

Angiostatic activity of synthetic inhibitors of urokinase type plasminogen activator

RAFAL SWIERCZ^{1,3}, EWA SKRZYPCZAK-JANKUN^{4,1}, MATT M. MERRELL², STEVEN H. SELMAN^{1,2,3}, and JERZY JANKUN^{1,2,3}

¹Urology Research Center, Departments of ²Urology, ³Physiology & Molecular Medicine, Medical College of Ohio, Toledo, Ohio 43614-2589; ⁴Instrumentation Center, College of Arts and Sciences, University of Toledo, Toledo, Ohio, 43606-3390.

Abstract. We hypothesize that tumor angiogenesis can be limited by the reduction of enzymatic activity of the urokinase type plasminogen activator. The proposed mechanism is elimination of proteolytic activity by the advancing tip of capillaries which utilize proteolysis to produce space needed for vessel expansion. To test our hypothesis, we have investigated the angiostatic activity of synthetic low molecular weight inhibitors of urokinase: amiloride, benzamidine, EGCG, B428, and B623 using the chicken embryo corioallantoic membrane (CAM) model. We found that all tested inhibitors of urokinase cause a significant reduction of angiogenesis.

Introduction

Many of the important physiological processes such as maturation of corpus luteum, endometrial regeneration, wound healing, and embryogenesis depend on angiogenesis. Angiogenesis is required in pathological processes such as cancer development. Neoplasms can grow to a few millimeters in diameter without a blood supply, but eventually must develop their own capillary network to survive (13). During the process of angiogenesis, tumor cells secrete a variety of growth factors, which stimulate the endothelial cells of adjacent blood vessel walls to proliferate, and migrate toward the tumor forming capillaries. The migrating tips of the developing blood vessels express large amounts of urokinase plasminogen activator (uPA) that create high proteolytic activity in proximity of the migrating vessel tip (1-4). Urokinase triggers the proteolytic cascade that results in generation of a high local concentration of plasmin. Active plasmin then degrades constituents of stroma as well as activates other proteases involved in extracellular matrix degradation (5, 6).

High proteolytic activity on the tip of capillary vessels is required to produce space needed for vessel expansion. The activity of uPA as it relates to angiogenesis appears to be independent of its activity in local tumor invasion and metastasis (19).

Folkman has shown that angiostatin restricts formation of new blood vessels by inhibiting proliferation of endothelial cells reducing the size and even completely eliminating established cancers in mice (7). In our previous study, we showed that uPA inhibitors reduce the size of transplantable cancers in the SCID mouse (19). Since the effect was observed in cancers that express and do not express uPA, we hypothesized that inhibitors prevent angiogenesis rather than directly inhibiting tumor growth. In this project, we tested the hypothesis that angiogenesis is reduced or inhibited if uPA enzymatic activity is abolished by inhibiting degradation of extracellular matrix (ECM) thereby limiting the extracellular space needed for angiogenesis. We used several well known inhibitors of uPA to determine their effects on angiogenesis. Of the models of angiogenesis, the most frequently used are the chicken embryo corioallantoic membrane assay (8), the rabbit corneal pocket (9), the hamster cheek pouch (10), and the in vitro assay of angiogenesis (11). We have chosen chick embryo CAM assay as a simple, reliable and inexpensive method of studying angiogenesis. We have investigated the angiostatic capacity of five synthetic low molecular weight inhibitors of uPA: amiloride, benzamidine, (-) epigallocatechin-3 gallate (EGCG), 4-iodine-benzo[b]thiophene-2-carboxamidine (B428), and 4-benzodioxolanylenyl-benzo[b]thiophene-2-carboxamidine (B623). We have found that all tested inhibitors of urokinase significantly reduce angiogenesis.

Materials and methods

Eggs. Fertilized Leghorn white eggs were purchased from the Embryo Division of Hertzfeld Poultry Farms, Inc. (Waterville, OH). Prior to incubation, eggs were stored at 4°C.

Correspondence to: Dr. Jerzy Jankun, Urology Research Center, Department of Urology, Medical College of Ohio, 3000 Arlington Avenue, Toledo, OH 43699-0008, U.S.A. Phone: (419) 383-3691; Fax: (419) 383-3168; E-mail: jerzy@golemiv.dh.mco.edu; Internet address: <http://golemiv.dh.mco.edu/~jerzy/>

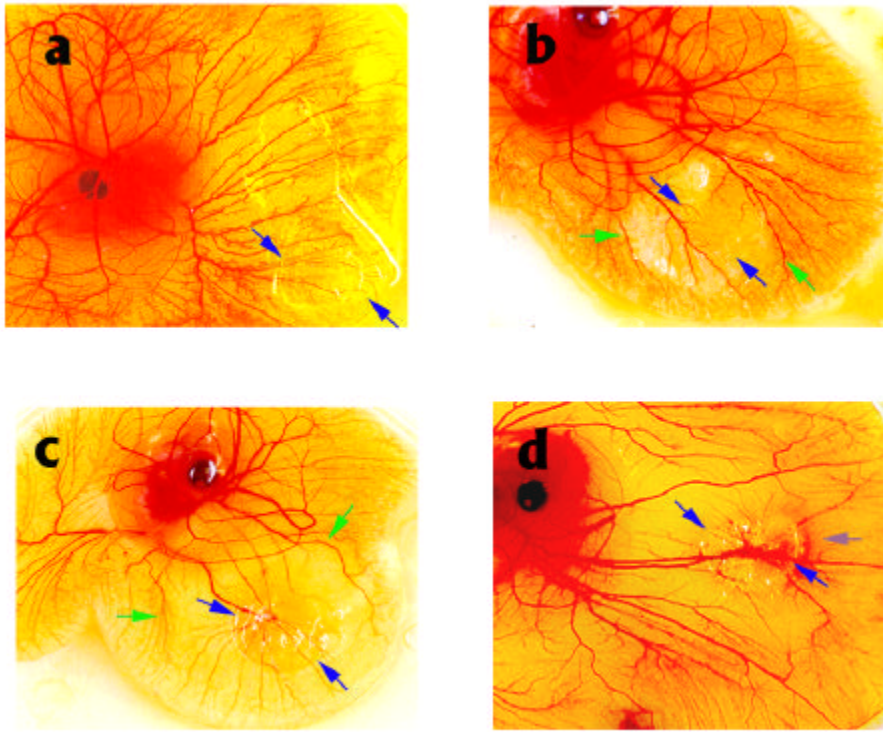


Fig. 1. Inhibition of angiogenesis by uPA inhibitors (a control, b amiloride - 30 µg/embryo, c B428 - 250 µg/embryo) and stimulation by VEGF (positive control). Green arrows indicate area of inhibited angiogenesis, blue ones shows approximate position of methylcellulose discs, magenta (d) area of increased angiogenesis.

Reagents and Chemicals. Amiloride, benzamidine, EGCG, vascular endothelial growth factor (VEGF) and methylcellulose were purchased from Sigma Chemical Co. (St. Louis, MO); B428 and B623 were the generous gifts from Eisai Research Institute (Andover, MA).

Preparation of the inhibitor solutions. Amiloride, and benzamidine were dissolved in sterilized deionized water to final concentrations of 10, 20, and 30 µg per 50 µl of water. B428 was dissolved or suspended in a 5% glucose solution to a final concentration of 5 mg/ml. B623 is less soluble than B428 and it was dissolved or suspended in a 5% glucose solution to a final concentration of 1 mg/ml. Later, B428 was diluted to 250, 125, and 62.5 µg per 50 µl of water, and B623 was diluted to 50, 25, and 12.5 µg per 50 µl of water. EGCG was diluted or suspended to 134, 67, and 35.5 µg per 50 µl of water. Vascular endothelial growth factor was reconstituted in PBS buffer (pH = 7.4) containing 0.1% of BSA to a concentration of 100 nM.

Preparation of methylcellulose disks. The disks were made by mixing 200 µl of 5% methylcellulose solution (in sterilized deionized water) with 50 µl of the inhibitor solution. Disks containing inhibitor were allowed to dry out under laminar flow after which they were implanted on the CAM of a 5-day-old chicken embryo.

Chicken chorioallantoic membrane (CAM) assay. The one-day-old fertilized eggs were incubated for three days in the water-jacketed

incubator (38°C, 85% humidity). Next, the eggs were cracked and the chick embryos with intact yolks were placed in plastic Petri dishes containing 10 ml of RPMI-1640 medium (38°C, 85% humidity, 3% of CO₂). After 3 days of incubation, the methylcellulose disk containing inhibitor was implanted on the CAMs of the individual embryos. After 48h of incubation, CAM of individual embryo was analyzed for formation of avascular zones and photographed. The angiostatic effect was determined as a percentage of the area of blood vessels under the methylcellulose disks (3-5 eggs for each concentration) in relation to the non-treated areas. Results were normalized to the control group.

Image processing and analysis. The area below the disk and two non-treated areas were scanned and saved on a computer disk as .tif files. Color images were converted into black/white images, contrast was enhanced and images were saved as 16-bitmap files using Paint software (Microsoft Corp., Redmond, WA). Next, black/white images were converted into false color (rainbow striped) images using Transform2 software (Fortner, Sterling, VA). Finally, the area of the blood vessels was calculated using T3D software (Fortner, Sterling, VA).

Statistical Analysis. Statistical analysis was performed using the one way ANOVA test followed by LSD test (12). All statistical calculations were performed using the SPSS software (Jandel Scientific Corp., Chicago, IL), and significance was established at the level of $P < 0.05$.

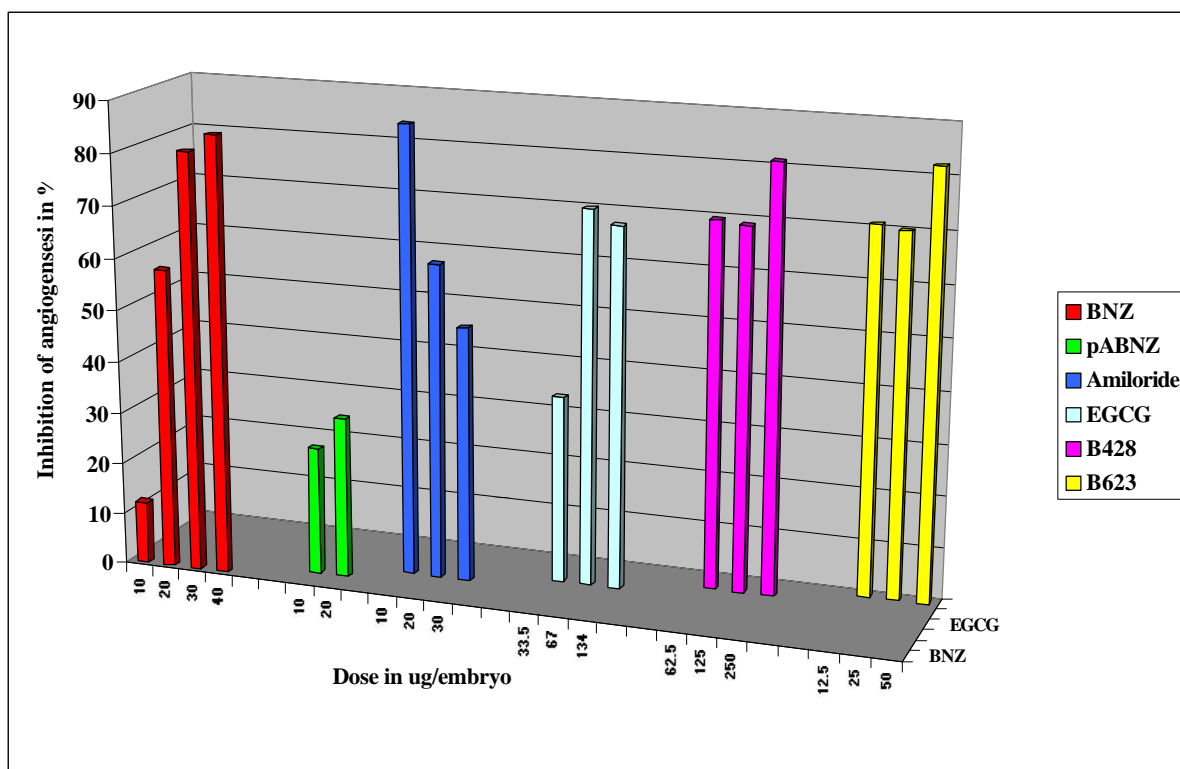


Fig. 2. Effect of different doses of uPA inhibitors on angiogenesis of 8-day old chorioallantoic membrane. Difference between treated and not-treated areas were statistically significant at the level of $P < 0.05$.

Results

All urokinase inhibitors tested reduce angiogenesis in the chick embryos as shown in Fig 1 and 2. We noticed that in case of EGCG, which possesses antioxidant activity, the methylcellulose disks quickly change color. EGCG produce a colorless or yellowish solution and the change of color to brown is a sign of a probable oxidation of this chemical. Therefore, inhibition of angiogenesis in this instance could be affected by these changes. Formation of embryonic neovascularization was significantly reduced under the methylcellulose disks in all cases as shown in Fig 1. Additionally, angiogenesis was observed in the large avascular zones outside of areas covered by methylcellulose disk containing the inhibitor. This effect was observed for B428 and amiloride. In contrast, control CAMs implanted on the empty methylcellulose disks without inhibitors did not develop avascular zones as determined by visual examination (Fig. 1a). However, computer image analysis revealed that some decrease in angiogenesis was observed and all results with uPA inhibitors were normalized to reflect this fact. As the positive control, we implanted methylcellulose disk containing VEGF. As it is demonstrated on Fig. 1d, dense areas of newly formed vessels were developed. In some cases we have observed, both in the control and in the treated group that the chick embryo died. Those experiments were repeated and only results observed on the surviving embryos were included in statistical evaluation. Whether or not the accidental death can be related to the toxicity of some uPA inhibitors or

simply due to the nature of this assay is outside the scope of this paper.

Discussion

Vascularization is a complex process of penetration of basement membranes by capillary vessels, proliferation of endothelium, and migration of endothelial cells toward a cancer mass that secretes a variety of growth factors (14). The role of various growth factors and proteolytic enzymes in the process of angiogenesis has been studied, characterized, and is well recognized, but relatively little has been reported about the role of urokinase plasminogen activator in neovascularization. All uPA inhibitors limited angiogenesis in the chick embryo model complementing other reports that inhibition of the components of the urokinase plasminogen activator, such as uPAR, or uPA itself could limit angiogenesis. Rosenberg has shown that binding of proteolytically inactive urokinase receptor ligands prevents cell surface plasminogen activation and consequently prevents angiogenesis in mouse model (15). He emphasized that uPA receptor focuses uPA initiated proteolytic activity on the migrating cell surface that is required for angiogenesis (20). Ignjatovic observed inhibition of angiogenesis in the rabbit cornea while treating animals with amiloride, one of the competitive inhibitors of urokinase plasminogen activator (9). The studies reported here establish that inhibitors of urokinase are similar in potency while compared to other inhibitors of angiogenesis. The dose required to obtain a ~50% reduction of vasularization was reported to be in the range from 4 $\mu\text{g}/\text{embryo}$ in case of ChDI to 500

µg/embryo in the case of suramin and 50% inhibitory doses of uPA inhibitors are well within these limits (17, 18).

In general, we observed that uPA inhibitors in the highest dose produced the highest inhibition of angiogenesis. However, it was not the case when amiloride was implanted on chick embryos. The lowest dose produced the highest reduction of angiogenesis. We envision this phenomenon as a false positive response; *e. g.* by two competing mechanism of inhibition and stimulation of angiogenesis by the same agent. It has been reported that in CAM assays almost any irritant or wound could produce false positives. This is not surprising in the sense that wound healing, irritant and inflammatory responses include an angiogenic component. Amiloride inhibits angiogenesis and at the same time could be an irritant that stimulates angiogenesis (21, 22).

The further study of uPA inhibitors is of great importance since inhibitors may lead to potential therapeutic agents for treatment of cancer and large number of other diseases that might be controlled by inhibition of the pathological angiogenesis.

Acknowledgments

This work was supported in part by grants from the American Diagnostica, Inc., Greenwich, CT, the MCO Foundation, Toledo, OH, and the OBR Research Challenge Grant. The authors want to express gratitude to Hania Kutcher for her excellent editorial work on this paper.

References

- Rabbani SA: Metalloproteases and urokinase in angiogenesis and tumor progression *In Vivo* 12: 135-142, 1998.
- Wilson MJ and Sinha AA: Human prostate tumor angiogenesis in nude mice: metalloprotease and plasminogen activator activities during tumor growth and neovascularization of subcutaneously injected matrigel impregnated with human prostate tumor cells. *Anat Rec* 249: 63-73, 1997.
- Gualandris A, Lopez Conejo T, Giunciuglio D, Albini A, Sabini E, Rusnati M, Dell'Era P and Presta M: Urokinase-type plasminogen activator overexpression enhances the invasive capacity of endothelial cells. *Microvasc Res* 53: 254-60, 1997.
- Pepper MS, Vassalli JD, Montesano R and Orci L: Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. *J Cell Biol* 105: 2535-2541, 1987
- Dvorak HF: Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 315: 1650-9, 1986.
- Goldfarb RH and Liotta LA: Proteolytic enzymes in cancer invasion and metastasis. *Semin Thromb Hemost* 12: 294-307, 1986.
- Folkman J: What is the evidence that tumors are angiogenesis dependent? *J Nat Cancer Inst* 82: 4-6, 1990.
- Ribatti D, Vacca A, Roncali L and Dammacco F: The chick embryo chorioallantoic membrane as a model for in vivo research on angiogenesis. *Int J Dev Biol* 40:1189-1197, 1996.
- Ignjatovic Z and Nikolic Lj: Inhibition of angiogenesis in the cornea with amiloride. *Srp Arh Celok Lek* 124: 120-123, 1996.
- Nishioka K and Katayama I: Angiogenic activity in culture supernatant of antigen-stimulated lymph node cells. *J Pathol*: 126: 63-69, 1978.
- Brown KJ, Maynes SF, Bezos A, Maguire DJ, Ford MD and Parish CR: A novel in vitro assay for human angiogenesis. *Lab Invest* 75: 539-55, 1996.
- Hazard-Murno B: Specific Statistical Techniques In: *Statistical Methods In Health Care Research*. Hazard-Murno B (Ed). Lippincott-Raven Publishers 1997, Philadelphia, New York.
- Lemoine NR, Wright NA: *The Molecular Pathology of Cancer*, Clod Spring Laboratory Press, 1993, Cold Spring.
- Moses MA, Sudhalter J, Langer R: Identification of an Inhibitor of Neovascularization from Cartilage, *Science*, 248, 1408-1410, 1990.
- Moses MA, Langer R: Inhibitors of Angiogenesis, *Biotechnology*, 9, 630-633, 1991.16. Min HY, Doyle LV, Vitt CR, Zandonella CL, Stratton-Thomas JR, Shuman MA, Rosenberg S: Urokinase Receptor Antagonists Inhibit Angiogenesis and Primary Tumor Growth in Syngeneic Mice. *Cancer Research*, 56: 2428-2433, 1996.
- Takano S, Gately S, Engelhard H, Tsanaclis AMC, Gross WF, Eidsvoog K, Neville M, Brem S: Angiosuppressive and Antiproliferative Actions of Suramin: Growth Factor Antagonist, Growth Factors, Peptides and Receptors, Edited by Noody TW, Plenum Press, New York, 1993.
- Moses MA, Sudhalter J, Langer R: Isolation and Characterization of an Inhibitor of Neovascularization from Scapular Chondrocytes, *The Journal of Cell Biology*, 19: 475-482, 1992.
- Jankun J, Keck RW, Selman SH, Skrzypczak-Jankun E, Swierz R: Inhibitors of Urokinase Restrict Growth of Prostate Cancer Xenografts in Severe Combined Immunodeficient Mice. *Cancer Research*, 57: 559-563, 1997.
- Rosenberg S: *The Role of Surface Bound Urokinase in Angiogenesis and Development of Specific Urokinase Receptor Antagonists*, New Cancer Strategies, Edited by Kuhl P, CHI, Waltham, 1993.
- Auerbach R, Auerbach W, Polanowski I: *Assays for Angiogenesis: A Review*, *Pharmac. Ther.* 51: 1-11, 1991.
- Karuri AR, Dobrowsky E, Tannock IF: Selective cellular acidification and toxicity of weak organic acids in an acidic microenvironment. *Br J Cancer*, 68:1080-1087 1993.