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Urokinase Plasminogen Activator in Injured Adventitia Increases the Number of Myofibroblasts and Augments Early Proliferation

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Key Words
Adventitial myofibroblasts · Arterial remodeling · Urokinase

Abstract
Myofibroblasts are involved in vessel remodeling during the development of hypertension as well as after angioplasty and aortocoronary grafting, but the mechanisms of myofibroblastic phenotypic modulation are not fully elucidated. We assessed the role of urokinase plasminogen activator (uPA) and its proteolytic activity in myofibroblast differentiation and the early proliferation following mechanical injury of the rat carotid adventitia. The effects of perivascular application of recombinant uPA (r-uPA), proteolytically inactive r-uPA(H/Q) and uPA neutralizing antibody were evaluated 4 days after surgical injury to the adventitia. The phenotype of adventitial cells was assessed using anti-α-smooth muscle actin (α-SM actin) antibody, anti-SM heavy chain myosin, anti-high-molecular-weight caldesmon, anti-smoothelin and anti-ED-1 antibodies, proliferation by the expression of proliferating cell nuclear antigen, and the size of the adventitia by quantitative morphometry. Four days after injury, the intensive immunostaining for urokinase appeared in the rat carotid artery adventitia. At the same time, the frequency of α-SM actin-positive adventitial cells was 1.8 ± 1.1% in uninjured arteries and 25.2 ± 5.4% in injured arteries ($p < 0.05$), and the respective frequency of ED-1-positive cells 1.5 ± 1.1 and 25.0 ± 5.2%. The application of exogenous r-uPA doubled the numbers of α-SM actin-positive adventitial cells to 55.7 ± 6.8% ($p < 0.05$). ED-1-positive cells and proliferating cell nuclear antigen-positive cells as well as the size of the adventitia were also significantly increased after r-uPA compared with injury alone. In contrast, the proteolytically inactive r-uPA(H/Q) did not affect any parameters. The application of uPA neutralizing antibody attenuated the frequency of α-SM actin-positive cells to 12.6 ± 3.5% ($p < 0.05$), the frequency of ED-1-positive cells, and the numbers of adventitial cells. r-uPA stimulation of cultured human skin fibroblasts significantly increased the α-SM actin content in a concentration-dependent manner. In contrast, r-uPA(H/Q) did not induce changes in α-SM actin content. We conclude that uPA, which is upregulated in the injured adventitia, can augment adventitial cell accumulation, including myofibroblasts, and adventitia growth early after injury of the rat carotid artery adventitia by mechanisms involving proteolysis.

Introduction

Recent data suggest that adventitia and perivascular tissue surrounding the artery are actively involved in vascular remodeling in a number of pathological conditions including restenosis after angioplasty and hypertension [1, 2]. The important characteristic feature of arterial remodeling in hypertension is the significant increase in

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adventitial fibroblast replication [3, 4]. In postangioplasty restenosis adventitial cells are involved in both the process of inward arterial remodeling and neointima formation after vessel injury [5, 6]. Gabbiani et al. [7, 8] have established that wound healing is associated with the transition from fibroblasts to myofibroblasts, which then proliferate, migrate and synthesize extracellular matrix (ECM) components such as collagen type I and III. The most reliable marker of differentiated myofibroblasts is the expression of α-smooth muscle actin (α-SM actin) [9]. Their putative function is generating a contractile force which, through the control of ECM reorganization, can lead to tissue contraction [10].

Thus, myofibroblast differentiation leading to fibrotic changes in the adventitia represents an important independent mechanism of vascular remodeling. The mechanisms of myofibroblastic modulation of fibroblasts are not fully understood. Mechanical stress, transforming growth factor-β_1 (TGF-β_1) and cellular fibronectin are the known regulators of fibroblast transition to myofibroblasts [8]. At the same time, it is well established that the proteolytic system is an important participant of tissue remodeling. Proteolysis is required for local degradation of the ECM during cell migration, replication, and ECM remodeling. Urokinase plasminogen activator (uPA) is known to stimulate proliferation of several cell types [11, 12] and to contribute to vessel remodeling after arterial injury [13–15]. Schafer et al. [16] suggested the importance of monocytes/macrophages and fibroblasts in uPA-mediated plasminogen activation in healing human skin wounds. Our previous studies [15] showed that uPA plays an important role in arterial remodeling after balloon injury in rats. uPA is expressed by myofibroblasts during wound healing [17] and is likely to be similarly important for adventitia remodeling by myofibroblasts following vascular wall injury.

In the present study, we investigated in vivo the effects of urokinase and the significance of its proteolysis property for early myofibroblastic modulation in injured rat carotid artery adventitia. We demonstrated that uPA could augment myofibroblast accumulation and adventitia growth early after injury by mechanisms dependent on its proteolytic properties.

Materials and Methods

r-uPAs and Antibodies

A recombinant wild-type human uPA (r-uPA) was produced as previously described [15]. After activation by plasmin, the proteolytic activity of r-uPA ranged from 1 to 1.2 × 10^5 U/mg protein [18]. A proteolytically inactive uPA, r-uPA(H/Q), was prepared by mutating His-204 within the catalytic center to Gln [18]. r-uPA(H/Q) was mapped with antibodies for different uPA epitopes. It did not possess proteolytic activity, measured using S2444 (Chromogenix, Essen, Germany). The ability of r-uPA-(H/Q) to bind to human uPA receptor is identical to r-uPA and it is as effective as r-uPA in displacing ^{125}I-r-uPA bound to cultured rat aortic medial smooth muscle cells (SMC; IC_{50}, approximately 20 nM) [18]. Both recombinant forms of uPA appeared as single proteins on SDS electrophoresis, with apparent molecular weights ranging from 40 to 43 kDa. Their purity was greater than 95%.

An anti-uPA monoclonal IgG1 antibody was prepared by immunizing mice with human urokinase, purified from urine as previously described [14]. The antibody has a high affinity for human and rat uPA and recognizes all forms of uPA. It is capable of neutralizing human r-uPA activity (1 μg/ml neutralizes 0.8 nM uPA). In rat tissues, it detects uPA with an apparent molecular weight of 48 kDa. Nonspecific total mouse IgG from pooled serum (Inpharm Inc., Moscow, Russia) was used as control for periadventitial anti-uPA antibody. Primary antibodies used for immunohistochemistry were mouse monoclonal raised against uPA (Inpharm Inc.), proliferating cell nuclear antigen (PCNA; PC 10) and SM myosin heavy chain (SM-MHC, G-4; Santa Cruz Biotechnology, Santa Cruz, Calif., USA), α-SM actin (Dako, Glostrup, Denmark), ED-1 (anti-monocytes/macrophages/dendritic cells; Serotec, Düsseldorf, Germany), smoothelin (Chemicon, Hofheim, Germany), high-molecular-weight caldesmon (h-caldesmon, smooth; Sigma-Aldrich, Moscow, Russia). Control nonimmune mouse IgG and biotinylated horse anti-mouse rat adsorbed antibody were purchased from Vector Laboratories (Grunberg, Germany).

Animals and Surgical Procedures

Male Wistar-Kyoto rats (4–5 months old) were obtained from a colony maintained at the Cardiology Research Center, Moscow, Russia. Their left common carotid artery was subjected to surgical procedures approved by the Cardiology Research Center Animal Care and Experimentation Committee and followed the criteria outlined in the 'Guide for the care and use of laboratory animals' (NIH publication No. 85–23). After anesthetizing the rats with ketamine hydrochloride (100 mg/kg body weight intraperitoneally; Gedeon Richter, Budapest, Hungary), a midline incision was made in the neck to expose the left external carotid artery. The exposed 15 mm of the left common carotid artery, immediately proximal to the carotid bifurcation, underwent the external mechanical injury of the adventitia using Adson-Brown shark teeth forceps (Fine Science Tools Inc., Heidelberg, Germany). The Adson-Brown shark teeth forceps was applied onto this artery, and its tips were fixed using rubber O-rings to keep them closed with a standard degree of pressure on the tips to injure only arterial adventitia and then slowly rotated while pulling the forceps towards the sternum and back. This was repeated three times, and then, 0.5 ml of the desired Pluronic solution was placed around the vessel as previously described [14]. The incisions were closed and the animals were allowed to recover.

Assessment of the significance of uPA for adventitia repair early after injury was investigated by applying to the adventitial side of 7–8 arteries 20 nmol/kg of either (1) r-uPA, or (2) proteolytically inactive r-uPA(H/Q), in which glutamine replaced histidine in position 204. We also examined the effects of applying a

uPA neutralizing monoclonal IgG antibody (500 μg/vessel) or control nonspecific mouse IgG (500 μg/vessel). The peptides were dissolved in 0.5 ml saline containing 40% gel F-127 (Pluronic, BASF, Berlin, Germany) [14]. Control vessels only received 0.5 ml of 40% Pluronic solution after adventitial injury. The uninjured left carotid arteries from sham-operated rats were also analyzed. Four days later, the animals were killed, and isolated arteries subjected to immunohistochemical and morphometric analyses.

Tissue Collection and Processing

The animals were deeply anesthetized with sodium pentobarbital (100 mg/kg body weight; Sanofi Santé Animale, Libourne, France). Then they were perfused (120 mm Hg) with saline solution, followed by 4% formaldehyde solution for 10 min [14]. Left and right common carotid arteries were removed, cleaned of extraneous material and cut into three equal segments before embedding in paraffin. Cross-sections (5 μm for immunohistochemistry and 10 μm for morphometry) were cut from each block, at 100- to 200-μm intervals.

Morphometry

Morphometry was carried out as previously described [15]. Briefly, cross-sectional areas of lumen, media and adventitia, defined as a collagen-rich area surrounding the media and located between periadventitial tissues and external elastic laminae, of formaldehyde-fixed Van Geisen-Verhoff-stained sections were measured by a blinded histologist, using a Zeiss microscope coupled to a ProgRess-3008 camera (Kontron Elektronik, Eching, Germany) and a computer with an Optimas morphometric program (Optimas Corporation, Carlsbad, Calif., USA). Morphometry was performed on five sections of the distal and five sections of the proximal segments of the arteries, and the data for each artery averaged.

Immunohistochemistry

Sections were deparaffinized with xylene, rehydrated and treated with 3% hydrogen peroxide to quench endogenous peroxidase. Serial sections were used to localize immunoreactive peptides [15]. The sections were incubated in 10% serum (ICN, Budapest, Hungary) from the same species as the secondary biotinylated antibodies, and then, with either the anti-uPA monoclonal antibody (10 μg/ml), the anti-myosin polyclonal antibody (10 μg/ml), the anti-h-caldesmon monoclonal antibody (2 μg/ml), the anti-ED-1 monoclonal antibody (3.3 μg/ml), the anti-PCNA monoclonal antibody (4.8 μg/ml), the anti-α-SM actin monoclonal antibody (3.3 μg/ml), the anti-smoothelin monoclonal antibody (10 μg/ml), or the appropriate control mouse or rabbit nonimmune IgGs in concentrations coinciding with those of each immune IgG for 1 h in a humidified chamber. After multiple washings in physiological buffered saline (pH 7.4), the sections were incubated with the appropriate biotinylated anti-mouse or anti-rabbit antibodies (15 μg/ml). Antigens were detected using the ABC method (Vector Laboratories Inc.) and the chromogen 3,3′-diaminobenzidine tetrahydrochloride, before lightly staining the sections with hematoxylin.

Assessment of Efficacy of Perivascular Administration

The efficacy of perivascular delivery of the uPA neutralizing antibody was assessed using a rat adsorbed biotinylated horse anti-mouse antibody, a polyclonal horse anti-goat antibody as negative control, and an avidin-biotin immunoperoxidase kit as previously described [15]. The delivery of control nonspecific total mouse IgG was also assessed. The efficacy of perivascular delivery of the recombinant forms of uPA was assessed 2 and 4 days after their application to the arteries, using r-uPA forms conjugated to biotin. r-uPAs applied to the vessels were later detected using (1) Western blots and NeutrAvidin (Pierce, Rockford, Ill., USA) and (2) immunohistochemistry with the streptavidin-peroxidase kit of the Vector Laboratories as previously described [15]. All substances were present in the adventitia of the arteries 2 days after their application, but by 4 days, they were no longer detectable (data not shown).

Cell Culture

Human fibroblasts were obtained post mortem from normal subcutaneous tissues of skin. Subcutaneous tissues were collected during autopsy, not later than 6 h after death, from healthy male individuals, aged 30–38 years at the Cardiology Research Center, Moscow, Russia. All subjects died of external causes (accidental death) and did not suffer from infectious or other disorders which could potentially affect subcutaneous fibroblasts. The procedures for collecting subcutaneous tissues for the studies were approved by the Cardiology Research Center Human Ethics Experimentation Committee. Tissues were excised, minced, washed in phosphate-buffered saline (PBS) and then treated at 35°C with a mixture of 0.1% trypsin and 100 units/ml 0.15% of type IV collagenase (Sigma-Aldrich) for 10 min. Isolated cells were plated on culture dishes in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and incubated for 2 h at 37°C in 5% CO_2. The purity of cell cultures was analyzed by immunostaining with anti-prolyl-4-hydroxylase antibody specific for fibroblasts and anti-von Willebrand factor antibodies specific for endothelial cells and anti-SM myosin antibodies for SMC. In a standard cell preparation, only 1–2% of cells were positively stained with anti-von Willebrand factor antibodies and 99% of cells were stained positively with anti-prolyl-4-hydroxylase antibodies. For all our studies, cells from passages 4–6 were used. Prior to treatment, cells were grown to 70% confluent and deprived in DMEM containing 0.1% FBS for 24 h. Then, 1–100 nM of r-uPA or r-uPA(H/Q) in DMEM supplemented with 0.15 FBS was added to the cells and incubation proceeded for 48 h.

Cell Lysates and Immunoblot Analysis

Primary cultures of fibroblasts were washed two times and scraped with ice-cold PBS. Cell suspension was centrifuged for 5 min at 1,000 *g*, and the cell pellet was lyzed for 10 min in 0.1 M Tris-HCl, pH 8.1, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF (100 μl of lysis buffer for one 100-mm culture plate). Then, lysates were centrifuged for 30 min at 14,000 *g*. Protein concentration in supernatants was determined according to the Bradford method using BSA as standard protein.

Lysates (80 μg of total protein) were separated by SDS-PAGE on 12% polyacrylamide gel under reducing conditions according to Laemmli and transferred to PVDF membrane according to Towbin. Membranes were blocked for 1 h at 25°C with blocking buffer (5% non-fat dry milk in PBS containing 0.05 Tween-20) and incubated for 1 h at 25°C with primary antibodies. Immunoblotting was done, as previously described [19], with anti-human α-SM actin monoclonal IgG (Sigma-Aldrich) diluted to

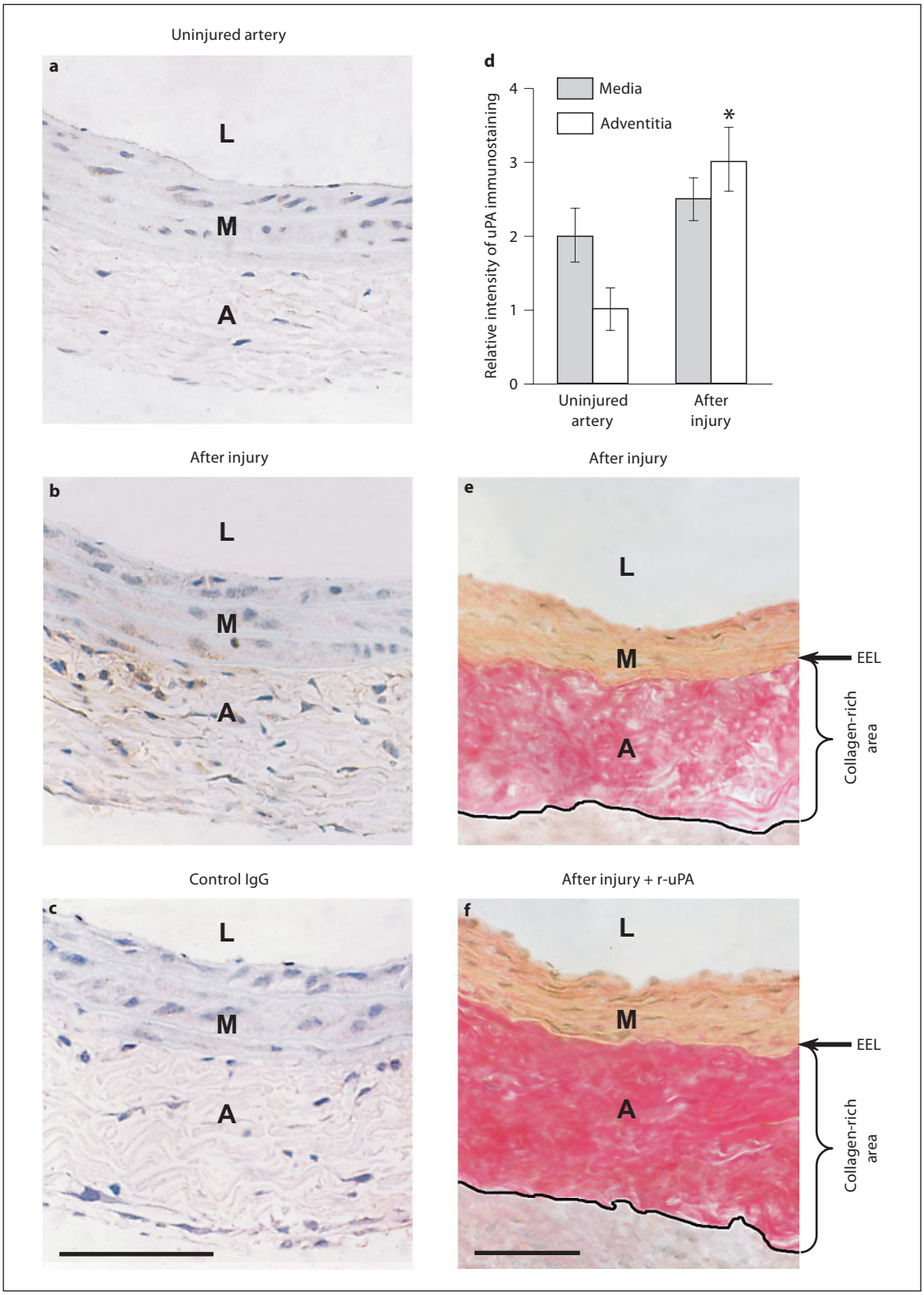

Fig. 1. a–c Photomicrographs showing the uPA immunolocalization. Cell nuclei are stained with hematoxylin. L = Lumen; M = media; A = adventitia. Scale bar = 30 μm. **a** In normal carotid arteries, uPA immunostaining is absent in the adventitia. **b** At 4 days after injury, strong uPA immunoreactivity (brown coloring) is apparent in the hypercellular adventitia. **c** Control showing

Table 1. Percentages of cells expressing various markers after treatment and total cell numbers in adventitia

Marker	Uninjured	Control	r-uPA	r-uPA(H/Q)	uPA antibody
α-SM actin	1.8 ± 1.1	25.2 ± 5.4*	55.7 ± 6.8**	30.1 ± 5.9	12.6 ± 3.5**
SM-MHC	0	8.4 ± 1.2*	14.3 ± 4.3**	7.9 ± 2.2	5.6 ± 2.1**
h-Caldesmon	0	0	0	0	0
Smoothelin	0	0	0	0	0
ED-1	1.5 ± 1.1	25.0 ± 5.2*	33.1 ± 4.5**	26.2 ± 6.1	16.7 ± 3.1**
Total cell number	104.5 ± 12.0	165.5 ± 8.1*	243.5 ± 26.1**	170.5 ± 25.5	124.5 ± 15.5**
Adventitial area, mm^2	0.077 ± 0.006	0.103 ± 0.011*	0.138 ± 0.013**	0.094 ± 0.008	0.089 ± 0.004

Data represent the mean ± SEM of the percentages of adventitial cells expressing either α-SM actin, SM-MHC, h-caldesmon, smoothelin or monocyte/macrophage marker (ED-1), as well as total numbers of adventitial cells per cross-section and changes in the adventitial area in at least 7 arteries 4 days after adventitial injury of the carotid artery and perivascular administration of either r-uPA, r-uPA(H/Q) or uPA neutralizing antibody. Controls represent vessels which only received pluronic gel in saline. The uninjured group represents left carotid arteries from sham-operated animals.

* $p < 0.05$, control versus uninjured artery, ** $p < 0.05$ versus control.

0.18 μg/ml, SM-MHC (G-4; Santa Cruz Biotechnology) diluted to 0.1 μg/ml, smoothelin (Chemicon) diluted to 1 μg/ml, and h-caldesmon (smooth; C 4562, Sigma-Aldrich) diluted 1:2,000. Blots were incubated with secondary anti-mouse IgG conjugated to horseradish peroxidase (0.08 μg/ml; Dianova, Hamburg, Germany). The chromogen 3,3′-diaminobenzidine tetrahydrochloride was used for antigen detection. Stained blots were scanned using a digital video camera (Kodak, Stuttgart, Germany), and images were analyzed with PCBAS 2.08. software.

Data Analyses

The immunohistochemical staining for uPA was graded in a semiquantitative blinded manner by 2 observers using a scale of 0–4, with 0 indicating no staining (background); +1, variable staining in a specific region; +2, weak staining; +3, consistent positive staining, and +4, pronounced positive staining (3 sections/rat) [15]. PCNA-positive cells and total cell numbers (i.e. hematoxylin-stained nuclei in the media and adventitia) were determined by counting (3 sections/rat). A PCNA labeling index was calculated using the following equation: PCNA labeling index = (PCNA – positive cells per 3 cross-sections/total cells per 3 cross-sections) × 100. The percentages of urokinase, α-SM actin, smoothelin, SM-MHC, h-caldesmon and ED-1-positive cells in the adventitia were determined in the same manner. All results are means ± SEM. Comparisons between multiple groups were performed using one-way ANOVA and the Student-Newman-Keuls test for multiple comparisons. Single comparisons were made using Student's t test. A value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed using Jandel SigmaStat.

Results

uPA Expression and Adventitial Cells after Periadventitial Injury

The uPA expression in the rat carotid artery adventitia was significantly upregulated 4 days after periadventitial injury compared with uninjured arteries (fig. 1a, d) and distributed uniformly throughout the adventitia (fig. 1b). The frequency of uPA-positive adventitial cells was increased from 1.4 ± 0.5% in uninjured arteries to 47.4 ± 3.2% in injured arteries ($p < 0.05$). Increases in the expression of PCNA immunoreactive peptides were also apparent 4 days after injury, and we observed an increased PCNA labeling index of 16.6 ± 3.2% compared with that in the uninjured vessels of 5.1 ± 2.5% ($p < 0.05$).

The phenotype of adventitial cells was assessed using anti-α-SM actin, anti-SM-MHC, anti-h-caldesmon, anti-smoothelin and anti-ED-1 antibodies (table 1). The α-SM actin expression in the adventitia was significantly upregulated 4 days after injury versus that in the uninjured

nonimmune mouse IgG control. **d** Expression of uPA in uninjured arteries and 4 days after injury to the rat carotid adventitia. The intensity of staining was graded from 0 to +4 as described in 'Materials and Methods', and the results for each animal were averaged. Results are means ± SEM of 7 animals in each group. * $p < 0.05$ versus uninjured arteries. **e** Histological figure depicting a vessel which received only pluronic gel in saline. **f** Histological figure depicting the effects of perivascular administration of r-uPA on carotid artery adventitia 4 days after adventitial injury. Sections are stained with Van Geisen-Verhoff stain. L = Lumen; M = media; A = adventitia. The adventitia was defined as a collagen-rich area located between periadventitial tissues and external elastic laminae (EEL). Scale bar = 30 μm.

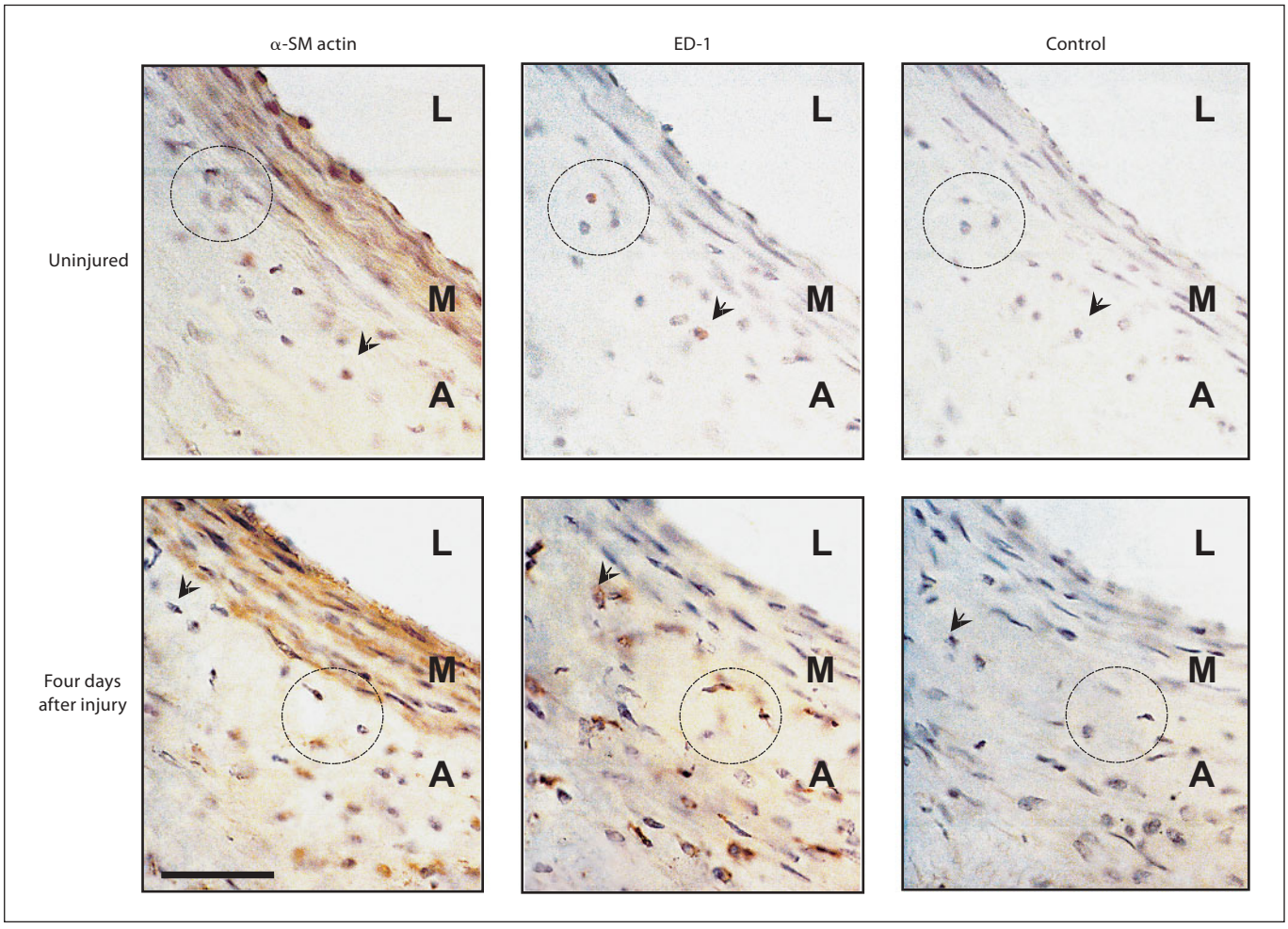

Fig. 2. Expression of α-SM actin and ED-1 immunoreactive peptides in serial sections of uninjured carotid arteries and antigen expression 4 days after injury. Immunoreactive peptides are represented by brown coloration. Controls represent nonimmune mouse IgG controls for the mouse monoclonal antibody. L = Lumen; M = media; A = adventitia. Arrows and rings indicate cells and groups of cells to demonstrate representative immunostaining differing myofibroblasts and leukocytes. Cell nuclei are stained with hematoxylin. Scale bar = 50 μm.

adventitia ($p < 0.05$; table 1, fig. 2). SM-MHC expression was not detected in uninjured adventitia and was significantly upregulated after injury ($p < 0.05$). Adventitial cells did not express h-caldesmon or smoothelin before or after injury (table 1). The overall frequency of ED-1 expression was significantly increased 4 days after injury compared with uninjured adventitia ($p < 0.05$; table 1, fig. 2). About 10 ± 3.7% of α-SM actin positive cells were also ED-1 positive (fig. 2, indicated by arrows).

uPAs and Adventitial Myofibroblasts

To examine the significance of uPA and its proteolytic activity in myofibroblast differentiation, we compared the frequency of α-SM actin-positive cells 4 days after various treatments of injured adventitia (table 1). The administration of r-uPA with wild-type structure significantly increased the α-SM actin labeling index ($p < 0.05$), while uPA neutralizing antibody attenuated the frequency of α-SM actin staining ($p < 0.05$). The proteolytically inactive r-uPA(H/Q) was ineffective ($p > 0.05$). Adventitial cells did not express h-caldesmon or smoothelin despite various treatments (table 1). The percentage of SM-MHC-positive cells in the adventitia was also upregulated after r-uPA ($p < 0.05$) and downregulated after uPA neutralizing antibody ($p < 0.05$). r-uPA(H/Q) did not affect SM-MHC expression. After r-uPA, the frequency of

ED-1-positive cells was significantly increased ($p < 0.05$), while the proteolytically inactive r-uPA(H/Q) was ineffective ($p > 0.05$). uPA neutralizing antibody attenuated the frequency of ED-1 staining ($p < 0.05$) (table 1).

uPA, Adventitia Size and Cell Proliferation

To further evaluate the relative importance of the proteolytic property of uPA in regulating adventitia growth and early myofibroblast proliferation, we compared the size of the adventitia, total cell numbers and PCNA labeling indexes in the injured adventitia 4 days after various treatments. Periadventitial r-uPA increased the size of the adventitia ($p < 0.05$ vs. control; table 1, fig. 1e, f), while uPA neutralizing antibody did not significantly change the adventitial area ($p = 0.05$ vs. control). The application of r-uPA(H/Q) did not affect the adventitial structure ($p > 0.05$ vs. control). None of the substances affected the vessel lumen or medial area compared with the vehicle ($p > 0.05$; data not shown).

In injured vessels, we observed an increased total adventitial cell number per cross-section versus uninjured vessels ($p < 0.05$). Following perivascular uPA, the total adventitial cell number per cross-section nearly doubled compared with control vehicle-treated vessels ($p < 0.05$), while r-uPA(H/Q) did not affect cell numbers in the adventitia ($p > 0.05$). uPA neutralizing antibody significantly decreased the adventitial cell number ($p < 0.05$; table 1).

Periadventitial r-uPA increased the PCNA labeling index in the adventitia to 51.3 ± 6.1% ($p < 0.01$ vs. 16.6 ± 3.2% in the control group). In contrast, neither proteolytically inactive r-uPA(H/Q) nor uPA neutralizing antibody affected the proliferation indexes versus control ($p > 0.1$ and $p > 0.05$, respectively).

uPA and Transformation of Fibroblasts into Myofibroblasts in Cell Culture

To further confirm the ability of urokinase to stimulate the transdifferentiation of fibroblasts into myofibroblasts, we assessed the effects of the native r-uPA on myofibroblastic transformation of cultured fibroblasts. We evaluated the relative content of α-SM actin in cultured cells after 48 h r-uPA stimulation in increasing concentrations (from 1 to 50 nM) using immunoblotting. Following r-uPA stimulation, the α-SM actin content was increased in accordance with uPA concentrations (fig. 3a), which provides evidence of the increase in the frequency of myofibroblasts in culture and can indicate the phenotypic modulation of fibroblasts into myofibroblasts. As shown in the Western blots of figure 3b, cultured fibroblasts were negative for three SMC differentiation markers – SM-MHC, smoothelin and h-caldesmon – whereas after r-uPA (10 nM) treatment, light expressions of SM-MHC with a parallel increase in α-SM actin expression

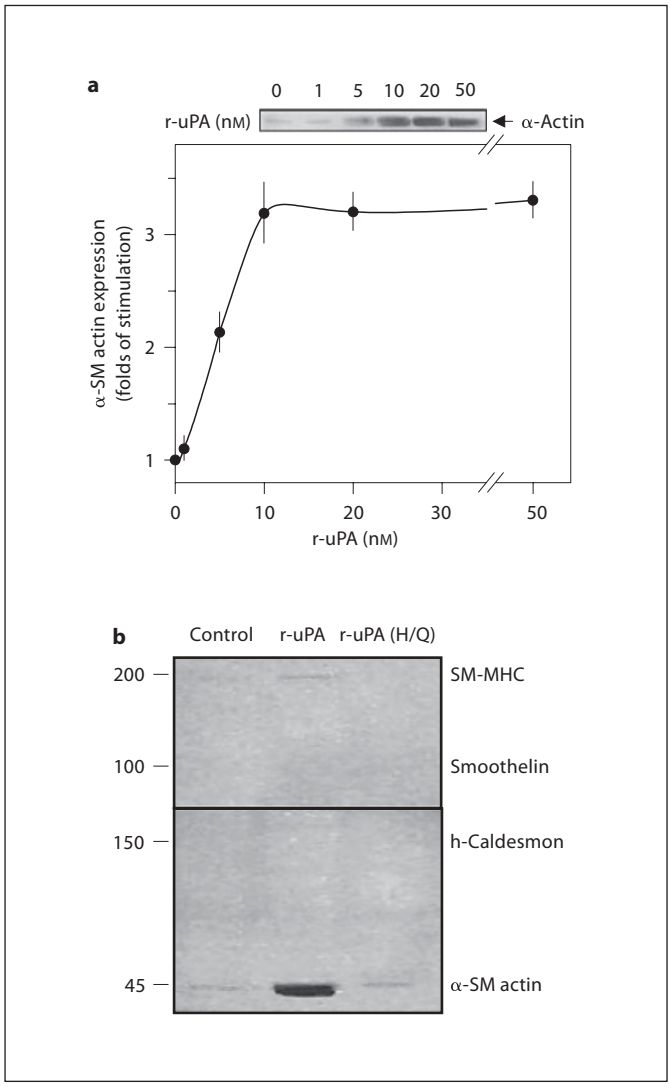

Fig. 3. a Concentration-dependent effects of r-uPA on α-SM actin content in cultured skin human fibroblasts (r-uPA; 1–50 nM). The content of α-SM actin in cell culture homogenates was assessed using immunoblotting. Results are expressed as fold stimulation relative to α-SM actin content in untreated cells, which was arbitrarily normalized to 1.0 for each experiment. Results are means ± SEM of 4 experiments. **b** Western blots showing SMC differentiation marker (SM-MHC, smoothelin, h-caldesmon and α-SM actin) expression in cultured skin human fibroblasts after either r-uPA or r-uPA(H/Q) (10 nM) treatment (representative of 4 experiments). The control group represents cells treated with saline.

but no smoothelin or h-caldesmon were observed in cultured fibroblasts. In contrast to r-uPA, the proteolytically inactive r-uPA(H/Q) did not induce changes in α-SM actin or SM-MHC content in cultured cells.

Discussion

To efficiently prevent pathological arterial narrowing, it is important to fully clarify the mechanisms implicated in unfavorable inward arterial remodeling. Recent studies indicate that adventitial myofibroblast accumulation with subsequent constrictive fibrosis might be one of the major causes of negative arterial remodeling [5, 6, 20]. It is well accepted that the uPA system can regulate cell proliferation and migration as well as participate in lumen narrowing in injured vessels and in the development of various human vascular disorders [13, 14, 21, 22]. Elevated uPA expression is now considered to cause constrictive arterial remodeling [23, 24]. To elucidate the role of uPA for adventitial cell functioning, we studied the effects of uPA for fibroblasts both in vivo and in vitro. Our results suggest that uPA is upregulated in injured rat carotid artery adventitia. Our data indicate that elevations in uPA after adventitial injury lead to increases in myofibroblast numbers and adventitia growth. These effects are highly dependent on the proteolytic activity of uPA. Our data on the effects of uPA on the content of α-SM actin in fibroblastic cell culture indicate the increase in the portion of myofibroblasts after uPA stimulation. These findings provide direct evidence of stimulation of myofibroblastic phenotypic modulation by urokinase.

We used well-established SMC differentiation markers such as α-SM actin, SM-MHC, smoothelin and h-caldesmon. These markers have been previously used for the definition of changes in fibroblast phenotype [5, 6, 25]. In particular, α-SM actin is the most reliable characteristic of myofibroblasts [1, 8, 9]. Myofibroblasts can also express SM myosin, but it was not always observed in microfilament bundles of myofibroblasts during wound healing nor after balloon catheter injury of the artery [5, 9]. h-Caldesmon, which is a cytoskeleton-associated actin-binding protein, has been reported to be one of the most specific myogenic markers, since myofibroblasts express a negligible amount of h-caldesmon [26]. Smoothelin is a well-accepted highly selective late differentiation marker of SMC [27]. Similarly to other models in our study, adventitial fibroblasts modulated into myofibroblasts were indicated by the positive α-SM staining, to a less extent by SM-MHC staining and the negative staining for h-caldesmon and smoothelin.

Several lines of evidence indicate that uPA appears to be the important determinant influencing adventitial cell proliferation and myofibroblastic modulation. First, exogenous uPA stimulated cell proliferation and the number of myofibroblasts in injured adventitia. Second, uPA neutralizing antibody attenuated α-SM actin expression by adventitial cells after injury. Third, uPA upregulated the content of α-SM actin in fibroblastic cell culture in a concentration-dependent manner.

An important role of uPA for myofibroblast function in tissue repair was suggested by findings of enhanced expression of uPA during wound healing inflicted on cultured human fibroblasts [17]. Other in vitro studies have demonstrated elevated uPA production by myofibroblasts, capable of extensive migration [28, 29]. Our data are consistent with previous findings of Garvin et al. [30] on high uPA expression by adventitial myofibroblasts during arterial remodeling of coronary arteries in transplanted heart.

uPA is now considered to be implicated in the regulation of cell proliferation and migration by means of its proteolytic as well as nonproteolytic properties [11, 12, 18, 31–33]. For example, the proteolytically inactive uPA derivatives have been shown to initiate proliferation in osteoblast-like cells [33], whereas in renal cells [31] and fibroblasts [12, 32], the presence of both growth factor-like and proteolytic domains of uPA were required for mitogenesis. Our previous studies showed that uPA-mediated plasmin proteolysis is an important contributor to SMC proliferation in vitro [11] as well as in vivo [14, 15] after arterial injury. In the present study, proteolytically inactive r-uPA(H/Q), despite of its ability to bind to the uPA receptor [18], did not increase early adventitia growth and did not affect the expression of myofibroblast markers, either in vivo or in vitro, contrasting markedly with the effects of proteolytically active r-uPA that indicate the importance of uPA proteolytic activity for myofibroblastic modulation. Proteolysis initiated via uPA has the potential to regulate cell differentiation, proliferation and migration by a number of mechanisms. Upregulation of metalloproteinases (MMPs) plays a role in the transformation of cells to myofibroblast-like cell phenotype [34]. The conversion of pro-MMPs to active MMPs is dependent on urokinase-generated plasmin activities, which in turn can degrade ECM proteins including collagens [21]. TGF-β_1 is one of the well-established regulators of myofibroblastic modulation [8, 35]. Matrix-bound growth factors, such as heparin-binding growth factor and latent

TGF-β_1, are released during ECM degradation and have the potential to further augment cell differentiation and proliferation [36, 37]. uPA-dependent plasmin proteolysis is one of the key activators of TGF-β_1 [36] that in turn may further contribute to the increases in myofibroblast numbers in injured adventitia. Plasmin proteolysis is involved in the regulation of TGF-induced endothelin-1 release [38] that can also promote myofibroblast activation [39]. Moreover, recent evidence suggests the role for plasmin in the stimulation of fibroblast proliferation via the upregulation of the expression of Cyr61, a growth factor-like gene, and the release of Cyr61 from the ECM [40]. The involvement of another serine protease – thrombin – in differentiation of fibroblasts to a myofibroblast phenotype in this model is also possible, as thrombin is a potent profibrotic mediator [41] and is known to be important for tissue repair after injury [42]. As soon as urokinase and thrombin can cleave each other, giving rise to derivatives with various properties, the role for interactions of urokinase and thrombin in myofibroblastic modulation of fibroblasts is not obvious and requires elucidation in further independent studies.

Our data on ED-1-positive cell accumulation after r-uPA treatment of injured adventitia and the opposite effect of uPA-neutralizing antibody are consistent with findings in uPA knock-out mice indicating that uPA mediates infiltration of leukocytes during arterial remodeling and neointima formation after injury [13] and during arteriogenesis [43]. Delayed monocyte recruitment into experimental venous thrombus was also associated with the absence of uPA [44]. Leukocytes are the important participants of vascular remodeling in a number of pathological conditions. The important role for leukocyte accumulation is postulated for neointima formation and restenosis [45]. Adventitial inflammatory responses and subsequent constrictive fibrosis have been proposed to be the major cause of constrictive negative remodeling after balloon injury [46, 47]. It was shown that inhibition of the inflammatory response in the adventitia limited the development of arterial constrictive remodeling by reducing adventitial fibrosis [46]. The inflammatory reaction was also suggested to underlie the pathogenesis of hypertension and the associated lesion formation [48]. Present results indicate a role of uPA in inflammatory cell accumulation in arterial adventitia that may represent one of the mechanisms of uPA control of adventitial remodeling.

To summarize, we have shown that addition of urokinase following injury of the rat carotid artery adventitia augments early myofibroblastic modulation, myofibroblasts and inflammatory cell accumulation in injured adventitia, as well as adventitia growth, by mechanisms dependent on its proteolytic properties.

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