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Advances in Brief

Endothelial Progenitor Cells as Putative Targets for Angiostatin

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Abstract

Angiostatin, a product of the proteolytic cleavage of plasminogen, possesses potent antitumor and antiangiogenic properties *in vivo*. Studies with cultured endothelial cells suggest that under certain conditions, angiostatin inhibits the migration and proliferation of these cells or, alternatively, increases their rate of apoptosis. In general, the effects of angiostatin have been considerably less potent *in vitro* than *in vivo*. One potential explanation for this disparity is that the *in vivo* target of angiostatin is not the mature endothelial cell. Recently, evidence has accumulated to show that circulating endothelial progenitor cells (EPCs) contribute to neovascularization. In this study, we have isolated EPCs from human subjects and demonstrated that, in contrast to that of mature endothelial cells, the growth of EPCs is exquisitely sensitive to angiostatin. These results suggest that angiostatin and related compounds may exert their biological effects by inhibiting the contribution of EPCs to angiogenesis and not by altering the growth of mature endothelial cells.

Introduction

The isolation of antiangiogenic molecules represents a potentially useful addition to cancer therapeutics. In particular, agents such as angiostatin (1) have potent *in vivo* antitumor properties when used alone (1–4) or in combination with other modalities of treatment (5). Angiostatin inhibits the growth of tumor vasculature, although the exact mechanism of this effect remains unclear. Studies with mature endothelial cells have demonstrated that in some instances, angiostatin appears to delay progression of the cell cycle (2), although in other instances, this effect has not been observed (6). Similarly, some studies have noted that angiostatin produces a decrease of about 20% in serum-stimulated endothelial cell growth (7), although again this effect has not been demonstrated in other studies (6). Finally, some reports have noted that angiostatin stimulates a small but significant increase in endothelial apoptosis (6, 8). Surprisingly, these *in vitro* effects appear significantly more modest than those observed *in vivo*. Recently, several reports (9–12) have described the existence of EPCs[2]. These bone-marrow-derived cells appear to circulate in reasonable abundance and contribute to neovascularization (13, 14). In this report, we have attempted to provide an explanation for the disparity of results previously obtained with angiostatin *in vivo* and *in vitro* by demonstrating that human EPCs are significantly more sensitive than mature endothelial cells to the growth inhibitory effects of angiostatin.

Materials and Methods

Isolation and Characterization of EPCs. Anonymous buffy coats were obtained from the NIH Blood Bank. PBNCs were obtained by subsequent purification over Ficoll gradients. After purification, 5×10^6 cells were plated per well of a 6-well fibronectin-coated plate. After 24 h, nonadherent cells were recovered and counted and subsequently replated on fibronectin-coated dishes at a density of 2.5×10^3 cells/mm². Media was changed every 3 days. The attached cells were assessed immunologically on day 7 after plating to confirm that they were similar to the previously characterized human EPC colonies (9). Indirect immunostaining was performed using antibodies directed against Flk-1 (Santa Cruz), Tie-2 (Santa Cruz), and CD31 (DAKO). For control immunohistochemical analysis, the primary antibodies were replaced by equal amounts of the corresponding normal mouse or rabbit IgG. The control procedure resulted in no appreciable staining (data not shown).

Assessment of Angiostatin Effects. Angiostatin was purchased from Angiogenesis Research Industries (Chicago, IL). Fresh angiostatin at the indicated concentration was added every 3 days. The number of EPC colonies was assessed by fixation and staining of the well (Diff-Quick Stain Set, Dade Diagnostics) followed by counting three separate wells per treatment condition. The growth of HUVECSs was determined from triplicate cultures using a Coulter counter. Given the difficulty in fully trypsinizing EPCs, the growth for these cells was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Dojindo Laboratories, Japan). HUVECSs were obtained from Clonetics and grown in endothelial growth medium (Clonetics) supplemented with 10% FCS. EPCs were maintained in M199 media (Life Technologies, Inc.) supplemented with 10% FCS.

Results and Discussion

Previous studies in humans, mice, rabbits, and dogs have demonstrated the existence of circulating EPCs (9–12). Human EPCs are derived from CD34-positive cells, although their isolation is enhanced if they are initially maintained with CD34-negative cells during isolation (9). Presumably, this reflects the secretion of certain trophic factors that increase the growth and/or viability of EPCs. When human PBNCs were plated on fibronectin-coated dishes, as previously described (9), a small percentage of cells became attached and subsequently became organized into small colonies over a period of several days. The number of colonies formed increased as a function of the initial plating density but varied from one donor to another (data not shown).

As previously described for other species, colonies obtained from attached human PBNCs subsequently gave rise to spindle-shaped cells (Fig. 1). Immunological characterization 1 week after initial isolation of these attached cells demonstrated that they stained positively for the endothelial markers CD31, Tie-2, and Flk-1 (Fig. 1). Therefore, as described in previous reports, peripheral blood contains circulating cells, termed EPCs, that can adhere to fibronectin and express a variety of endothelial cell markers after a period of 7 days in culture.

We next sought to assess the effects of angiostatin on human EPCs. As demonstrated in Fig. 2*A*, the number of EPC colonies observed after plating on fibronectin was decreased by angiostatin in a concen-
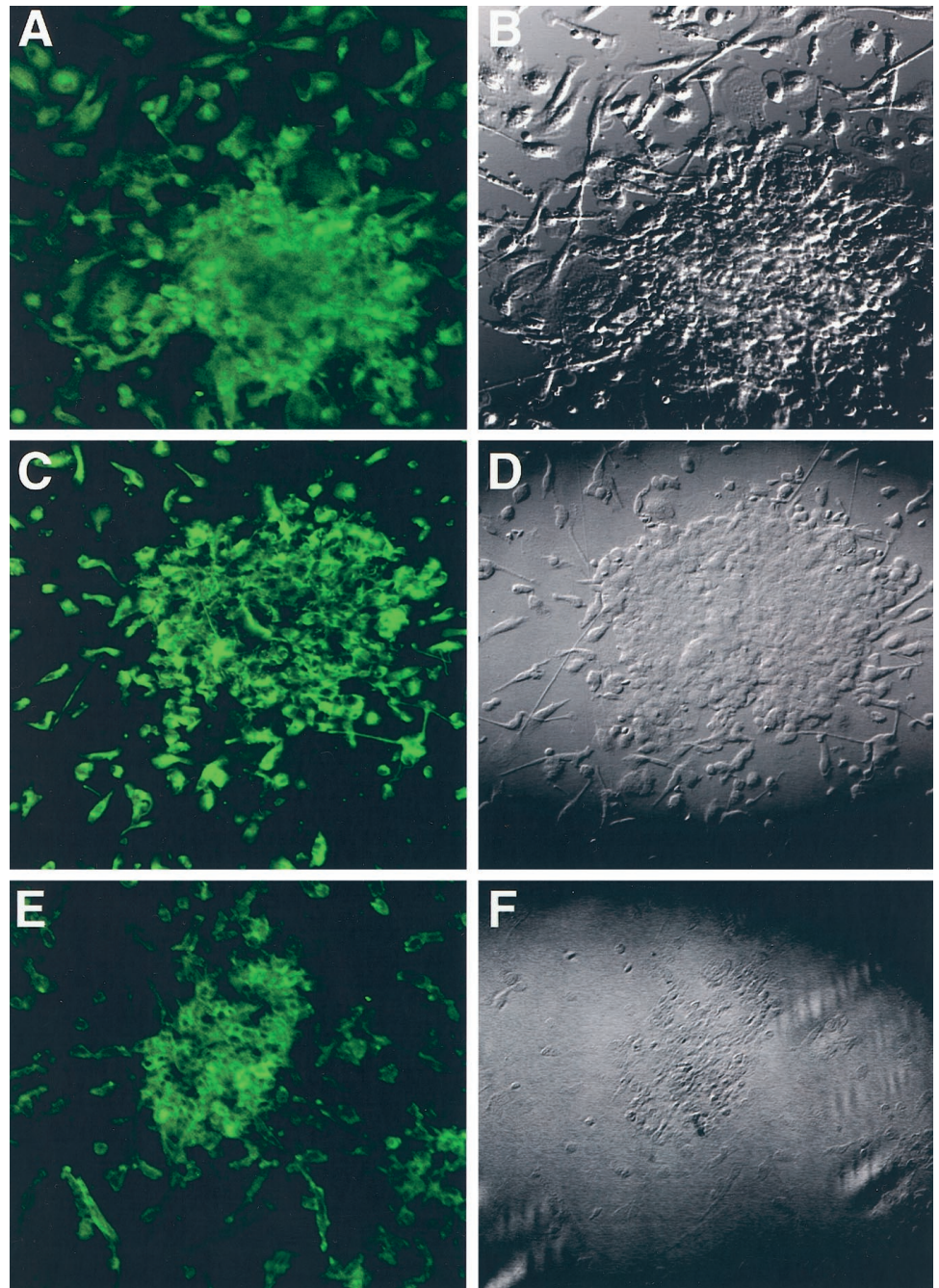
Fig. 1. Immunological characterization of human EPCs. PBNCs were plated on fibronectin, and after 7 days, attached cells were fixed and assayed by immunohistochemistry. Reactivities for CD31, Tie-2, and Flk-1 are shown in *A*, *C*, and *E*, respectively. The corresponding phase-contrast images of attached EPCs are shown in *B*, *D*, and *F*, respectively. No staining was observed when the primary antibody was replaced with the corresponding control mouse or rabbit IgG (data not shown).

tration-dependent fashion. Interestingly, those colonies that did form in the presence of angiostatin, in contrast to control colonies, gave rise to fewer spindle-shaped cells (Fig. 2, *B* and *C*). In an effort to further quantify these effects, we measured the growth of EPCs over a 3-day period. As shown in Fig. 2*D*, although control EPCs had close to an 8-fold increase in cell number during this period, EPCs exposed to angiostatin only increased ~2-fold. In contrast to the effects of angiostatin on EPCs, we observed essentially no alterations on human endothelial cell cultures. As demonstrated in Fig. 2*E*, the growth of endothelial cells increased ~9-fold over a 3-day period and was unaffected by the addition of angiostatin.

In summary, we demonstrated that cells with the characteristics of EPCs can be readily isolated from human peripheral blood. When exposed to angiostatin, the growth of these cells is significantly inhibited, whereas under similar conditions, the growth of mature endothelial cells is not effected. Studies in animal models have suggested that the numbers of circulating EPCs are increased following ischemia and that these cells are recruited to sites of neovascularization (13, 14). The role of circulating EPCs in tumor angiogenesis has been less well-studied. Recently, however, by using a mouse tumor model with genetically marked bone marrow, it has been possible to demonstrate a significant incorporation of EPCs into tumor neovasculature (15). These *in vivo* results, coupled with our *in vitro* data, suggest that strategies using the inhibition of EPC growth may be useful as a sensitive bioassay for the identification of novel antiangiogenic compounds. In addition, given that EPCs are significantly more sensitive than mature endothelial cells to the effects of angiostatin at least *in vitro*, it is tempting to speculate that EPCs represent an important, if not the most important, *in vivo* target of angiostatin.
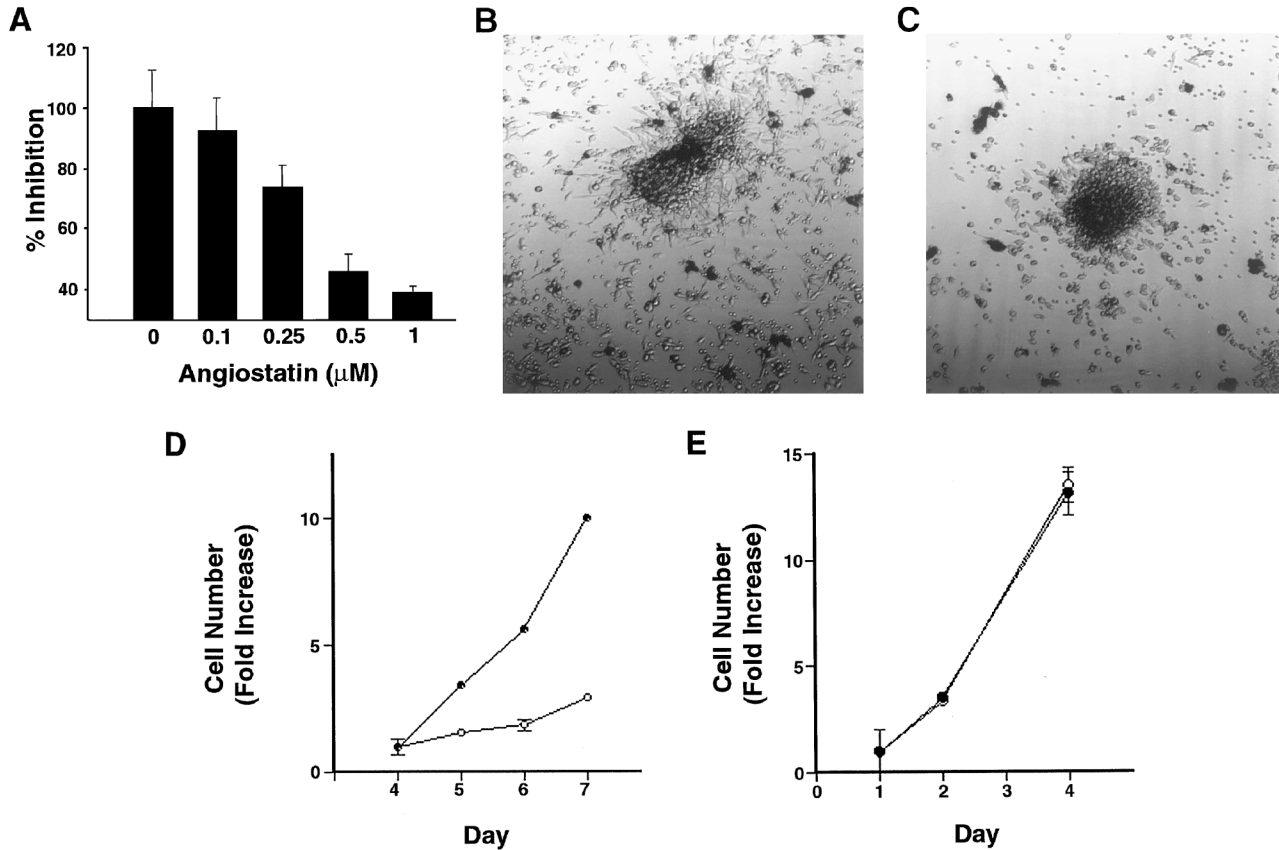
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Fig. 2. Effects of angiostatin on EPCs and HUVECSs. *A*, number of EPC colonies observed on day 7 in cells treated with the indicated concentration of angiostatin. *Bars*, mean ± SD. *B*, normal morphology of the EPC colony with surrounding spindle-like cells. *C*, morphology of the angiostatin-treated (1 μM) EPC colony. *D*, growth of human EPCs with (*open circle*) or without (*closed circle*) 1 μM angiostatin. EPCs were plated in the presence or absence of angiostatin (day 0), and their subsequent growth rate from day 4 to day 7 was evaluated. Results are the mean ± SD of triplicate cultures. *E*, growth of HUVECSs with (*open circles*) or without (*closed circles*) 1 μM angiostatin. Results are the mean ± SD of triplicate cultures.

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