Synergistic Activity of Recombinant Human Endostatin in Combination with Adriamycin: Analysis of in Vitro Activity on Endothelial Cells and in Vivo Tumor Progression in an Orthotopic Murine Mammary Carcinoma Model

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ABSTRACT

Purpose: Current combination treatment strategies in malignancy are designed to evaluate the use of cytotoxic drugs and antiangiogenic agents. Endostatin, a fragment of collagen XVIII, was initially isolated from a murine hemangioblastoma cell line. Soluble rhEndostatin produced in a Pichia pastoris expression system inhibits endothelial cell function and cord formation. Additional studies of this in vitro combination included a determination of Adriamycin-induced cardiotoxicity and in vitro effects on human umbilical vein endothelial cell proliferation and cord formation.

Results: For single-agent activity, optimal tumor growth inhibition was observed after s.c. administration of 50 mg/kg/day rhEndostatin or 5 mg/kg Adriamycin injected i.v. every 4 days. Combination of Adriamycin with optimal or suboptimal doses of rhEndostatin resulted in synergistic inhibition of DA-3 tumor growth. Importantly, unlike other antiangiogenic agents, rhEndostatin did not exacerbate the cardiotoxicity of Adriamycin. The synergistic interaction between rhEndostatin and Adriamycin was also observed in vitro for inhibition of human umbilical vein endothelial cell proliferation and inhibition of cord formation.

Conclusions: These data suggest that the synergy observed with rhEndostatin in combination with Adriamycin is exerted at the level of the endothelial cell and can result in enhanced tumor growth inhibition. The potential benefit of Adriamycin used in combination with rhEndostatin is being considered for clinical evaluation.

INTRODUCTION

With the recognition that the progressive growth of primary neoplasms and metastatic lesions is dependent on angiogenesis, considerable research effort has focused on manipulation of the tumor vascular compartment as a potential therapeutic target. This, in turn, led to the identification and subsequent development of a diverse array of agents that target and exert an antiangiogenic, antivascular, or angiostatic effect on the neovascular processes that accompany tumor progression. Such agents exert significant inhibitory effects on endothelial cells with a downstream effect on tumor growth in a number of experimental models. One such molecule, endostatin, a M_r 20,000 proteolytic fragment of the COOH terminus of collagen XVIII, was initially isolated from a murine hemangioblastoma cell line (1). Soluble rhEndostatin produced in a Pichia pastoris expression system inhibits endothelial cell function in vitro and exerts an antiangiogenic and antitumor effect in multiple in vivo systems without toxicity or drug resistance (2–5). Furthermore, recently completed Phase I clinical trials with rhEndostatin demonstrated treatment to be safe and well tolerated in advanced cancer patients and demonstrated some antitumor activity. Of the 74 patients participating in Phase I studies, 2 patients had minor responses (40% and 17% reduction in tumor volume for 22 and 11 months, respectively), and two patients had mixed responses for 14 or 5 months. Additionally, 8 patients were noted to have stable disease for 14 or 5 months. These results did not correlate with endostatin dose, and in all cases endostatin was administered by i.v. bolus infusion (6–8). In an additional study, treatment with endostatin dose-dependently decreased blood flow in tumors as measured by positron emission tomography scan. Furthermore, endostatin induced endothelial cell and tumor cell apoptosis in tumor biopsies (9).

The anticipated benefits of developing therapeutic strategies capable of targeting both the tumor and its associated neovascular compartment have received intense interest. Adria-
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mycin is a standard chemotherapeutic agent that is used in the treatment programs of multiple cancer types including breast and ovarian cancer, Hodgkin’s and non-Hodgkin’s lymphoma, and soft tissue sarcomas. However, the clinical efficacy and utility of Adriamycin at conventional treatment doses are often compromised due to inherent myelosuppression and cardiotoxicity and development of cellular resistance (10–14). Strategies to enhance the therapeutic efficacy of conventional Adriamycin treatment have included combinations with the anti-erbB2 agent, trastuzumab (15–17). The enhancement of anthracycline antitumor activity in patients treated concurrently with trastuzumab has been suggested to involve an antiangiogenesis secondary pathway, but unfortunately this combination has also been linked to an increased incidence of heart failure (18–21). These studies have suggested, however, that a combination of Adriamycin with a specific antiangiogenic agent, such as rhEndostatin, may result in greater antitumor benefit in the absence of enhanced cardiotoxicity.

In the present study, we have investigated the combination of rhEndostatin and Adriamycin as a treatment strategy to target an orthotopically growing murine mammary carcinoma and its supporting vascular compartment. We demonstrate that when given together, rhEndostatin and Adriamycin act synergistically to inhibit endothelial cell function and tumor development in the absence of augmented Adriamycin-induced toxicity. This is the first account of synergy observed between rhEndostatin and Adriamycin in a clinically relevant orthotopically implanted mammary tumor model. The potential benefits of combining rhEndostatin and Adriamycin may effectively increase the therapeutic potential of each drug, and this approach warrants clinical investigation.

MATERIALS AND METHODS

Cell Lines and Growth Conditions. HUVECs (Clonetics, Walkersville, MD) were maintained in endothelial cell growth medium EGM-2 (Clonetics) supplemented with 1% l-glutamine (BioWhittaker, Walkersville, MD) and bovine brain extract in 75-cm² cell culture flasks (Corning Costar, Corning, NY) at 37°C in a humidified atmosphere of 5% CO₂/95% air.

The DA-3 murine breast adenocarcinoma was obtained from the National Cancer Institute Central Repository (Frederick, MD). Tumor cells were grown in DMEM (Biowhittaker) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 200 mM l-glutamine (BioWhittaker), 100× MEM vitamin mixture (BioWhittaker), and 100× nonessential amino acid solution (BioWhittaker) as monolayer cultures at 37°C in a humidified atmosphere of 5% CO₂/95% air. DA-3 cells were certified free of Mycoplasma and the pathogenic murine viruses reovirus type 3, pneumonia virus of mice, K virus, Thelier’s encephalitis virus, Sedai virus, minute virus of mice, mouse adeno-virus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus.

Orthotopic Syngeneic DA-3 Primary Tumor. DA-3 tumor cells in exponential growth phase were harvested by a short trypsinization [0.25% trypsin-verse (EDTA) solution for 3–5 min at 37°C]. The tumor cells were washed with PBS (BioWhittaker) and resuspended to 1.0 × 10⁶ cells/ml in PBS. The tumor cells (0.1 ml) were injected into the mammary fat pad of female Balb/ByJ 6–8-week-old mice. In conducting the research in this report, the investigators adhered to the Guide for Laboratory Animals and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council. Tumor volume was determined using the following formula: L × W² × 0.52. Tumor growth was monitored on a daily basis, and treatments were initiated when tumors reached approximately 100 mm³. For the dose-response experiments, mice (n = 5 mice/group) were treated with the following therapeutic agent at the indicated doses: (a) rhEndostatin (100 mg/kg s.c., bis in die; 50, 5, or 0.5 mg/kg, s.c., daily dosing); (b) Adriamycin (doxorubicin HCl; Sigma Aldrich, St. Louis, MO; 5, 2.5, or 1 mg/kg, i.v., every 4 days for 3 weeks); (c) CP buffer [rhEndostatin vehicle control; 132 mM sodium phosphate, 34 mM citrate (pH 6.2), 100 μl, s.c., daily]; and (d) 0.9% NaCl (Adriamycin vehicle control; 100 μl, i.v., every 4 days for 3 weeks). For the combination studies the following therapeutic regimens were used: mice (n = 5 mice/group) were treated with rhEndostatin (50, 5, or 0.5 mg/kg, s.c. daily dosing) with or without Adriamycin (5 mg/kg, i.v., every 4 days for 3 weeks). Included in the combination studies were cohorts of animals receiving CP buffer, 0.9% NaCl, or no treatment. For all experiments, tumor measurements were taken every 7 days. Mean tumor volumes were determined. At the completion of the experiment, final tumor measurements were assessed, and mice were sacrificed. FIVs were determined by dividing mean tumor volumes in treated animals by that of controls.

Adriamycin-Induced Cardiotoxicity in Vivo. Balb/ByJ female mice (n = 5 mice/group) received i.v. injection (0.2 ml) with 5 mg/kg Adriamycin and were treated daily (0.1 ml, s.c.) with 50 mg/kg rhEndostatin or CP buffer (0.1 ml, s.c.) for 5 weeks. Mice were monitored on a weekly basis for weight loss. At 5 weeks, mice were euthanized, and hearts were removed, fixed in 10% buffered formalin, sectioned, and stained with H&E (Pathology Associates, Inc., Frederick, MD). Morphological evaluation of cardiac lesions was performed and scored based on severity and extent of the damage observed. Severity was defined as follows: 1, sarcomplastic microvacuolizations and/or inclusions and interstitial or cellular edema; and 2, same as 1 plus sarcomplastic macrovacuolizations or atrophy, necrosis, fibrosis, endocardial lesions, and thrombi. Extent was defined as follows: 0, no lesions; 0.5, less than 10 single altered myocytes in the whole heart section; 1, scattered single altered myocytes; 2, scattered small groups of altered myocytes; 3, widely spread small groups of altered myocytes; 4, confluent groups of altered myocytes; and 5, most cells damaged. The product of the severity and the extent of the damage observed in each mouse was used to calculate the severity score (from 0 to 10). The method of quantitation is derived from Bertazzoli et al. (10).

In Vitro Proliferation Assay: DA-3 Tumor Cells. DA-3 tumor cells grown as described above were harvested by a brief trypsinization and plated at 5000 cells/well in 96-well plates. Two h after plating, rhEndostatin (100, 10, or 1 μg/ml) or Adriamycin (100, 10, 1, 0.1, 0.01, or 0.001 μg/ml) were added to the cells and incubated for 72 h at 37°C, 5% CO₂. For combination studies, both rhEndostatin (100, 10, or 1 μg/ml) and Adriamycin (100, 10, 1, 0.1, or 0.01 μg/ml) were added...
Fig. 1  Inhibition of orthotopic DA-3 tumor development by rhEndostatin or Adriamycin administered as single agents. Murine DA-3 tumors implanted orthotopically into the mammary fat pad of female mice were grown to a volume of 100 mm³. A, mice were treated daily s.c. with rhEndostatin at 100, 50, 5, and 0.5 mg/kg. Tumor volumes were assessed every 4–7 days. Control animals were treated with CP buffer as a vehicle control. Statistical significance was determined using Student’s t test. (†, P < 0.004; ‡, P < 0.02). Error bars indicate SDs.

Fig. 2  Combination effects of rhEndostatin and Adriamycin on DA-3 tumor growth. Female Balb/cByJ mice transplanted with DA-3 cells were treated with 50, 5, and 0.5 mg/kg rhEndostatin with or without Adriamycin (5 mg/kg). Tumor volumes were measured 32 days after tumor inoculation. T/C, ratio of tumor volume of treated animals to tumor volume of vehicle control-treated animals. This experiment is representative of three experiments. Statistical differences were determined using Student’s t test (†, P < 0.05). Error bars indicate SD.

In Vitro Proliferation Assays: HUVECs. Briefly, HUVECs at passage 2–5 were washed with PBS and trypsinized with a 0.05% solution of trypsin-verse mixture (BioWhittaker). Cells were suspended in endothelial cell basal medium (EBM-2; Clonetics) supplemented with 2% heat-inactivated fetal bovine serum (HyClone) and 2 mM l-glutamine at a concentration of 2.5 × 10⁶ cells/ml and plated in 96-well (100 µl/well) culture plates (Costar, Cambridge, MA) and incubated for 24 h at 37°C in 5% CO₂. Cells were incubated with either rhEndostatin (100, 10, or 1 µg/ml), Adriamycin (100, 10, 1, 0.1, 0.01, and 0.001 µg/ml), or media alone for 20 min. For combination studies, both rhEndostatin (100, 10, or 1 µg/ml) and Adriamycin (100, 10, 1, 0.1, 0.01, and 0.001 µg/ml) were incubated with cells for 20 min. The cells were then stimulated with 10 ng/ml human recombinant FGF-2 (R&D Systems, Minneapolis, MN) or media alone for 72 h. Cell proliferation was measured by BrdUrd incorporation using an ELISA (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s instructions.

Endothelial Cell Cord Formation Assay in Vitro. Matrigel (no phenol red (BD Biosciences, Bedford, MA)] was thawed at 4°C overnight and kept on ice during use. Matrigel (65 µl) was added to each well of a 96-well tissue culture plate and incubated at 37°C for 30 min to allow the Matrigel to solidify. HUVECs at passage 2–5 were washed with PBS (BioWhittaker) and trypsinized with a 0.05% solution of trypsin-verse mixture (BioWhittaker). Cells were suspended in EBM-2 (Clonetics) supplemented with 2% heat-inactivated fetal bovine serum (HyClone) and 2 mM l-glutamine (BioWhittaker) at a concentration of 1 × 10⁵ cells/ml. Before the combination studies, we first determined dose responses for rhEndostatin (100 µg, 10 µg, and 1 µg) and Adriamycin (100 µg, 10 µg, 1 µg, 0.1 µg, and 0.01 µg) alone. Serial dilutions of rhEndostatin or Adriamycin were prepared at 2X the desired final concentration and mixed with cell suspension at a 1:1 ratio. Each sample was then added to the Matrigel-coated 96-well plate (200 µl/well) along with CP buffer and 0.9% NaCl buffer as vehicle controls for rhEndostatin and Adriamycin, respectively. All samples were plated in triplicate. Plates were incubated for 16 h at 37°C. After incubation, the plates were assessed microscopically and quantitated using a macro written for Image Pro Plus (Media Cybernetics, Silver Spring, MD). The software analyzed the number of junctions formed by the HUVECs in each well, cell surface area, and junction length. Suboptimal doses of rhEndostatin (1 µg/ml) and Adriamycin (0.1 µg/ml or 0.01 µg/ml) were combined and plated in triplicate. Each plate also included each drug alone at the same concentrations and the appropriate controls.
To characterize effects in vitro. Results were analyzed for statistical significance using the two-tailed Student's t test. Analysis of multiple drug effects in vitro and in vivo was performed using the method of Chou and Talalay based on the median effect principle in combination with the CalcuSyn program (Biosoft, Cambridge, United Kingdom; Ref. 22). Dose-response curves were generated for rhEndostatin and Adriamycin alone to determine the median effects plots. Variable ratios of drug concentrations and mutually exclusive equations were used to determine the CI (equations from Ref. 23). This type of analysis allows drugs with distinct mechanisms of action to be assessed in combination and allows for different drug interactions to be observed. A CI of <1 designates synergy (more than additive), a CI of 1 demonstrates summation (additive), and a CI of >1 indicates antagonism (less than additive).

**RESULTS**

**Effects of rhEndostatin or Adriamycin as Single Agents on an Orthotopic DA-3 Mammary Tumor.** To characterize the effects of rhEndostatin on the growth of an orthotopically implanted syngeneic DA-3 tumor, we treated tumor-bearing animals with four different doses of rhEndostatin (0.5, 5, or 50 mg/kg/day quaque die or 100 mg/kg/day, bis in die, given s.c.) or CP buffer as a vehicle control. rhEndostatin inhibited the growth of DA-3 tumor in a dose-dependent fashion (Fig. 1A). Treatment of mice with 100 and 50 mg/kg rhEndostatin inhibited tumor growth by 57% and 54%, respectively (P < 0.004). Treatment of mice with 5 and 0.5 mg/kg inhibited DA-3 tumor growth by 25% and 14%, respectively. These values were not statistically different from controls.

Adriamycin was also analyzed for antitumor activity at three different doses (5, 2.5, and 1 mg/kg × 4 quaque die) in DA-3 tumor-bearing animals. We determined the maximum tolerated dose at the dosing regimen described to be 5 mg/kg. Mice treated with 10 mg/kg were moribund within 16 days (data not shown). Both 5 and 2.5 mg/kg doses of Adriamycin inhibited DA-3 tumor growth by approximately 56% (P < 0.004), and the 1 mg/kg dose of Adriamycin delayed tumor growth by 34% (P < 0.02; Fig. 1B).

**Effect of Combined Therapy with rhEndostatin and Adriamycin on DA-3 Tumor Growth.** To assess whether rhEndostatin combined with Adriamycin would have an enhanced inhibitory effect on tumor growth, both optimal (50 mg/kg/day) and suboptimal (5 and 0.5 mg/kg/day) doses of rhEndostatin were combined with Adriamycin (5 mg/kg) to treat DA-3 tumor-bearing mice. Daily treatment of mice with rhEndostatin as a single agent inhibited tumor growth in a dose-dependent fashion: 50, 5, and 0.5 mg/kg of rhEndostatin inhibited tumor growth by 40%, 4%, and 4%, respectively (day 21 measurement). Adriamycin at 5 mg/kg every 4 days demonstrated an inhibitory effect on DA-3 tumor growth of 54%. Combination of rhEndostatin (50, 5, or 0.5 mg/kg) and Adriamycin (5 mg/kg) resulted in a greater than expected inhibition of tumor growth: 92%, 82%, and 57%, respectively (Fig. 2). To evaluate whether the inhibition was synergistic, statistical analysis was performed. Table 1 shows FTVs for each group at day 21. The method of Chou and Talalay in conjunction with CalcuSyn was used to determine that combination therapy of an optimum rhEndostatin dose (50 mg/kg) with Adriamycin (5 mg/kg) showed more than an additive antitumor effect (CI = 0.001). There was a 4-fold improvement in antitumor activity in the combination group than expected if the combination was simply additive. A CI of <1 indicates a synergistic effect. Furthermore, an ineffective antitumor dose of rhEndostatin (5 mg/kg), combined with Adriamycin, resulted in synergistic in-

**Table 1** Combination therapy with rhEndostatin and Adriamycin

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<th>Combination treatment</th>
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<td>Dose (mg/kg)</td>
<td>FTV (a)</td>
<td>Dose (mg/kg)</td>
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<tr>
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\(\text{FTV} = \frac{\text{mean FTV of rhEndostatin}}{\text{mean FTV of Adriamycin}}\)

\(\text{CI} < 1\) designates synergy (more than additive), a CI of 1 demonstrates an additive effect, and a CI of >1 indicates an antagonistic effect.

**Fig. 3** Effect of combination therapy on Adriamycin-induced cardiotoxicity. Female mice were treated i.v. with 5 mg/kg Adriamycin every 4 days for 5 weeks in the presence or absence of daily s.c. treatment with 50 mg/kg rhEndostatin. After treatment, mice were euthanized, and hearts were fixed in formalin, sectioned, and stained with H&E. Severity of cardiotoxicity was displayed as mean severity score determined as described in the text (see “Materials and Methods”). This experiment is representative of two experiments. Differences were not statistically significant as determined by Student’s t test. Error bars indicate 1 SD.
hibition of DA-3 tumor growth (CI = 0.021). Combination of rhEndostatin (0.5 mg/kg) with Adriamycin did not result in synergistic inhibition.

Effect of rhEndostatin on Adriamycin-Induced Cardiotoxicity. To determine whether increasing the therapeutic effect of Adriamycin with rhEndostatin also increased the toxicity, we assessed cardiotoxicity by histological evaluation in mice treated with the combination. Mice were treated every 4 days with 5 mg/kg Adriamycin i.v. with or without daily s.c treatment with rhEndostatin at 50 mg/kg for 5 weeks. At 5 weeks, mice were euthanized, and hearts were removed, fixed, sectioned, stained with H&E, and reviewed for morphological pathologies. All mice that received Adriamycin had sarcoplasmic microvacuolizations and degenerative cardiac lesions (defined by atrophy, myolysis, and necrosis). In addition, a thickening of the endocardium was observed. Cardiotoxicity was quantified from histological sections as mean severity score by methods described previously (10). The addition of rhEndostatin did not increase the extent or severity of Adriamycin-induced cardiotoxicity: there was no significant change in the morphological appearance of the lesions or the severity of Adriamycin-induced toxicity (Fig. 3).

Effect of rhEndostatin or Adriamycin on DA-3 Tumor Cell Proliferation in Vitro. To evaluate the nature of the synergistic effect observed in vivo, evaluation of the combination of rhEndostatin and Adriamycin in vitro was performed. We assessed the sensitivity of DA-3 tumor cells to both agents alone or in combination. As expected, rhEndostatin failed to demonstrate an inhibitory effect on tumor cells regardless of the dose administered (Fig. 4A). Adriamycin, however, inhibited DA-3 tumor cell proliferation in a dose-dependent manner, with a half maximal inhibitory concentration (IC50) of 0.04 μg/ml (Fig. 4A). Combination of 100, 10, or 1 μg/ml rhEndostatin with 100, 10, 1, 0.1, or 0.01 μg/ml Adriamycin showed no enhanced inhibitory activity above that observed with Adriamycin alone (Fig. 4B). End point analysis of cell proliferation at 72 h was performed by BrdUrd ELISA.

Effect of rhEndostatin or Adriamycin on HUVEC Proliferation in Vitro. Next we assessed the sensitivity of HUVECs to both rhEndostatin and Adriamycin as single agents or in combination. Both rhEndostatin and Adriamycin, added before FGF-2 stimulation, inhibited the proliferation of HUVECs as measured by BrdUrd incorporation at 72 h (IC50 = approximately 100 and 3 μg/ml, respectively; Fig. 5). Under the culture conditions of this proliferation assay, viability for all doses of rhEndostatin and doses of ≤10 μg/ml for Adriamycin

Fig. 4 Effects of rhEndostatin and Adriamycin on DA-3 tumor cell proliferation in vitro. A, DA-3 tumor cells were plated on 96-well tissue culture plates in the presence of Adriamycin (■), and rhEndostatin (▲) as single agents. B, DA-3 tumor cells were cultured in the presence of various concentrations of Adriamycin alone (■), or in combination with 100 μg/ml rhEndostatin (▲), 10 μg/ml rhEndostatin (□), or 1 μg/ml rhEndostatin (△). Proliferation of DA-3 tumor cells was assessed by BrdUrd ELISA at 72 h. Each point represents the mean of triplicate sample wells ± 1 SD. This experiment is representative of three experiments.

Fig. 5 Effect of combination of rhEndostatin and Adriamycin on HUVEC proliferation. HUVECs were cultured with various concentrations of rhEndostatin alone (■), Adriamycin alone (◇), or Adriamycin with 1 μg/ml rhEndostatin (▲) for 20 min before stimulation with FGF-2 (10 ng/ml). Proliferation was assessed by BrdUrd incorporation at 72 h. Each point represents the mean of triplicate sample wells ± 1 SD. Combination of 1 μg/ml rhEndostatin with 0.01, 0.1, or 1 μg/ml Adriamycin was statistically different from either agent alone (P < 0.05). Statistical significance was determined using Student’s t test. This experiment is representative of three experiments.
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was >95%. At 100 μg/ml Adriamycin, viability dropped to 80%. Combination of 1 μg/ml rhEndostatin with 100, 10, 1, 0.1, or 0.01 μg/ml Adriamycin showed an enhanced inhibitory effect upon addition of both components. Combination of three non-inhibitory doses of Adriamycin (1, 0.1, and 0.01 μg/ml) with 1 μg/ml rhEndostatin induced inhibition of HUVEC proliferation beyond the effects of either molecule alone (P < 0.05).

**Effect of rhEndostatin or Adriamycin on Endothelial Cell Function.** To evaluate the effect of combination treatment on endothelial cells, rhEndostatin and Adriamycin were tested for their ability to inhibit the development of endothelial cell cord formation on a Matrigel matrix. Incubation of rhEndostatin with HUVECs plated on Matrigel-coated 96-well plates at 50, 25, and 5 μg/ml resulted in a dose-dependent inhibition of cord formation (Fig. 6A). Interestingly, Adriamycin at 100, 10, 1, 0.1, and 0.01 μg/ml also inhibited the differentiation of endothelial cells into cord structures (Fig. 6B). Under these culture conditions, neither rhEndostatin (10 μg/ml) nor Adriamycin (10 μg/ml) exerted toxic effects on the endothelial cells as assessed by viability counts with trypan blue (>95% viability).

To determine whether an enhanced inhibitory effect could be observed after simultaneous exposure to both drugs, we added 1 μg/ml rhEndostatin with 0.01 μg/ml Adriamycin in a cord formation assay. Endostatin at this dose was ineffective in inhibiting endothelial cell cord formation, and Adriamycin at 0.01 μg/ml inhibited cord formation by 21% (Fig. 7, B and C, respectively). When combined, however, these doses of rhEndostatin and Adriamycin inhibited cord formation to a greater extent than either agent alone (80% inhibition; Fig. 7D). To determine whether the increased inhibition of endothelial cord formation by rhEndostatin and Adriamycin was synergistic or simply additive, we carried out an experiment in which we examined the combination of different concentrations of Adriamycin with the 1 μg/ml dose of rhEndostatin (Table 2). In Table 2, the “Expected Value” is the calculated fractional junction formation expected if rhEndostatin and Adriamycin exerted an additive effect. We observed 88% inhibition at 1 μg of rhEndostatin + 1 μg of Adriamycin, 71% inhibition at 1 μg of rhEndostatin + 0.1 μg of Adriamycin, and 51% inhibition at 1 μg of rhEndostatin + 0.01 μg of Adriamycin. The CI for these dose combinations was 0.056, 0.022, and 0.005 respectively, where a CI of <1 is indicative of a synergistic effect.

**DISCUSSION**

In the present study, we demonstrate that concomitant treatment of mice bearing a mammary carcinoma with rhEndostatin and a conventional therapeutic dose of Adriamycin results in a synergistic inhibition of tumor growth. Importantly, our results also documented a biological dose of rhEndostatin, one without direct antitumor effects when administered alone, that enhanced the antitumor activity of Adriamycin when used in combination. Moreover, we showed that the observed synergistic interaction was detected in vitro as assessed by inhibition of endothelial cell proliferation and differentiation, but not by tumor cell proliferation. Whereas previous studies reported that combined treatment with rhEndostatin and doxorubicin in the C26 colon carcinoma liver metastases model resulted in additive therapeutic effects (24), our results are the first to show synergistic activity of this combination in an orthotopically implanted mammary tumor model. The current studies directly support the incorporation of rhEndostatin into AdriaMycin treatment regimens as a means to improve the clinical utility of each drug.

Clinical implementation of combination strategies to enhance the therapeutic effectiveness of doxorubicin has been hindered by either nonoverlapping or overlapping toxicities. These drug interactions necessitated either a dose reduction of the cytotoxic, in the case of the former, or were associated with unmanageable toxicity, in the latter situation. These findings were also observed with combination strategies using doxorubicin and nonspecific antiangiogenic agents. Two such drugs, Herceptin and thalidomide, when combined with doxorubicin, were associated with increased risk of heart failure and deep venous thrombosis, respectively (19, 20, 25, 26). In the present studies, the combination of rhEndostatin with a conventional...
treatment regimen of Adriamycin did not exacerbate the anthracycline-induced cardiotoxicity. Whereas the rhEndostatin used in these studies could lead to the generation of neutralizing antibodies, it is unlikely that the antibodies would induce a selective effect on cardiac tissue and not on tumor stroma, where synergy is observed. The lack of augmented toxicity observed in the present studies may be attributed to the specific effects of rhEndostatin on activated endothelial cells. Indeed, data from recently completed Phase I studies of rhEndostatin showed no toxicity at the doses studied with evidence of both antiangiogenic and antitumor effects (6–9). Taken together, our data suggest that the combination of rhEndostatin and Adriamycin will result in enhanced therapeutic activity in the absence of augmented toxicity.

Table 2: Combination of rhEndostatin and Adriamycin in endothelial cell cord formation assay

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<th>rhEndostatin (µg/ml)</th>
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<th>FJF</th>
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*FJF, fractional junction formation = (mean junctions formed experimental)/(mean junctions formed control).

"A CI of <1 denotes a synergy (more than additive), a CI of 1 demonstrates an additive effect, and a CI of >1 indicates an antagonistic effect.

Targeting the tumor endothelium is an important strategy for cancer therapy. Use of cytotoxic drugs in a low-dose metronomic therapy involves giving chemotherapy at suboptimum doses at a higher frequency specifically to potentiate the antiangiogenic effect (27). Combination strategies using metronomic chemotherapy and antiangiogenic therapy enhance anti-tumor activity by targeting multiple agents toward the endothelial cell (28). Metronomic therapy, however, is not currently an accepted clinical application for cytotoxic drugs. Our approach combines a clinically relevant dose of Adriamycin with rhEndostatin to target both the tumor cell and related endothelium. This interaction between rhEndostatin and Adriamycin resulted in synergistic antitumor activity in vivo that may be exerted at the level of the tumor-associated endothelium and not the malignant cells. This conclusion is based on the following observations. First, in vitro proliferation of DA-3 mammary carcinoma cells was not affected by treatment with rhEndostatin. Secondly, Adriamycin exhibited a dose-dependent inhibition of tumor cell proliferation (IC50 = 10 ng/ml), and this activity was unaffected by coincubation with rhEndostatin. These data demonstrate that rhEndostatin neither had a direct effect on the tumor cell nor acted as a biochemical modulator of Adriamycin cytotoxic activity. However, both Adriamycin at noncytotoxic concentrations and rhEndostatin showed a dose-dependent inhibition of endothelial cell proliferation and differentiation. Moreover, the in vivo synergy between rhEndostatin...
Endostatin Synergizes with Adriamycin and Adriamycin in the DA-3 tumor model was recapitulated in these *in vitro* assays of endothelial cell function. Thus, these results suggest that the synergistic activity of rhEndostatin and Adriamycin may act on the tumor-associated endothelial cell. Further investigation into this possibility is warranted.

Taken together, these data suggest the combination of Adriamycin with rhEndostatin can increase the therapeutic effectiveness of each drug, yielding a greater antitumor benefit in the absence of augmented toxicity. Furthermore, this combination strategy resulted in synergy at the endothelial cell level. Finally, this approach uses a dose of Adriamycin in combination with rhEndostatin that may be translated into clinical use and warrants clinical investigation.

**REFERENCES**


Synergistic Activity of Recombinant Human Endostatin in Combination with Adriamycin: Analysis of in Vitro Activity on Endothelial Cells and in Vivo Tumor Progression in an Orthotopic Murine Mammary Carcinoma Model

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