GENERAL PATHOLOGY AND PATHOPHYSIOLOGY

Urokinase Stimulates Inflammatory Response in Damaged Vascular Wall during *in Vivo* Arterial Remodeling

O. S. Plekhanova, M. Yu. Menshikov, M. A. Solomatina, E. I. Ratner, V. A. Tkachuk, Ye. V. Parfyonova

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 145, No. 1, pp. 15-19, January, 2008
Original article submitted April 13, 2007

Perivascular application of urokinase to the ballooned artery promoted the growth of neointima and constrictive remodeling of the vessel and stimulated the inflammatory response in the damaged vascular wall *in vivo*. Recombinant tissue plasminogen activator did not induce these changes. Our results indicate that urokinase is involved in the regulation of the inflammatory response during *in vivo* remodeling of the damaged vascular wall.

Key Words: *urokinase; neointima; remodeling; restenosis; balloon angioplasty*

Vascular wall remodeling is a critical stage in the pathogenesis of vascular diseases, including atherosclerosis, restenosis after balloon angioplasty, and arterial hypertension [2,4,13]. The development of restenosis is mediated by two mechanisms: neointima formation and negative geometric remodeling of the damaged arterial segment [9]. Urokinase plasminogen activator (uPA) is an essential component of the vascular response to damage [1,2]. Our previous studies showed enhanced synthesis of urokinase by cells of the vascular wall in atherosclerotic plaque [3] and in damaged vessels during angioplasty; urokinase contributes to the development of restenosis, stimulates growth of the neointima, and promotes constrictive remodeling of the artery [1,2,12]. Migration, proliferation, and apoptosis in vascular cells (smooth muscle cells, SMC; leukocytes; and fibroblasts) and remodeling of the extracellular matrix (EM) serve as the key stages in remodeling of the vascular wall, contribute to the formation of neointima and neoadventitia, and cause constriction of the artery [4,13]. These processes are regulated by proteases, adhesion molecules, and integrins. Cytokines secreted primarily by monocytes/macrophages infiltrating the vascular wall after damage are also involved in this regulation [2,6]. The nonspecific inflammatory response of the vascular wall to damage is an important pathogenetic stage of restenosis and atherosclerosis [7]. In the early period after damage, this reaction is provided by neutrophils that increase the formation of reactive oxygen species, trigger gene expression, and contribute to cell proliferation [11]. Prolonged inflammatory response results from infiltration of the vascular wall by monocytes secreting a variety of cytokines and growth factors [7]. The role of monocyte infiltration in restenosis was demonstrated in experimental studies and clinical trials [7,10]. The role of plasminogen activators in the inflammatory response during *in vivo* remodeling of the damaged vascular wall remains unclear. Tumor necrosis factor-α (TNF-α) is an im-

Russian Cardiology Research-and-Production Complex, Federal Agency for Health Care and Social Development, Moscow. ***Address for correspondence:*** yeparfyon@cardio.ru. Ye. V. Parfyonova

0007-4888/08/1451-0010

portant mediator of inflammation. TNF-α is produced by monocytes/macrophages, neutrophils, and structural components of the vascular wall (SMC and fibroblasts) and induces the formation and secretion of proinflammatory factors (interleukin-10, corticosterone, and prostaglandins). Transition of membrane-bound TNF-α into active soluble form is induced by TNF-α-converting enzyme (TACE) belonging to the metalloproteinase family [8].

Here we studied the effect of urokinase on the inflammatory response in the vascular wall (*e.g.*, formation of TNF-α and converting enzyme) after experimental balloon damage to the artery. This process was compared with early stages of arterial remodeling.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats. The animals were divided into 2 groups (14-16 rats per group). Balloon damage to the left common carotid artery was induced under general anesthesia with 10 mg/kg calipsol [1,2]. Recombinant urokinase and tissue plasminogen activator in 0.5 ml 40% pluronic F-127 gel (20 nmol/kg, Boehringer Ingelheim Pharma KG) were applied periadventitially [12]. In control rats, pluronic gel without the test agents was applied after ballooning of the vessel. The arteries were extracted on days 2 and 4 after surgery under general anesthesia with 2 mg/kg pentobarbital. The vessels were washed with cold Hanks solution (40°C), frozen in liquid nitrogen, and stored at -70°C to perform the polymerase chain reaction (PCR). For immunohistochemical study, the animals were fixed by perfusion of 4% paraformaldehyde through the left ventricle [1]. The carotid arteries were extracted and embedded into paraplast (Sigma) as described elsewhere [1].

Sections (4 μ) were prepared and deparaffinized in decreasing concentrations of xylene and ethanol. Target protein-bound antibodies were routinely visualized in the reaction of avidin—biotin—horseradish peroxidase (Vector Laboratories) with diaminobenzidine tetrahydrochloride (Sigma) [1]. Sections were stained with hematoxylin and embedded into Canadian balsam (Sigma) [1]. Monoclonal antibodies against PCNA (4.8 mg/ml) and ED-1 (3.3 mg/ml), nonimmune mouse immunoglobulins (Vector Laboratories), rabbit serum (Sigma), and secondary biotinilated rabbit antibodies against goat immunoglobulins (Vector Laboratories) were used.

To study cell proliferation in the damaged and intact vessels, the sections were stained for proliferating cell marker (PCNA protein). This protein is associated with δ-polymerase and synthesized in the early G1 and S phase of the cell cycle. The count of PCNA-positive cells and total number of cells were estimated under a light microscope (×20). The proliferation index was calculated as follows: $I=n\times100/N$, where n is the count of antigen-positive cells; and N is the total number of cells. The proliferation indexes for the intima and media of the damaged left carotid arteries were compared with those of the intact right carotid arteries.

For evaluation of vascular wall infiltration with monocytes/macrophages and leukocytes, the sections of vessels were stained with antibodies against ED-1 and CD45, respectively. The count of positive cells and total number of cells were estimated under a light microscope.

Expression of mRNA for TNF-α and TACE was studied on days 2 and 4 after ballooning by the reverse transcription PCR [1]. β-Actin protein expression was assayed to estimate the amount of RNA in each reaction.

The data are presented as the arithmetic mean and coverage error of the sample mean. The significance of differences between general indexes was evaluated by analysis of variance (ANOVA, minimum significance level $p=0.05$) using Jandel SigmaStat software for statistical data processing.

RESULTS

Application of recombinant urokinase to the vessel on day 4 after balloon angioplasty was followed by a 2-fold increase in the total number of cells in the neointima. Cell count in the media and adventitia increased by 50 and 70%, respectively, compared to the control (Table 1). The total number of cells in the neointima significantly decreased, while the count of cells in the media and adventitia remained unchanged after application of tissue plasminogen activator (Table 1).

A significant increase in cell proliferation (compared to the control group with gel application) was revealed only in the neointima of animals after application of urokinase to the carotid arteries (Table 1). Periadventitial application of plasminogen activators had little effect on the ratio of PCNA-positive cells in the media and adventitia (Table 1).

Antigen ED1-positive cells (monocytes/macrophages) were found in various layers of the artery in control animals on day 4 after ballooning (Table 1). Infiltration of the neointima with monocytes/macrophages was somewhat more pronounced after application of urokinase. These parameters remained unchanged after treatment with tissue plasminogen activator (compared to the control group).

TABLE 1. Effect of Plasminogen Activators on Cell Accumulation, Proliferation, and Infiltration of Rat Carotid Artery with Monocytes/Macrophages on Day 4 after Experimental Balloon Angioplasty (*M*±*m*)

Vascular wall		Control (pure gel)	Plasminogen activator	
			urokinase	tissue
Neointima	total cell number	84.7±8.6	155.9±33.4*	17.6±4.9*
	PCNA, %	26.7±1.4	36.9±2.3*	30.0±1.9
	ED1, %	17.8±3.5	24.0±4.2	17.0±2.5
Media	total cell number	152.4±12.9	234.3±57.6*	193.8±45.2*
	PCNA, %	21.0±1.9	18.1±1.8	21.9±1.4
	ED1, %	12.9±2.1	17.2±3.5	13.5±2.4
Adventitia	total cell number	171.8±13.8	303.7±69.7*	233.5±45.2*
	PCNA, %	28.4±2.8	27.3±3.7	29.5±3.2
	ED1, %	25.0±5.2	33.1±4.5*	26.2±6.1

Note. Each group included at least 7 animals. *$p<0.05$ compared to the control.

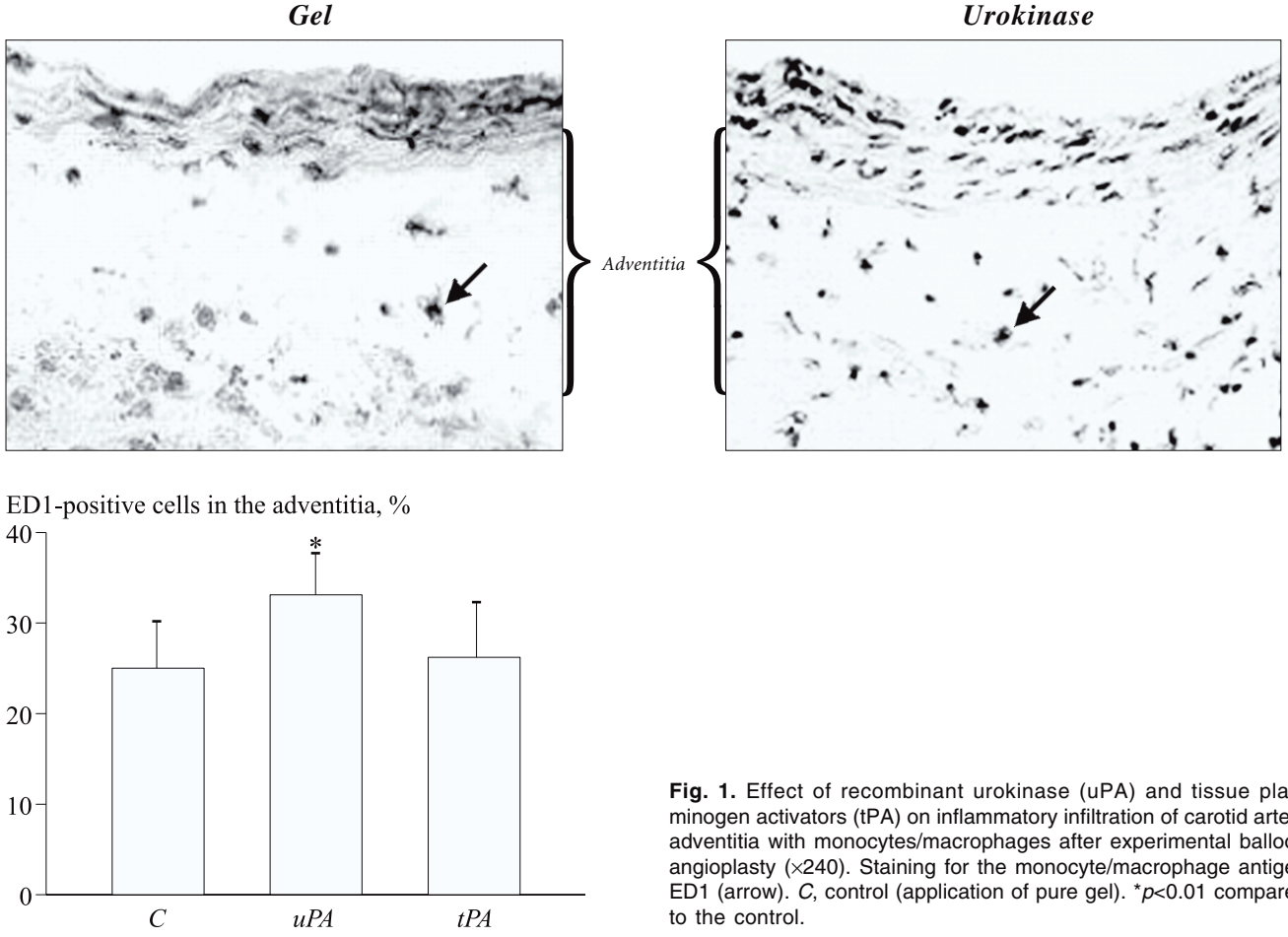

Fig. 1. Effect of recombinant urokinase (uPA) and tissue plasminogen activators (tPA) on inflammatory infiltration of carotid artery adventitia with monocytes/macrophages after experimental balloon angioplasty (×240). Staining for the monocyte/macrophage antigen ED1 (arrow). *C*, control (application of pure gel). *$p<0.01$ compared to the control.

Periadventitial application of plasminogen activators had little effect on the count of ED1-positive cells in the media. Changes in the adventitia contribute to the development of constrictive remodeling. Monocyte infiltration of the adventitia in animals was more pronounced after application of urokinase (Fig. 1). Application of tissue plasminogen activator induced only a slight increase in the ratio of ED1-positive cells in the adventitia compared to the control (Table 1).

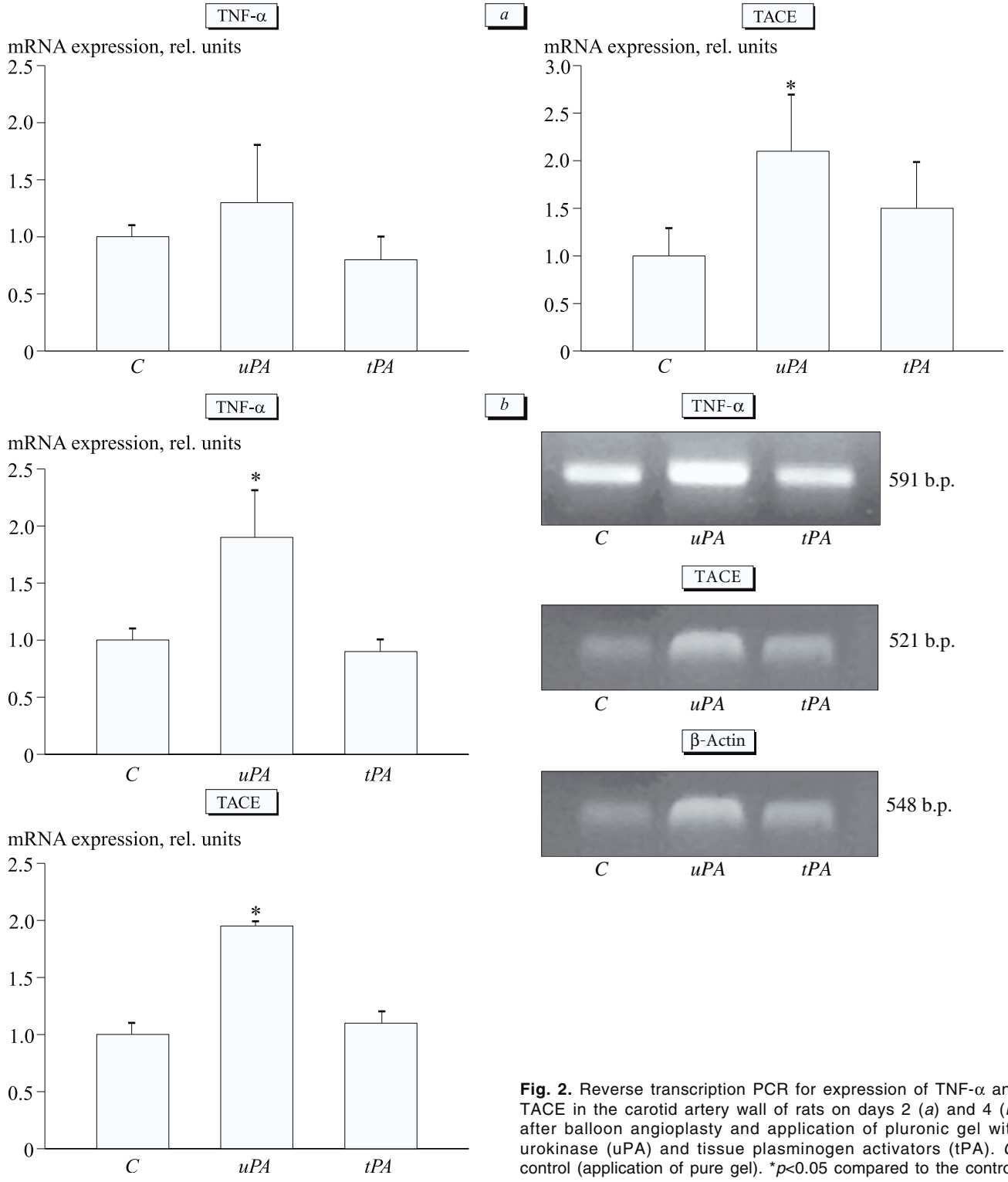

Fig. 2. Reverse transcription PCR for expression of TNF-α and TACE in the carotid artery wall of rats on days 2 (*a*) and 4 (*b*) after balloon angioplasty and application of pluronic gel with urokinase (uPA) and tissue plasminogen activators (tPA). *C*, control (application of pure gel). *$p<0.05$ compared to the control.

Monocyte recruitment in vessel is a mechanism for the action of urokinase on vascular remodeling, since monocytes serve as the source of cytokines and growth factors. These data are supported by the results of our previous experiments. Urokinase increased the expression of mRNA for TNF-α and TACE in the vascular wall. These compounds belong to a group of major proinflammatory factors secreted by monocytes/macrophages. Expression of these proteins was not detected in intact vessel, but increased after vascular damage. Application of urokinase was followed by a significant increase in

TACE expression on days 2 and 4 after ballooning. TNF-α expression also increased on day 4. However, these changes were not observed after application of tissue plasminogen activator (Fig. 2).

The inflammatory response to damage is a general mechanism for neointima formation and arterial remodeling [10,14]. Experiments on rabbits with balloon angioplasty showed that the development of postinflammatory adventitial fibrosis is the major cause of constrictive arterial remodeling [15]. Prevention of the inflammatory response in the adventitia abolishes constrictive remodeling [5].

It can be hypothesized that inflammatory infiltration and fibrosis of the adventitia contribute to constrictive remodeling at the late stage after application of urokinase to the vessel. The urokinase-induced formation of TNF-α probably plays an important role in this process. Fibrous and rigid adventitia compresses the vessel, which prevents compensatory vasodilation in response to neointima growth. Hence, urokinase is a specific factor for the inflammatory response in the vascular wall. These changes constitute a mechanism for negative arterial remodeling under conditions of increased urokinase concentration. A complex balanced control over the expression of urokinase and proinflammatory genes in the damaged region of the vascular wall can prevent unfavorable remodeling of the vascular wall.

This work was supported by the Russian Foundation for Basic Research (grants No. 06-04-49303-a, 05-04-49108-a, and 06-04-08138-ofi) and State Contract with the Belgorod State University (No. 73/2006). We are grateful to E. M. Tararak (Professor), O. P. Il'inskaya (Candidate of Biological Sciences), and N. I. Kalinina (Candidate of Biological Sciences) for their help in this work.

REFERENCES

1. Ye. V. Parfyonova, O. S. Plekhanova, N. I. Kalinina, *et al.*, *Kardiologiya*, No. 9, 69-77 (2000).
2. O. Plekhanova, N. I. Kalinina, E. A. Volynskaya, *et al.*, *Ros. Fiziol. Zh.*, **86**, No. 1, 18-27 (2000).
3. M. A. Solomatina, O. S. Plekhanova, O. P. Il'inskaya, *et al.*, *Tsitologiya*, **46**, No. 4, 352-360 (2004).
4. S. Arribas, C. Hillier, C. Gonzales, *et al.*, *Hypertension*, **30**, No. 6, 1455-1464 (1997).
5. J. M. Breuss, M. Cejna, H. Bergmeister, *et al.*, *Circulation*, **105**, No. 5, 633-638 (2002).
6. M. Hoshiga, C. E. Alpers, L. L. Smith, *et al.*, *Circ. Res.*, **77**, No. 6, 1129-1135 (1995).
7. U. Ikeda, *Cur. Vasc. Pharmacol.*, **1**, No. 1, 6dI70 (2003).
8. A. A. Khalil, J. C. Hall, F. A. Aziz, *et al.*, *ANZ J. Surg.*, **76**, No. 11, 1010-1016 (2006).
9. M. J. Mulvany, G. L. Baumbach, and C. Aalkjaer, *Hypertension*, **28**, No. 3, 505-506 (1996).
10. M. Nakatani, Y. Takeyama, M. Shibata, *et al.*, *Cardiovasc. Pathol.*, **12**, No. 1, 40-48 (2003).
11. E. Okamoto, T. Couse, H. De Leon, *et al.*, *Circulation*, **104**, No. 18, 2228-2235 (2001).
12. Ye. Parfyonova, O. Plekhanova, M. Solomatina, *et al.*, *J. Vasc. Res.*, **41**, No. 3, 268-276 (2004).
13. R. Schwartz, *Am. J. Cardiol.*, **81**, No. 7A, 14E-17E (1998).
14. R. Schwartz, T. D. Henry, *et al.*, *Rev. Cardiovasc. Med.*, **3**, No. 5, S4-S9 (2002).
15. M. E. Staab, S. S. Srivatsa, A. Lerman, *et al.*, *Int. J. Cardiol.*, **58**, No. 1, 31-40 (1997).