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Modulation of Bovine Microvascular Endothelial Cell Proteolytic Properties by Inhibitors of Angiogenesis

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Abstract A tightly controlled increase in extracellular proteolysis, restricted both in time and space, is an important component of the angiogenic process, while anti-proteolysis is effective in inhibiting angiogenesis. By focussing on the plasminogen activator (PA)-plasmin system, the objective of the present studies was to assess whether previously described inhibitors of angiogenesis modify bovine microvascular endothelial cell proteolytic properties. We demonstrate that although synthetic angiostatic steroids (U-24067 and U-42129), heparin, suramin, interferon alpha-2a, and retinoic acid are all inhibitors of in vitro angiogenesis, each of these agents has distinct effects on the plasminogen-dependent proteolytic system. Specifically, angiostatic steroids and interferon alpha-2a reduce urokinase-type PA (u-PA) and PA inhibitor-1 activity, while heparin and retinoic acid increase u-PA activity. Suramin reduces cell-associated u-PA activity and greatly increases PAI-1 production at doses which induce monolayer disruption. These findings demonstrate that a spectrum of alterations in extracellular proteolysis is associated with anti-angiogenesis, and that anti-angiogenesis and anti-proteolysis are not necessarily correlated. A reduction in extracellular proteolysis would be expected to reduce invasion, whereas an increase in proteolysis might modulate the activity of inhibitory cytokines, which in turn could reduce endothelial cell proliferation and migration and inhibit angiogenesis. The spectrum of effects on different elements of the PA system observed in response to the agents assessed suggests that the role of modulations in extracellular proteolytic activity in anti-angiogenesis is likely to be varied and complex.

Key words: urokinase-type plasminogen activator, plasminogen activator inhibitor-1, angiostatic steroids, heparin, suramin, interferon alpha-2a, retinoic acid

Angiogenesis is the formation of new capillary blood vessels by a process of sprouting from pre-existing vessels. In addition to its role during development and in the physiology of female reproductive function, wound healing, and collateral blood vessel formation in ischaemia, angiogenesis occurs in pathological situations such as proliferative retinopathy and hemangioma formation. Angiogenesis is also necessary for the continued growth of solid tumors and contributes to the hematogenous spread of tumor cells and the formation of metastasis [reviewed by Folkman and Klagsbrun, 1987; Zetter, 1988]. The identification of agents which either promote or inhibit angiogenesis and the elucidation of their mechanisms of action is therefore of clinical relevance.

Angiogenesis begins with localized breakdown of the basement membrane of the parent vessel. Endothelial cells then migrate into the surrounding matrix within which they form a capillary sprout. Sprout elongation occurs as a result of further migration and of endothelial cell proliferation proximal to the migrating front. Fusion with the tip of another maturing sprout produces a capillary loop. A functional capillary results once a lumen has been formed, and maturation is completed by reconstitution of the basement membrane [reviewed by Folkman and Klagsbrun, 1987; Zetter, 1988]. Alterations in at least three endothelial cell functions thus occur during this series of events: 1) modulation of interactions with the extracellular matrix, which requires alterations of cell matrix contacts and the production of matrix degrading proteolytic

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enzymes; 2) an initial increase and subsequent decrease in locomotion (migration), which allows the cells to translocate towards the angiogenic stimulus and to stop once they reach their destination; and 3) an increase in proliferation which provides new cells for the growing and elongating new vessel, and a subsequent return to the quiescent state once the vessel is formed.

During the process of endothelial cell invasion, matrix degrading proteolytic enzymes are directly involved in overcoming the mechanical barriers imposed by the surrounding extracellular matrix [Moscatelli and Rifkin, 1988]. These enzymes also modulate cytokine activity either by direct proteolytic activation of latent cytokines such as transforming growth factor-β (TGF-β), or indirectly by releasing matrix-bound cytokines such as basic fibroblast growth factor (bFGF) and thereby increasing their bioavailability [Flaumenhaft and Rifkin, 1992]. One of the most extensively studied extracellular proteolytic enzyme systems, which is relevant to both matrix degradation and modulation of cytokine activity, is the plasminogen activator (PA)-plasmin system [Vassalli et al., 1991]. The central component of this system is plasmin, a protease of tryptic specificity which degrades certain matrix components (such as laminin and proteoglycans) and also activates metalloprotease zymogens (which degrade collagens and certain other glycoproteins). Plasmin is generated from its inactive precursor plasminogen, by the activity of two PAs, urokinase-type PA (u-PA) and tissue-type PA (t-PA). u-PA activity can be localized to the cell surface through binding to a specific high-affinity receptor (u-PAr), and u-PA and t-PA are subject to inhibition by specific physiological PA inhibitors (PAIs), PAI-1 and PAI-2.

Although it has consistently been observed that endothelial cell migration and invasion are associated with increased extracellular proteolytic activity, protease inhibitors play an equally important albeit permissive role during angiogenesis by preserving matrix integrity [Montesano et al., 1987]. This has lead to the notion that a precise protease-antiprotease equilibrium allows for localized pericellular matrix degradation during cell migration, while at the same time protecting the extracellular matrix against inappropriate destruction [Montesano et al., 1990; Pepper et al., 1990; Pepper and Montesano, 1990]. A tightly controlled increase in extracellular proteolysis, restricted both in time and space, is therefore an important component of the angiogenic process, and this has led to the notion that anti-proteolysis could be effective in inhibiting angiogenesis. Indeed, exogenously added protease inhibitors do inhibit angiogenesis both in vivo and in vitro [Mignatti et al., 1989; Moses et al., 1990].

In the studies reported in this paper we have asked whether previously described inhibitors of in vivo angiogenesis, namely, the synthetic angiostatic steroids U-24067 and U-42129 [Folkman et al., 1983; Ingber et al., 1986; Ingber and Folkman, 1988; Wilks et al., 1991], heparin [Folkman et al., 1983; Crum et al., 1985; Jakobson and Hahnenberger, 1991], suramin [Gagliardi et al., 1992; Pesenti et al., 1992], interferon alpha-2a [Sidky and Borden, 1987], and retinoic acid [Ingber and Folkman, 1988; Oikawa et al., 1989; Szmurlo et al., 1992], alter the proteolytic properties of bovine microvascular endothelial (BME) cells. Using an in vitro model of angiogenesis in which endothelial cells can be induced to invade a three-dimensional collagen gel within which they form capillary-like tubes [Montesano and Orci, 1985], we first assessed the effect of these agents on the angiogenic response induced by bFGF [Montesano et al., 1986], one of the most completely characterized angiogenic factors to date [reviewed by Klagsbrun and D'Amore, 1991]. We next determined their effect on BME cell proteolytic properties by focussing on the PA system. Finally, we assessed the effect of these agents on endothelial cell proliferation and migration in conventional two-dimensional assays. We demonstrate that all of the anti-angiogenic agents tested inhibit in vitro angiogenesis, and that while alterations in migration and proliferation correlate with the inhibitory effects of these agents on in vitro angiogenesis, each agent has distinct effects on different elements of the plasminogen-dependent proteolytic system.

MATERIALS AND METHODS

Reagents

Recombinant human bFGF provided by Dr P. Sarmientos (Farmitalia Carlo Erba, Milan, Italy) was dissolved in serum-free alpha MEM containing 1 mg/ml BSA (fatty acid free, Sigma Chemical Co., St. Louis, MO) to give a stock solution of 300 ng/ml. U-24067 (6α-fluoro-17,12-dihydroxy-16α-methylpregna-4,9,(11)-diene-3,20-dione-21- acetate) and U-42129 (6α-fluoro-17, 12-dihydroxy-16β-methylpregna-4,9,(11)-diene-

3,20-dione) were dissolved in DMSO to give stock solutions of 2 mM which were protected from light. Heparin from porcine intestinal mucosa purchased from Sigma Chemical Co. (St. Louis, MO) or Hepar Inc. (Franklin, OH) was dissolved in distilled water to give a stock solution of 10 mg/ml. Suramin (Germanin) purchased from Bayer AG (Leverkusen, Germany) was dissolved in PBS to give a stock solution of 100 mM. Interferon alpha-2a (Roferon-A3) purchased from Hoffman-La Roche AG (Grenzach-Wyhlen, Germany) was dissolved in the diluent provided to give a stock solution of 3×10^6 IU/ml (14 mg/ml). Intermediate dilutions of U-24067, U-42129, Sigma heparin and Hepar heparin, suramin, and interferon alpha 2a were prepared in serum-free alpha MEM or PBS containing 1 mg/ml BSA (fatty acid free, Sigma Chemical Co., St. Louis, MO). All-*trans*-retinoic acid purchased from Sigma Chemical Co. (St. Louis, MO) was initially dissolved in absolute ethanol to obtain a 12 mM solution. This solution, protected from light, was diluted in complete culture medium to yield the final concentrations required.

Cell Culture

Adrenal cortex-derived bovine microvascular endothelial (BME) cells, provided by Dr. M.B. Furie and Dr. S.C. Silverstein (Columbia University, New York, NY) [Furie et al., 1984], were grown in minimal essential medium (MEM), alpha modification (Gibco AG, Basel, Switzerland), supplemented with 15% heat-inactivated donor calf serum (DCS, Flow Laboratories, Baar, Switzerland), penicillin (500 U/ml), and streptomycin (100 μg/ml). Cells were subcultured at a 1:4 split ratio in 1.5% gelatin-coated tissue culture dishes or flasks (Falcon Labware, Becton-Dickinson Company, Lincoln Park, NJ), and all experimental manipulations were performed upon reaching confluence (5–7 days).

In Vitro Angiogenesis Assay

BME cells were seeded onto three-dimensional collagen gels, prepared in 18 mm tissue culture wells as previously described [Montesano and Orci, 1985], at 5×10^4 cells/well in 500 μl complete medium. Upon reaching confluence (approximately 5 days) bFGF (10 ng/ml) and anti-angiogenic compounds were added; the latter were added 2 h before adding bFGF. Medium was changed and bFGF and anti-angiogenic compounds were added every 2–3 days. After 7 days, randomly selected fields measuring 1.0 mm × 1.4 mm were photographed in each well at a single level beneath the surface monolayer by phase contrast microscopy, using a Nikon Diaphot TMD inverted photomicroscope. Invasion was quantitated as previously described [Pepper et al., 1993b]. Results are shown as the mean additive length ± SEM (in μm) of endothelial cell cords from three randomly selected photographic fields per experiment, for each of at least 3 experiments per condition. Mean values were compared using Student's unpaired *t*-test, and a significant p value was taken as $P < 0.05$.

Zymography and Reverse Zymography

Confluent monolayers of BME cells in 35 mm tissue culture dishes were washed twice with serum-free alpha-MEM and 1.5 ml serum-free alpha MEM containing Trasylol (200 KIU/ml) (Bayer AG, Zurich, Switzerland) was added. Anti-angiogenic compounds were added alone or with bFGF (10 ng/ml); where relevant, anti-angiogenic compounds were added 1 h before bFGF. 15 h later, cell extracts and culture supernatants were prepared, and analyzed by zymography and reverse zymography as previously described [Vassalli et al., 1984; Pepper et al., 1990]. Over the 15 h assay period in serum free medium, cell number is relatively stable with less than 15% variation under a variety of conditions (data not shown); samples were therefore analyzed on the basis of volume equivalents of cell extract or culture supernatant. In the case of suramin, where cell numbers varied greatly due to detachment, cells were counted in a parallel series of dishes and samples loaded on the basis of cell equivalents.

RNA Preparation, In Vitro Transcription, and Northern Blot Hybridization

Total cellular RNA was prepared from confluent monolayers of BME cells in 100 mm culture dishes exposed to anti-angiogenic compounds and/or to bFGF (10 ng/ml); where appropriate, anti-angiogenic compounds were added 1–2 h before bFGF. The last medium change was always 24 h before starting the experiment. RNA was extracted at the indicated time points; where anti-angiogenic compounds were co-added with bFGF, time zero was taken as the time of addition of bFGF. RNA preparation, Northern blots, in vitro transcription, hybridization, and post-hybridization washes were as previously described [Pepper et al., 1990]. For Northern blots,

5 μg total cellular RNA was loaded per lane. [^{32}P]-labelled cRNA probes were prepared from bovine urokinase-type plasminogen activator (u-PA) [Krätzschmar et al., 1993], bovine u-PA receptor (u-PAr) [Krätzschmar et al., 1993], human tissue-type PA (t-PA) [Fisher et al., 1985], and bovine PA inhibitor-1 (PAI-1) [Pepper et al., 1990] cDNAs as previously described [Pepper et al., 1990, 1993a]. Autoradiographs were scanned with a GenoScan laser scanner (Genofit, Geneva, Switzerland). Results are expressed relative to control cultures at the corresponding time point.

RESULTS AND DISCUSSION

The purpose of the studies reported in this paper was a) to determine the effect of previously described inhibitors of in vivo angiogenesis on the in vitro angiogenic response using bovine microvascular endothelial (BME) cells, and b) to document the effects of these inhibitors on the PA proteolytic system of the same cells at concentrations which inhibited angiogenesis in vitro. u-PA has been implicated in processes of cell migration and tissue remodelling, while t-PA is believed to be involved mainly in intravascular thrombolysis [Moscatelli and Rifkin, 1988; Vassalli et al., 1991]. In addition, bFGF, which has been used as the angiogenic stimulus in these studies, increases u-PA, u-PAr, and PAI-1 expression in BME cells, with very little effect on t-PA [Montesano et al., 1986; Moscatelli et al., 1986; Saksela et al., 1987; Pepper et al., 1990, 1993a; Mignatti et al., 1991]. For these reasons, our present study has focussed primarily on u-PA and PAI-1. It should be noted that BME cell-associated u-PA is mainly receptor bound [Pepper et al., 1993a]. Alterations in cell-associated u-PA activity as detected by zymography are therefore a reflection of alterations in u-PAr binding capacity and/or u-PA synthesis. Once u-PAr binding sites are saturated, increased u-PA synthesis will be reflected as increased activity in the culture supernatant.

Angiostatic Steroids and Heparin

Early reports on the inhibitory effects of steroids on tumor-associated angiogenesis [Shubik et al., 1976; Gross et al., 1981] were followed in 1985 by the description of a class of steroids, termed "angiostatic steroids," which required heparin to be effective [Crum et al., 1985]. U-24067 and U-42129, the synthetic angiostatic steroids used in our present studies, have previously been shown to inhibit angiogenesis in vivo; according to some authors, their activity was strictly heparin dependent [Folkman et al., 1983; Ingber et al., 1986; Ingber and Folkman, 1988], while according to others, it was not [Wilks et al., 1991; J.W. Wilks, unpublished observation]. We have observed that U-24067 and U-42129 inhibit angiogenesis in vitro, and that this effect is heparin independent (Figs. 1 and 2, and data not shown). Our findings suggest that endothelial cells may be one of the targets for these steroids in vivo, and that in the complex environment in vivo, heparin may target angiostatic steroids to endothelial cells [Thorpe et al., 1993].

To address the mechanism of inhibition by these steroids, we assessed their effect on the PA system of BME cells. Both U-24067 and U-42129 decreased basal and bFGF-induced u-PA and PAI-1 activity (Fig. 3). After 4 h exposure to 10 μM U-24067, basal levels of u-PA, u-PAr, and PAI-1 mRNA were decreased 70%, 39%, and 17%, respectively, while bFGF-induced levels of the same mRNAs were decreased 67%, 27%, and 45%, respectively; co-addition of bFGF and U-24067 increased t-PA mRNA levels approximately 2-fold (data not shown). The effects of U-24067 and U-42129 on the BME cell PA system differ from those observed with another angiostatic steroid, medroxyprogesterone acetate (MPA) [Gross et al., 1981]. MPA decreased PA activity in bovine endothelial cells [Ashino-Fuse et al., 1989], which could be accounted for by an increase in PAI-1 [Blei et al., 1993]. These results demonstrate that different angiostatic steroids affect the endothelial cell PA system in different ways.

Heparin alone has been shown to have variable effects on angiogenesis in vivo: it may thus stimulate [Ribatti et al., 1987; Norrby and Sörbo,

Fig. 1. Effects of U-24067, suramin and retinoic acid on bFGF-induced in vitro angiogenesis. Confluent monolayers of BME cells on the surface of three-dimensional collagen gels were treated with U-24067 (10 μM), suramin (100 μM), or retinoic acid (10 μM) in the presence or absence of bFGF (10 ng/ml) for 7 days. In response to bFGF, the cells invaded the underlying matrix, and by focussing beneath the surface monolayer, capillary-like tubes were observed; the presence of a lumen is indicated (arrow heads). U-24067 and retinoic acid decreased invasion and tube formation; in addition, in the presence of retinoic acid BME cells became elongated and spindle shaped. In contrast, suramin at the dose indicated (100 μM) potentiated the angiogenic effect of bFGF and induced the formation of tube-like structures with widely patent lumina (arrow heads). All images are phase contrast micrographs; ×25.

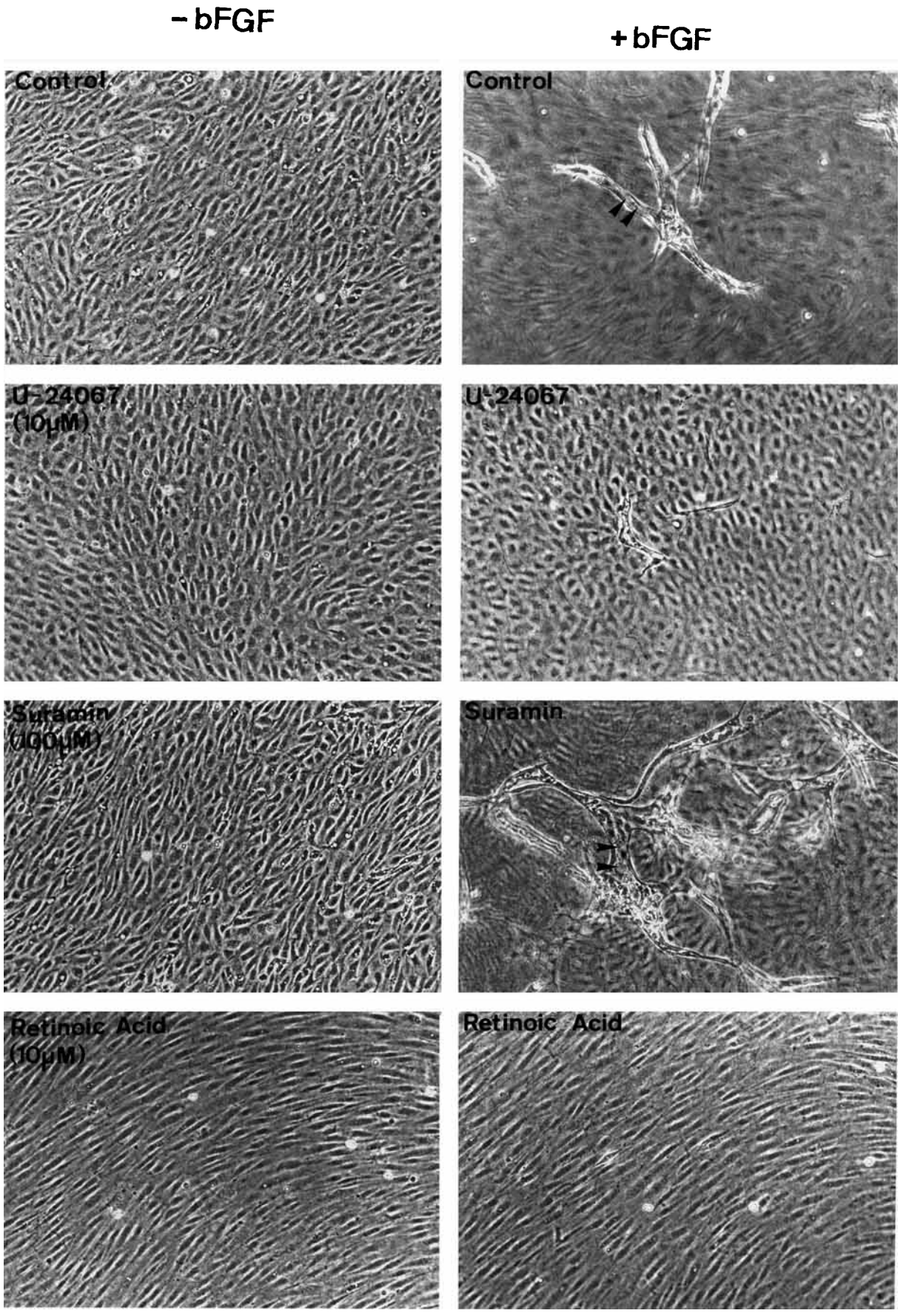
- bFGF
+ bFGF
Control
Control
U-24067 (10μM)
U-24067
Suramin (100μM)
Suramin
Retinoic Acid (10μM)
Retinoic Acid

Figure 1.

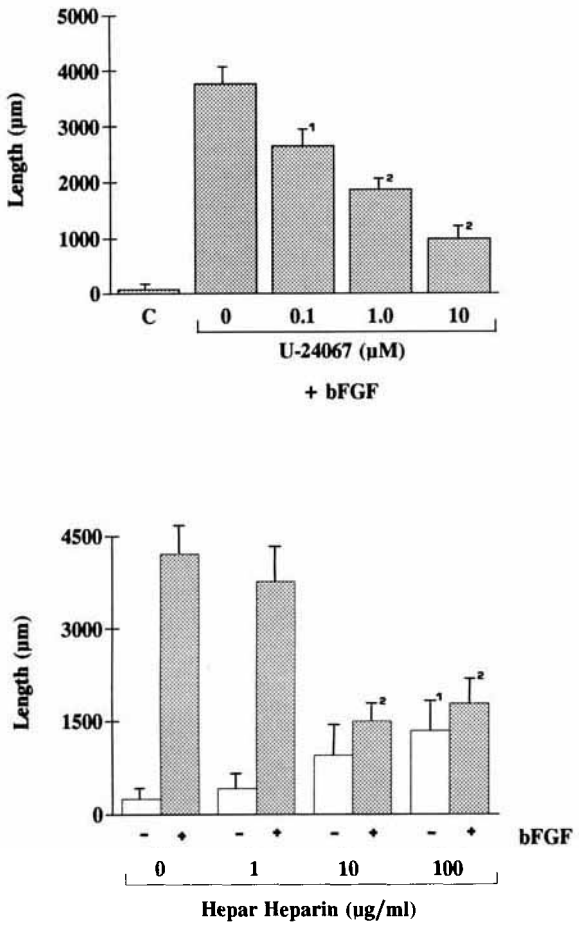

Fig. 2. Quantitative assessment of the effect of U-24067 and Hepar heparin on bFGF (10 ng/ml)-induced in vitro angiogenesis. Values are mean ± SEM. C, control, nontreated cultures. Invasion was significantly decreased by U-24067 at 0.1 μM ($P^1 < 0.05$; $P^2 < 0.001$). Basal levels were unaffected by U-24067 at the concentrations shown (data not shown). Hepar heparin on its own induced spontaneous invasion, although this only reached statistical significance at 100 μg/ml ($P^1 < 0.05$). In contrast, Hepar heparin decreased bFGF-induced invasion at 10 and 100 μg/ml ($P^2 < 0.001$).

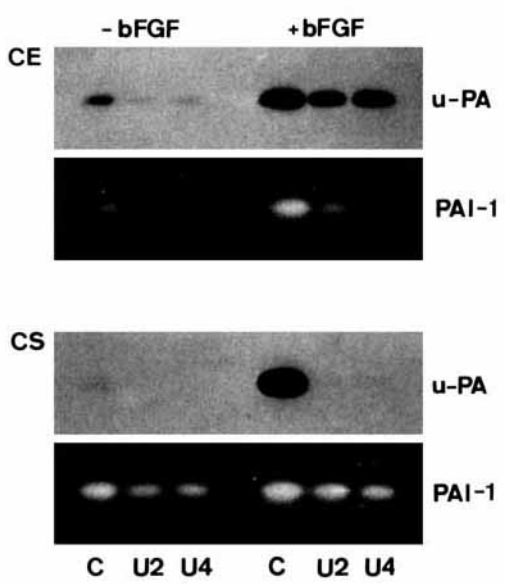

Fig. 3. Effect of U-24067 (U2) and U-42129 (U4) on BME cell u-PA and PAI-1 activity. BME cell u-PA activity was analyzed by zymography and characterized on the basis of inhibition of its catalytic activity by amiloride [Vassalli and Belin, 1987; Pepper et al., 1987]. PAI-1 activity was detected by reverse zymography; we have previously shown by immunoprecipitation studies with anti-bovine PAI-1 antibodies that the PAI produced by BME cells is PAI-1 [Pepper et al., 1991]. Both steroids (10 μM) decreased basal and bFGF (10 ng/ml)-induced u-PA and PAI-1 activity in BME cell extracts (CE) and culture supernatants (CS).

1992], inhibit [Wilks et al., 1991; Jakobson and Hahnenberger, 1991], or have no effect [Taylor and Folkman, 1982; Castellot et al., 1982]. Here we have observed that in vitro, heparin from two different sources (Sigma and Hepar) slightly increases spontaneous invasion when added alone, but markedly inhibits invasion induced by exogenous bFGF (Fig. 2, and data not shown).

With respect to the PA system, we have found that heparin increases bFGF-induced u-PA activity in the culture supernatant of BME cells, without affecting u-PA mRNA levels; heparin had no effect on PAI-1 activity or mRNA levels (Fig. 4, and data not shown). It has previously been demonstrated that heparin increases both basal and aFGF-induced PA activity in rat epididymal fat pad endothelial cells [Castellot et al., 1986] and t-PA antigen levels in human umbilical vein endothelial (HUVE) cells [Konkle and Ginsburg, 1988]. Although heparin increases the activation of plasminogen by both u-PA and t-PA [Andrade-Gordon and Strickland, 1986], it is unlikely that this accounts for our observations, as heparin would dissociate from u-PA during SDS-polyacrylamide gel electrophoresis, and would therefore not affect u-PA's plasminogen activating activity. However, since heparin binds with high affinity to u-PA [Andrade-Gordon and Strickland, 1986] and prevents denaturation and proteolytic degradation of other molecules like acidic and basic FGF [Gospodarowicz and Cheng, 1986; Rozengart et al., 1988; Saksela et al., 1988; Sommer and Rifkin, 1989], it is possible that our findings reflect a heparin-mediated increase in u-PA stability. This is consistent with the observation that heparin increases t-PA antigen detection in normal human plasma [Wun and Capuano, 1987]. Heparin is a poor inhibitor of u-PA binding to its receptor [Behrendt et al., 1993].

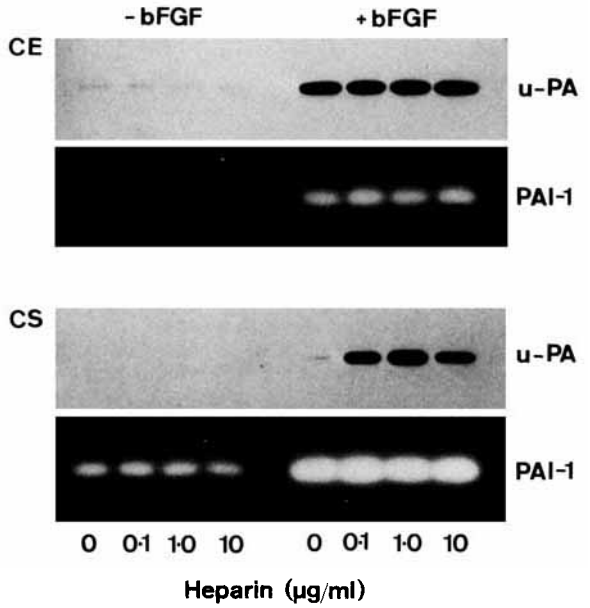

Fig. 4. Effect of heparin on BME cell u-PA and PAI-1 activity. Sigma heparin (0.1, 1, and 10 μg/ml) increased u-PA activity in the culture supernatant (CS) but not the cell extract (CE) of bFGF (10 ng/ml)-treated cells, without affecting the activity of PAI-1. In three separate experiments, Sigma heparin (10 μg/ml) did not alter mRNA levels of u-PA or any other component of the PA system (data not shown).

TABLE I. Effects of Anti-Angiogenic Agents on Wound-Induced Two-Dimensional Migration*

		Values relative to control
U-24067	1 μM	79%
	10 μM	74%
Heparin (Sigma)	1 μg/ml	64%
	10 μg/ml	59%
	100 μg/ml	26%
Suramin	100 μg/ml	115%
	300 μg/ml	56%
Interferon alpha-2a	1 IU/ml	92%
	10 IU/ml	84%
	100 IU/ml	80%
	1,000 IU/ml	74%
Retinoic acid	0.01 μM	90%
	0.1 μM	83%
	1 μM	72%
	10 μM	51%

*Confluent monolayers of BME cells in gelatin-coated 35 mm culture dishes were wounded with a razor blade to mark the original wound edge, washed twice with serum-free alpha-MEM, and serum-free alpha MEM/0.1% gelatin and the compound to be tested added. In experiments with suramin, monolayers were wounded in complete medium (i.e., in the presence of serum), and the medium not changed thereafter. After 15 h, monolayers were fixed and stained with 0.1% crystal violet in 20% ethanol for 30 min. Randomly selected fields measuring 1.0 mm × 1.4 mm were photographed using a Nikon Diaphot TMD inverted photomicroscope, and the total number of cells which had crossed the original wound edge determined. Experiments were performed in duplicate culture dishes; values are expressed relative to controls (where controls = 100%) and were calculated from the mean of three randomly selected fields per dish, i.e., six fields per condition.

We also show that U-24067, U-42129, and both Sigma and Hepar heparins inhibit BME cell proliferation and wound-induced migration (Tables I and II). The inhibitory effects of angiostatic steroids on endothelial cell proliferation, and the potentiating effect of heparin on this inhibition, have been reported [Sakamoto et al., 1986; Sakamoto and Tanaka, 1988; Cariou et al., 1988]. Heparin is known to potentiate the mitogenic effect of aFGF (reduces the ED_{50}) and to lower the K_d for binding to its receptor [Thornton et al., 1983; Schreiber et al., 1985]; this effect of heparin is not seen with bFGF [Saksela et al., 1988; Sommer and Rifkin, 1989]. On its own however, heparin has been reported to inhibit endothelial cell proliferation [Gospodarowicz et al., 1986; Rosenbaum et al., 1986]. Previous reports have indicated that heparin may increase [Azizkhan et al., 1980], decrease [Klein-Soyer et al., 1989], or have no effect [Sato and Rifkin, 1988] on endothelial cell migration.

Although it has been reported that the activity of U-24067 and U-42129 is strictly heparin dependent [Folkman et al., 1983; Ingber et al., 1986; Ingber and Folkman, 1988], others have found that U-24067 has angiostatic activity when tested alone on the CAM [Wilks et al., 1991] and inhibits the growth of B16 melanoma in mice when administered as a single agent (J.W. Wilks, unpublished observation). Wilks et al. [1991] did however find that heparin (which on its own had a small angiostatic effect) enhanced the inhibitory effect of U-24067. We have observed that at 1 μg/ml, Sigma heparin abrogates the anti-angiogenic effect of U-24067 in our three-dimensional system; when higher concentrations of heparin (10 and 100 μg/ml) were co-added with U-24067, in vitro angiogenesis was inhibited, although this effect was no greater than that observed with heparin alone (data not shown). Our in vitro findings therefore fail to corroborate the potentiating effect of heparin on the inhibitory activity of U-24067 in vivo. The reasons for these differences are not known.

TABLE II. Effects of Anti-Angiogenic Agents on bFGF-Induced BME Cell Proliferation *

		Values relative to bFGF-induced proliferation
U-24067	10 μM	31%
U-42129	10 μM	42%
Heparin (Sigma)	10 μg/ml	52%
Heparin (Hepar)	10 μg/ml	50%
Suramin	100 μg/ml	54%
	300 μg/ml	16%
Interferon alpha-2a	1 IU/ml	132%
	10 IU/ml	131%
	100 IU/ml	127%
	1,000 IU/ml	116%
Retinoic acid	0.01 μM	101%
	0.1 μM	45%
	1 μM	13%
	10 μM	3%

*BME cells were seeded at 1×10^4 cells per 23 mm well of a 12-well tissue culture plate (Costar, Cambridge, MA) in 1.5 ml complete medium. Anti-angiogenic compounds were added 2 h later, and bFGF (10 ng/ml) was added after a further 1 h (i.e., 3 h after plating). Medium was changed and bFGF and anti-angiogenic compounds added after 2 days. After a further 2 days (i.e., 4 day assay), cells were dissociated by exposure to trypsin and counted in a FACScan Analyzer (Becton-Dickinson, San José, CA). Experiments were performed in duplicate wells; mean values are expressed relative to bFGF-induced proliferation (taken to be 100%), and were calculated as follows:

$$\frac{(\text{cultures treated with bFGF and angiogenesis inhibitor} - \text{control cultures}) \times 100}{(\text{cultures treated with bFGF alone} - \text{control cultures})}.$$

Suramin

In addition to the variable in vivo and in vitro responses to heparin noted above, a high degree of variability in the heparin-dependency of the anti-angiogenic effect of angiostatic steroids has been reported [reviewed by Wilks, 1992]. This has been attributed principally to the heterogeneous composition of commercially available heparin [Folkman et al., 1983, 1989]. For example, heparin from Sigma was required at five times the concentration of heparin from Hepar to achieve an optimal angiostatic effect when co-administered with hydrocortisone [Folkman et al., 1989]. It should be noted however that in the in vitro studies described in this paper, we have obtained very similar results with heparin from both sources (data not shown). The variability of the heparin response observed in vivo led to the search and subsequent identification of beta cyclodextrin tetradecasulfate [Folkman et al., 1989] and suramin [Wilks et al., 1991; Gagliardi et al., 1992] as effective heparin substitutes, both of which are chemically defined molecules and homogeneous in composition.

Although suramin has traditionally been used in the treatment of parasitic diseases, it has recently been used with a variable degree of success as an antitumor compound [La Rocca et al., 1990; Stein, 1993]. Suramin interferes with the activity of a large number of enzymes and growth factors [La Rocca et al., 1990; Stein, 1993], and it has been suggested that the inhibitory effect of suramin on tumor growth is due in part to its anti-angiogenic effect [Wilks et al., 1991; Gagliardi et al., 1992; Pesenti et al., 1992]. Here we show that suramin has a biphasic effect on in vitro angiogenesis: at 100 μM, suramin increased bFGF-induced invasion, while at 300 μM invasion was inhibited (Fig. 5). At the invasion-potentiating dose of suramin (100 μM), BME cells invaded the underlying collagen gel from the surface monolayer to form anastomosing tube-like structures with widely patent lumina (Fig. 1). The biphasic effect of suramin in non-endothelial cell types has previously been noted: low doses (10–100 μM) stimulated, whereas higher doses (0.1–1.0 mM) inhibited proliferation [Els et al., 1990; Guo et al., 1990; Olivier et al., 1990; Pienta et al., 1991] and colony formation in soft agar [Coffey et al., 1987]. We have consistently observed that in the presence of 15% serum (used throughout the studies reported in this paper), suramin at concentrations of 500 μM and greater induces monolayer disruption, i.e., cell retraction and detachment, both on gelatin-coated tissue culture dishes and on the surface of three-dimensional collagen gels; this effect was observed at a 5-fold lower concentration (100 μM) in the absence of serum (data not shown). For this reason, the effect of suramin at concentrations greater than 300 μM on bFGF-induced invasion of collagen gels (assay performed in 15% serum) was not assessed. Suramin "toxicity" has previously been reported in endothelial [250 μM suramin in 0.5% serum, Coomber, 1993] and non-endothelial [Guo et al., 1990; Pienta et al., 1991] cell types.

Suramin inhibits bFGF-receptor interactions [Coffey et al., 1987; Sato and Rifkin, 1988; Middaugh et al., 1992] and removes bFGF bound to low affinity matrix binding sites and high affinity receptors in BAE cells [Sato and Rifkin, 1988]. Heparin has been reported to abrogate the anti-angiogenic effect of suramin in vivo

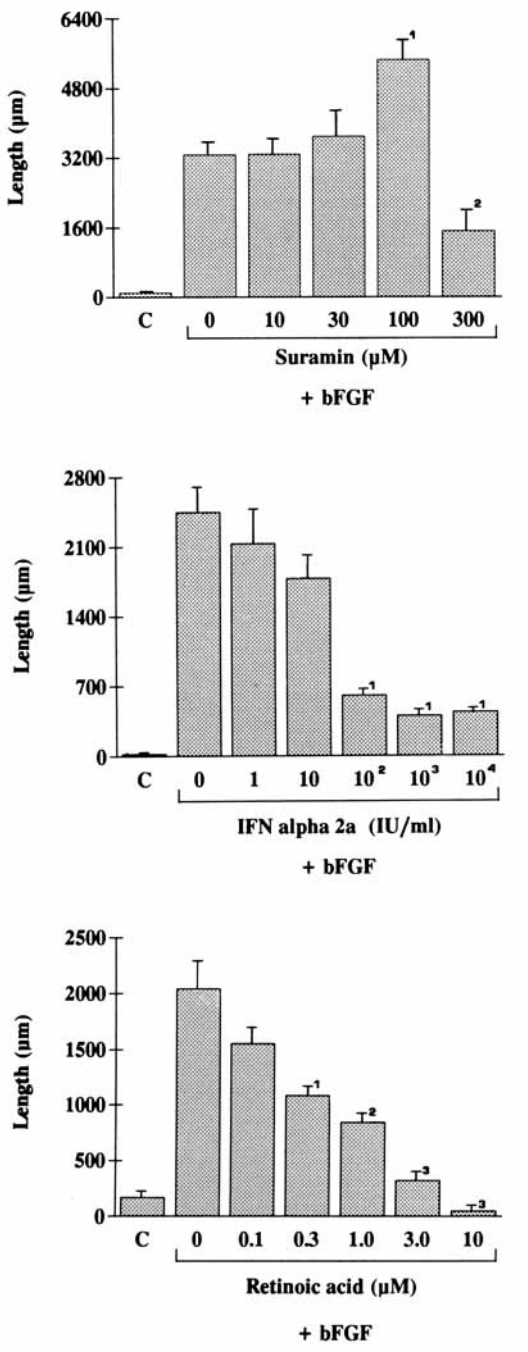

Fig. 5. Quantitative assessment of the effect of suramin, interferon alpha-2a, and retinoic acid on bFGF-induced in vitro angiogenesis. Values are mean ± SEM. C, control, non-treated cultures. The effect of suramin was biphasic: at 100 µM, suramin significantly increased invasion ($P^1 < 0.005$), whereas at 300 µM invasion was significantly inhibited ($P^2 < 0.02$). Interferon alpha-2a decreased invasion in a dose-dependent manner, with an ED_{50} of approximately 30 IU/ml ($P^1 < 0.001$). Retinoic acid also decreased invasion in a dose-dependent manner, with an ED_{50} of approximately 300 nM ($P^1 < 0.05$; $P^2 < 0.005$; $P^3 < 0.001$). Basal levels of invasion were unaffected by suramin, interferon alpha-2a, and retinoic acid at the concentrations shown (data not shown).

[Gagliardi et al., 1992; Pesenti et al., 1992]. This may be related to the observation that heparin competes for the binding of suramin to FGFs [Middaugh et al., 1992], since the biological functions of FGFs are maintained (bFGF) and enhanced (aFGF) after binding to heparin, while suramin impairs bFGF activity. With respect to bFGF-induced invasion of collagen gels, we have observed that Sigma heparin (1–100 µg/ml) partially overcomes the inhibitory effect of 300 µM suramin (data not shown), suggesting that this effect of suramin is due to its interaction with exogenously added bFGF. In addition, although monolayer disruption (100 µM suramin in the absence of serum) was unaffected by co-added Sigma heparin (1–100 µg/ml), this could be partially reversed by co-addition of 10 ng/ml bFGF (data not shown), suggesting that this "toxic" effect is related to suramin's interaction with endogenous bFGF.

Suramin had two major effects on the PA system of BME cells. First, cell-associated u-PA activity was decreased [as described by Sato and Rifkin, 1988] at doses which inhibit in vitro angiogenesis, and this activity could be recovered in the culture supernatants of the same cells (Fig. 6). These findings are likely to be related to the observation that suramin inhibits the binding of u-PA to its cell surface receptor [Behrendt et al., 1993]. Second, suramin greatly increased PAI-1 activity and mRNA levels in BME cells at doses which induced monolayer disruption (Figs. 6 and 7). Other forms of "stress" like hypoxia and hyperthermia have previously been reported to increase the expression of PAI-1 in endothelial cells [Wojta et al., 1988, 1991].

We also observed that suramin inhibited BME cell proliferation (Table II), which is consistent with the observation that suramin reversibly inhibits DNA synthesis in BAE cells [Sato and Rifkin, 1988]. In contrast, we observed that suramin had a biphasic effect on wound-induced migration, which correlates well with its biphasic effect on in vitro angiogenesis (Table I and Fig. 5). Previous studies have reported an inhibitory effect of suramin on endothelial cell migration [Sato and Rifkin, 1988; Coomber, 1993].

It has been reported that suramin potentiates the antiangiogenic effect of U-24067 and other angiostatic steroids [Wilks et al., 1991; Gagliardi et al., 1992]. Using our three-dimensional invasion model, we observed that U-24067 (1 and 10 µM) significantly decreased the potenti-

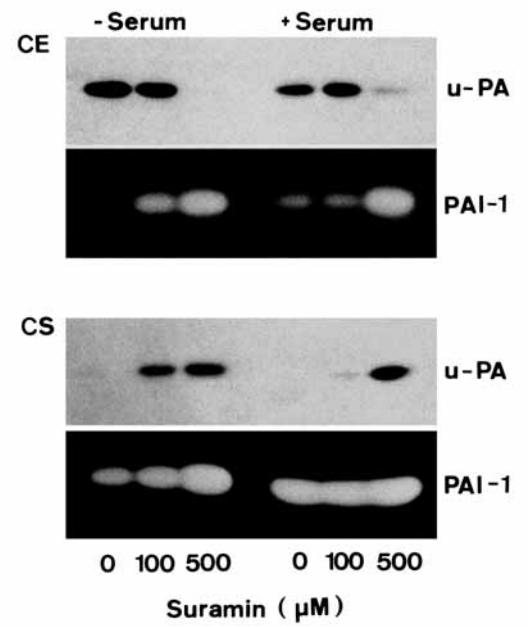

Fig. 6. Effect of suramin on BME cell u-PA and PAI-1 activity. Suramin had two major effects on the PA system of BME cells. First, both in the absence and presence of serum, suramin (100 μM and 500 μM, respectively) decreased u-PA activity in cell extracts (CE), which could be recovered in the culture supernatants (CS) of the same cells. Second, suramin greatly increased PAI-1 activity at 100 μM in the absence and 500 μM in the presence of serum, concentrations at which BME cell retraction and detachment occurred (data not shown).

ating effect of 100 μM suramin on bFGF-induced BME cell invasion; when U-24067 was co-added with lower doses of suramin (10 and 30 μM), the inhibition of bFGF-induced invasion was comparable to that seen with U-24067 alone (data not shown). Our in vitro findings therefore fail to corroborate the potentiating effect of suramin on the inhibitory effect of U-24067 in vivo. The reasons for these differences are not known.

Interferon Alpha-2a

Recently, interferon alpha-2a has been used in the treatment of childhood hemangiomatous diseases [White et al., 1989; Orchard et al., 1989; White et al., 1991; Ezekowitz et al., 1992]. This has been related in part to its ability to inhibit experimental angiogenesis in vivo [Sidky and Borden, 1987; Dvorak and Gresser, 1989]. Here we show that interferon alpha-2a inhibits bFGF-induced BME cell invasion and tube formation in vitro with an ED_{50} of approximately 30 IU/ml (Fig. 5). In contrast to our findings, Maheshwari et al. [1991] found that interferon alpha-2a stimulated matrigel-induced HUVE cell cord formation; this process was inhibited by

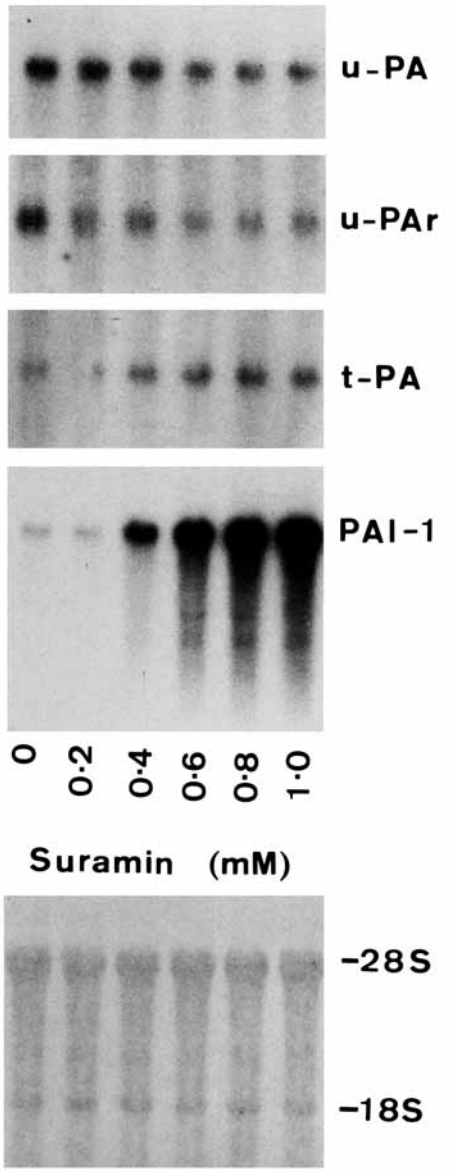

Fig. 7. Effect of suramin on BME cell u-PA, u-PAr, t-PA, and PAI-1 mRNA levels. Suramin increased PAI-1 mRNA levels in a dose-dependent manner: when BME cells were exposed to 400 μM suramin for 8 h, PAI-1 mRNA was increased 16-fold, and this increased to 116-fold above control values at 1.0 mM. In the same samples, u-PA and u-PAr mRNAs were decreased by 50–80%, while t-PA mRNA was increased approximately 2-fold.

human interferon gamma [Maheshwari et al., 1991], confirming the inhibitory effect of this cytokine on in vitro angiogenesis reported by others [Tsuruoka et al., 1988; Niedbala and Picarella, 1992]. The reasons for these differences may be due to differences in the in vitro assays employed.

We also demonstrate that interferon alpha-2a decreases basal and bFGF-induced u-PA and

TABLE III. Effects of Anti-Angiogenic Agents on BME Cell In Vitro Angiogenesis, PA System, Migration, and Proliferation (Growth)*

	Angiogenesis		u-PA Activity	u-PAr	PAI-1 Activity			
	In vivo[a]	In vitro	mRNA	mRNA	mRNA	Migration	Growth	Comments
Steroids (U-24067/ U-42129)	↓	↓	↓	↓	↓	↓	↓	
Interferon alpha-2a	↓	↓	↓	→	↓	↓	↑ (↓)[b]	
Heparin	↓ ↑ →	↓	↑[1]	→	→	↓ (↑ →)[b]	↓	1. mRNA, no effect
Retinoic acid	↓	↓	↑[1]	↑	↑	↓	↓	1. Synergism with bFGF
Suramin	↓	biphasic	↓[1]	↓	↑[2]	biphasic	↓	1. Also ↓ binding to u-PAr 2. Monolayer *disruption*

* ↓, decrease; ↑, increase; →, no effect.
[a]References cited in text.
[b]Where previous findings differ from our own, these are shown in parentheses; references cited in text.

PAI-1 activity in BME cells (Fig. 8), and that this is accompanied by a reduction in levels of the corresponding mRNAs: after 18 h of exposure to 1,000 IU/ml interferon alpha-2a, basal, and bFGF-induced u-PA mRNA levels were reduced by 33% and 27%, and basal and bFGF-induced PAI-1 mRNA levels by 81% and 90%, respectively; u-PAr levels were unaffected, while co-addition of bFGF and interferon alpha-2a slightly increased t-PA mRNA (data not shown). Interferon gamma has been reported to antagonize tumor necrosis factor (TNF)- and interleukin-1α-induced u-PA activity and mRNA increase in HUVE and human foreskin microvascular endothelial cells [Niedbala and Picarella; 1992; Wojta et al., 1992], as well as the ability of TNF to augment u-PA-mediated HUVE cell degradation of the extracellular matrix [Niedbala and Picarella, 1992]; this effect was specific for u-PA as interferon gamma did not antagonize TNF-induced PAI-1 antigen and mRNA increase. In contrast, interferon alpha failed to alter the TNF-induced increase in levels of plasminogen-dependent caseinolytic activity in HUVE cells [Niedbala and Picarella, 1992]. The reasons for these differences may be related to differences in the cell types used.

With respect to proliferation, we observed that interferon alpha-2a increased bFGF-stimulated BME cell growth (Table I). This is in agreement with the findings of Cozzolino et al. [1993], who recently reported that interferon alpha slightly increased HUVE cell proliferation, and that this

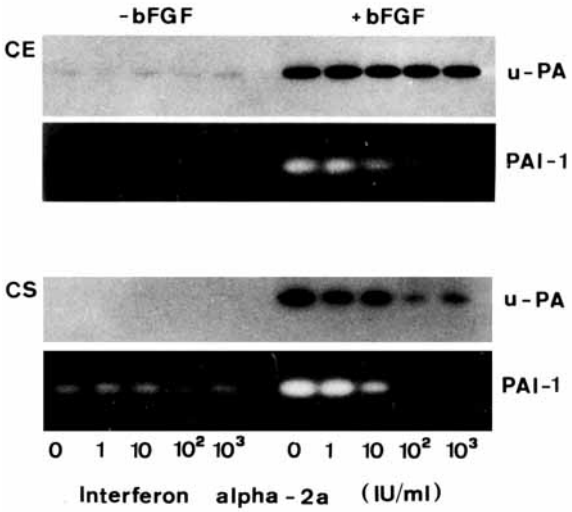

Fig. 8. Effect of interferon alpha-2a on BME cell u-PA and PAI-1 activity. Interferon alpha-2a decreased bFGF (10 ng/ml)-induced u-PA activity in culture supernatants (CS) and basal and bFGF-induced PAI-1 activity in CS and cell extracts (CE) of BME cells, with maximal inhibition at 100 IU/ml.

was mediated by enhanced bFGF synthesis and release. This is however in opposition to other reports in which interferon alpha-2a was shown to inhibit bovine aortic and human dermal microvascular endothelial cell proliferation in the range used in our present studies [Heyns et al., 1985; Ruszczak et al., 1990]. In contrast to its effect on proliferation, we observed that interferon alpha-2a decreased wound-induced BME cell migration in a dose-dependent manner

(Table II). Human leukocyte interferon has previously been shown to inhibit BME cell migration [Brouty-Boyé and Zetter, 1980].

Retinoic Acid

It has been reported that retinoids inhibit angiogenesis in vivo [Ingber and Folkman, 1988; Oikawa et al., 1989; Szmurlo et al., 1992]. Here we demonstrate that retinoic acid inhibits bFGF-induced endothelial cell invasion and tube formation in vitro with an ED_{50} of approximately 300 nM (Figs. 1 and 5).

We have observed that retinoic acid increases expression of all components of the BME PA system (Figs. 9 and 10), which is in agreement with previous reports [Inada et al., 1985; Kojima et al., 1986; Kooistra et al., 1991; Thompson et al., 1991; Medh et al., 1992; Krätzschmar et al., 1993]. However, the most striking effect was observed when retinoic acid was co-added with bFGF: this induced a potent synergistic increase in u-PA, with a similar although less marked increase in t-PA; this synergistic effect was not observed with u-PAr and PAI-1 (Figs. 9 and 10).

We also show that retinoic acid decreases bFGF-stimulated BME cell proliferation and two-dimensional wound-induced migration in a dose-dependent manner (Tables I and II). This

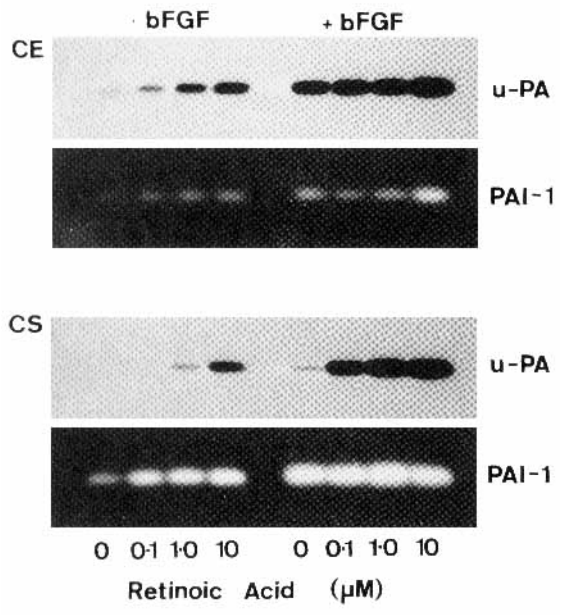

Fig. 9. Effect of retinoic acid on BME cell u-PA and PAI-1 activity. Retinoic acid (0.1, 1, and 10 μM) increased basal levels of u-PA and PAI-1 activity in cell extracts (CE) and culture supernatants (CS) of BME cells. When retinoic acid was co-added with bFGF a potent synergistic increase in u-PA activity was observed, which was most evident in the CS.

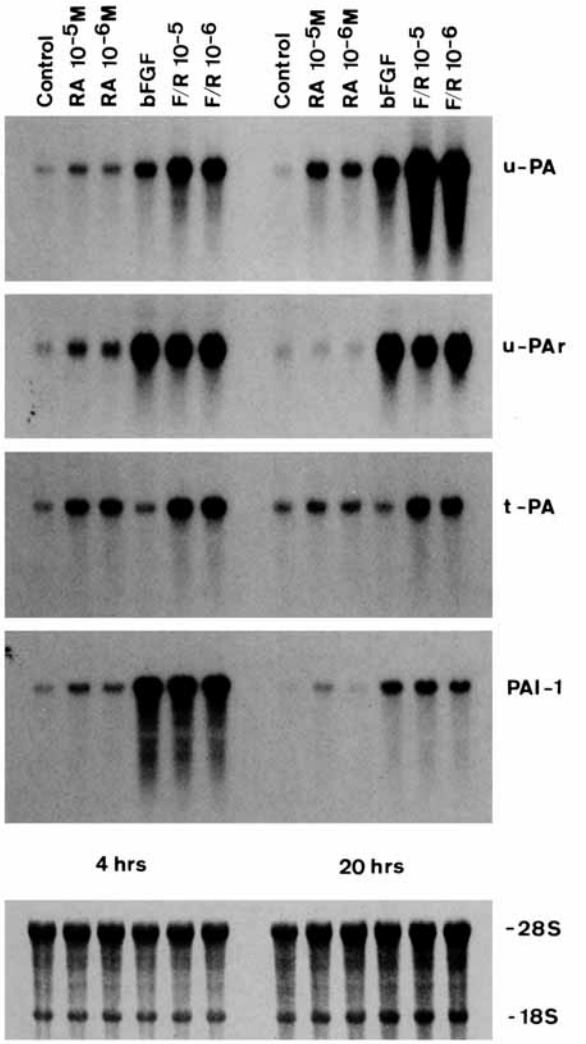

Fig. 10. Effect of retinoic acid on BME cell u-PA, u-PAr, t-PA, and PAI-1 mRNA levels. Retinoic acid (RA, 1 and 10 μM) increased mRNA levels of all components of the PA system, although differences in kinetics were observed; with the exception of t-PA, these increases were less than those induced by bFGF. The most striking effect was observed when retinoic acid (R) was co-added with bFGF (F): this induced a potent synergistic increase in u-PA mRNA levels. Thus, when retinoic acid was co-added at 10 μM, at the 20 h time point u-PA mRNA levels were increased 12.2- and 3.4-fold above levels induced by retinoic acid or bFGF alone, while co-addition of retinoic acid at 1 μM increased u-PA mRNA levels 14.4- and 2.8-fold above levels induced by retinoic acid or bFGF alone. A similar although less marked effect was observed with t-PA mRNA. This synergistic effect was not observed with u-PAr and PAI-1 mRNAs.

is in agreement with previous reports demonstrating that retinoids inhibit endothelial cell proliferation [Kojima et al., 1986; Braunhut and Palomares, 1991] and migration [Kojima and Rifkin, 1993], although Melnykovych and Clowes [1981] have reported that retinoids stimulate endothelial cell growth.

Kojima and Rifkin [1993] have recently reported that the inhibitory effect of retinoids on endothelial cell proliferation and migration is due to the effect of plasmin-activated TGF-β. We have previously shown that TGF-β1 has a biphasic effect on angiogenesis in our in vitro model [Pepper et al., 1993b]. Here we demonstrate that the inhibitory effect of retinoic acid on invasion in this model occurs over a wide range of concentrations, and clearly lacks the biphasic effect of exogenously added TGF-β1. It is therefore unlikely that retinoic acid-mediated inhibition in our system is due to activation of TGF-β1, although we cannot exclude the possibility that this may be due to activation of other isoforms of TGF-β. It will be important in future studies to determine whether the inhibitory effect of retinoids in our system can be abrogated by antibodies to TGF-β.

Summary and Conclusions

Angiogenesis is dependent on precisely controlled sequential alterations in a number of endothelial cell functions which include endothelial cell-matrix interactions, extracellular proteolysis, proliferation, and migration, each of which are potential targets for anti-angiogenic strategies. A large degree of variability has previously been described in the migratory and proliferative responses of endothelial cells to most of the agents we have employed. Since differences in cell types and culture conditions could account for this variability, we considered it important to measure these parameters in the BME cells used in our in vitro angiogenesis assay, despite the fact that the effects of most of the agents we have employed have previously been described. With respect to migration, we observed an excellent correlation between inhibition of bFGF-induced invasion of collagen gels and inhibition of migration in a widely used two-dimensional assay. A less consistent correlation was observed with proliferation. This suggests that although three-dimensional collagen gels are required for histotypic capillary-like tube formation, the two-dimensional migration assay might serve as an appropriate substitute in the screening of potential pharmacological inhibitors of angiogenesis.

Although an increase in proteolysis is required for extracellular matrix degradation during angiogenesis, this must be accompanied by a balanced increase in protease inhibitors to prevent excessive matrix degradation [Pepper and Montesano, 1990]. Alterations in protease and/or inhibitor activity might therefore be expected to perturb normal capillary morphogenesis [Montesano et al., 1990; Pepper et al., 1990]. From results reported in this and other correlative studies, a spectrum of alterations in extracellular proteolysis which accompany anti-angiogenesis is emerging. Thus endothelial cell proteolysis is decreased either as a consequence of an increase in PAI-1 by the angiostatic steroid MPA [Blei et al., 1993] and by leukemia inhibitory factor, which inhibits in vitro angiogenesis (M.S. Pepper, N. Ferrara, L. Orci and R. Montesano, manuscript submitted), or as a consequence of a decrease in u-PA in response to 2-methoxyestradiol (Pepper et al., in preparation), a natural estrogen metabolite with anti-angiogenic properties [Fotsis et al., 1994]. Both u-PA and PAI-1 are decreased by the angiostatic steroids U-24067 and U-42129 (this paper), interferon alpha-2a (this paper), and genistein, a dietary-derived inhibitor of in vitro angiogenesis [Fotsis et al., 1993]. Suramin reduces cell-associated u-PA activity [Sato and Rifkin, 1988; Behrendt et al., 1993] at doses which inhibit angiogenesis in vitro (this paper), and greatly increases PAI-1 production at doses which induce monolayer disruption (this paper). However, it has also been shown that endothelial cell proteolysis is increased by heparin [Castellot et al., 1986; this paper] and retinoic acid [Inada et al., 1985; Kojima et al., 1986; Kooistra et al., 1991; Thompson et al., 1991; Medh et al., 1992; this paper]. Thus, although it has been well described that exogenously added protease inhibitors inhibit angiogenesis both in vivo and in vitro [Mignatti et al., 1989; Moses et al., 1990], the results summarized above demonstrate that it does not necessarily follow that agents which inhibit angiogenesis will reduce extracellular proteolysis. A reduction in extracellular proteolysis would be expected to reduce the ability of endothelial cells to overcome the mechanical barriers imposed by the surrounding extracellular matrix. However, since proteases also modulate cytokine activity, an increase in proteolysis could activate latent inhibitory cytokines, which in turn would inhibit endothelial cell proliferation and migration and indirectly reduce extracellular proteolytic activity [Flaumenhaft et al., 1992]. The spectrum of effects on different elements of the PA system observed in response to the agents assessed suggests that the role of modulations in extracellular proteolytic activity in anti-angiogenesis is likely to be varied and complex.

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REFERENCES

Andrade-Gordon P, Strickland S (1986): Interaction of heparin with plasminogen activators and plasminogen: Effects on the activation of plasminogen. Biochemistry 25:4033–4040.

Ashino-Fuse H, Takano Y, Oikawa T, Shimamura M, Iwaguchi T (1989): Medroxyprogesterone acetate, an anticancer and anti-angiogenic steroid, inhibits the plasminogen activator in bovine endothelial cells. Int J Cancer 44:859–864.

Azizkahn RG, Azizkhan JC, Zetter BR, Folkman J (1990): Mast cell heparin stimulates migration of capillary endothelial cells in vitro. J Exp Med 152:931–944.

Behrendt N, Rønne E, Danø K (1993): Binding of urokinase-type plasminogen activator to its cell surface receptor is inhibited by low doses of suramin. J Biol Chem 268:5985–5989.

Blei F, Wilson EL, Mignatti P, Rifkin DB (1993): Mechanism of action of angiostatic steroids: Suppression of plasminogen activator activity via stimulation of plasminogen activator inhibitor synthesis. J Cell Physiol 155:568–578.

Braunhut SJ, Palomares M (1991): Modulation of endothelial cell shape and growth by retinoids. Microvasc Res 41:47–62.

Brouty-Boyé D, Zetter B (1980): Inhibition of cell motility by interferon. Science 208:516–518.

Cariou R, Harousseau JL, Tobelem G (1988): Inhibition of human endothelial cell proliferation by heparin and steroids. Cell Biol Int Rep 12:1037–1047.

Castellot JJ, Kambe AM, Dobson DE, Spiegelman BM (1986): Heparin potentiation of 3T3-adipocyte stimulated angiogenesis: Mechanism of action on endothelial cells. J Cell Physiol 127:323–329.

Castellot JJ, Karnovsky MJ, Spiegelman BM (1982): Differentiation-dependent stimulation of neovascularization and endothelial cell chemotaxis by 3T3 adipocytes. Proc Natl Acad Sci USA 79:5597–5601.

Coffey RJ, Leof EB, Shipley GD, Moses HL (1987): Suramin inhibition of growth factor receptor binding and mitogenecity in AKR-2B cells. J Cell Physiol 132:143–148.

Coomber BL (1993): Centrosome reorientation in regenerating endothelial monolayers requires bFGF. J Cell Biochem 52:289–296.

Cozzolino F, Torcia M, Lucibello M, Ziche M, Platt J, Fabiani S, Brett J, Stern D (1993): Interferon-α and interleukin 2 synergistically enhance basic fibroblast growth factor synthesis and induce release, promoting endothelial cell growth. J Clin Invest 91:2504–2512.

Crum R, Szabo S, Folkman J (1985): A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230:1375–1378.

Dvorak HF, Gresser I (1989): Microvascular injury in pathogenesis of interferon-induced necrosis of subcutaneous tumors in mice. J Natl Cancer Inst 81:497–502.

Els MJJ, Berns EMJJ, Schuurmans ALG, Bolt J, Lamb DJ, Foekens JA, Mulder E (1990): Antiproliferative effects of suramin on androgen responsive tumor cells. Eur J Cancer 26:470–474.

Ezekowitz RAB, Mulliken JB, Folkman J (1992): Interferon alfa-2a therapy for life-threatening hemangiomas of infancy. N Engl J Med 326:1456–1463.

Fisher R, Waller EK, Grossi G, Thompson D, Tizard R, Schleuning W (1985): Isolation and characterization of the human tissue-type plasminogen activator structural gene including its 5′ flanking region. J Biol Chem 260: 11223–11230.

Flaumenhaft R, Abe M, Mignatti P, Rifkin DB (1992): Basic fibroblast growth factor-induced activation of latent transforming growth factor β in endothelial cells: Regulation of plasminogen activator activity. J Cell Biol 118:901–909.

Flaumenhaft R, Rifkin DB (1992): The extracellular regulation of growth factor action. Mol Biol Cell 3:1057–1065.

Folkman J, Klagsbrun M (1987): Angiogenic factors. Science 235:442–447.

Folkman J, Langer R, Linhardt RJ, Haudenschild C, Taylor S (1983): Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. Science 221:719–725.

Folkman J, Weisz PB, Joullié MM, Li WW, Ewing WR (1989): Control of angiogenesis with synthetic heparin substitutes. Science 243:1490–1493.

Fotsis T, Pepper M, Aldercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L (1993): Genistein, a dietary-derived inhibitor of in vitro angiogenesis. Proc Natl Acad Sci USA 90:2690–2694.

Fotsis T, Zhang Y, Pepper MS, Aldercreutz H, Montesano R, Nawroth PP, Schweigerer L (1994): 2-methoxyestradiol, an endogenous estrogen metabolite, inhibits angiogenesis and suppresses tumor growth. Nature 368:237–239.

Furie MB, Cramer EB, Naprstek BL, Silverstein SC (1984): Cultured endothelial cell monolayers that restrict the transendothelial passage of macromolecules and electrical current. J Cell Biol 98:1033–1041.

Gagliardi A, Hadd H, Collins DC (1992): Inhibition of angiogenesis by suramin. Cancer Res 52:5073–5075.

Gospodarowicz D, Massoglia S, Cheng J, Fujii DK (1986): Effect of fibroblast growth factor and lipoproteins on the proliferation of andothelial cells derived from bovine adrenal cortex, brain cortex, and corpus luteum. J Cell Physiol 127:121–136.

Gospodarowicz D, Cheng J (1986): Heparin protects basic and acidic FGF from inactivation. J Cell Physiol 128:475–484.

Gross J, Azizkhan RG, Biswas C, Bruns RR, Hsieh DST, Folkman J (1991): Inhibition of tumor growth, vascularization, and collagenolysis in the rabbit cornea by medroxyprogesterone. Proc Natl Acad Sci USA 78:1176–1180.

Guo X-J, Fantini J, Roubin R, Marvaldi J, Rougon G (1990): Evaluation of the effect of suramin on neural cell growth and N-CAM expression. Cancer Res 30:5164–5170.

Heyns A du P, Eldor A, Vlodavsky I, Kaiser N, Fridman R, Panet A (1985): The antiproliferative effect of interferon and the mitogenic activity of growth factors are independent cell cycle events. Exp Cell Res 161:297–306.

Inada Y, Hagiwara H, Kojima S, Shimonaka M, Saito Y (1985): Synergism of vitamins A and C on fibrinolysis. Biochem Biophys Res Commun 130:182–187.

Ingber D, Folkman J (1988): Inhibition of angiogenesis through modulation of collagen metabolism. Lab Invest 59:44–51.

Ingber D, Madri JA, Folkman J (1986): A possible mechanism for inhibition of angiogenesis by angiostatic steroids: Induction of capillary basement membrane dissolution. Endocrinology 119:1768–1775.

Jakobson AM, Hahnenberger R (1991): Antiangiogenic effect of heparin and other sulphated glycosaminoglycans in the chick embryo chorioallantoic membrane. Pharmacol Toxicol 69:122–126.

Klagsbrun M, D'Amore PA (1991): Regulators of angiogenesis. Annu Rev Physiol 53:217–239.

Klein-Soyer C, Beretz A, Cazenave J-P, Wittendorp-Rechenmann E, Vonesch J-L, Rechenmann RV, Driot F, Maffrand J-P (1989): Sulfated polysaccharides modulate effects of acidic and basic fibroblast growth factors on repair of injured confluent human vascular endothelium. Arteriosclerosis 9:147–153.

Kojima S, Hagiwara H, Shimonaka M, Saito Y, Inada Y (1986): Synergism of retinoids and L-ascorbic acid in producing plasminogen activator in endothelial cells. Biomed Res 7:155–159.

Kojima S, Rifkin DB (1993): Mechanism of retinol-induced activation of latent transforming growth factor-β in bovine endothelial cells. J Cell Physiol 155:323–332.

Konkle BA, Ginsburg D (1988): The addition of endothelial cell growth factor and heparin to human umbilical vein endothelial cell cultures decreases plasminogen activator inhibitor-1 expression. J Clin Invest 82:579–585.

Kooistra T, Opdenberg JP, Toet K, Hendriks HFJ, van den Hoogen RM, Emis JJ (1991): Stimulation of tissue-type plasminogen activator synthesis by retinoids in cultured human endothelial cells and rat tissues in vivo. Thromb Hem 65:565–572.

Krätzschmar J, Haendler B, Kojima S, Rifkin DB, Schleuning W-D (1993): Bovine urokinase-type plasminogen activator and its receptor: cloning and induction by retinoic acid. Gene 125:177–183.

La Rocca RV, Stein CA, Myers CE (1990): Suramin: Prototype of a new generation of antitumor compounds. Cancer Cells 2:106–115.

Maheshwari RK, Srikantan V, Bhartiya D, Kleinman HK, Grant DS (1991): Differential effects of interferon gamma and alpha on in vitro model of angiogenesis. J Cell Physiol 146:164–169.

Medh RD, Santell L, Levin EG (1992): Stimulation of tissue plasminogen activator production by retinoic acid: synergistic effect on protein kinase C-mediated activation. Blood 80:981–987.

Melynkovych G, Clowes KK (1981): Growth stimulation of bovine endothelial cells by vitamin A. J Cell Physiol 109: 265–270.

Middaugh CR, Mach H, Burke CJ, Volkin DB, Dabora JM, Tsai PK, Bruner MW, Ryan JA, Marfia KE (1992): Nature of the interaction of growth factors with suramin. Biochemistry 31:9016–9024.

Mignatti P, Mazzieri R, Rifkin DB (1991): Expression of urokinase receptor in vascular endothelial cells is stimulated by basic fibroblast growth factor. J Cell Biol 113: 1193–1202.

Mignatti P, Tsuboi R, Robbins E, Rifkin DB (1989): In vitro angiogenesis on the human amniotic membrane: Requirements for basic fibroblast growth factor-induced proteases. J Cell Biol 108:671–682.

Montesano R, Orci L (1985): Tumor-promoting phorbol esters induce angiogenesis in vitro. Cell 42:469–477.

Montesano R, Pepper MS, Möhle-Steinlen U, Risau W, Wagner EF, Orci L (1990): Increased proteolytic activity is responsible for the aberrant morphogenetic behaviour of endothelial cells expressing middle T oncogene. Cell 62: 435–445.

Montesano R, Pepper MS, Vassalli J-D, Orci L (1987): Phorbol ester induces cultured endothelial cells to invade a fibrin matrix in the presence of fibrinolytic inhibitors. J Cell Physiol 132:509–516.

Montesano R, Vassalli J-D, Baird A, Guillemin R, Orci L (1986): Basic fibroblast growth factor induces angiogenesis in vitro. Proc Natl Acad Sci USA 83:7297–7301.

Moscatelli D, Presta M, Rifkin DB (1986): Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis and migration. Proc Natl Acad Sci USA 83:2091–2095.

Moscatelli D, Rifkin DB (1988): Membrane and matrix localization of proteases: A common theme in tumor invasion and angiogenesis. Biochim Biophys Acta 948:67–85.

Moses MA, Sudhalter J, Langer R (1990): Identification of an inhibitor of neovascularization from cartilage. Science 248:1408–1410.

Niedbala MJ, Picarella MS (1992): Tumor necrosis factor induction of endothelial cell urokinase-type plasminogen activator mediated proteolysis of extracellular matrix and its antagonism by γ-interferon. Blood 79:678–687.

Norrby K, Sörbo J (1992): Heparin enhances angiogenesis by a systemic mode of action. Int J Exp Pathol 73:147–155.

Oikawa T, Hirotani K, Nakamura O, Shudo K, Hiragun A, Iwaguchi T (1989): A highly potent antiangiogenic activity of retinoids. Cancer Lett 48:157–162.

Olivier S, Formento P, Fischel JL, Etienne MC, Milano G (1990): Epidermal growth factor receptor expression and suramin cytotoxicity in vitro. Eur J Cancer 26:867–871.

Orchard PJ, Smith CM, Woods WG, Day DL, Dehner LP, Shapiro R (1989): Treatment of hemangioendotheliomas with alpha interferon. Lancet 2:565–567.

Pepper MS, Belin D, Montesano R, Orci L, Vassalli J-D (1990): Transforming growth factor beta-1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. J Cell Biol 111:743–755.

Pepper MS, Montesano R (1990): Proteolytic balance and capillary morphogenesis. Cell Diff Dev 32:319–328.

Pepper MS, Montesano R, Orci L, Vassalli J-D (1991): Plasminogen activator inhibitor-1 is induced in microvascular endothelial cells by a chondrocyte-derived transforming growth factor-beta. Biochem Biophys Res Commun 176: 633–638.

Pepper MS, Sappino A-P, Stöcklin R, Montesano R, Orci L, Vassalli J-D (1993a): Upregulation of urokinase receptor expression on migrating endothelial cells. J Cell Biol 122: 673–684.

Pepper MS, Vassalli J-D, Montesano R, Orci L (1987): Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. J Cell Biol 105:2535–2541.

Pepper MS, Vassalli J-D, Orci L, Montesano R (1993b): Biphasic effect of transforming growth factor-β1 on in vitro angiogenesis. Exp Cell Res 204:356–363.

Pesenti E, Sola F, Mongelli N, Grandi F, Spreafico F (1992): Suramin prevents neovascularization and tumor growth through blocking of basic fibroblast growth factor activity. Br J Cancer 66:367–372.

Pienta KJ, Isaacs WB, Vindivich D, Coffey DS (1991): The effects of basic fibroblast growth factor and suramin on cell motility and growth of rat prostate cancer cells. J Urol 145:199–202.

Ribatti D, Roncali L, Nico B, Bertossi M (1987): Effects of exogenous heparin on the vasculogenesis of the chorioallantoic membrane. Acta Anat 130:257–253.

Rosenbaum J, Tobelem G, Molho P, Bârzu T, Caen JP (1986): Modulation of endothelial cell growth induced by heparin. Cell Biol Int Rep 10:437–446.

Rosengart TK, Johnson WV, Friesel R, Clark R, Maciag T (1988): Heparin protects heparin-binding growth factor-1 from proteolytic inactivation in vitro. Biochem Biophys Res Commun 152:432–440.

Ruszczak Z, Detmar M, Imcke E, Orfanos CE (1990): Effects of rIFN alpha, beta, and gamma on the morphology, proliferation, and cell surface antigen expression of human dermal microvascular endothelial cells. J Invest Dermatol 95:693–699.

Saksela O, Moscatelli D, Rifkin DB (1987): The opposing effects of basic fibroblast growth factor and transforming growth factor beta on the regulation of plasminogen activator in capillary endothelial cells. J Cell Biol 105:957–963.

Saksela O, Moscatelli D, Sommer A, Rifkin DB (1988): Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. J Cell Biol 107:743–751.

Sakamoto N, Tanaka NG (1988): Mechanism of the synergistic effect of heparin and cortisone against angiogenesis and tumor growth. Cancer J 2:9–13.

Sakamoto N, Tanaka NG, Tohgo A, Ogawa H (1986): Heparin plus cortisone acetate inhibit tumor growth by blocking endothelial cell proliferation. Cancer J 1:55–58.

Sato Y, Rifkin DB (1988): Autocrine activities of basic fibroblast growth factor: Regulation of endothelial cell movement, plasminogen activator synthesis and DNA synthesis. J Cell Biol 107:1199–1205.

Schreiber AB, Kenney J, Kowalski WJ, Friesel R, Mehlman T, Maciag T (1985): Interaction of endothelial cell growth factor with heparin: Characterization by receptor and antibody recognition. Proc Natl Acad Sci USA 82:6138–6142.

Shubik P, Feldman R, Garcia H, Warren BA (1976): Vascularization induced in the cheek pouch of the syrian hamster by tumor and nontumor substances. J Natl Cancer Inst 57:769–774.

Sidkey YA, Borden EC (1987): Inhibition of angiogenesis by interferons: Effects on tumor- and lymphocyte-induced vascular responses. Cancer Res 47:5155–5161.

Sommer A, Rifkin DB (1989): Interaction of heparin with basic fibroblast growth factor: Protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. J Cell Physiol 138:215–220.

Stein CA (1993): Suramin: A novel antineoplastic agent with multiple potential mechanisms of action. Cancer Res 53: 2239–2248.

Szmurlo A, Marczak M, Jablonska S, Bollag W (1992): Antitumor action of retinoids: Inhibition of tumor cell line-induced angiogenesis and prevention of tumors in mice. Dermatology 184:116–119.

Taylor S, Folkman J (1982): Protamine is an inhibitor of angiogenesis. Nature 297:307–312.

Thompson LA, Nelles L, Collen D (1991): Effect on retinoic acid on the synthesis of tissue-type plasminogen activator and plasminogen activator inhibitor-1 in human endothelial cells. Eur J Biochem 201:627–632.

Thornton SC, Mueller SM, Levine EM (1983): Human endothelial cells: Use of heparin in cloning and long-term serial cultivation. Science 222:623–625.

Thorpe PE, Derbyshire EJ, Andrade SP, Press N, Knowles PP, King S, Watson GJ, Yang Y-C, Rao-Betté M (1993): Heparin-steroid conjugates: New angiogenesis inhibitors with antitumor activity in mice. Cancer Res 53:3000–3007.

Tsuruoka N, Sugiyama M, Tawaragi Y, Tsujimoto M, Nishihara T, Goto T, Sato N (1988): Inhibition of in vitro angiogenesis by lymphotoxin and interferon-γ. Biochem Biophys Res Commun 155:429–435.

Vassalli J-D, Belin D (1987): Amiloride selectively inhibits the urokinase-type plasminogen activator. FEBS Lett 214: 187–191.

Vassalli JD, Dayer J-M, Wohlwend A, Belin D (1984): Concomitant secretion of prourokinase and of a plasminogen activator-specific inhibitor by cultured human monocytes-macrophages. J Exp Med 159:1653–1668.

Vassalli J-D, Sappino A-P, Belin D (1991): The plasminogen activator/plasmin system. J Clin Invest 88:1067–1072.

White CW, Sondheimer HM, Crouch EC, Wilson H, Fan LL (1989): Treatment of pulmonary hemangiomatosis with recombinant interferon alfa-2a. N Engl J Med 320:1197–1200.

White CW, Wolf SJ, Korones DN, Sondheimer HM, Tosi MF, Yu A (1991): Treatment of childhood angiomatous diseases with recombinant interferon alfa-2a. J Pediatr 118: 59–66.

Wilks JW (1992): Steroid inhibitors of angiogenesis. In N.J. Alexander and C.d'Arcangues (eds): "Steroid Hormones and Uterine Bleeding." Washington D.C.: AAAS Press, pp 159–169.

Wilks JW, Scott PS, Vrba LK, Cocuzza JM (1991): Inhibition of angiogenesis with combination treatments of angiostatic steroids and suramin. Int J Radiat Biol 60:73–77.

Wojta J, Holzer M, Hufnagl P, Christ G, Hoover RL, Binder BR (1991): Hyperthermia stimulates plasminogen activator inhibitor type 1 expression in human umbilical vein endothelial cells in vitro. Am J Physiol 139:911–919.

Wojta J, Jones RL, Binder BR, Hoover RL (1988): Reduction in pO_2 decreases the fibrinolytic potential of cultured bovine endothelial cells derived from pulmonary arteries and lung microvasculature. Blood 71:1703–1706.

Wojta J, Zoellner H, Gallicchio M, Hamilton JA, McGrath K (1992): γ-interferon counteracts interleukin-1a stimulated expression of urokinase-type plasminogen activator in human endothelial cells in vitro. Biochem Biophys Res Commun 188:463–469.

Wun T-C, Capuano A (1987): Immunoradiometric quantitation of tissue plasminogen activator-related antigen in human plasma: Crypticity phenomenon and relationship to plasma fibrinolysis. Blood 69:1348–1353.

Zetter B (1988): Angiogenesis State of the art. Chest 93: 159S–166S.