We have shown previously that a polymeric form of fibronectin is strongly antimetastatic when administered systemically to tumor-bearing mice. The polymeric fibronectin, sFN, is formed in vitro by treating soluble fibronectin with a 76-aa peptide, III1-C, which is derived from the first type III repeat in fibronectin. Here we show that the III1-C peptide and sFN also reduce tumor growth in mice, and that this effect correlates with a low density of blood vessels in the tumors of the treated mice. III1-C also polymerized fibrinogen, and the fibrinogen polymer, sFBG, had antitumor and antiangiogenic effects similar to those of sFN. Mice that had been injected s.c. with three different types of human tumor cells and treated with biweekly i.p. injections of III1-C, sFN, or sFBG over a 5-week period had tumors that were 50–90% smaller than those of control mice. Blood vessel density in the tumors of the treated mice was reduced by 60–80% at the end of the experiment. Xenograft tumors from a human breast carcinoma line (MDA-MB-435) were particularly susceptible to these treatments. Metastasis into the lungs from the primary s.c. tumors also was inhibited in the mice treated with III1-C and the two polymers. The III1-C peptide is an antiangiogenic and antimetastatic agent. Because of its ability to suppress tumor growth, angiogenesis, and metastasis, we have named the III1-C peptide anastellin (from anaste/ll(o) (Greek), inhibit, force a retreat).

A fibronectin fragment inhibits tumor growth, angiogenesis, and metastasis

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Fibronectin is a prototypic extracellular matrix (ECM) protein that is deposited by various types of cells into an adhesive fibrillar meshwork of protein (1). Fibronectin, and ECM in general, control many cellular functions, including growth, migration, differentiation, and survival. The signals that control these behaviors are transmitted from the ECM to the cell by integrins, a family of transmembrane receptors (2, 3). Malignant cells often bypass the ECM-integrin signaling system; they are not bound by the spatial constraints imposed by the ECM on normal cells, and they no longer require ECM contact for survival (4).

In past studies, we have looked for ways to restore the matrix control in malignant cells and developed a polymeric form of fibronectin, sFN (5). sFN is generated by treating soluble fibronectin with a 76-aa fibronectin fragment, the III1-C peptide, anastellin, which comes from the first type III repeat of the fibronectin polypeptide (5). The first type III repeat contains a site that is important in fibronectin self-assembly into fibrils (6–8). The isolated III1 repeat and its fragments may interfere with the intramolecular binding interactions that keep fibronectin in its soluble configuration (7), and disruption of those interactions may induce the molecule to undergo assembly into fibrils (5). Alternatively, the III1 fragments could change the conformation of the fibronectin molecule in such a way that cryptic fibronectin–fibronectin binding sites that are capable of driving fibril assembly are exposed (9). The III1 fragments used to induce sFN formation remain part of the sFN polymer. The polymer is 10-fold more strongly adhesive to cells than fibronectin coated onto plastic without polymerization (5). We also have shown that sFN has profound antimetastatic effects when administered systemically to mice bearing various types of tumors (10). The sFN-inducing fragment, anastellin, was tested in parallel with sFN, but it had only a marginal effect on metastasis. The antimetastatic effects were obtained without any significant reduction in the growth rate of the primary tumors.

Because these early in vivo studies were focused on metastasis, they used animals carrying large primary tumors. The use of sFN and anastellin to treat smaller tumors at higher doses has now revealed additional antitumor activities in these compounds. We show here that anastellin and sFN curtail both the growth and metastasis of various types of xenograft tumors in mice and that the antitumor activity of these compounds is related to their ability to inhibit tumor angiogenesis.

Materials and Methods

Proteins. Anastellin (the III1-C fibronectin fragment) and III11-C (control fragment from the 11th type III repeat) were prepared as recombinant His-tagged proteins in bacteria and purified as described (5, 10). Human plasma fibronectin was from Chemicon, and human fibrinogen was from Sigma. We converted fibronectin to sFN by mixing 100 µg of fibronectin in 100 µl PBS with 300 µg of anastellin in 100 µl PBS (10). III1-C also binds to fibrinogen (7), and substitution of fibrinogen (from human plasma, fraction I; Sigma) for fibrinogen yielded polymerized fibrinogen (“superfibrinogen,” sFBG). Protein polymerization was monitored by measuring the optical density at 620 nm. The sFN and sFBG preparations were freshly made for each experiment. The protein solutions were sterilized by filtering through 0.2-µm membrane before polymerization.

Tumor Cell Culture and Harvesting. The C8161 melanoma, KRIB osteosarcoma, and MDA-MB-435 breast carcinoma human tumor cell lines were used to establish human xenograft tumors in nude mice as described (10, 11). Before use, the cells were grown in the continuous culture for no more than three consecutive passages. Actively growing cells were detached from culture plates with PBS/2.5 mM EDTA or trypsin-EDTA (0.25% trypsin/1 mM Na-EDTA; GIBCO/BRL). The detached cells were resuspended in DMEM, counted, and examined for viability by trypan blue exclusion. The cells were injected into mice as described in the next section. A portion of the cells that were used in the injections was seeded back into a culture plate to determine plating efficiency. The viability was higher than 99%, and the plating efficiency was greater than 95%.

Tumor Inoculation. Two-month-old immunodeficient BALB/c nu/nu female mice (Harlan–Sprague–Dawley) were used for the experiments. To obtain s.c. tumors, we injected 10⁶ tumor cells in 200 µl of DMEM into the right posterior flank of the mice;
the mice were randomized and divided into experimental groups, with five or six mice per group. At 3 weeks after the tumor cell implantation, when nearly all of the mice had developed palpable tumors, the mice were treated with i.p. injections (200 \( \mu \)l) of various proteins in PBS or with PBS alone. The treatments were administered twice a week until the experiment was terminated.

To monitor tumor growth during the treatment, we estimated tumor size by measuring biweekly the longest and shortest diameters of the tumor and by averaging the two measurements. At the end of the experiment (usually 8 weeks after tumor cell implantation and 5 weeks after the start of treatments), the mice were anesthetized and perfused through the heart with PBS, and the tumors and the lungs were excised and weighed. The lungs also were examined for the number of metastatic foci. The tissues were fixed in 4% paraformaldehyde for 24 h and stored in 70% ethanol. Paraffin embedding, sectioning, and immunostaining for blood vessels with anti-CD31 were carried out in the Burnham Institute Histology Facility. The rat anti-mouse CD31 antibody was from PharMingen.

**Results**

**Systemic Treatment with sFN or the sFN-Inducing Anastellin Peptide Inhibits Tumor Growth.** In previous work, we found that systemic treatment with sFN, a polymer of fibronectin that is prepared by mixing fibronectin and anastellin, had a strong antimetastatic effect when used to treat mice bearing large (0.5 g in weight) tumors. This effect was achieved even though sFN had little or no effect on the growth of the primary tumors (10). In the present study, we started the treatments at an earlier stage of tumor development and found that sFN significantly inhibited the growth of C8161 human melanoma xenograft tumors (Fig. 1). Surprisingly, anastellin given alone at the dose used to prepare sFN had a similar, albeit perhaps somewhat weaker, antitumor activity as sFN.

**Anastellin-Induced Fibrinogen Polymer.** Our finding that anastellin could inhibit tumor growth without being mixed ex vivo with fibronectin raised the question of whether anastellin might form an active complex with fibrinogen in vivo. A fibrinogen fragment that overlaps with anastellin binds to both fibrinogen and fibronectin (7), and fibrinogen is more abundant in plasma than is fibronectin. Indeed, we found that adding anastellin to a fibrinogen solution in vitro caused turbidity, producing a polymeric compound, sFBG (Fig. 2). However, a higher concentration of fibrinogen than fibronectin was needed to produce the same degree of polymerization with anastellin. In subsequent tumor treatment experiments, we compared sFN and sFBG at the same approximate levels of polymerization. The solubility of fibrinogen limited the amount of sFN that could be administered to a mouse, but anastellin alone does not have this limitation. Thus, we also tested anastellin at a higher dose.

**Comparison of the Antitumor Effects of Anastellin, sFN, and sFBG.** Comparing the antitumor activities of the various anastellin compounds, we found that sFBG inhibited tumor growth approximately as effectively as anastellin alone or sFN. Similar results were obtained with three different tumors, C8161 (not shown), KRIB human osteosarcoma (Fig. 3), and MDA-MB-435 human breast carcinoma (Fig. 4). In some experiments, a peptide analogous to anastellin but derived from the 11th type III repeat of fibronectin (III11-C) was used as an additional control. This peptide does not bind to fibronectin (5) and had no effect on the growth of the breast carcinoma tumors (Fig. 4). In other experiments, we also included as controls nonpolymerized fibrinogen and fibrinogen at the same concentration.
The tumors were significantly smaller at the end of the experiment in the mice treated with anastellin, sFN, or sFBG (Fig. 3). The doses per injection were as follows: anastellin, 300 μg; sFN, 500 μg; sFBG, 500 μg. Tumor sizes (mean and SEM) in each treatment group (six mice per group) at the indicated time point are shown. The tumors of the mice treated with anastellin, sFN, or sFBG were significantly smaller at the end of the experiment than those of the mice treated with vehicle alone (anastellin, P < 0.01; sFN, P < 0.05; sFBG, P < 0.01).

Anastellin, sFN, and sFBG Inhibit Tumor Angiogenesis. We hypothesized that the decreased tumor growth obtained with anastellin, sFN, and sFBG could be caused by inhibition of tumor angiogenesis. We therefore analyzed the density of blood vessels by staining sections of tumors collected at the end of the experiments shown in Figs. 1, 3, and 4. Staining for the endothelial marker CD31 showed greatly reduced blood vessel density in the tumors of the mice treated with anastellin, sFN, and sFBG relative to the vehicle-treated controls. The results were similar for all of the three tumor types; Fig. 5 shows examples of the staining results with KRIB tumors, and quantitative results are shown for the MDA-MB-435 tumors in Fig. 6. In contrast to the tumor results, the density of CD31 positive vessels in the lungs of the mice that received the various treatments showed no discernible differences (not shown). These results suggest that the inhibition of tumor growth by anastellin, sFN, and sFBG is caused at least in part by suppression of tumor angiogenesis.

Anastellin, sFN, and sFBG Inhibit Metastasis. We also examined the lungs of the tumor-bearing mice for evidence of metastasis. All of the vehicle-treated mice with C8161 tumors and most of the mice with KRIB tumors developed macroscopic lung metastases; the MDA-MB-435 tumors did not metastasize within the time frame of our experiments. In agreement with earlier results, only a few mice with C8161 or KRIB tumors developed metastatic foci in the lungs when treated with sFN (10) or anastellin (Table 1). SFBG was studied less extensively but seemed to have an effect on metastasis similar to that of sFN and anastellin.

Discussion
We describe here a antiangiogenic protein, anastellin. Anastellin is a fibronectin fragment that binds to and polymerizes fibronectin and fibrinogen. Our results show that systemic treatment of mice with intraperitoneally injected anastellin or its fibronectin or fibrinogen polymers suppresses the growth of s.c. tumors and that this effect of anastellin is likely to be caused by its antiangiogenic activity. Anastellin also inhibits metastasis.

We previously have shown that sFN, a fibronectin polymer induced by anastellin, has a potent antimetastatic activity in experimental and spontaneous metastasis models (10). In that study, systemic treatment of the tumor-bearing mice with sFN or anastellin had no significant effect on tumor growth. In contrast, we found in the present study that tumor growth was inhibited by these compounds, as well as by SFBG, a fibrinogen polymer generated by treating soluble fibrinogen with anastellin. The likely explanation for the difference between the previous result and the antitumor effect we observed here is in the timing of the treatment. In the metastasis study, the tumors were large at the time when the treatment was started, whereas smaller tumors were treated in the present study. The large tumors might have been beyond the point where antiangiogenic treatment would still be effective. Furthermore, the dose might not have been optimal. Although a complete dose–response study remains to be performed, we believe that anastellin is a promising candidate for clinical trials as an antitumor agent.
be done, the present results suggest that doubling the dose of anastellin made it more potent.

Our results indicate that the inhibitory effect of anastellin and the fibronectin and fibrinogen polymers is mediated by an antiangiogenic activity of these compounds. This effect seems to be different from the antimetastatic activity we observed earlier (10), where only sFN was clearly active. Here, anastellin was approximately as effective as sFN in suppressing tumor growth and tumor angiogenesis.

The antiangiogenic effects of anastellin, sFN, and sFBG are likely to have been primarily responsible for the inhibition of tumor growth that we have demonstrated here. The blood vessel density in the tumors of the treated mice was only about 20% of that in control tumors. As vascularization is a prerequisite of tumor growth (12), the low number of blood vessels must have been a major impediment to tumor growth in the treated mice.

Inhibition of angiogenesis with its suppressive effect on tumor growth possibly underlies the antimetastatic effect of anastellin and the polymers we observed in this study. First, we found that the number of metastases correlated with the size of the primary tumor and the number of blood vessels in it. The reduced vasculature in the tumor could make it more difficult for tumor cells to enter the circulation. Another possible contributing factor is the reduced ability of tumor cells that have gained access to the circulation to establish metastatic colonies. Earlier studies have shown that sFN inhibits lung colonization by tumor cells injected into the circulation (10). As anastellin exhibited this activity, or did so weakly, this inhibitory activity could explain why sFN might be somewhat more active in reducing metastasis than is anastellin, at least with the C8161 tumors. Anastellin also did not inhibit spontaneous metastasis in the previous study, suggesting that the antimetastatic activities of sFN observed in the previous study depended on a mechanism other than antiangiogenic activity. Preliminary data suggest that that effect is related to accelerated removal of tumor cells from the circulation (unpub-
Anastellin is a representative of a growing class of antiangiogenic substances that are derived from ECM and blood proteins by proteolysis or other modifications. These substances include angiostatin (13), endostatin (14), heparin-binding fragments of fibronectin (15, 16), and a modified form of antithrombin (17). As is the case with these substances, the mechanism of the antiangiogenic activity of anastellin is unknown. However, we do know that each of these substances binds to one or more adhesion proteins. Angiostatin and its parent protein plasminogen can bind vitronectin (18), endostatin has been shown to bind to fibulins and nidogen-2 (19), and the antiangiogenic form of antithrombin is similar to the modified antithrombin that binds to vitronectin (20). Anastellin not only binds to fibronectin and fibrinogen in serum (7), it polymerizes these proteins in vitro (ref. 5 and the present study) and likely in vivo. Fibronectin and fibrinogen, and each of the other ligands for the various antiangiogenic substances we have listed, are adhesion proteins containing the arginine–glycine–aspartic acid cell adhesion sequence (21). Moreover, they all bind to the αvβ3 integrin, which is expressed at high levels in angiogenic endothelial cells and which plays an important role in angiogenesis (22). On the basis of these considerations, we propose a common mechanism of action in vivo for the known antiangiogenic protein fragments: they polymerize an arginine–glycine–aspartic acid–containing protein, the resulting polymers bind to the αvβ3 integrin on angiogenic endothelial cells, and the polymers inhibit cell proliferation and cause apoptosis. The polymers possibly act by affecting ECM formation (23) or by arginine–glycine–aspartic acid–mediated activation of intracellular caspases (24, 25).

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Fig. 6. Quantification of decreased blood vessel density in MDA-MB-435 tumors from mice treated with anastellin, sFN, or sFBG. Blood vessels in tumor sections stained with anti-CD31 were counted from five microscopic fields for all tumors in the experiment shown in Fig. 4. The mean and SEM for the number of blood vessels in the tumors from the six mice in each treatment group are shown. The blood vessel density was significantly reduced in each of the anastellin (P < 0.02), sFN (P < 0.02), and sFBG (P < 0.01) groups relative to the vehicle alone group and the other control group that received III-11C.