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The role of Urokinase in Vascular Cell Migration and in Regulation of Growth and Branching of Capillaries

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Abstract—The urokinase system, represented by a plasminogen activator of urokinase type (urokinase, uPA), urokinase receptor (uPAR), and inhibitors of plasminogen activator (PAI-1 and PAI-2), plays an important role in the regulation of vascular wall functioning. Urokinase signaling initiates proteolytic cascade and degradation of the extracellular matrix; and also activates intracellular signaling in vascular cells. This study is the first to reveal a urokinase-mediated fundamental mechanism that regulates the growth trajectory and branching morphogenesis of blood vessels. This mechanism may be of particular importance during vessel growth in early embryogenesis and in the adult during tissue regeneration.

Keywords: urokinase, urokinase receptor, angiogenesis, guidance receptors, branching of vessels

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The urokinase system comprise a group of proteins involved in the processes of activation of plasminogen and fibrinolysis (Collen, 1999; Stepanova et al., 2008). These proteins include urokinase uPA, a serine protease that activates plasminogen, converting it to active plasmin, and urokinase receptor uPAR (Tkachuk et al., 1996; Collen, 1999). Three domains have been identified in the structure of urokinase: the N-terminal domain, similar to epidermal growth factor (growth domain); the kringle domain; and the C-terminal proteolytic domain. The growth domain provides a high affinity of uPA binding to its receptor uPAR. The kringle domain is involved in stabilization of the urokinase complex with uPAR receptor, and its interactions with extracellular matrix components. Urokinase receptor uPAR is a three-domain protein anchored to the membrane via glycosylphosphatidylinositol, which mediates its high surface mobility. UPAR is important for the regulation of directed cell movement. High mobility of the urokinase receptor enables the uPA/uPAR complex to concentrate on the leading edge of the cell, where high levels of proteolytic activity are required (Blasi and Carmeliet, 2002; Gardsvoll et al., 2006).

Urokinase system also includes urokinase inhibitors, PAI-1 and PAI-2 proteins, and a number of components involved in interaction of urokinase and its receptor with other cell proteins (integrins, vitronectin, gp-120, LRP/α2-MR, chemokine receptors, and others) (Goretzki and Mueller, 1998; Behrendt, 2004; Franco et al., 2006). In addition to activation of plasminogen, urokinase has the ability to induce a number of signal processes in the cell, leading to cell migration and proliferation (Tang et al., 1998; Parfyonova et al., 2009; Carriero et al., 2011). Several studies have reported the effects of urokinase and its receptor on the stimulation of differentiation and proliferation of smooth muscle and endothelial cells during vascular remodeling (Falkenberg et al., 2002; Menshikov et al., 2006; Plekhanova et al., 2006).

Results of our previous studies using an experimental model of restenosis revealed that activity of urokinase system in migrating and proliferating vascular cells, as well as exogenous administration of recombinant urokinase into the wall of a damaged vessel, stimulates development of neointima and neoadventita, cell migration, proliferation, and phenotypic transformation of vascular cells (Resnati et al., 1996; Tang et al., 1998; Parfyonova et al., 2009). It has also been shown that the urokinase system stimulates angiogenesis (Heymans et al., 1999; Colombo et al., 2007). This is determined by its activating effect on migration of endothelial and vascular smooth muscle cells due to localization of uPA/uPAR to the leading edge of vascular cells and consequent effect on local proteolysis of the extracellular matrix (Blasi and Carmeliet, 2002; Gardsvoll, 2006).

The processes of active vascular growth and remodeling are closely coupled with activation of angiogenic factors deposited in extracellular matrix, such as VEGF, HGF, bFGF, angiopoietin, and other growth factors and chemoattractants, and are completed with

[1] *Abbreviations*: uPA—urokinase-type activator of plasminogen or urokinase, uPAR—urokinase receptor, SMC—smooth muscle cells, BSA—bovine serum albumin, PBS—phosphate buffered saline solution.

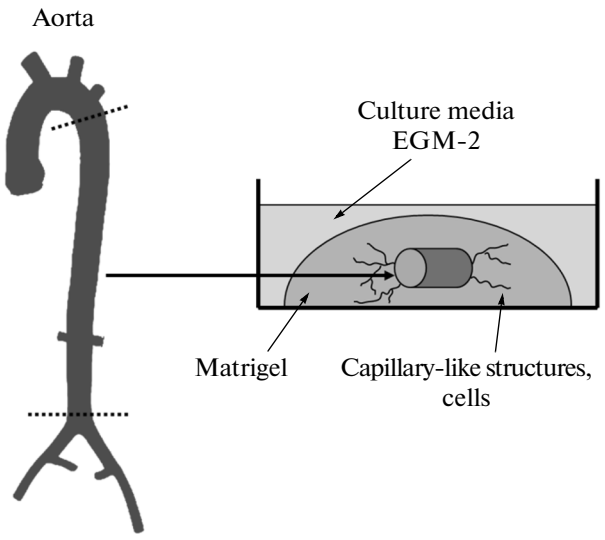

Fig. 1. Three-dimensional model of abdominal mouse aortic explant in Matrigel cultured in EGM-2 medium. Dashed line on the left shows outlines of the areas of resection of abdominal aorta.

formation of functionally mature blood vessels (Rubina et al., 2009; Carmeliet, 2000). Notably, growth trajectory and branching of blood vessels cannot be explained only by the gradient of growth factors and chemoattractants, since during the growth of blood vessels, surrounding cells produce factors that cause both positive and negative chemotaxis.

Determination of the direction of vessel growth involves so-called guidance receptors: interaction of guidance receptors with ligands on the cells of growing blood vessels, and cells or tissue, through which they grow, results in the formation of complex branched vasculature (Carmeliet, 2003; Melani and Weinstein, 2010). Despite the fact that the role of guidance receptors in the regulation of the trajectory of the growing blood vessels has been demonstrated, the mechanisms of their function in regulation of vascular branching are still not known. Urokinase is classified as a molecular guidance system involved in regulation of angiogenesis. However, its role in the regulation of pathfinding and branching of blood vessels is not completely clear.

In this study, we investigated the possible involvement of the components of the urokinase system in these processes. We found that the urokinase system not only affects the migration of vascular cells and formation of capillary-like structures, but also regulates the branching of vascular structures and determines the phenotype of vascular smooth muscle cells. This is the first report that describes the participation of urokinase and urokinase receptor in the process of branching and morphogenesis of blood vessels.

MATERIALS AND METHODS

Working with laboratory animals. All experiments were carried out in accordance with the "Rules of Work with Experimental Animals," Decree of the Ministry of Higher and Secondary Special Education of the Soviet Union no. 742 from November 13, 1984, and the rules adopted by the Council on Bioethics of the Department of Fundamental Medicine at Moscow State University. We used mice 4–8 weeks old that lacked the gene for urokinase (PLAU mice) obtained in the laboratory of Dr. Carmeleit in C57/B6 background (Herbert et al., 1997), and wild-type C57 mice.

Formation of capillary-like structures in the three-dimensional mouse explant model of ring aorta in Matrigel. To assess the effect of urokinase on the growth and branching of blood vessels, we used the previously described model (Nicosia, Ottinetti, 1990) with modifications (Rubina et al., 2007). To isolate mouse aorta, mice were lethally anesthetized with isoflurane by inhalation anesthesia (IsoFlo®, USP) and then bled by decapitation. The abdominal aorta was cleaned from the surrounding tissues; tied with threads distally, in the region of the distal aortic arch, and proximally in the bifurcation area; and cut with scissors (Fig. 1). Isolated aorta was placed in a Petri dish with sterile phosphate buffered saline (PBS). Plates were transferred to a culture unit, and further manipulations were performed under sterile conditions. Pieces of aorta were cut into rings 3 mm in length and placed in wells of eight-well culture plates with thin glass bottom (Lab-Tek Chambered Coverglass, Thermo Scientific, catalog number 155 411, United States). Pieces of aorta were supplemented with 60–80 μL of cold (4°C) Matrigel (BD Matrigel™ Basement Membrane Matrix, Germany). In some experiments, Matrigel was complemented with uPAR blocking antibody (R & D Systems, catalog number MAB531, United Kingdom) or nonimmune IgG at equivalent concentration as a control. Samples were placed in incubator (37°C, 5% CO_2) for 10 min for Matrigel polymerization. Then, wells were supplemented with EGM-2 culture medium for endothelial cells (Lonza, United States). The medium was exchanged for a fresh one every 5 days. Visualization of migrating cells was carried out using dark-field microscopy at low magnification (magnification obj. 5× or 10×).

Immunofluorescent staining of aortic explants. To prevent depolymerization of Matrigel, all procedures were carried out at 37°C. For immunofluorescence staining, samples in wells were washed with warm PBS (Sigma-Aldrich, United States) and fixed with 4% formaldehyde for 24 h (Panreac, Spain). For permeabilization of Matrigel, wells were supplemented with 1% Triton X-100 (Triton® X-100, Peroxide Free, Panreac, Spain) for 24 h and then washed for 24 h in PBS. To block non-specific staining, samples were treated

for 24 h with 5% solution of bovine serum albumin (BSA, Sigma-Aldrich, United States) in PBS, containing 1% Triton X-100. Wells were furthermore supplemented with a dilution of primary mouse antibody against smooth muscle actin (α-SMA, Sigma-Aldrich) or against CD31 mouse endothelial cell antigen (BD, United States). Incubation in solution with primary antibodies was carried out for 24 h; they were then washed in PBS for 24 h and placed in solution with secondary antibodies conjugated to AlexaFluor 594 fluorochrome (Molecular Probes, United States). After 24-h incubation, samples were washed from the second antibody and nuclei counterstained with DAPI. Next, after PBS washes, samples were placed in PBS containing 0.001% sodium azide (NaN_3). Images were obtained using a Leica confocal laser scanning microscope (TCS SP5, Germany) equipped with a Plan-Apo objective lens for 10x magnification. DAPI and AlexaFluor® 594 Fluorescent dyes, were sequentially visualized at laser wavelengths of 405 and 594 nm, respectively. All immunofluorescence images were acquired at identical microscope settings. Each figure presents reproducible results of three independent experiments. For image analysis, we used the MetaMorph 5.0 software (Universal Imaging, United States).

Protein electrophoresis and Western blotting. Specimens of aorta were placed in cold PBS and incubated on ice for 30 min. Samples were then covered with liquid nitrogen and triturated with pestle in a porcelain mortar until complete homogenization. Homogenates were transferred to Eppendorf tubes, weighed, filled with equivalent amount of lysis buffer (100 mM Tris-HCl pH8.1, 1% Triton X-100, 20 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, protease inhibitors (Pierce) 1 : 100, PMSF 1 : 100) and incubated for 15 min. Protein concentration in the lysate was determined by Bradford methodology. Protein electrophoresis was performed under denaturing conditions in SDS Laemmli buffer in a 10% polyacrylamide gel. After transferring proteins from gel to PVDF-membrane (Millipore, Germany), membranes were pre-incubated in 5% skim milk solution in PBS containing 0.05% Tween-20 (Pierce, United States) to block nonspecific protein binding. Then, membranes were incubated successively with primary (against mouse actin or uPAR) and secondary antibody (peroxidase conjugated) at room temperature for 60 min. Imaging was performed using a two-component ECL system (Pierce) followed by exposure to X-ray film (BioMax, Kodak, United States). Reproducible results of three independent experiments are presented.

Preparation of conditioned medium containing murine urokinase. We used HEK293 cell line to prepare conditioned medium comprising functionally active mouse urokinase. Cells were transfected with a plasmid for expression of mouse urokinase (pcNDA3.1/uPA) using Lipofectamine 2000 reagent (Invirogen, United States) in serum-free Opti-MEM medium (Gibco, United Kingdom). Medium was collected and centrifuged at 3500 rev/min for 10 min. Supernatant was transferred to clean tubes and concentrated tenfold using Centricon filters (YM-50, Millipore, Germany), which retain proteins with molecular weight less than 50 kDa. The concentration of urokinase was determined by ELISA using Mouse PLAU/Urokinase/UPA ELISA Kit (LifeSpan BioSciences, United States) in accordance with the manufacturer's instructions.

Statistical analysis was performed using the Statistica 6.0 software environment. Graphs present mean and standard error. Differences were considered significant at a significance level of less than $p < 0.05$.

RESULTS

To assess the effects of urokinase on growth and branching of blood vessels, we used an ex vivo three-dimensional (3D) model of mouse abdominal aortic culture explants (RAE, ring aorta explant) in Matrigel (Fig. 1). This system represents a unique model of angiogenesis in vivo as it allows to test the impact of various substances introduced in Matrigel, the rate of migration of vascular cells, and the growth dynamics of capillary-like structures. Using this method, we determined the direction and rate of migration of two types of vascular cells, smooth muscle and endothelial, and defined the growth trajectory and branching characteristics of capillary-like structures that extend from aortic explants into the Matrigel. The dynamics of cell migration and growth of capillary-like structures were followed for 14 days by light microscopy. In this work, we used explants of aorta from wild-type (uPA+/+) mice and a mouse strain with a knock-out for urokinase gene PLAU (uPA−/−). Genetic studies were complemented with immunoblocking analysis using antibodies against uPAR receptor (anti-uPAR), which prevent its specific binding to urokinase. Three-dimensional aortic explants were stained with antibodies to CD31 to visualize migrating endothelial cells and formation of capillary-like structures. Staining with anti-α-SMA antibodies was carried out to visualize smooth muscle cells (SMCs).

Examination of the dynamics of vascular cell migration from aortic explants into Matrigel revealed that, PLAU mice, the number of migrating cells, as well as the rate of their migration, is substantially lower than in control. Figure 2a shows an image of aortic explants 14 days after the beginning of the experiment. Arrows indicate directions of cell migration from the explant. It can be seen that aortic cells from mice null for urokinase gene (uPA−/−) achieve a significantly shorter migration path compared to control explants (uPA+/+). Statistically significant quantification of these results is presented in Figs. 2b and 2c. Thus, vascular cell migration from explants is dependent on the expression of urokinase. "Switching off" the urokinase

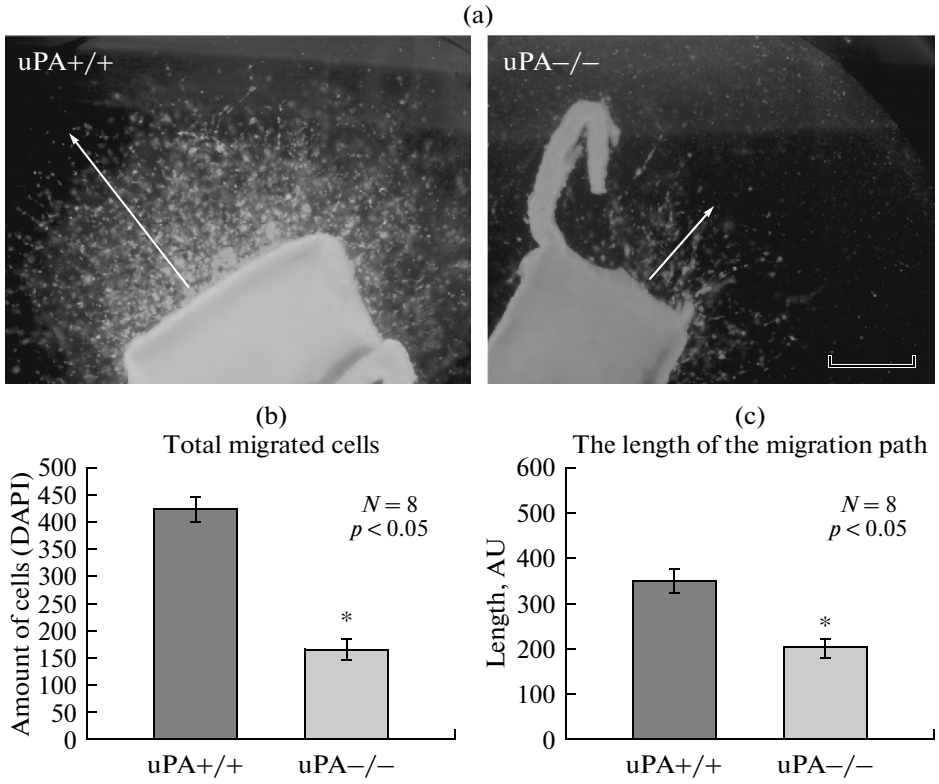

Fig. 2. Effect of urokinase on cell migration from mouse aortic explants. (a) Three-dimensional culture of abdominal aorta in Matrigel. After 14 days in culture, the number of vascular cells that migrated from aorta explants from PLAU mice in Matrigel was significantly lower than in controls (uPA+/+). The length of the migration path is indicated by the length of the arrow. Scale: 250 μm; (b) quantification of migrating cells, (c) length of the path of migrating cells. Presented data are based on statistical analysis of eight explants; differences are significant ($p < 0.05$).

gene in PLAU mice significantly reduces both spontaneous cell migration (the total number of cells around explants) and as well as their rate of migration (migration path length).

Analysis of uPAR expression by Western blotting demonstrated that urokinase gene knockout also results in a decrease of expression urokinase receptor. Figure 3 presents the results of uPAR expression at protein level in aorta samples obtained from three mice. These results indicate that reduction of migration activity of vascular cells derived from uPA–/– mice can be caused not only by absence of urokinase, but also by lower expression of uPAR.

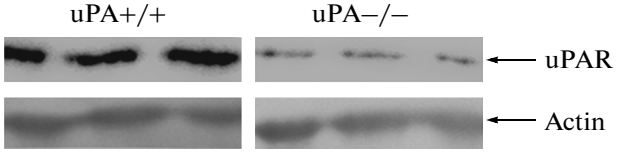

Fig. 3. Western blot analysis of the expression of urokinase receptor (uPAR) in three mice aortas. uPA+/+ is uPAR expression in the aorta of control mice; uPA–/– is expression of uPAR in the aorta of mice deprived of urokinase gene. Presented data are normalized to the content of actin. Reproducible results of three independent experiments are shown.

Visualization of cells migrating from explants into Matrigel under a light microscope does not give a complete picture of what kind of vascular cells crawl from the aorta. In order to identify the type of vascular cell that is most affected by the urokinase system, we conducted immunofluorescence analysis of aortic explants on the 14th day of culturing ex vivo in Matrigel with antibodies against CD31 (endothelial cells marker) and anti-α-SMA (SMC marker). Figure 4 shows immunofluorescent labeling of aortic explants using antibodies to CD31 (Fig. 4a) and antibodies to α-SMA (Fig. 4b) followed by three dimensional imaging under with a confocal microscope.

Results of immunofluorescent staining indicated that in mice null for uPA, the number of migrating SMC is significantly reduced, while migration of endothelial cells remains unchanged. To confirm these data, we conducted statistical analysis of the obtained images. The number of migrating cells was measured by the number of nuclei stained with DAPI in the region surrounding explants in Matrigel. Quantitative evaluation confirmed the assumption that the absence of urokinase inhibits SMC migration from explants

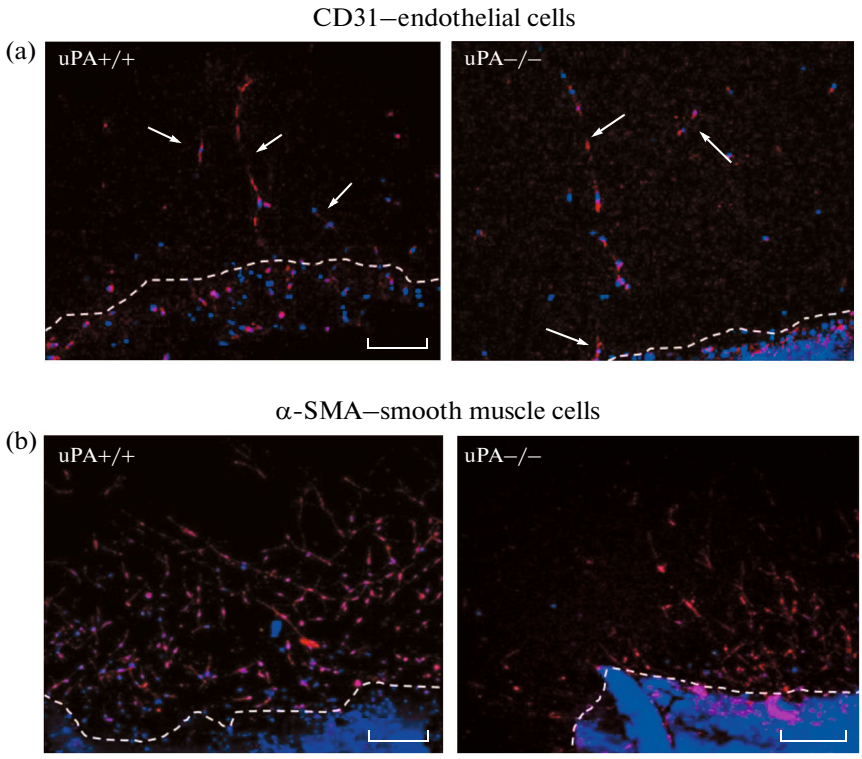

Fig. 4. Immunofluorescent analysis of three-dimensional culture of cells migrating from mouse abdominal aortic explant into Matrigel. Samples of aorta from control mice (uPA+/+) and urokinase knock-out mice (uPA–/–) were stained with antibodies against CD31 (a, red) and anti-α-SMA (b, red). Nuclei were counterstained with DAPI (blue). Arrows in a indicate CD31-positive capillary-like structures; dashed line marks the tissue explant. Three-dimensional visualization of fluorescent staining was performed using a confocal microscope. Reproducible results of three independent experiments are presented. Scale: 250 μm.

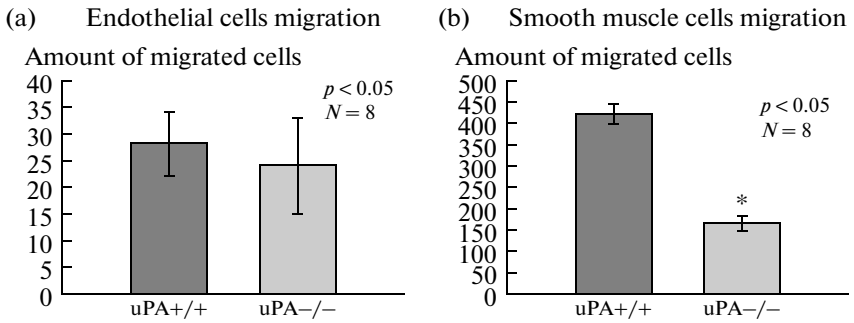

Fig. 5. Effect of urokinase (uPA) on the migration of endothelial cells (a) and smooth muscle cells (b). (a) Lack of uPA has no effect on migration of endothelial cells; (b) knock-out of urokinase gene leads to the suppression of smooth muscle cells migration. Cell migration in Matrigel was evaluated based on staining to tissue specific markers. A statistically significant result based on examination of eight explants is presented (b, $p < 0.05$).

into Matrigel (Fig. 5b), yet has little effect on migration of endothelial cells (Fig. 5a). A statistically significant difference ($p < 0.05$) was observed in the case of SMC, which migrated from explants derived from PLAU mice compared with the controls. Thus, urokinase is important for SMC migration, as it is the amount of crawling SMCs that is significantly lower in aortic explants derived from PLAU-mice compared to controls. The number of migrating endothelial cells in explants obtained from control and PLAU mice did not differ significantly.

Next, we used antibodies that block urokinase receptor to determine the effects of uPAR on directed vascular cell migration and formation of capillary-like branched structures as well as the influence of uPAR on vascular cell phenotype. Blocking antibody was

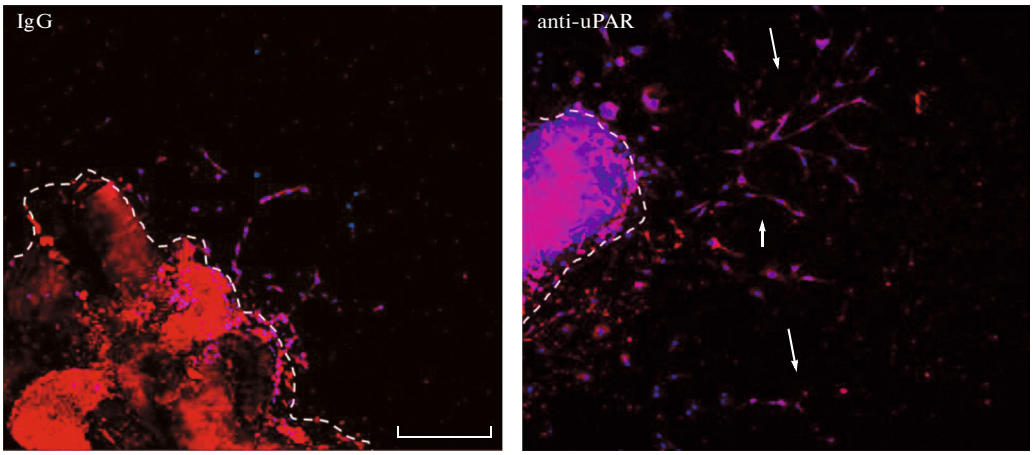

Fig. 6. Blocking of uPAR causes increased branching of capillary-like structures. Samples of aortic explants were fixed and stained with antibodies to identify endothelial cells (CD31, red). Nuclei were counterstained with DAPI (blue). Arrows indicate branching points of CD31-positive structures in the background of blocking of the urokinase receptor. Confocal microscopy. IgG is preimmune antibodies; anti-uPAR is antibodies blocking uPAR. Dashed line shows the area of tissue explant. Reproducible results of three independent experiments are presented. Scale: 250 μm.

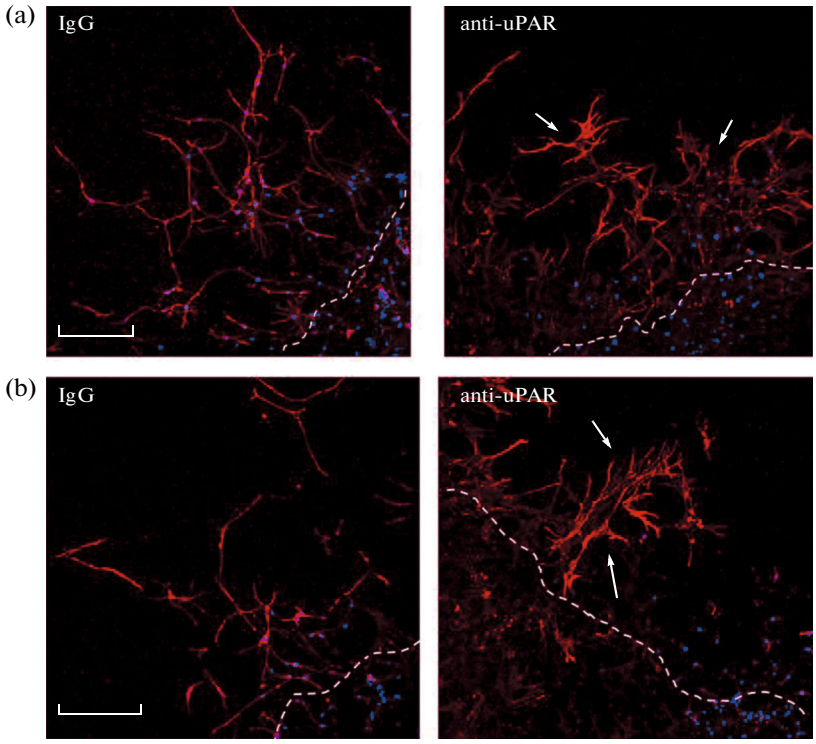

Fig. 7. Changes in the phenotype of smooth muscle cells (SMCs) caused by blocking of uPAR. Aortic explants from (a) control mice and (b) mice lacking urokinase gene were fixed and stained with antibodies against α-SMA to identify SMCs (red). Nuclei were counterstained with DAPI (blue). Arrows indicate flattened SMCs in the context of blocking uPAR. Other explanations are the same as in Fig. 6.

added to Matrigels upon planting of aortic explants obtained from control mice (uPA+/+) and mice lacking the urokinase gene (uPA–/–). After 14 days in culture, aortic explants were stained using antibodies to CD31 and α-SMA, respectively, and visualized by confocal microscopy. Figure 6 shows the results of staining of endothelial cells that migrated in Matrigel from explants derived from control mice in the context of blocking uPAR. Figure 7 shows the results of the immunofluorescent staining of SMC cells that

migrated into Matrigel migrated from explants derived from both types of mice (control uPA+/+ and mutant for the urokinase gene uPA–/–) in the context of uPAR blocking.

We found that using antibodies that block functional activity of uPAR resulted in an increase in branching of capillary-like structures (Fig. 6) and a change in smooth muscle cell morphology (Figs. 7a, 7b). In control explants, migrating SMCs had a typical spindle shape, which reflects their promigratory phenotype (Fig. 7a). Blocking of uPAR results in acquisition of a flattened form by SMCs, which indicates that cells loose their polarized phenotype, characteristic of directed cell migration (Fig. 7b). Explants from mice in which urokinase function was abrogated by uPAR blocking also demonstrated a change in SMC morphology. However, while, in control uPA+/+ explants, some SMCs retained a spindle shape (Fig. 7a, anti-uPAR), in explants from mice knock-out for the urokinase gene uPA–/– such cells disappeared almost entirely (Fig. 7b, anti-uPAR).

To further elucidate the role of the uPA/uPAR in the regulation of cell migration, exogenous urokinase and/or uPAR blocking antibody were added to Matrigels to the culture system of aortic explants. UPA was added to Matrigels and to the culture medium at a final concentration of 50 ng/mL, while uPAR blocking antibodies were administrated into Matrigels at the concentration of 25 μg/mL, and BSA and nonimmune IgG antibody were added at equivalent concentrations as controls. In the course of this experiment, we assessed spontaneous cell migration (total number of cells that migrated from explants into Matrigel) and the rate of cell migration (migration path length). We found that addition of uPA activates not just spontaneous migration of vascular cells (number of postmigratory cells in Fig. 8a, $p < 0.05$), but also increases the rate of migration (migration path length in Fig. 8b). The total number of cells and the speed of cell migration from aortic explants obtained from PLAU-mice were significantly lower than from controls ($p < 0.05$). Thus, uPAR blocking had a statistically significant effect on these two processes: a decrease in both, spontaneous cell migration and the rate of cell migration in explants obtained from PLAU-mice compared to controls (anti-uPAR in Fig. 8, $p < 0.05$). This results strongly indicate that stimulatory effect of urokinase on vascular cell migration is due to its specific interaction with its receptor uPAR.

Thus, examination of the role of urokinase system in the regulation of cell migration, directional growth and branching of capillary-like structures, using ex vivo vascular ringlet model revealed that migration of SMCs is dependent on the expression of urokinase system. Urokinase activated SMC migration and increased the rate of cell migration. In turn, blocking of uPAR inhibited both of these processes. Furthermore, blocking of the uPAR enhanced the branching of capillary-like structures formed by endothelial cells and promoted loss of the postmigratory phenotype in SMCs.

DISCUSSION

It is an important problem today to identify the molecular mechanisms regulating the growth of blood vessels and the formation of extensive vascular networks. Vascularization, just as is innervation, is an integral part of morphogenesis and regeneration of tissues and organs. Full recovery of organ function is impossible without restoring its blood supply. Thus, identification of molecular mechanisms that regulate angiogenesis is essential for understanding the processes of morphogenesis and regeneration, as well as the development of effective methods to control these processes. Development of such methods is difficult without resolving the fundamental problem of complete decoding of the mechanisms that regulate formation and growth of blood vessels. Understanding these mechanisms is also necessary for advancement in engineering of tissues and organs.

Previous reports have indicated that urokinase is the key regulator of angiogenesis (Parfyonova et al., 2002; Tkachuk et al., 2013). Experiments in animal models of hindlimb ischemia and myocardial infarction demonstrated stimulatory effects of administration of a plasmid construct for expression of urokinase on angiogenesis (Traktuev et al., 2007). It has been shown that uPA stimulates formation of capillaries and arterioles, reduces infarct size and prevents the necrosis in an ischemic limb. It is believed that urokinase stimulates neovascularization by participating in the processes of extracellular proteolysis and tissue remodeling through activation of latent angiogenic factors and growth factors deposited in the extracellular matrix, activation of matrix metalloproteinases, stimulation of migration, and proliferation of endothelial and vascular smooth muscle cells, as well as by attracting monocytes (Parfyonova et al., 2002). In vitro upregulation of urokinase in endothelial cells enhances their invasive potential. Use of a recombinant form of urokinase, lacking proteolytic activity, inhibits migration of endothelial cells in culture and formation of capillary-like structures in Matrigel (Basire et al., 2006; Traktuev et al., 2007).

The results obtained in this study using a three-dimensional ex vivo model of aortic explants are consistent with the previous reports. Our data indicate that urokinase is required for migration of vascular cells. In PLAU-mice with an "off" state of the urokinase gene, the rate of vascular cell migration is significantly reduced relative to control. It should be noted that, in PLAU-mice, the urokinase receptor is expressed at a lower level, which may contribute to reducing the migration potential of vascular cells.

It has been shown previously that the use of antibodies blocking urokinase receptor suppresses angiogenic effects of growth factors, such as fibroblast

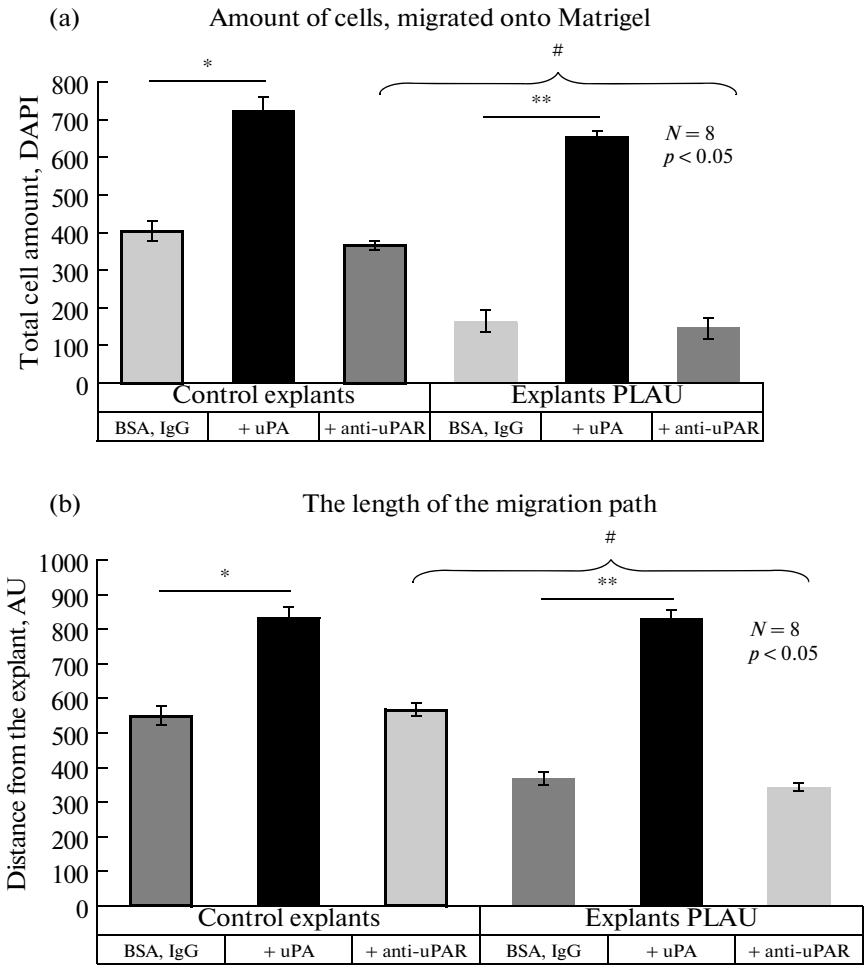

Fig. 8. (a) Stimulation of spontaneous migration by urokinase, and (b) the rate of migration of vascular cells. (a) Results of statistical calculations of the number of cells that migrated from aortic explants into Matrigel on the 14th day after the start of the experiment and (b) the average length of migration path. Addition of urokinase (+uPA) stimulates migration rate and increase the number of migrating cells in both types of explants, compared to control (BSA, IgG). Asterisks indicate significant differences, $p < 0.05$. Blocking of uPAR (anti-uPAR) significantly reduced spontaneous migration and migration rate (comparison of cells migrated from explants without urokinase with control explants (grid, $p < 0.05$)). A statistically significant result based on examination of eight explants is presented.

growth factor (bFGF) and vascular endothelial growth factor (VEGF), on endothelial cell migration and formation of capillary-like structures in fibrin matrix (Kroon et al., 1999). In contrast to these data, our results indicate that blocking the uPAR receptor does not affect endothelial cell migration from the explant, but significantly inhibits SMC migration and causes a change in their phenotype from promigratory to flattened.

Although the role of uPA and its receptor in vascular remodeling and angiogenesis has been fairly well characterized, the mechanisms of guidance function of the urokinase receptor, that defines the trajectory of vessel growth, are almost completely unknown. This study is the first to demonstrate that blocking of the uPAR receptor results in an increase in the degree of branching of capillary-like structures. Angiogenesis is a multistep process of development of new functional blood vessels and capillaries from preexisting ones. The initial stage of this process involves activation of endothelial cells, expression of proteases in them, degradation of the extracellular matrix, and cell migration and proliferation, leading to the formation of vascular sprouts. Subsequently, newly formed vascular structures are stabilized by the attraction of pericytes and SMC, and form a complex three-dimensional functional mature vasculature (Parfyonova and Tkachuk, 2007). At the initial stages of angiogenesis, angiogenic factors induce cell specialization: only one of many endothelial cells becomes a "tip-cell," and this phenotype is suppressed in neighboring cells that acquire "stalk" phenotype. It has been proposed that tip cells convert the combination of different guidance signals of the microenvironment into directed migration. Tip cells are thought to determine the direction of growth of the entire vessel and the site of the next branching

event (De Smet et al., 2009). The fact that blocking of the urokinase receptor leads to an increase in the degree of branching of the forming capillary-like CD31-positive structures from aortic explants may reflect a failure in the tip/stalk specialization of endothelial cells, or a loss of the ability of tip cells to adequately recognize guidance signals. Confirmation of this hypothesis requires further studies.

Thus, in this study, using a mouse model aortic ex vivo explant culture, we have shown that, blocking the uPA/uPAR system (in animals, genetic knock-outs of urokinase or by using anti-uPAR antibody), leads to flattened SMC phenotype and disruption in their directional movement. We propose that this processes depend not only on uPA, but also on its receptor. Urokinase knockout in uPA–/– mice also results in a decreased expression of the urokinase receptor, and the combined effect of the loss of urokinase and its receptor leads to the change in the SMC phenotype and reduction in their migratory potential. In addition to the SMC effect of the urokinase system, we also detected an increase in the branching of capillary-like CD31-positive structures, which may reflect a decrease in the ability of endothelial cells to recognize guidance signals of the microenvironment.

In the present study for the first time we revealed the fundamental mechanism of regulation of the growth trajectory and branching of blood vessels with the participation of urokinase and its receptor. These mechanisms may be important in the processes of vascular growth in early embryogenesis and in the adult during tissue repair (regeneration).

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