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Inhibitory effect of suramin in rat models of angiogenesis in vitro and in vivo

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Abstract The aim of the present study was to test the ability of the chemotherapeutic agent suramin to inhibit angiogenesis in experimental models in vitro and in vivo. In the culture of rat aortic rings on fibronectin, suramin dose-dependently inhibited vascular cell growth, achieving the maximal effect (mean – 88% versus controls, $P < 0.05$) at 400 μg/ml. Image analysis showed that suramin could inhibit microvessel sprouting in fibrin from rat aortic rings as evaluated by the ratio between the cellular area and the mean gray value of the sample (sprouting index); suramin at 50 μg/ml significantly reduced the sprouting index from the control value of 0.35 ± 0.04 to 0.14 ± 0.02 mm^2/gray level ($P < 0.05$). Likewise, the area occupied by cells was 19.2 ± 1.8 mm^2 as compared with 41.8 ± 4.2 mm^2 in controls ($P < 0.05$). In the rat model of neovascularization induced in the cornea by chemical injury, suramin at 1.6 mg/eye per day reduced the length of blood vessels (0.7 ± 0.1 mm as compared with 1.5 ± 0.1 mm in controls, $P < 0.05$). In the same model the ratio between the area of blood vessels and the total area of the cornea (area fraction score) was decreased by suramin from 0.19 ± 0.02 in controls to 0.03 ± 0.003 ($P < 0.05$). Suramin given i.p. at 30 mg/kg per day markedly inhibited the neovascularization induced in the rat mesentery by compound 48/80 or conditioned medium from cells secreting the angiogenic protein fibroblast growth factor-3 (FGF-3). The area fraction score in control rats treated with compound 48/80 was 0.31 ± 0.03, and this was reduced to 0.07 ± 0.01 by suramin ($P < 0.05$). After i.p. administration of FGF-3 the area fraction score was reduced by suramin from 0.29 ± 0.03 to 0.05 ± 0.01 ($P < 0.05$). These results provide evidence that suramin exerts inhibitory effects on angiogenesis in both in vitro and in vivo models.

Key words Angiogenesis · Suramin · Experimental models · Image analysis

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Introduction

Angiogenesis, the development of new capillaries from preexisting blood vessels, is a complex process involving extensive interplay between cells, soluble factors, and extracellular matrix components. Neovascularization is controlled by the balance between stimulatory and inhibitory factors and by the interaction of endothelial cells with their microenvironment, particularly with the proteoglycans of the extracellular matrix (for a review see Cockerill et al. [6]). The imbalance toward stimulatory factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF), may result in the formation of a pathologic blood vessel network, a distinctive feature of malignant tissue. Indeed, the proliferation of a neoplasm might be sustained in part by the paracrine or autocrine stimulation of tumor cells by growth factors and matrix proteins released by the proliferating capillary endothelium or tumor cells and by the delivery of an adequate blood supply (for a review see Folkman [15]).

Suramin, a symmetric polysulfonated naphthylurea, was introduced into clinical use for the treatment of

onchocerciasis [18]. More recently, suramin has been investigated as an antineoplastic drug for the treatment of adrenal [22] and prostate cancer [23]. Suramin antagonizes several growth factors in vitro, including PDGF [20], insulin-like growth factor I (IGF-1) [32], epidermal growth factor (EGF) [28], bFGF [7], transforming growth factor-β (TGF-β), and VEGF [27]. Furthermore, suramin inhibits several enzymatic systems such as topoisomerase II, protein kinase C, DNA polymerase, ATPase, phosphoinositol kinase, and diacyl glycerol kinase [35].

Because of the key role of angiogenesis in tumor growth, the use of pharmacologic inhibitors of neovascularization is of great interest for the treatment of human cancer. The effect of suramin on angiogenesis has been documented in the chick chorioallantoic membrane (CAM) assay [16, 31] and in mice bearing the bFGF-secreting reticulosarcoma M 5076 [31]. The data presented in the literature, however, derive from a limited number of experimental models of angiogenesis, especially in the CAM assay, and offer a restricted view of the therapeutic potential of suramin in the management of pathologic neovascularization. To improve the existing knowledge on the chemotherapeutic activity of suramin and to characterize in more detail its anti-angiogenic potential, we tested this drug on in vitro and in vivo rat models of neovascularization.

Materials and methods

Materials and animals

Cell culture media and supplements, compound 48/80, and all other chemicals not listed in this section were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). Plastics for cell culture were supplied by Costar (Cambridge, Mass., USA). The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay kit (Cell Titer 96) was purchased from Promega (Madison, Wis., USA). Suramin was obtained from Bayer (Leverkusen, Germany) and was dissolved in 0.9% NaCl as a vehicle before use. $AgNO_3$/KNO_3 (75:25, w/w) applicators were purchased from Graham-Field Surgical (New Hide Park, N.Y., USA). Female Wistar rats weighing 200–250 g were obtained from Nossan (Milan, Italy) and were allowed unrestricted access to food and tap water; their care and handling were undertaken in accordance with the recommendations of the European Economic Community on animal experimentation.

Aortic sprouting on fibronectin

The procedure described by Diglio et al. [12] was followed in the present study. In brief, rats were anesthetized by intraperitoneal injection of urethane (1 g/kg) and a thoracotomy was performed. The thoracic aorta was excised and, after the removal of adventitia, 1-mm-long rings were cut. The bottom of each well of sterile 96-well cluster plate was coated with 20 μg of fibronectin and the rings of aorta were positioned in the center of each well containing 150 μl of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and suramin at 50–400 μg/ml or drug vehicle. Explants were then incubated at 37 °C in an atmosphere containing 5% CO_2 for 7 days. The aortic rings were then removed and cell proliferation was estimated by the MTT assay by reading of the absorbance at 570 nm with a model 450 Microplate Reader (Biorad, Richmond, Calif., USA). The percentage of reduction in cell proliferation relative to controls was expressed as the mean value ± SE of three independent experiments.

Aortic sprouting in fibrin gel

The method described by Nicosia and Ottinetti [24] was adopted with minor modifications. In brief, the bottom of each well of a 24-well cluster plate was coated with 1.2 mg of fibrinogen and 0.5 NIH U of thrombin dissolved in 400 μl of serum-free medium. After the fibrin gel had formed, the rings of aorta, obtained as described above, were embedded in the center of the matrix by an additional 400 μl of clotting fibrinogen. Next, 1 ml of DMEM medium supplemented with 10% FBS was added to each well and the cultures were incubated at 37 °C in an atmosphere containing 5% CO_2. ε-Aminocaproic acid was added to the culture medium at 300 μg/ml during the first 3 days, followed by 50 μg/ml for the remainder of the experiment, to inhibit fibrinolysis. The explants were treated with suramin at 50–800 μg/ml; the drug was added to the culture medium at the beginning of the 4th day of culture and was left until the end of the experiment (10th day); controls received DMEM medium only. Ten rings of aorta per treatment were photographed with a phase-contrast Leitz MD IL microscope (Leica, Heerbrugg, Switzerland) for image analysis.

Angiogenesis in the rat cornea induced by chemical injury

The experimental procedure of Proia et al. [33] was used with minor modifications. In brief, rats were deeply anesthetized with ether and both corneas, as previously described by Culton et al. [9], were cauterized with an $AgNO_3$/KNO_3 applicator for 7 s at a 3-mm distance from the corneoscleral limbus, which was followed by a single topical application of erythromycin ointment on the surface of the cornea [10]. Rats were treated 30 min later with a solution containing suramin at 10 mg/ml in ophthalmic vehicle (0.9% NaCl and 2.5% carboxymethylcellulose); treatments were done four times daily for a total dose of 1.6 mg/eye per day; control rats received vehicle only. After 6 days of treatment, each rat was killed by i.p. injection of 1 g/kg urethane; the upper body was perfused with 50 ml of Ringer's solution and then with 20 ml of a mixture of 11% gelatin and 10% filtered India ink in Ringer's solution. The gelatin mixture within the blood vessels was solidified by freezing of the eyes by dichlorodifluoromethane. Samples of cornea were fixed in 10% phosphate-buffered formaldehyde (pH 7.4) for 24 h, and three full-thickness peripheral radial cuts were made to allow flattening of the cornea. The samples were placed on a glass slide in mounting medium for microscopy, and ten images of corneas per treatment were photographed for image analysis. In this model a marked immunostaining to bFGF is obtained after 12 and 24 h [3].

Angiogenesis in the rat mesentery induced by compound 48/80

The mast-cell-degranulating compound 48/80 is a condensation product of *p*-methoxy-*N*-methylphenethylamine with formaldehyde and is highly angiogenic when injected i.p. in the rat [26]. In brief, compound 48/80 was given i.p. twice daily to animals starting at 1 mg/kg, and the dose was increased by 1 mg/kg daily to reach 5 mg/kg; control rats received vehicle alone. Suramin at 30 mg/kg per day was injected i.p. starting from the 1st day of compound 48/80 challenge; the treatment with suramin was well tolerated as demonstrated by observations of body weight and food intake. At the end of the study, animals were killed by anesthetic overdose and the peritoneal cavity was exposed. Three mesenteric windows, the triangular portions of mesentery delimited proximally by the vascular branches of mesenteric vessels and distally by the small bowel, were randomly dissected from each of ten animals after ligation of the vascular peduncle and were photographed with a Leitz stereomacroscope (Leica, Heerbrugg, Switzerland) for image analysis.

Angiogenesis in the rat mesentery induced by conditioned medium of MCF-10A$^{int\text{-}2}$ cells

Conditioned medium from *int*-2-transfected MCF-10A cells (MCF-10A$^{int\text{-}2}$) has been shown to induce neovascularization in the rat mesentery [8]. In brief, MCF-10A$^{int\text{-}2}$ cells were grown at 60–70% confluence for 5 days in DMEM/F-12 medium (1:1) containing 10% horse serum. The culture medium was collected and centrifuged at 2000 *g* for 10 min, and 1 ml was injected i.p. into rats twice daily for 10 days. Animals received i.p. suramin at 30 mg/kg per day for 10 days concurrently with conditioned medium injection, and at day 11 the rats were killed by injection of 1 g/kg urethane and laparotomized. Three mesenteric windows were randomly dissected from each of ten animals as described above and were photographed with a Leitz stereomacroscope (Leica, Heerbrugg, Switzerland) for image analysis.

Image analysis and data processing

Photographs obtained from the angiogenesis assays were digitized in a 512 × 512-pixel matrix using a color video camera TK-1280E (JVC, Tokyo, Japan) and a microcomputer processor. Digitized pictures were visualized on a high-resolution color display (Sampo, Tao-Yuan Hsien, Taiwan). The true-color image-analysis software package KS 300 v.1.2 (Kontron Elektronik GmbH, Eching, Germany) was run for interactive manipulation, quantification of the images, and data collection. Geometric calibrations were set with a sample of known dimensions, and a gray-scale analysis was performed to measure the density of the image that was in the range of 0–255, where 0 was black (presence of vascular structures) and 255 was white (absence of vascular structures). The threshold levels for each sample were set interactively by the software in the respective red, green, and blue (RGB) image partitions that form the full-color image. The area occupied by the vessels was calculated by the computer in a binary picture that resulted after application of the RGB-discrimination step.

In the fibrin culture of aortic rings the average length of microvessel sprouts was calculated as measured from the explant to the farthest edge of cell growth. The mean gray level of the sprouting area was then measured and the parameter sprouting index was defined as:

$$\textit{Sprouting index} = \textit{Sprouting area}/\textit{Mean gray level of sprouting area}.$$

To evaluate the neovascularization of the injured rat corneas we used the interactive threshold method. The area of the whole cornea was measured with a freehand command and the area fraction score was obtained as follows:

$$\textit{Area fraction score} = \textit{Area of new blood vessels}/\textit{Overall area of the cornea}.$$

The contour of blood vessels invading the cornea from the corneoscleral limbus was enhanced with a sharpening filter, which was used twice to enhance the contrast of adjacent pixels, and with a smoothing filter to eliminate extraneous noise in the image. The vessel length was then measured by assessment of the nonlinear distance between two points. In the digitized images of mesenteric windows the area fraction score was interactively calculated as follows:

$$\textit{Area fraction score} = \textit{Area of new blood vessels}/\textit{Overall area of the mesenteric window}.$$

The results (mean values ± SEM for n observations) of MTT assays and image analysis were subjected to statistical analysis by analysis of variance (ANOVA) followed by the Student-Newman-Keuls test; the level of significance was set at $P < 0.05$. The 50% inhibitory concentration (IC_{50}) of suramin on aortic sprouting was calculated by nonlinear least-squares fitting of the data using a computer program (GraphPad PRISM, San Diego, Calif., USA).

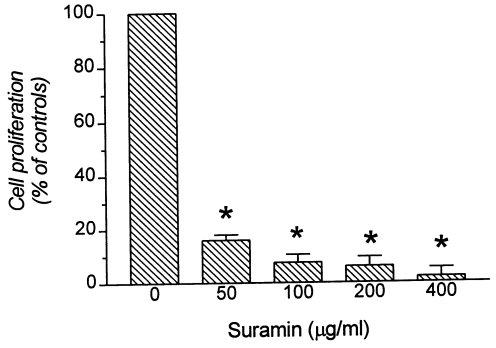


Fig. 1 Inhibition by suramin of vascular cell growth on fibronectin in the rat aortic ring explant assay. The proliferation of microvascular-like sprouts treated with suramin was estimated by the MTT assay and expressed as a percentage of control values. Data represent mean values for 3 independent experiments ± SE (*vertical bars*). *$P < 0.05$ versus controls

Results

Suramin inhibits aortic sprouting on fibronectin

Aortic rings formed microvascular-like sprouts after 4 days in culture; the sprouts were composed of overlapping vascular cells that extended radially from the explants and grew on the fibronectin-precoated surface of plastic wells. The attachment, proliferation, and migration of cells were greatly improved by precoating with fibronectin as compared with uncoated wells. The vascular cell population was composed of smooth-muscle and endothelial cells that proliferated rapidly for 4 days after the explant and formed a monostrate on the surface of the culture microwells, reaching a plateau at the 7th day. Treatment with suramin resulted in a concentration-dependent inhibition of the proliferation rate of vascular cells; their growth in the presence of suramin at 400 μg/ml was 2 ± 3.4% of the control value ($n = 3$, $P < 0.05$; Fig. 1). The mean IC_{50} of suramin on vascular cell proliferation was 17.7 ± 1.4 μg/ml.

Suramin inhibits aortic sprouting in fibrin gel

The aortic sprouting within the fibrin matrix was characterized by numerous microvessels around the aortic ring (Fig. 2, left). Maximal growth of the three-dimensional microvascular network occurred during the 1st week and reached a plateau at 10 days after the explant (Fig. 2, left). Vascular cells organized radially to form microvessels that underwent continuous remodeling. Suramin markedly inhibited the production of new microvessels by producing a decrease in the density and length of vascular sprouts (Fig. 2, right). The inhibition was concentration-dependent; at a suramin concentration of 50 μg/ml the sprouting index was 0.14 ± 0.02 as compared with 0.35 ± 0.04 mm^2/gray level in controls, and this value decreased to 0.014 ± 0.01 mm^2/gray level on treatment of rats with

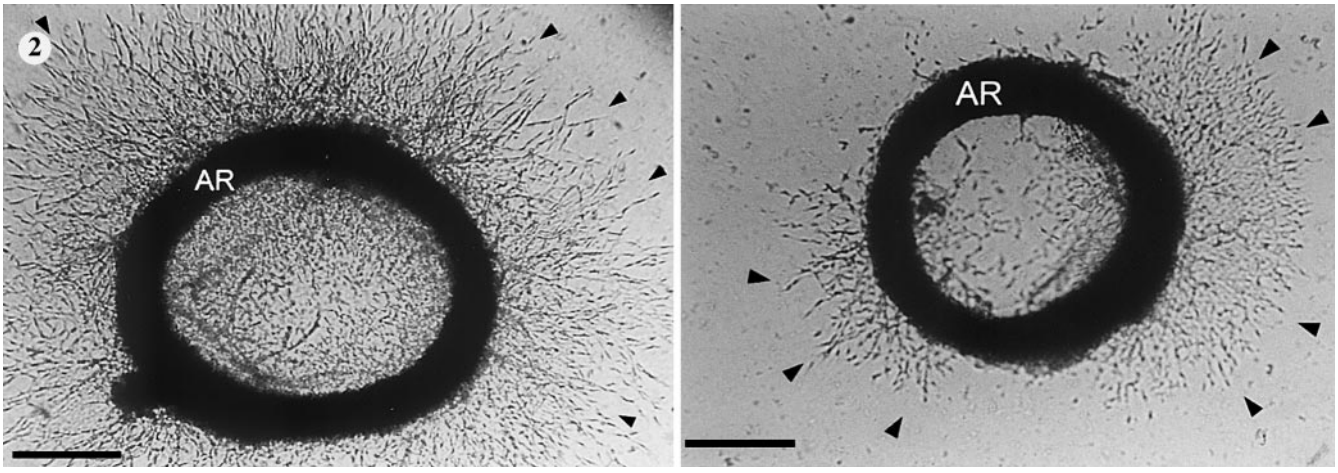


Fig. 2 Microscopic picture of a representative sample of aortic ring embedded in fibrin that shows microvessel structures extending radially from the explant (*left*, *arrowheads*). Suramin at 400 μg/ml markedly reduced the proliferation and migration of vascular cells within the extracellular matrix (*right*, *arrowheads*) (*AR* Aortic ring). *Bars* 1 mm

suramin at 400 μg/ml ($n = 10$, $P < 0.05$ versus controls), with the calculated IC_{50} being 36.8 ± 2.9 μg/ml. The area occupied by cells was 19.2 ± 1.8 mm^2 as compared with the control value of 41.8 ± 4.2 mm^2, and this area diminished to 2.4 ± 0.2 mm^2 following suramin administration at 400 μg/ml ($n = 10$, $P < 0.05$ versus controls). As measured from the aortic ring to the farthest edge of microvessel growth, the radius was 1.5 ± 0.2 mm at a suramin concentration of 50 μg/ml as compared with 2.8 ± 0.3 mm in controls, and this value was reduced to 0.34 ± 0.04 mm following treatment with suramin at 400 μg/ml ($n = 10$, $P < 0.05$ versus controls; Fig. 3).

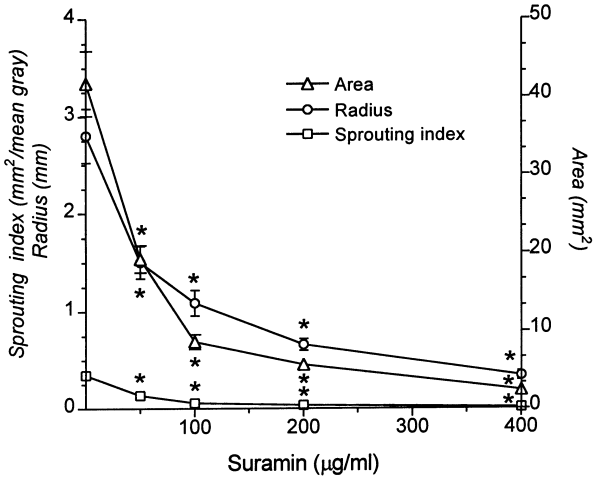


Fig. 3 Inhibition by suramin of aortic sprouting within the fibrin extracellular matrix. The effect of suramin is expressed by the area occupied by cells, the radius, and the sprouting index. *Points* represent mean values for 10 experiments ±SE (*vertical bars*). *$P < 0.05$ versus controls

Suramin inhibits angiogenesis in the rat cornea induced by chemical injury

Angiogenesis following chemical damage by $AgNO_3$/KNO_3 was characterized by numerous vessels originating from the limbar vascular arcades and invading the adjacent cornea after 2–3 days. The pericorneal vessels were dilated and showed tortuosities, and the conjunctive appeared thickened and edematous. Vessels continued to grow toward the burned area, and after 6 days a dense vascular network extending from the corneoscleral limbus to the cauterized site was observed in the corneas of control animals (Fig. 4, left). In the presence of suramin, vascular sprouts were reduced in number and their spread was limited to the cornea adjacent to the scleral limbus (Fig. 4, right). The mean length of new blood vessels was 1.5 ± 0.1 mm in corneas of control rats (Fig. 5); in contrast, topical treatment with suramin at 1.6 mg/eye per day was associated with a marked reduction in the length of new

Fig. 4 Angiogenesis in the $AgNO_3$/KNO_3-cauterized cornea of control rats. Numerous blood vessels originating from the limbar vascular arcades invade the adjacent avascular cornea toward the site of cautery (*left*, *arrowheads*). Inhibition of corneal angiogenesis by suramin applied at 1.6 mg/eye per day for 6 days (*right*). Few vessel loops penetrate into the cornea (*arrowheads*) (*S* Sclera, *C* cornea, *asterisk* lesion). *Bars* 3 mm

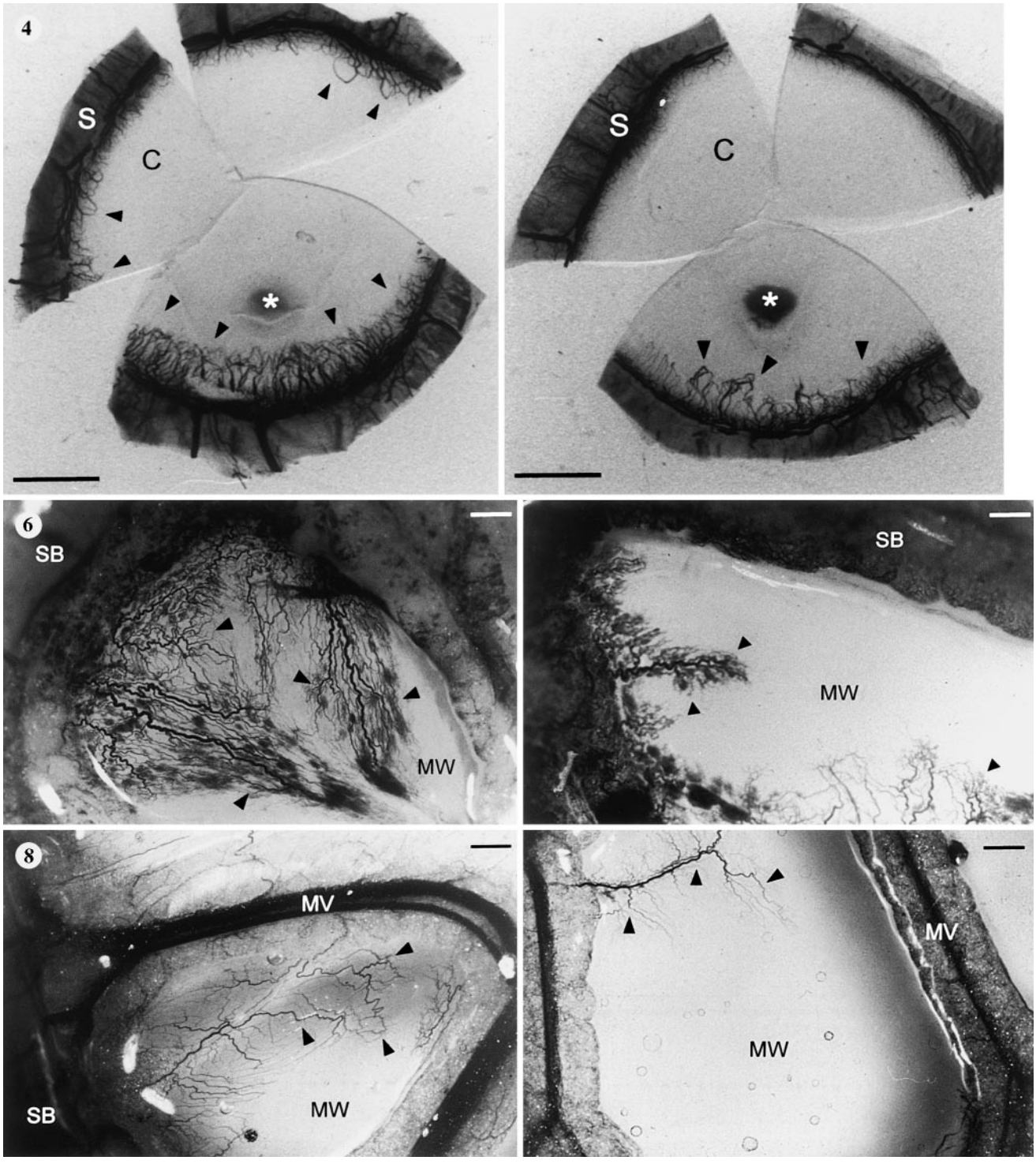
Fig. 6 Stereomacroscopic appearance of a mesenteric window of a rat treated twice daily for 5 days with compound 48/80. New large blood vessels (*arrowheads*) invaded the thickened mesentery with numerous dilations, tortuosities, and hemorrhages (*left*). In a rat treated with compound 48/80 and suramin at 30 mg/kg per day, few vessels grew within the mesentery (*right*, *arrowheads*) (*MW* Mesenteric window, *SB* small bowel). *Bars* 1 mm

Fig. 8a,b Stereomacroscopic appearance of a mesenteric window of a rat treated i.p. twice daily for 10 days with conditioned medium from *int-2*-infected MCF-10A cells. A dense capillary network (*arrowheads*) originating from the main mesenteric vessels developed through the stromal layer of the mesentery (*left*). In the rat treated with conditioned medium and suramin at 30 mg/kg per day, few capillaries invaded the mesenteric window (*right*) (*MV* Mesenteric vessel, *MW* mesenteric window, *SB* small bowel). *Bars* 1 mm

blood vessels growing toward the lesion (0.7 ± 0.1 mm, $n = 10$, $P < 0.05$ versus controls; Fig. 5). In this model the area fraction score of controls proved to be 0.19 ± 0.02; after topical administration of suramin the area fraction score decreased to 0.03 ± 0.003 ($n = 10$, $P < 0.05$ versus controls; Fig. 5).

Suramin inhibits angiogenesis in the rat mesentery induced by compound 48/80

In the mesentery of normal rats, blood vessels are macroscopically undetectable and small vascular structures can be detected only by microscopy. Intraperitoneal administration of compound 48/80 induced the formation of new large blood vessels within a



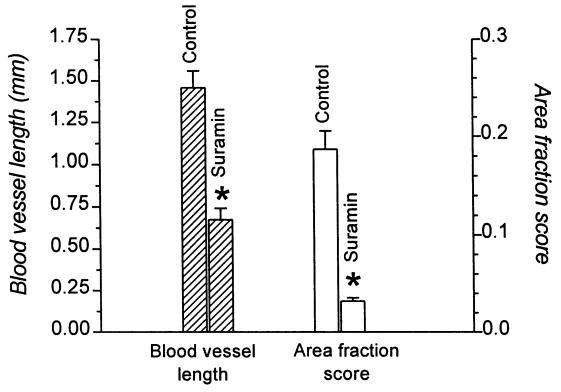


Fig. 5 Inhibition of angiogenesis by suramin in the rat cornea after chemical damage. The inhibition of angiogenesis was measured by the blood vessel length and the area fraction score. *Columns* represent mean values for 10 experiments ±SE (*vertical bars*). $^{*}P < 0.05$ versus controls

thickened mesentery (Fig. 6, left). Vascular growth was associated with hemorrhages of the peritoneal lining, and the new blood vessels presented numerous dilations and tortuosities (Fig. 6, left). After i.p. administration of suramin at 30 mg/kg per day, a few vessels penetrated into the mesentery and formed short loops (Fig. 6, right). The mean area fraction score in control rats treated with compound 48/80 was 0.31 ± 0.03 (Fig. 7); in suramin-treated animals this parameter was reduced to 0.07 ± 0.01 ($n = 10$, $P < 0.05$ versus controls; Fig. 7). The mesentery of rats injected with a solution of 0.9% NaCl presented normal, avascular membranous mesentery after the same period of treatment.

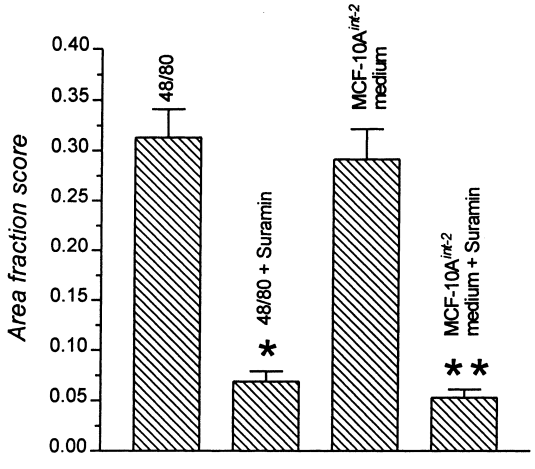


Fig. 7 Inhibition of neovascularization by suramin in the rat mesentery after i.p. injections of compound 48/80 or conditioned medium from MCF-10A^{int-2} cells. The inhibitory effect of suramin at 30 mg/kg per day was quantified by the area fraction score. *Columns* represent mean values for 10 experiments ±SE (*vertical bars*). $^{*}P < 0.05$ versus compound 48/80; $^{**}P < 0.05$ versus conditioned medium from MCF-10A^{int-2} cells

Suramin inhibits angiogenesis in the rat mesentery induced by conditioned medium of MCF-10A^{int2} cells

A dense capillary network originating from the preexisting branches of the mesenteric blood vessels developed through the stromal layer of the mesentery after i.p. injection of conditioned medium from MCF-10A^{int2} cells (Fig. 8, left). At variance with the angiogenesis induced by compound 48/80, hemorrhages were not detected (Fig. 8, left). The antiangiogenic effect of suramin given i.p. at 30 mg/kg per day was demonstrated by the presence of a few capillaries confined to the perivascular fat tissue with marginal involvement of the mesenteric windows (Fig. 8, right). In control rats the area fraction score was 0.29 ± 0.03; i.p. treatment with suramin significantly reduced this parameter to 0.05 ± 0.01 ($n = 10$, $P < 0.05$ versus controls; Fig. 7).

Discussion

The pharmacologic suppression of angiogenesis is an important field of investigation for the development of novel anticancer agents targeting the vascular endothelium and being potentially effective against a wide variety of human tumors [13]. Although the chemotherapeutic activity of suramin in the treatment of some malignancies, particularly prostate cancer, is well established, the inhibition of angiogenesis by this drug has been obtained in a limited number of experimental models, particularly the CAM assay [17]. The present study provides evidence that suramin is an angiogenesis inhibitor in both in vitro and in vivo models, including aortic sprouting, chemically injured rat cornea, and neovascularization in the rat mesentery as induced by compound 48/80 and medium from cells secreting FGF-3.

For assessment of the direct inhibitory effect of the drug on the proliferation of normal vascular cells, suramin was tested on two different models in vitro: the culture of aortic rings on fibronectin and their cultivation in fibrin. Furthermore, these models offer the advantages of better control of experimental conditions, including the concentration and time of drug exposure, and allow the construction of a concentration effect plot. In the first method, suramin was found to suppress vascular sprouts at concentrations starting from 50 μg/ml and ranging up to 400 μg/ml. This cytotoxic effect is in agreement with previous reports showing inhibition of the proliferation of bovine microvascular endothelial cells [30] and rabbit aortic smooth-muscle cells [2] by suramin. The antiproliferative effect of suramin could be dependent on its ability to block several enzymatic systems involved in cell proliferation, such as DNA polymerase α and δ, ATPase, and nuclear DNA topoisomerase II [35]. In addition, suramin has been shown to inhibit the biologic activity of PDGF, a potent mitogen for smooth-muscle cells [20] that participates in angiogenic processes.

The aortic ring model in fibrin offered the possibility to study the vascular cell growth in a three-dimensional extracellular matrix, where cells migrate and produce microvessel-like structures that interact with the fibrin microenvironment. In this model, suramin produced a marked reduction in microvessel formation, which could be explained by the suppression of endothelium-mediated proteolysis of the matrix [30]. Indeed, suramin has been shown to suppress the production of the urokinase-type plasminogen activator (uPA), a protease required for endothelial cell migration in bovine capillary endothelial cells [30, 37]. In the present study the pharmacologic inhibition of vascular cell proliferation in vitro was confirmed in experimental models of angiogenesis in vivo. Suramin significantly reduced neovascularization in the rat cornea after chemical injury as well as in mesentery exposed to injections of compound 48/80 and conditioned medium from MCF-10A^{int2} cells secreting the angiogenic factor FGF-3.

The cornea assay is a valid method for evaluation of the effect of drugs and growth factors affecting angiogenesis [25]. The mechanisms of corneal angiogenesis have been extensively studied, and various mediators appear to be involved in this process, particularly bFGF [1], a mitogenic factor for endothelial cells [19]. The production of bFGF in the corneal epithelium was significantly enhanced after chemical cauterization, and the increase appeared to be time-dependent. Corneas sampled from eyes at 12 h after the cautery displayed positive bFGF immunostaining, which was significantly increased in these eyes as compared with undamaged corneas [3]. The release of bFGF may represent the signal for the development of a capillary network as reported for inflammatory macrophages [36]. In the present study, topical administration of suramin inhibited corneal angiogenesis induced by chemical damage, in agreement with a previous investigation using the corneal micropocket technique containing bFGF as the angiogenic stimulus [37]. When bFGF was complexed with heparin the effect was not observed [37], confirming the hypothesis that suramin exerts its activity through interaction with heparin-binding growth factors, particularly bFGF.

These findings could be explained on the basis of the polyanionic structure of suramin, which seems to be essential for the interaction with various heparin-binding growth factors [14] and for inhibition of the interaction of bFGF with its receptors [4, 7]. Indeed, suramin can remove the growth factors bound to low-affinity matrix-binding sites and to high-affinity receptors in bovine aortic endothelial cells [34].

Other charged molecules show the capacity to bind and inactivate angiogenic growth factors. The sulfonic derivatives of dystamicin A prevent the binding of bFGF and PDGF-β to their receptors in vitro and tumor angiogenesis in vivo [5]. Protamine, a 4.3-kDa arginine-rich cationic protein, binds heparin, inhibits endothelial cell migration and proliferation in vitro, and produces a decrease in tumor neovascularization in vivo [13].

The findings of this study indicate that suramin might interfere with the early stages of angiogenesis as previously described in the CAM [11], most likely via its ability to bind and inactivate angiogenic heparin-binding growth factors as demonstrated in the rat mesentery model of neovascularization induced by FGF-3, a potent angiogenic peptide [8]. In addition, suramin has been shown to be capable of reducing the in vivo growth of the murine M5076 sarcoma secreting bFGF, with maximal activity being observed when the compound was given from the 1st day after tumor transplant, thus supporting the hypothesis that the drug binds to and inactivates bFGF [31]. In the present study, suramin was capable of inhibiting the mesenteric angiogenesis induced by compound 48/80, an in vivo model characterized by a neoangiogenesis process that appears to be dependent on the release of several mediators, including PDGF, aFGF, and bFGF [29].

Although evidence of direct inhibition of angiogenic factors by suramin was not provided in the experimental models of the present study, the inhibitory effect of suramin on heparin-binding growth factors could be the most relevant mechanism of the pharmacodynamic effect of the drug. Animal studies have confirmed that suramin inhibits the angiogenesis induced by bFGF [11], VEGF [21], and hepatocyte growth factor; however, the drug is ineffective against the neovascularization produced by platelet-derived endothelial cell growth factor (PD-ECGF) [13], a peptide that is not bound by heparin.

In conclusion, the present report shows that suramin is an effective inhibitor of angiogenesis as demonstrated by in vitro assays and confirmed in in vivo models; the effects of the drug on the growth of blood vessels could contribute to its antitumor effect.

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References

1. Adamis AP, Meklir B, Joyce NC (1991) In situ injury-induced release of basic-fibroblast growth factor from corneal epithelial cells. Am J Pathol 139: 961–966
2. Asada Y, Tsuneyoshi A, Marutsuka K, Sumiyoshi A (1994) Suramin inhibits intimal thickening following intimal injury in the rabbit aorta in vivo. Cardiovasc Res 28: 1166–1169
3. Benelli U, Bocci G, Danesi R, Lepri A, Bernardini N, Bianchi F, Lupetti M, Dolfi A, Campagni A, Agen C, Nardi M, Del Tacca M (1998) The heparan sulfate suleparoide inhibits rat corneal angiogenesis and in vitro neovascularization. Exp Eye Res 67: 133–142
4. Braddock PS, Hu DE, Fan T-PD, Stratford IJ, Harris AL, Bicknell R (1994) A structure-activity analysis of antagonism of the growth factor and angiogenic activity of basic fibroblast growth factor by suramin and related polyanions. Br J Cancer 69: 890–898
5. Ciomei M, Pastor IW, Mariani M, Sola F, Grandi M, Mongelli N (1994) New sulfonated distamycin A derivatives with bFGF complexing activity. Biochem Pharmacol 47: 295–302

6. Cockerill GW, Gamble JR, Vadas MA (1995) Angiogenesis: models and modulators. Int Rev Cytol 159: 113–160
7. Coffey RJ, Leof EB, Shipley GD, Moses ML (1987) Suramin inhibition of growth factor receptor binding and mitogenicity in ARK-2B cells. J Cell Physiol 132: 143–148
8. Costa M, Danesi R, Agen C, Di Paolo A, Basolo F, Del Bianchi S, Del Tacca M (1994) MCF-10A cells infected with the *int*-2 oncogene induce angiogenesis in the chick chorioallantoic membrane and in the rat mesentery. Cancer Res 54: 9–11
9. Culton M, Chandler DB, Proia AD, Hickingbotham D, Klintworth GK (1990) The effect of oxygen on corneal neovascularization. Invest Ophthalmol Vis Sci 31: 1277–1281
10. D'Amato RJ, Loughnan MS, Flynn E, Folkman J (1994) Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci USA 91: 4082–4085
11. Danesi R, Del Bianchi S, Soldani P, Campagni A, La Rocca RV, Myers CE, Paparelli A, Del Tacca M (1993) Suramin inhibits bFGF-induced endothelial cell proliferation and angiogenesis in the chick chorioallantoic membrane. Br J Cancer 68: 932–938
12. Diglio CA, Grammas P, Giacomelli F, Wiener J (1989) Angiogenesis in rat aorta ring explant cultures. Lab Invest 60: 523–531
13. Fan T-PD, Jaggar R, Bicknell R (1995) Controlling the vasculature, angiogenesis, anti-angiogenesis and vascular targeting of gene therapy. Trends Pharmacol Sci 16: 57–66
14. Firsching A, Nickel P, Mora P, Allolio B (1995) Antiproliferative and angiostatic activity of suramin analogues. Cancer Res 55: 4957–4961
15. Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Med 1: 27–31
16. Gagliardi ART, Hadd H, Collins DC (1992) Inhibition of angiogenesis by suramin. Cancer Res 52: 5073–5075
17. Gagliardi ART, Kassack M, Kreimeyer A, Muller G, Nickel P, Collins DC (1998) Antiangiogenic and antiproliferative activity of suramin analogues. Cancer Chemother Pharmacol 41: 117–124
18. Hawking F (1989) Suramin with special reference to onchocerciasis. Adv Pharmacol Chemother 15: 289–322
19. Hayek A, Culler FL, Beattie GM, Lopez AD, Cuevas P, Baird A (1987) An in vivo model for study of the angiogenic effects of basic fibroblast growth factor. Biochem Biophys Res Commun 147: 876–880
20. Hosang M (1985) Suramin binds to platelet growth factor and inhibits its biological activity. J Cell Biochem 29: 265–273
21. Hu DE, Fan T-PD (1995) Suppression of VEGF-induced angiogenesis by the protein tyrosine kinase inhibitor, lavendustin A. Br J Pharmacol 114: 262–268
22. La Rocca RV, Stein CA, Danesi R, Jamis-Dow CA, Weiss GH, Myers CE (1990) Suramin in adrenal cancer: modulation of steroid hormone production, cytotoxicity in vitro and clinical antitumor effect. J Clin Endocrinol Metab 71: 497–504
23. Myers CE, Cooper M, Stein CA, La Rocca RV, Walther MM, Weiss GH, Choyke P, Dawson N, Steinberg S, Uhrich MM, Cassidy J, Kohler DR, Trepel J, Linehan DR (1992) Suramin: a novel growth factor antagonist with activity in hormone-refractory metastatic prostate cancer. J Clin Oncol 10: 881–889
24. Nicosia RF, Ottinetti A (1990) Growth of microvessels in serum-free matrix culture of rat aorta. Lab Invest 63: 115–121
25. Niederkorn JY, Ubelaker JE, Martin JM (1990) Vascularization of corneas of hairless mutant mice. Invest Ophthalmol Vis Sci 31: 948–953
26. Norrby K, Jakobsson A, Sorbo J (1986) Mast-cell-mediated angiogenesis: a novel experimental model using the rat mesentery. Virchows Arch [Cell Pathol] 52: 195–206
27. Olander JV, Connolly DT, DeLarco JE (1991) Specific binding of vascular permeability factor to endothelial cells. Biochem Biophys Res Commun 175: 68–76
28. 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
29. Peacock DJ, Banquerigo ML, Brahn E (1992) Angiogenesis inhibition suppresses collagen arthritis. J Exp Med 175: 1135–1138
30. Pepper MS, Vassalli J-D, Wilks JW, Schweigerer L, Orci L, Montesano R (1994) Modulation of bovine microvascular endothelial cell proteolytic properties by inhibitors of angiogenesis. J Cell Biochem 55: 419–434
31. Pesenti E, Sola F, Mongelli N, Grandi M, Spreafico F (1992) Suramin prevents neovascularization and tumour growth through blocking of basic fibroblast growth factor activity. Br J Cancer 66: 367–372
32. Pollack M, Richard M (1990) Suramin blockade of insulin-like growth factor I-stimulated proliferation of human osteosarcoma cells. J Natl Cancer Inst 82: 1349–1352
33. Proia AD, Chandler DB, Haynes WL, Smith CF, Suvarnamani C, Erkel FH, Klintworth GK (1988) Quantitation of corneal neovascularization using computerized image analysis. Lab Invest 58: 473–479
34. 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
35. Stein CA (1993) Suramin: a novel antineoplastic agent with multiple potential mechanisms of action. Cancer Res 53: 2239–2248
36. Sunderkotter C, Roth J, Sorg C (1990) Immunohistochemical detection of bFGF and TNF-α in the course of inflammatory angiogenesis in the mouse cornea. Am J Pathol 137: 511–515
37. Takano S, Gately S, Neville ME, Herblin WF, Gross JL, Engelhard H, Perricone M, Eidsvoog K, Brem S (1994) Suramin, an anticancer and angiosuppressive agent, inhibits endothelial cell binding of basic fibroblast growth factor, migration, proliferation, and induction of urokinase-type plasminogen activator. Cancer Res 54: 2654–2660