fisher, Pittsburgh, PA). For hematocrit levels, samples were spun down for 10 min in a microhematocrit centrifuge (Clay-Adams), and the amount of hematocrit was determined using an international microcapillary reader (IEC, Needham, MA). To obtain creatinine levels, a kit was purchased from Sigma Diagnostics and used according to the manufacturer’s instructions.

3 The abbreviations used are: PCNA, proliferating cell nuclear antigen; MVD, microvessel density; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
RESULTS

Anginex Inhibits Tumor Growth in Vivo. Mice inoculated with MA148 ovarian carcinoma cells were randomized and treated systemically with anginex for 28 days using osmotic minipumps starting the day of inoculation. A control peptide, βpep28, which is 91% sequentially homologous and 67% identical to anginex, was used to control for peptide content. Another set of animals was treated with vehicle containing BSA to control for protein content. As illustrated in Fig. 1, treatment with anginex resulted in a dose-dependent inhibition of tumor growth that was maximal at 10 mg/kg/day as compared with vehicle-treated animals. At this dose, anginex inhibited about 70% of tumor growth. At half this dose, tumor growth was inhibited by only 50%, whereas a higher dose (20 mg/kg/day) did not result in enhanced efficacy (Fig. 1). Tumors from βpep28-treated mice did not differ in size from tumors in the BSA-vehicle-treated animals. Moreover, treatment with BSA or βpep28 did not result in altered tumor growth as compared with treatment with saline alone (data not shown).

Anginex Inhibits Tumor Growth of Established Tumors and Improves Conventional Chemotherapy. Because initial animal experiments were performed using an experimental setup in which treatment was started at the time of tumor inoculation (a system that models treatment of minimal residual disease), anginex was also tested using the MA148 model in an intervention setup where treatment was initiated after tumor establishment. In these experiments, anginex inhibited tumor growth by approximately 50% (Fig. 2B).

In an attempt to improve the efficacy of platinum-based chemotherapy, anginex was administered to tumor-bearing mice that were concurrently treated with a suboptimal dose of carboplatin. Carboplatin treatment resulted in an effective reduction of tumor growth; however, when it was combined with anginex, no tumor mass could be palpated in these mice (Fig. 2A). One week after termination of treatment the tumor reestablished.

Anginex and Angiostatin Act Synergistically to Inhibit Tumor Growth. Using optimized treatment regimes for angiostatin (9) and anginex, we found that both anginex and angiostatin, administered separately, inhibited tumor growth comparably by approximately 50% in the same ovarian tumor model. On the basis of those findings, the interaction between angiostatin and anginex was examined. Combination therapy of angiostatin with anginex resulted in enhanced tumor growth inhibition (80%; Fig. 2B), which was deemed to be synergistic (Table 1).

Toxicity. Animals treated with anginex (alone or in combination regimens) did not show any sign of toxicity as assessed by unaltered behavior, weight gain during experiments, normal hematocrit and creatinine levels, and macro- and microscopic morphology of internal organs on autopsy. Body weights of mice were monitored as an indirect measurement of general toxicity. In experiments in which carboplatin was administered, the weights of mice actually fell initially and subsequently increased on termination of exposure to carboplatin. This was taken as a sign of mild reversible toxicity. Anginex did not augment this toxicity. One day after the termination of treatment, blood was drawn, and hematocrit and creatinine levels were determined as a measure of bone marrow and kidney toxicity, respec-
Hematocrit levels reported as a percentage of RBCs (vehicle, 50.2 ± 2.9; anginex, 51.3 ± 2.5; carboplatin, 49.3 ± 2.8; and combination 47.2 ± 2.4) and creatinine levels reported in μM (vehicle, 46.8 ± 8; anginex, 48 ± 1.4; carboplatin, 55.5 ± 12.6; and combination 42 ± 5.3) showed no significant differences in the study involving carboplatin. The study combining anginex and angiostatin treatment showed similar hematocrit levels reported as a percentage of RBCs (vehicle, 49 ± 1.7; anginex, 49.2 ± 2.6; angiostatin, 47.8 ± 2.1; and combination, 48.3 ± 1.9) and creatinine levels reported in μM (vehicle, 46.8 ± 6.4; anginex, 48 ± 1.4; angiostatin, 41 ± 0.4; and combination, 39.2 ± 5.9).

**Histological Analysis of MVD, Cell Death, and Proliferation.**

Anginex treatment resulted in a decrease of tumor MVD, suggesting that the antitumor activity of anginex is the result of angiogenesis inhibition. Angiostatin demonstrated a similar result. Although anginex and angiostatin acted synergistically on tumor growth inhibition, this was not reflected in the MVD assessment (Fig. 3B).

Aside from vessel density (including number, size, and length; see Ref. 12), the digital approach discriminates branch points, end points, and vessel lengths. Some of these architectural parameters did change in combination treatment relative to single-agent treatment. For example, combination treatment revealed a synergistic reduction in the number of branch points (data not shown). Tumors from anginex-treated

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### Table 1  Combination therapy of anginex with carboplatin and angiostatin FTV relative to untreated controls

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<th>Day b</th>
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<th>Observed d</th>
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a FTV, fractional tumor volume (mean tumor volume experimental)/(mean tumor volume control).
b Day after tumor cell transplantation.
c (Mean FTV of anginex) / (mean FTV of other experimental group).
d Obtained by dividing the expected FTV by the observed FTV. A ratio of greater than 1 indicates a synergistic effect; a ratio of less than 1 indicates a less than additive effect.

determined by using Student’s t test relative to the vehicle group: *P < 0.05; **P < 0.01.

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**Fig. 3.** Immunohistochemistry analyses. After snap freezing tumor tissues, 10-μm sections were made and stained for MVD by using anti-CD-31 antibodies (A and B), cell death by TUNEL (C and D), and proliferation by PCNA (E and F), all expressed in number of white pixels. The procedure and quantification were described previously (12). A, C, and E show quantification of studies involving anginex and carboplatin. B, D, and F show quantification of studies involving anginex, angiostatin, or combination therapy. Error bars, SEs. As determined by using Student’s t test relative to the vehicle group: *P < 0.05; **P < 0.01; ***P < 0.01.

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ANGINEX, A POTENT IN VIVO TUMOR GROWTH INHIBITOR.
animals showed a convincing MVD reduction ($P < 0.01$), whereas tumors from carboplatin-treated animals showed a smaller, albeit significant, MVD reduction ($P < 0.05$; Fig. 3A). Tumors treated with the combination of anginex and carboplatin could not be stained because all tumors disappeared 3 weeks after initiation of treatment.

Although combination of anginex and angiostatin also showed a synergistic effect in the increased amount of cell death ($P < 0.03$), as determined by TUNEL analysis, angiostatin by itself did not trigger increased cell death compared with the vehicle group (Fig. 3D). As expected, carboplatin did show an increase in the amount of cells undergoing cell death ($P < 0.01$). A down-regulation in proliferation, as determined by PCNA staining, was revealed in all treated groups compared with controls and was significant for anginex- and combination-treated groups ($P < 0.01$; Fig. 3, E and F).

**DISCUSSION**

Induction of angiogenesis by malignant cells has been shown to play a pivotal role in the process of tumor proliferation and metastasis (13). Inhibition of angiogenesis is, therefore, a promising way to arrest tumor growth and prevent metastasis. Because of the need for new antitumor agents with improved potency, stability, selectivity, and ease of delivery, we used a novel approach in designing the β-sheet-forming peptide anginex (8). Anginex acts specifically on activated endothelial cells to trigger apoptosis, presumably by preventing cell adhesion and subsequent induction of anoikis (8). Here, we demonstrate that anginex is an antiangiogenic compound with antitumor activity when administered systemically as a single-agent therapeutic.

Because antiangiogenic agents can potentiate cytotoxic cancer therapies (7), anginex was tested in combination with the chemotherapeutic carboplatin. Platinum agents are the most widely used drugs in the first line of defense against ovarian cancer (14, 15). In a recent study, single-agent carboplatin proved to be just as effective as carboplatin plus paclitaxel in women requiring chemotherapy for ovarian cancer. The favorable toxicity profile of carboplatin alone suggested that this is a reasonable option as a single-agent chemotherapeutic (16). An additional advantage of carboplatin is that, in contrast to other agents such as taxanes cyclophosphamide and vincristine, it is not an antiangiogenic by itself. Because carboplatin has been shown to be a very powerful anticancer drug in the model used here, a suboptimal dose was used. Both anginex and carboplatin inhibited tumor growth, but the combination of the two blocked tumor growth completely, and palpable tumors regressed to undetectable sizes in all animals. Moreover, tumors remained undetectable until at least 1 week after termination of the treatment, after which tumors reestablished themselves, indicating the continued presence of microscopic disease, which was unresponsive to carboplatin and apparently independent of angiogenesis.

Whereas numerous investigators have focused on the antiangiogenic effects from endostatin in their tumor model studies, we chose to use angiostatin because in the same MA148 tumor mouse model, Yokoyama et al. (9) found that angiostatin was considerably more effective at inhibiting tumor growth than endostatin. Here, we found that although treatment with angiostatin or anginex showed essentially the same capacity to inhibit tumor growth, combination of the two produced a dramatically enhanced inhibitory effect. This synergy between angiostatin and anginex suggests that their mechanisms of action are different and that they can augment each other as antiangiogenic agents. These data, therefore, provide a validation for combination therapy, if not for mixtures of angiogenesis inhibitors, to improve the treatment of cancer.

Immunohistochemical assessment of MVD indicated that tumor growth inhibition by anginex is explained by its antiangiogenic activity. Angiostatin showed the same trend. Combination therapy, however, did not yield an increased MVD reduction. This may be explained by the fact that differences in architecture are not represented in the MVD value assessed either digitally or manually. The digital approach discriminates for architectural parameters, some of which did change in combination treatment relative to single-agent treatment. For example, combination treatment revealed a synergistic reduction in the number of branch points (data not shown).

This study adds credence to the proposal that cancer treatment using antiangiogenesis agents is more effective when performed in combination with other agents. However, it might be that only mixtures of antiangiogenic compounds would provide sufficient potency to be evaluated properly in early clinical trials. Currently, clinical evaluation of antiangiogenic compounds is in its infancy, and although a stand-alone approach using an antiangiogenic agent shows promise, combination therapy may provide for the best evaluation of these agents and, in the end, may be more beneficial. The present results suggest that combination of antiangiogenic agents with chemotherapeutic agents will produce a greater effect than combinations of antiangiogenic agents alone. At the very least, this study indicates that combining the antiangiogenic compound anginex with conventional chemotherapy allows the dosage of the chemotherapeutic to be reduced while maintaining the ability to effect tumor growth reduction.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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