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In vivo angiogenic activity of urokinase: role of endogenous fibroblast growth factor-2

Domenico Ribatti[1], Daria Leali[2], Angelo Vacca[3], Roberta Giuliani[2], Anna Gualandris[2,*], Luisa Roncali[1], Maria Luisa Nolli[4] and Marco Presta[2,‡]

[1]Institute of Human Anatomy, Histology and Embryology, University of Bari, Piazza G. Cesare 11, 70124 Bari, Italy
[2]Unit of General Pathology and Immunology, Department of Biomedical Sciences and Biotechnology, University of Brescia, via Valsabbina 19, 25123 Brescia, Italy
[3]Department of Biomedical Sciences and Human Oncology, University of Bari, Piazza G. Cesare 11, 70124 Bari, Italy
[4]Biosearch Italia S.p.A., 21040 Gerenzano, Varese, Italy
*Present address: Department of Cell Biology, New York University Medical Center, 550 First Avenue, 10016 New York, NY, USA
‡Author for correspondence (e-mail: presta@med.unibs.it)

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SUMMARY

In vitro experimental evidences suggest that the proteolytic degradation of the extracellular matrix (ECM) by activation of the urokinase-type plasminogen activator (uPA)/plasmin system may affect growth factor activity and bioavailability. However, no direct in vivo observations were available to support this hypothesis. Here we demonstrate that endothelial GM 7373 cells overexpressing human uPA (uPA-R5 cells) cause the release of ^{125}I-labeled fibroblast growth factor-2 (FGF2) from endothelial ECM in a plasmin-dependent manner. Accordingly, uPA-R5 cells are angiogenic in vivo when applied on the top of the chorioallantoic membrane (CAM) of the chick embryo. In contrast, mock-transfected Neo2 cells are unable to release ECM-bound ^{125}I-FGF2 and are poorly angiogenic. Neovascularization elicited by uPA-R5 cells is significantly reduced by neutralizing anti-FGF2 antibodies to values similar to those observed in Neo2 cell-treated CAMs. Accordingly, purified human uPA stimulates neovascularization of the CAM in the absence of an inflammatory response. The angiogenic activity of uPA is significantly inhibited by neutralizing anti-FGF2 antibodies or by pretreatment with phenylmethylsulfonyl fluoride. The non-catalytic, receptor-binding amino-terminal fragment of uPA is instead non angiogenic. Taken together, the data indicate that uPA is able to induce angiogenesis in vivo via a plasmin-dependent degradation of ECM that causes the mobilization of stored endogenous FGF2.

Key words: Angiogenesis, Chick embryo, Extracellular matrix, FGF, Fibrinolysis, Urokinase

INTRODUCTION

Angiogenesis is the growth of new capillary blood vessels from preexisting ones in response to angiogenic stimuli. During this process, production of proteases is of pivotal importance for the degradation of basement membrane and for the invasion of perivascular extracellular matrix (ECM) by endothelial cells (Mignatti and Rifkin, 1993).

Several experimental evidences point to a role for the plasminogen activator (PA)/plasmin system in angiogenesis (Mignatti, 1993; Pepper et al., 1996). For instance, inhibition of urokinase-type PA (uPA) activity reduces prostaglandin E1-induced blood vessel growth in the rabbit cornea (Avery et al., 1990) and plasmin inhibitors inhibit FGF2-induced neovascularization in vivo in the mouse gelatin-sponge assay (Bastaki et al., 1997). Also, uPA mRNA expression correlates with neovascularization in ovarian follicles, corpus luteum, and maternal decidua (Bacharach et al., 1992). In vitro, the capacity of endothelial cells to form capillary-like structures on type I collagen is inhibited by anti-uPA antibody and by plasmin inhibitors (Yasunaga et al., 1989) and uPA-overexpression enhances the chemoinvasive capacity of cultured endothelial cells (Gualandris et al., 1997).

The hypothesis that the fibrinolytic system may play a role during angiogenesis is supported also by the observation that cytokines and angiogenic factors, including tumor necrosis factor α, fibroblast growth factor-2 (FGF2), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF), upregulate uPA (Moscatelli and Rifkin, 1988; Pepper et al., 1991; Bussolino et al., 1992; Koolwijk et al., 1996). Secreted uPA binds to its receptor (uPAR) on the endothelial cell surface whose expression is also upregulated by FGF2 and VEGF (Mignatti et al., 1991; Mandriota et al., 1995). uPAR interacts with the non-catalytic amino-terminal region (ATF) of single-chain pro-uPA. The bound zymogen is proteolytically activated in the two-chain form. This causes an acceleration of plasminogen activation which results in the degradation of major ECM components and activation of other

proteinases (Mignatti and Rifkin, 1993). Modulation of uPA and uPAR expression plays a central role in growth factor-induced human endothelial cell morphogenesis in 3-D fibrin gels (Koolwijk et al., 1996).

FGF2 is a heparin-binding polypeptide that induces proliferation, migration, and protease production in cultured endothelial cells and neovascularization in vivo (Basilico and Moscatelli, 1992). FGF2 interacts with endothelial cells through tyrosine-kinase FGF receptors and low affinity, high capacity heparan sulfate proteoglycan receptors (HSPGs) present on the cell surface and in the ECM (Johnson and Williams, 1993; Rusnati and Presta, 1996). Accordingly, FGF2 is associated with endothelial ECM in vitro (Vlodavsky et al., 1987; Rogelj et al., 1989) and basement membranes in vivo (Folkman et al., 1988; DiMario et al., 1989; Hageman et al., 1991). Newly synthesized FGF2 is stored in the ECM from where it is released to induce long-term stimulation of target cells (Bashkin et al., 1989; Presta et al., 1989; Rogelj et al., 1989). Because of its association with heparan sulfate, released FGF2 is protected from inactivation and endowed with a larger radius of diffusion (Gospodarowicz and Cheng, 1986; Flaumenhaft et al., 1990; Coltrini et al., 1993).

In vitro studies have shown that plasmin releases ECM-bound FGF2 by degrading the core protein of HSPGs (Saksela and Rifkin, 1990; Whitelock et al., 1996). This releasing mechanism is strictly controlled by cytokines such as TGF-β and FGF2 itself that affect the synthesis of uPA and its inhibitors in cultured endothelial cells, thus modulating the activation of the proenzyme plasminogen to plasmin (Saksela and Rifkin, 1988). These observations raise the hypothesis that the fibrinolytic system may modulate neovascularization not only by facilitating the invasive phase of the angiogenesis process but also by favoring the mobilization of stored extracellular FGF2. However, no in vivo experimental evidence exists to support this hypothesis.

To address this point, we took advantage of the observation that the chicken embryo chorioallantoic membrane (CAM) expresses FGF2 mRNA and protein (Ribatti et al., 1998) and that endogenous FGF2 plays a limiting role in the development of the vascular system of this embryonic membrane (Ribatti et al., 1995). Thus, the CAM may represent an unique in vivo system to assess the hypothesis that exogenous uPA may affect neovascularization via an endogenous FGF2-dependent mechanism of action. In agreement with this hypothesis, our findings demonstrate that uPA-overexpressing endothelial cells and purified uPA exert a potent angiogenic effect on the CAM which depends on the catalytic activity of the enzyme and is reversed by neutralizing anti-FGF2 antibody. To our knowledge this represents the first in vivo experimental evidence about the capacity of uPA to affect angiogenesis via an increased mobilization of endogenous FGF2.

MATERIALS AND METHODS

Materials

Recombinant human FGF2 was expressed in *Escherichia coli* and purified as described (Coltrini et al., 1993). Anti-FGF2 antibodies were provided by D. B. Rifkin (New York University Medical Center, New York, NY). Purified human uPA (30,000 i.u./mg of protein) was from Calbiochem (San Diego, CA). The 135 amino acid residue ATF (<1,000 i.u./mg of protein) was purified from human uPA (Corti et al., 1989).

Cells

uPA-R5 cells were originated from fetal bovine aortic endothelial GM 7373 cells by transfection with pRSV-uPA harboring the human *uPA* cDNA (Gualandris et al., 1997). Cells were grown in Eagle's minimal essential medium (EMEM), 10% fetal calf serum (FCS), vitamins, essential and non-essential amino acids. Bovine aortic endothelial cells were cultured in EMEM plus 10% FCS, 2% essential amino acids, and 2% vitamins. Chicken embryo fibroblasts were grown in EMEM plus 5% newborn CS and 10% tryptose phosphate broth.

uPA immunoprecipitation and uPA activity assay

After 30 minutes of preincubation in methionine-free medium, confluent uPA-R5 and Neo2 cells were labeled for 24 hours with [^{35}S]methionine (100 μCi/ml). Then, conditioned media were sequentially immunoprecipitated with anti-human uPA antiserum and then with anti-plasminogen activator inhibitor type-1 antiserum (Gualandris et al., 1997). Immunocomplexes were analyzed on SDS-8% polyacrylamide gels under nonreducing conditions and autoradiographed.

For uPA activity assay, confluent cells were incubated for 18-20 hours in fresh medium containing 0.4% FCS. Then, conditioned media were collected and uPA activity was measured by using the plasmin chromogenic substrate H-D-norleucyl-hexahydrotyrosil-lysine-p-nitroanilide-acetate (American Diagnostic, Greenwich, CT, USA) (Gualandris and Presta, 1995).

Dot-blot casein-zymography was performed by spotting 200 μl aliquots of purified uPA or ATF (1 μg/ml in 0.1% BSA/PBS) onto nitrocellulose membranes. Then, membranes were overlaid onto a casein plasminogen-agar plate containing a mixture of 1% agarose in 0.1 M Tris-HCl, pH 8.1, 2.4% low fat milk powder as a source of casein, and 4% chicken serum as a source of chicken plasminogen. Chicken serum was omitted from control plates. The lysis spots, developed after incubation at 37°C in a humidified atmosphere, were photographed against a dark background.

Preparation of extracellular matrix-coated dishes and release of ^{125}I-FGF2

FGF2 was iodinated as described (Rusnati et al., 1993) at 800 cpm/fmol. Bovine aortic endothelial cells were grown in 24-well plates. Five to seven days after confluence, cell layers were removed by exposure to 0.5% Triton X-100 plus 20 mM NH_4OH for 5 minutes at room temperature, followed by four washes in PBS (Falcone et al., 1993). Next, matrices were incubated for 2 hours at 4°C with 10 ng/ml ^{125}I-FGF2 plus 0.2% gelatin. After extensive washing, uPA-R5 and Neo2 cells were seeded on the top of radiolabeled matrices at 80,000 cells/cm^2 in fresh medium added with 10% heat-inactivated FCS in the absence or in the presence of 1 μg/ml of human plasminogen. At different times of incubation at 37°C, media were collected and released ^{125}I-FGF2 was measured with a γ-counter. Radioactivity released in the absence of cells was subtracted from all the values.

FGF2 production by uPA-R5 cells

Confluent uPA-R5 and Neo2 cells were incubated in fresh medium for 3 days. Conditioned media were collected and monolayers were washed with 2.0 M NaCl in PBS to elute HSPG-bound FGF2 (Rusnati et al., 1993). Then, cells were scraped with a rubber policeman and sonicated on ice at 50 Watts for 20 seconds in PBS. Quantification of intracellular, HSPG-bound, and released FGF2 was performed using the Quantikine^{TM} FGF basic Immunoassay (R&D Systems, Minneapolis, MN).

uPA binding to chick embryo fibroblasts

This was performed as described (Mignatti et al., 1991). Briefly, confluent cell cultures in 24-well plates were washed twice with PBS

and treated with 50 mM glycine-HCl, 0.1 M NaCl, pH 3.0, for 3 minutes at room temperature to remove endogenous, surface-bound uPA. Then, cultures were washed twice with 1 mg/ml BSA in PBS (PBS/BSA). 60 ng/well of purified uPA or ATF were then added in 250 μl of ice-cold PBS/BSA and the cells were incubated for 2 hours at 4°C with or without 250 μg/ml suramin. Parallel cell cultures were incubated with uPA and 600 ng/well of ATF. All the cultures were then washed three times with PBS/BSA and incubated with 100 μl/well of 50 mM glycine-HCl, 0.1 M NaCl, pH 3.0, for 3 minutes at room temperature to elute surface-bound molecules. Acid washes were neutralized immediately and tested for uPA activity as described above.

CAM assay

Gelatin sponges (1 mm^3; Gelfoam, Upjohn Company, Kalamazoo, USA) were placed on the CAM at day 8 and adsorbed with a 3 μl suspension of uPA-R5 or Neo2 cells in PBS (18,000 cells per sponge) (Ribatti et al., 1997). In some experiments, cell suspensions were mixed with anti-FGF2 antibody (400 ng/embryo) before implantation. In a second set of experiments, sponges were adsorbed with 1 μg/sample of human uPA or ATF with or without anti-FGF2 antibody. Sponges containing vehicle alone or 1 μg of recombinant FGF2 were used as negative and positive controls, respectively. At day 12, CAMs were photographed in ovo. In some experiments, blood vessels entering the sponge within the focal plane of the CAM were counted by two observers in a double-blind fashion at ×50 magnification (Brooks et al., 1994). Then, all CAMs were processed for light microscopy. Briefly, the embryos and their membranes were fixed in ovo in Bouin's fluid. The sponges, the underlying and immediately adjacent CAM portions were removed and paraffin embedded. Eight μm serial sections were cut according to a plane parallel to the surface of the CAM and stained with 0.5% aqueous solution of toluidine blue.

The angiogenic response was assessed histologically by a planimetric method of 'point counting' (Ribatti et al., 1997). Briefly, every third section within 30 serial slides from each specimen were analyzed under a 144-point mesh inserted in the eyepiece of the photomicroscope. The total number of intersection points occupied by transversally cut vessels (3-10 μm diameter) were counted at ×250 magnification inside the sponge and at the boundary between the sponge and the surrounding CAM mesenchyme in 6 randomly chosen microscopic fields for each section. Mean values ± s.d. were determined for each analysis. The vascular density was indicated by the final mean number of the occupied intersection points. Statistical analysis was performed using Student's *t*-test for unpaired data. The same experimental procedure was utilized to quantify the mononuclear cell infiltrate.

RESULTS

uPA-dependent release of ^{125}I-FGF2 from extracellular matrix

To assess whether uPA upregulation would cause the release of matrix-bound FGF2, we utilized an endothelial cell clone (uPA-R5) originated by the transfection of endothelial GM 7373 cells with an expression vector harboring the human *uPA* gene under the control of a viral promoter (Gualandris et al., 1997). uPA-R5 cells produce significant amounts of uPA with a consequent increase in their invasive capacity in vitro (Gualandris et al., 1997). Indeed, immunoprecipitation of the conditioned media of metabolically labeled cells demonstrates that uPA-R5 cells synthesize higher amounts of uPA protein in respect to Neo2 cells, whereas the two cell lines produce similar amounts of plasminogen activator inhibitor type-1 protein (Fig. 1A, inset). Accordingly, the uPA activity present in the conditioned medium of uPA-R5 cells is 10 times higher than in the control Neo2 clone (Fig. 1A) and corresponds to approximately 15.0 and 1.5 i.u. of uPA/10^6 cells/24 hours for the two cell lines, respectively.

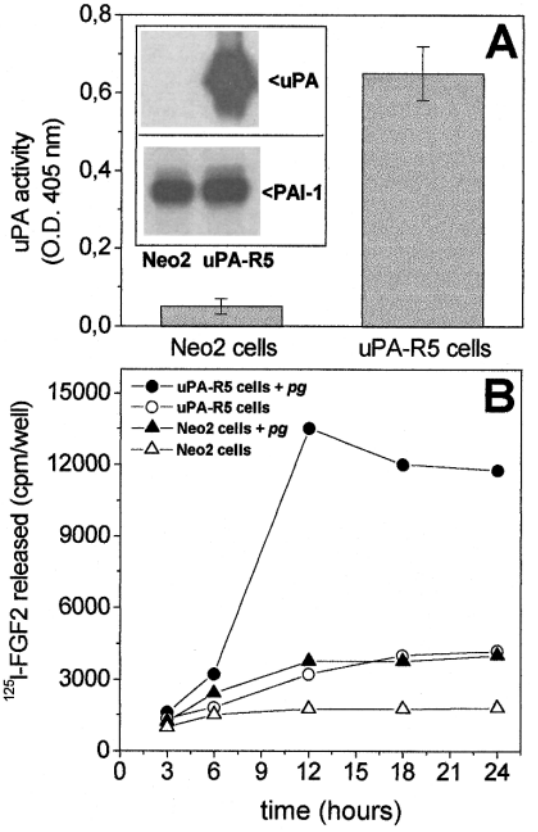

Fig. 1. uPA production and release of ECM-bound ^{125}I-FGF2 by uPA-R5 cells. (A) Chromogenic uPA activity assay performed on 10 μl of conditioned medium from uPA-R5 and Neo2 cells. Data are the mean ± s.e.m. of 3 determinations. Inset: Neo2 and uPA-R5 cells were metabolically labeled with [^{35}S]methionine for 24 hours. Then, their conditioned medium was sequentially immunoprecipitated with anti-uPA and anti-plasminogen activator inhibitor type-1 antibodies. Radiolabeled M_r 50,000 uPA (top) and M_r 54,000 PAI-1 (bottom) bands were visualized by autoradiography. No bands were detected by immunoprecipitation with non-immune serum (not shown). (B) Endothelial cell matrices were incubated for 2 hours at 4°C with 10 ng/ml ^{125}I-FGF2 plus 0.2% gelatin. After extensive washing, uPA-R5 (●,○) and Neo2 cells (△,▲) were seeded on the top of matrices at 80,000 cells/cm^2 in fresh medium added with 10% heat-inactivated FCS in the absence (○,△) or in the presence (●,▲) of 1 μg/ml of human plasminogen (*pg*). At different times of incubation at 37°C, media were collected and released ^{125}I-FGF2 was measured with a γ-counter. Radioactivity released at the same time points in the absence of cells was subtracted from all the values. Each value is the mean of three determinations.

On this basis, we monitored the capacity of uPA-R5 and Neo2 cells to release ^{125}I-FGF2 bound to bovine aortic endothelial ECM. As shown in Fig. 1B, Neo2 cells cause a very limited release of ^{125}I-FGF2 when added to ECM-coated wells both in the absence and in the presence of plasminogen. In contrast, uPA-R5 cells cause a dramatic increase of the amount of released ^{125}I-FGF2. This increase is strictly dependent upon the addition of plasminogen to the culture medium (Fig. 1B)

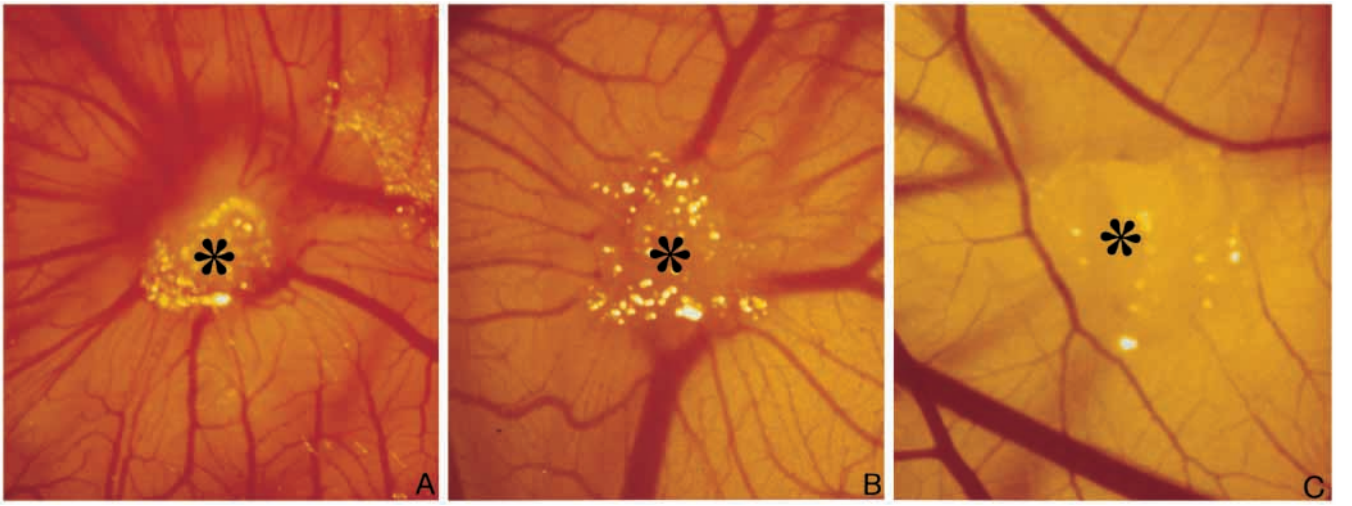

Fig. 2. Effect of uPA-R5 cells on CAM neovascularization. Cells were delivered at 18,000 cells per embryo on the top of the CAM at day 8 by using a gelatin sponge implant. Macroscopic observation of the membranes was performed at day 12. (A) Gelatin sponge adsorbed with uPA-R5 cells and surrounded by allantoic vessels (*n*=27) that develop radially towards the implant (asterisk) in a 'spoked-wheel' pattern. The allantoic vessels are less numerous (*n*=17) in the specimen treated with Neo2 cells (B) whereas no vascular reaction (*n*=2) is detectable around the vehicle-treated sponge (C). Original magnification, ×50.

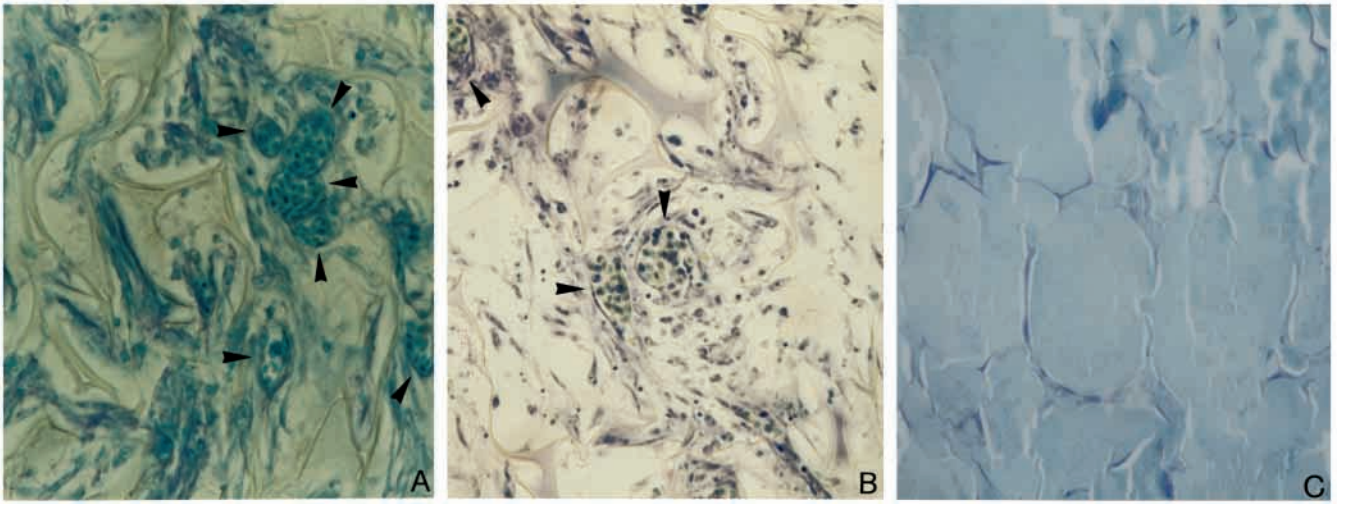

Fig. 3. Histological analysis of CAM grafted with uPA-R5 cells. CAMs were grafted with uPA-R5 cells or Neo2 cells. At day 12, CAMs were processed for light microscopy. A highly vascularized tissue is recognizable among the trabeculae of uPA-R5 cell-treated sponges (A). The tissue consisted of newly formed blood vessels (arrowheads) and of infiltrating fibroblasts within an abundant network of collagen fibers. The vessels are less numerous in the Neo2 cell-treated sponges (B) and absent in implants treated with PBS (C). Original magnification, ×250.

and is prevented by the uPA inhibitor amiloride (1 mM, data not shown), thus confirming the role of uPA in this process.

uPA-overexpressing cells induce a FGF2-dependent angiogenic response in the chick embryo CAM

FGF2 is expressed in the CAM where it plays a rate-limiting role in vascularization during development (Ribatti et al., 1995). To assess the possibility that uPA-R5 cells might induce angiogenesis by mobilizing endogenous FGF2, suspensions of uPA-R5 cells or Neo2 cells (18,000 cells per embryo) were delivered on the top of day 8 CAMs by using a gelatin sponge implant (Ribatti et al., 1997). Under these experimental conditions the two cell lines secrete approximately 1.0 and 0.1 i.u. of uPA throughout the experimental period, respectively. Macroscopic observation of the membrane at day 12 showed that the gelatin sponges adsorbed with uPA-R5 cells were surrounded by allantoic vessels that developed radially towards the implant in a 'spoked-wheel' pattern (Fig. 2A). The allantoic vessels were less numerous in the specimens treated with Neo2 cells (Fig. 2B) whereas no vascular reaction was detectable around the sponges treated with PBS only (Fig. 2C). At the microscope level, a highly vascularized tissue was recognizable among the trabeculae of uPA-R5 cell-treated sponges (Fig. 3A). The tissue consisted of newly formed blood vessels, mainly capillaries with 3-10 μm diameter growing perpendicularly to the plane of the CAM, and of infiltrating

Table 1. Angiogenic activity of uPA-R5 cells in the chick embryo CAM*

Treatment	Number of intersection points (mean ± s.d.)	Microvessel density (%)
PBS	0±1	0
Neo2 cells	5±2‡	4.6
Neo 2 cells + anti-FGF2 Ab	3±1§	2.2
uPA-R5 cells	11±1	8.3
uPA-R5 cells + anti-FGF2 Ab	6±2‡,§,¶	5.4
FGF2	31±2	22.6

*Neo2 cells and uPA-R5 cells (18,000 cells/embryo) were absorbed into gelatin sponges in the absence or in the presence of neutralizing anti-FGF2 antibody (400 ng/sponge) and grafted onto the CAM at day 8 (10 embryos per group). Recombinant FGF2 (1 μg/sponge) was used as a positive control. The angiogenic response was assessed histologically by a planimetric method of 'point counting' at day 12 as described in Materials and Methods.

‡Statistically different from uPA-R5 cells (P<0.01).

§Not statistically different from Neo 2 cells (P>0.05).

¶Statistically different from Neo 2 cells + anti-FGF2 Ab (P<0.05).

fibroblasts within an abundant network of collagen fibers. The vessels were less numerous in the Neo2 cell-treated sponges (Fig. 3B) and were absent among trabeculae of implants treated with PBS (Fig. 3C).

In agreement with the macroscopic and microscopic observations, a higher microvessel density was detectable within the sponges treated with uPA-R5 cells than in those treated with Neo2 cells or vehicle (P<0.01, Student's t-test) when the angiogenic response was quantified by a morphometric method of 'point counting' (Table 1).

To assess whether the stronger angiogenic response elicited by uPA-R5 cells was due to an increased mobilization of endogenous FGF2, these cells were added to the CAM in the presence of neutralizing, affinity-purified anti-FGF2 antibodies. Under these experimental conditions, anti-FGF2 antibodies abolish the angiogenic activity exerted by 400 ng of recombinant FGF2 (Ribatti et al., 1995). In agreement with our hypothesis, anti-FGF2 antibodies reduced the angiogenic response elicited by uPA-R5 cells to values similar to those measured in Neo2 cell-treated CAMs (Table 1). Anti-FGF2 antibody limitedly affected also the angiogenic activity of Neo2 cells even thought the effect was not statistically significant. This may be due to a limited mobilization of endogenous FGF2 caused by the basal levels of uPA activity released by Neo2 cells (see Fig. 1A). Similar results were obtained when the cells were applied on the CAM at day 6 and the angiogenic response was quantified at day 10 (data not shown).

To rule out the possibility that the observed effects were the mere consequence of a different expression of FGF2 by uPA- and mock-transfected cells, the amount of cell-associated and released FGF2 was measured in cell cultures by an immunoassay. As shown in Table 2, both cell types produce and release negligible amounts of FGF2. It can be calculated that the cells implanted onto the CAM express no more than 10 pg of FGF2 during the assay, an amount of growth factor 1,000 times smaller than the minimal amount required to elicit a significant angiogenic response in the CAM (Nguyen et al., 1994).

It is interesting to note that uPA-overexpressing cells added with neutralizing anti-FGF2 antibodies retain a limited angiogenic activity that is more potent than that exerted by

Table 2. FGF2 production by Neo2 and uPA-R5 cells

	FGF2 (pg/μg of protein)*		
	Intracellular	HSPG-bound	Free
Neo2 cells	3.5±0.6	0.1±0.1	0.2±0.1
uPA-R5 cells	3.9±0.4	0.3±0.1	0.2±0.1

*FGF-2 levels (mean ± s.d.) were measured in cell cultures at confluence using a FGF2 immunoassay as described in Materials and Methods.

Neo2 cells tested under the same experimental conditions. This suggests that released uPA may induce the mobilization of endogenous angiogenic factors other than FGF2 and/or that uPA may per se elicit a limited angiogenic response (see below).

Purified human uPA is angiogenic in the chick embryo CAM

The data described above indicate that mobilization of endogenous FGF2 by uPA is responsible, at least in part, for the observed effects on CAM vascularization triggered by uPA-R5 cells. To confirm these findings, the angiogenic activity of purified human uPA was evaluated. As shown in Table 3, human uPA exerts a dose-dependent angiogenic response in the CAM. The minimal amount of enzyme able to exert a significant angiogenic activity is equal to 250 ng per egg, corresponding approximately to 7.5 i.u. of uPA activity.

The capacity of uPA and its uPAR-binding, NH_2-terminal fragment ATF to promote diacylglycerol production, cell growth, chemotaxis, and matrix invasion of cultured endothelial cells led to the hypothesis that uPAR occupancy and activation is responsible for the angiogenic activity of uPA (Fibbi et al., 1998). To assess this hypothesis in vivo, we compared the angiogenic activity of enzymatically active and inactive human uPA. For this purpose, purified human uPA was pre-incubated for 30 minutes at room temperature with the serine-protease inhibitor phenylmethylsulfonyl fluoride (PMSF) or vehicle. PMSF is an irreversible uPA inhibitor (Moscatelli, 1986). Therefore, at variance with reversible serine-protease inhibitors (e.g. amiloride), its action is not susceptible to possible dilution and/or wash-out effects that may occur after delivery of the uPA-inhibitor complex to the CAM. After incubation, uPA samples were dialyzed extensively to remove free PMSF and their angiogenic activity was evaluated in the CAM. As shown in Table 4, preincubation

Table 3. Angiogenic activity of purified human uPA in the chick embryo CAM*

Treatment	No. of blood vessels
PBS	3±2
FGF2 (1.0 μg/sponge)	37±5‡
uPA (0.1 μg/sponge)	5±1
uPA (0.25 μg/sponge)	12±3‡
uPA (0.5 μg/sponge)	15±2‡
uPA (1.0 μg/sponge)	26±4‡

*uPA was absorbed into gelatin sponges implanted onto the CAM at day 8 (3 embryos per group). PBS- and FGF2-treated embryos were used as negative and positive controls, respectively. The angiogenic response was assessed macroscopically at day 12 by counting the number of blood vessels entering the sponge as described in Materials and Methods.

‡Statistically different from PBS (P<0.01).

Table 4. Characterization of the angiogenic activity of human uPA*

Treatment	Angiogenic response		Mononuclear cell infiltrate	
	No. of intersection points (mean ± s.d.)	Microvessel density (%)	No. of intersection points (mean ± s.d.)	Mononuclear cell density (%)
PBS	1±1	0.7	5±2	4.9
uPA	12±2	9.7	4±2	4.2
uPA + PMSF	4±1‡	2.8	ND	ND
uPA + anti-FGF2 Ab	5±1‡	4.2	ND	ND
ATF	1±1	0.7	ND	ND

*uPA and ATF (both at 1 μg/sponge) were absorbed in the absence or in the presence of neutralizing anti-FGF2 antibody (400 ng/sponge) into gelatin sponges implanted onto the CAM at day 8 (10 embryos per group). In one group, uPA was pre-treated with 1 mM PMSF and dialyzed extensively to remove free PMSF before experimentation. The angiogenic response and the mononuclear cell infiltrate were assessed histologically by a planimetric method of 'point counting' at day 12 as described in Materials and Methods. ND: not determined.

‡Statistically different from uPA (P<0.01).

of uPA with 1 mM PMSF significantly inhibits the angiogenic activity of the enzyme.

No significant increase of the mononuclear cell infiltrate was observed in uPA-treated sponges in respect to vehicle-treated samples, ruling out the possibility that the angiogenic activity exerted by purified uPA was the mere consequence of the triggering of an inflammatory response (Table 4). Moreover, the angiogenic activity exerted by purified uPA was reduced significantly by neutralizing anti-FGF2 antibodies (Table 4). Thus, the data indicate that purified uPA exerts an FGF2-dependent angiogenic activity in the CAM and that this effect depends, at least in part, upon the catalytic activity of the enzyme.

To further substantiate this hypothesis, we compared the angiogenic activity of purified human uPA to that exerted by human ATF (amino acid residues 1-135). The lack of enzymatic activity of the ATF preparation was confirmed by a chromogenic assay with human plasminogen as a substrate (not shown) and by dot-blot casein zymography with chick plasminogen (Fig. 4, inset). It must be pointed out that human uPA retains a significant enzymatic activity in the presence of chick plasminogen (Fig. 4, inset), even though this activity is reduced when compared to that exerted with human plasminogen as a substrate (not shown).

Since uPA/uPAR interaction is species specific in the human-mouse system (Estreicher et al., 1989) but not in the human- or mouse-bovine systems (Mignatti et al., 1991), we evaluated first the capacity of human uPA to interact with chick embryo cells. To this purpose, chick embryo fibroblasts were incubated in vitro at 4°C with purified human uPA in the absence or in the presence of a molar excess of ATF (Mignatti et al., 1991). At the end of the incubation, cells were washed extensively and the enzyme bound to the cell surface was eluted with an acid wash. Eluates were then assayed for uPA activity using a plasmin chromogenic assay. Non specific binding was evaluated by incubating the cells with uPA in the presence of 250 μg/ml suramin (Behrendt et al., 1993). As shown in Fig. 4, purified uPA binds to chick embryo fibroblasts and the binding can be inhibited to a significant extent by a molar excess of ATF.

These data indicate that chick embryo uPAR can interact with human uPA, as observed for bovine uPAR (Mignatti et al., 1991). Nevertheless, human ATF was ineffective when assayed for its angiogenic capacity in the chick embryo CAM (Table 4).

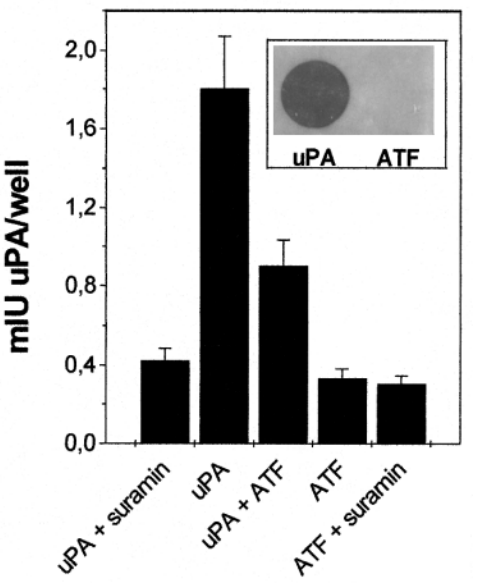

Fig. 4. uPA binding to chick embryo fibroblasts. Confluent cell cultures in 24-well plates were acid treated to remove endogenous, surface-bound uPA. Then, cells were incubated for 2 hours at 4°C with 60 ng/well of purified uPA or ATF in the absence or in the presence of 250 μg/ml suramin. Parallel cell cultures were incubated with 60 ng of uPA in the presence 600 ng of ATF (uPA + ATF). After washing, surface bound uPA was eluted with 50 mM glycine-HCl, 0.1 M NaCl, pH 3.0, immediately neutralized, and tested for enzymatic activity with a chromogenic assay. Data are the mean ± s.e.m. of 3 determinations. Inset: Dot-blot casein-zymography of purified uPA and ATF. 200 ng of purified uPA or ATF were spotted onto a nitrocellulose membrane. Then, membrane was overlaid onto a casein plasminogen-agar plate containing chicken serum, incubated at 37°C, and photographed against a dark background.

DISCUSSION

Here we demonstrate that human uPA released by transfected uPA-overexpressing endothelial cells or administered as a purified protein is able to induce neovascularization of the chicken embryo CAM. In both cases, neovascularization is significantly reduced by the simultaneous administration of neutralizing, affinity-purified anti-FGF2 antibodies, thus

implicating endogenous FGF2 in mediating the angiogenic activity of uPA.

FGF2 is characterized by the capacity to interact with HSPGs present on the cell surface and in the ECM and the biological implications of this interaction are manifold (for a discussion of this point see Rusnati and Presta, 1996). Among them, ECM may act as a physiological reservoir for extracellular FGF2. Indeed, FGF2 has been found associated with ECM in vitro and in vivo (Vlodavsky et al., 1987; Folkman et al., 1988; DiMario et al., 1989; Rogelj et al., 1989; Hageman et al., 1991). In vitro studies have shown that various enzymes, including heparanase, thrombin, collagenases, and plasmin release ECM-bound FGF2 by degrading the glycosaminoglycan chain or the core protein of HSPGs (Saksela and Rifkin, 1990; Falcone et al., 1993; Whitelock et al., 1996). Our data show that uPA-overexpressing endothelial uPA-R5 cells, but not mock-transfected Neo2 cells, cause the mobilization of ECM-bound ^{125}I-FGF2 in a plasminogen-dependent manner, thus confirming the capacity of uPA/plasmin to facilitate the release of the immobilized growth factor.

Several experimental evidences suggest that the balance between storage and release of angiogenic FGF2 in ECM, as well as the integrity of the matrix, may regulate the biological effects of this growth factor on endothelium. For instance, interleukin-1-induced degradation of ECM in cultured chondrocytes, with consequent release of extracellular FGF2, has been implicated in the neovascularization of the synovia of arthritic patients (Tamura et al., 1996). Also, uPA production and FGF2 release by macrophage derived-foam cells have been implicated in the development of the atherosclerotic lesion (Falcone et al., 1993). Finally, FGF2 mobilization from injured corneal epithelial basement membrane by increased plasmin activity (Salonen et al., 1987) has been suggested to have a role in corneal neovascularization (Folkman et al., 1988).

Even though these data strongly suggest that uPA can facilitate angiogenesis by increasing the bioavailability of ECM-stored FGF2, no direct in vivo experimental evidences were available to support this hypothesis. To this respect, neovascularization of the CAM of the chicken embryo represents an unique experimental system to assess this hypothesis. Indeed, FGF2 protein is present in the CAM where it plays a limiting role in blood vessel growth during development (Ribatti et al., 1995). Accordingly, chorionic epithelial cells and endothelial cells of the CAM mesoderm express FGF2 mRNA (Ribatti et al., 1998). Moreover, the presence of significant amounts of FGF2 in the chorioallantoic fluid, that parallel the levels of FGF2 present in the CAM, demonstrates that FGF2 is released by these cells and accumulates in the extracellular environment (Ribatti et al., 1995).

By utilizing this experimental system, we have shown that neutralizing anti-FGF2 antibodies reduce significantly the angiogenic activity exerted by uPA-R5 cells and purified human uPA, thus implicating extracellular endogenous FGF2 in the growth of newly formed blood vessels stimulated by uPA. However, the incapacity of anti-FGF2 antibody to fully suppress the angiogenic ability of purified uPA and uPA-R5 cells, even when tested under experimental conditions that fully inhibit the angiogenic activity of exogenous recombinant FGF2, suggests that more factors besides FGF2 might be implicated in protease-triggered CAM neovascularization. Indeed, a variety of angiogenic growth factors, including different members of the FGF family, some isoforms of VEGF and placenta growth factor, HGF, and interleukin-8 share the capacity to interact with HSPGs of the ECM (Rusnati and Presta, 1996) and exert an angiogenic response in the CAM (Oh et al., 1997; Ziche et al., 1997). This hypothesis is supported by the observation that uPA-R5 cells and Neo2 cells retain an angiogenic potential apparently related to their levels of uPA expression also in the presence of neutralizing anti-FGF2 antibodies (see Table 1). It is also interesting to note that both purified human uPA and uPA-R5 cells exert an angiogenic response in the CAM that is less potent than that exerted by exogenous recombinant FGF2 (see Table 1 and 3), suggesting that the levels of endogenous angiogenic growth factor(s) available to the protease action may represent a limiting factor in this experimental system.

uPA exerts a significant angiogenic activity when delivered onto the CAM in a single administration or when continuously released by uPA-R5 transfectants during the 4 days of experimentation. In the former experimental conditions, 250 ng of uPA (corresponding to approximately 7-8 i.u. of uPA activity) exert a significant angiogenic activity. In the latter experimental conditions, as few as 18,000 uPA-R5 cells, releasing approximately 1.0 i.u. of uPA activity throughout the experimental period, are sufficient to induce an angiogenic response. It must be pointed out that the amount of uPA released by uPA-R5 transfectants is similar to the amount of enzyme released by parental cells when stimulated in vitro by recombinant FGF2 (Gualandris et al., 1997), thus indicating that the levels of uPA produced by our transfectants are biologically significant. Our data are in keeping with previous observations showing that purified human uPA exerts an angiogenic response in the rabbit cornea when administered at doses ranging from 10 to 500 ng per implant (see Berman et al., 1982; Fibbi et al., 1998). Even though an accurate quantitative comparison among the various experimental systems cannot be performed due to their biological heterogeneity and the different enzymatic potency of human uPA with plasminogen from different sources (human uPA being tested in rabbit or chick experimental models), the bulk of data indicate that uPA can elicit a significant angiogenic stimulus in the absence of a detectable inflammatory response (see Table 4, and Fibbi et al., 1998).

Recent observations have shown that uPA/uPAR interaction induces a pro-angiogenic phenotype in cultured endothelial cells that can be triggered also by the uPAR-binding ATF (Fibbi et al., 1998). However, the inability of ATF to induce angiogenesis in the CAM indicates that the proteolytic activity of uPA is of pivotal importance in mediating its angiogenic capacity in vivo. This is confirmed by the reduced capacity of the PMSF-uPA complex to induce CAM neovascularization. Accordingly, irreversible inhibition of uPA by the active site inhibitor Phe-Ala-Arg-chloromethyl ketone prevents its angiogenic activity in the rabbit cornea (Berman et al., 1982). Also, the capacity of plasmin (Iruela-Arispe et al., 1995) and thrombin (Tsopanoglou et al., 1993) to induce neovascularization in the CAM further support the notion of ECM proteolysis as a trigger for new blood vessel growth. Nevertheless, our observations do not rule out the possibility that uPAR occupancy may affect endothelial cell behavior also

in the CAM, as suggested by the residual angiogenic activity exerted by PMSF-treated uPA. Relevant to this point, previous studies have shown that purified uPA exerts an uPAR-dependent mitogenic activity on human fibroblasts that cannot be mimicked by ATF alone, suggesting that both receptor occupancy and catalytic activity may play a role in the biological activity of uPA (De Petro et al., 1994).

Transgenic mice lacking uPA and/or tissue-type PA (Carmeliet et al., 1994) or plasminogen (Bugge et al., 1995) develop normally, demonstrating that the proteolytic cascade triggered by plasminogen activation is not a fundamental component for neovascularization during development. On the other hand, uPA/plasmin inhibitors affect angiogenesis in vitro and in vivo in different experimental models (Mignatti et al., 1989; Yasunaga et al., 1989; Avery et al., 1990; Bastaki et al., 1997). Also, the reduced rate of growth of murine experimental tumors by anti-fibrinolytic drug-treatment may depend on a reduced neovascularization (Astedt and Tropè, 1980; Corasanti et al., 1982; Teuscher and Pester, 1984). Finally, an altered fibrinolytic status affects polyoma middle T-induced vascular tumor formation (Sabapathy et al., 1997). Our observations indicate for the first time that uPA/plasmin-dependent degradation of ECM, with the consequent mobilization of stored angiogenesis factors, may represent one of the mechanisms by which the fibrinolytic system modulates angiogenesis in vivo.

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