Urokinase Increases the Content and Activity of Matrix Metalloproteinases 2 and 9 during *in Vivo* Constrictive Arterial Remodeling

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Perivascular application of urokinase to ballooned artery stimulating the formation of neointima and constrictive arterial remodeling increased the content and activity of matrix metalloproteinases 2 and 9 *in vivo*. Application of recombinant tissue plasminogen produced no such changes. It was demonstrated that urokinase stimulates expression of matrix metalloproteinases *in vivo*.

Key Words: *urokinase; matrix metalloproteinases; neointima; remodeling; balloon angioplasty*

Remodeling of the vascular wall is a critical stage in the pathogenesis of vascular diseases, including arterial hypertension, atherosclerosis, and restenosis after balloon angioplasty [2,6,11,12]. Urokinase plasminogen activator (urokinase) plays a key role in arterial remodeling after balloon angioplasty [2,7]. Our previous studied showed that urokinase stimulates the formation of neointima and constrictive arterial remodeling after experimental balloon angioplasty. However, tissue plasminogen activator decreases the count of neointimal cells and prevents negative remodeling of the artery after balloon injury [3-5]. Migration and proliferation of vascular cells (smooth muscle cells, SMC; leukocytes; and fibroblasts) and remodeling of the extracellular matrix are the key stages in remodeling of the vascular wall. They are accompanied by the formation of neointima and neoadventitia and narrowing of the artery lumen [6,11,12]. These processes are regulated by growth factors and protease system of plasminogen activators, plasmin, and matrix metalloproteinases (MMP) [2,13,14]. Cell migration requires activation of extracellular proteolysis under the influence of not only plasmin and urokinase, but also of MMP. Plasmin initiates transformation of inactive MMP into active compounds [1,7,8,10,13]. The study of transgenic animals not having the genes for MMP-2 and MMP-9 showed that MMP modulate migration of cells in the vascular wall, formation of neointima, and narrowing of the artery lumen after injury [9]. Administration of nonselective synthetic MMP inhibitors (batimastat and marimastat) and transfection with type 1 and 2 tissue inhibitors of MMP (TIMP-1 and TIMP-2) suppress the formation of neointima and prevent narrowing of the lumen in damaged artery [7,9]. It remains unclear whether the differences in the effect of urokinase and tissue plasminogen activator in the vascular wall are *in vivo* mediated by changes in the activity and expression of MMP.

Here we studied the effect of urokinase on expression and activity of MMP after experimental balloon injury of the artery. We assayed the early process of neointima formation and arterial remodeling.

MATERIALS AND METHODS

Experiments were performed on 70-72 male Wistar rats. Balloon injury of the left common carotid artery

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was produced under general anesthesia with 10 mg/kg calipsol [3-5]. Urokinase or tissue plasminogen activator (Boehringer Ingelheim Pharma KG, 20 nmol/kg) in 0.5 ml 40% pluronic gel was applied to the periadventitial space [4]. Control rats were treated only with pluronic gel after ballooning of the vessel. On days 2 and 4 after surgery, the arteries were extracted under general anesthesia with 2 mg/kg pentobarbital. For a zymographic study the vessels were washed with cold Hanks solution (4°C), frozen in liquid nitrogen, and stored at -70°C. For immunohistochemical and morphometric study the animals were perfused with 4% formaldehyde through the left ventricle [3,4]. The carotid arteries were routinely extracted and embedded into paraplast (Sigma) [3,4]. Immunohistochemical study and morphometry was performed on 4- and 10-μ sections, respectively.

For morphometry, the sections were deparaffinized with xylene and ethanol in decreasing concentrations, stained with 0.1% toluidine blue for a few minutes, routinely dehydrated by immersion, and embedded into Canadian balsam (Sigma). Morphometry of histological sections from the carotid arteries was performed using KS-100 2.0 software (ic/Windows Release 2.0, Copyright 1995, Kontron Elektronik GmbH). The area of media and adventitia and lumen of the vessel were measured with Optimas 6.1 software (Optimas Corporation) [3,4].

For immunohistochemical staining we used polyclonal goat and rabbit antibodies to MMP-2 and MMP-9 (Research Diagnostics, 0.02 mg/ml), respectively; nonimmune rabbit and goat Ig (Vector Lab.); goat serum (ICN); rabbit serum (Sigma); secondary biotinylated goat antibodies to rabbit Ig; and secondary biotinylated rabbit antibodies to goat Ig (Vector Lab.). Antibodies bound to the target proteins were visualized in the reaction of avidin-biotin-horseradish peroxidase complex (Vector Lab.) with diaminobenzidine tetrahydrochloride (Sigma) [3,5].

Zymography was performed by the standard method [1]. PAAG/SDS electrophoresis (with 0.2% gelatin) was carried out without mercaptoethanol. Proteins were separated in 7.5% PAAG (layer width 0.75 mm). The gel was washed with 2.5% Triton X-100 and incubated in a buffer containing 50 mmol NaCl, 0.05% Bridge 35 (Sigma), and 10 mmol $CaCl_2$ for 18 h. Gel staining with Coomassie G-250 was performed using a solution containing 0.25% CBB R-250, 40% methanol, and 10% acetic acid for 1 h. Zymograms were densitometried using Science Image software. Proteolytic activity was detected as an uncolored area on completely stained gel. This parameter was expressed in relative units.

For evaluation of MMP expression we used semiquantitative analysis of sections after immunohisto-

TABLE 1. Effect of Plasminogen Activators on MMP Expression in Rat Carotid Artery after Experimental Balloon Angioplasty

Parameter	Intact artery	Day 2			Day 4					
		media			media			neointima		
		control	UPA	TPA	control	UPA	TPA	control	UPA	TPA
MMP-2	1.25±0.25	2.5±0.3	3.75±0.25*	2.0±0.4	2.75±0.25	3.75±0.25*	1.75±0.25	2.75±0.25	3.5±0.3*	1.75±0.25*
MMP-9	0	1.5±0.3	2.5±0.3*	1.5±0.25	1.75±0.25	2.75±0.25*	1.5±0.3	1.75±0.25	2.75±0.25*	1.5±0.3

Note. UPA, urokinase plasminogen activator; TPA, tissue plasminogen activator. *$p<0.05$ compared to the control.

chemical treatment [3,5]. Expression of antigens was expressed in relative units: 0, no antigen; 1, weak or irregular staining; 2-4, regular staining of different degree. The data are expressed as the arithmetic mean and error. The significance of differences was estimated by Student's *t* test at a minimum significance level of p=0.05.

RESULTS

On section of intact vessels insignificant expression of MMP-2 was detected in the media around SMC (Table 1). Expression of MMP-2 in the media significantly increased 2 days after injury and reached maximum on day 4 (Table 1). Developing neointimal cells appeared on the inner elastic membrane 4 days after injury. Intensive specific staining was seen around these cells (Table 1). MMP-2 activity in the vascular wall increased 2 and 4 days after injury (Figs. 1, *a*, 2, *a*). Expression of MMP-9 was not detected in intact artery, it appeared on day 2 after injury, but was less intensive than expression of MMP-2 (Table 1). Expression of MMP-9 in the media and neointima peaked 4 days after injury (Table 1). MMP-9 activity was not found under normal conditions and 2 days after injury, but detected on day 4 (Figs. 1, *d*, 2, *c*).

These data suggest that expression and activity of MMP increase after injury. Published data show that changes in the expression and activity of MMP are temporally and spatially coincide with migration of vascular SMC and variations in urokinase expression [5]. Urokinase expression in the neointima was most significant on day 4, which coincided with the start of medial SMC migration into neointima and period of maximum proliferation of neointimal cells. Expression of urokinase and MMP is not necessarily associated with migration of SMC into the neointima after injury. Our results suggest that urokinase is involved in the regulation of MMP expression during vascular remodeling.

For evaluation of the effects of urokinase and tissue plasminogen activator on MMP content in the intact artery we studied expression and activity of MMP after periadventitial application of these agents. Application of urokinase significantly increased the content of MMP-2 (p<0.05, Table 1) and activities of MMP-2 (72 kDa) and its active form (59 kDa, p<0.05; Figs. 1, *a*, 2, *a*) in the media and neointima on day 4 after injury. Urokinase increased MMP-9 content in the media and neointima (p<0.05, Table 1) on day 2 and particularly on day 4 after injury. MMP-9 activity (92 kDa) remained practically unchanged on days 2 and 4 after injury and application of urokinase. However, activity of active MMP-9 significantly increased 4 days after injury (85 kDa, p<0.05; Figs. 1, *d*, 2, *c*). Therefore, urokinase potentiated activation of MMP-2. Application of tissue plasminogen activator produced no changes in vessels 2 days after injury. At the same time, the content and activity of MMP-2 decreased on day 4 after this treatment (72 kDa, p<0.05; Table 1, Figs. 1, *a*, 2, *a*). These changes can modulate

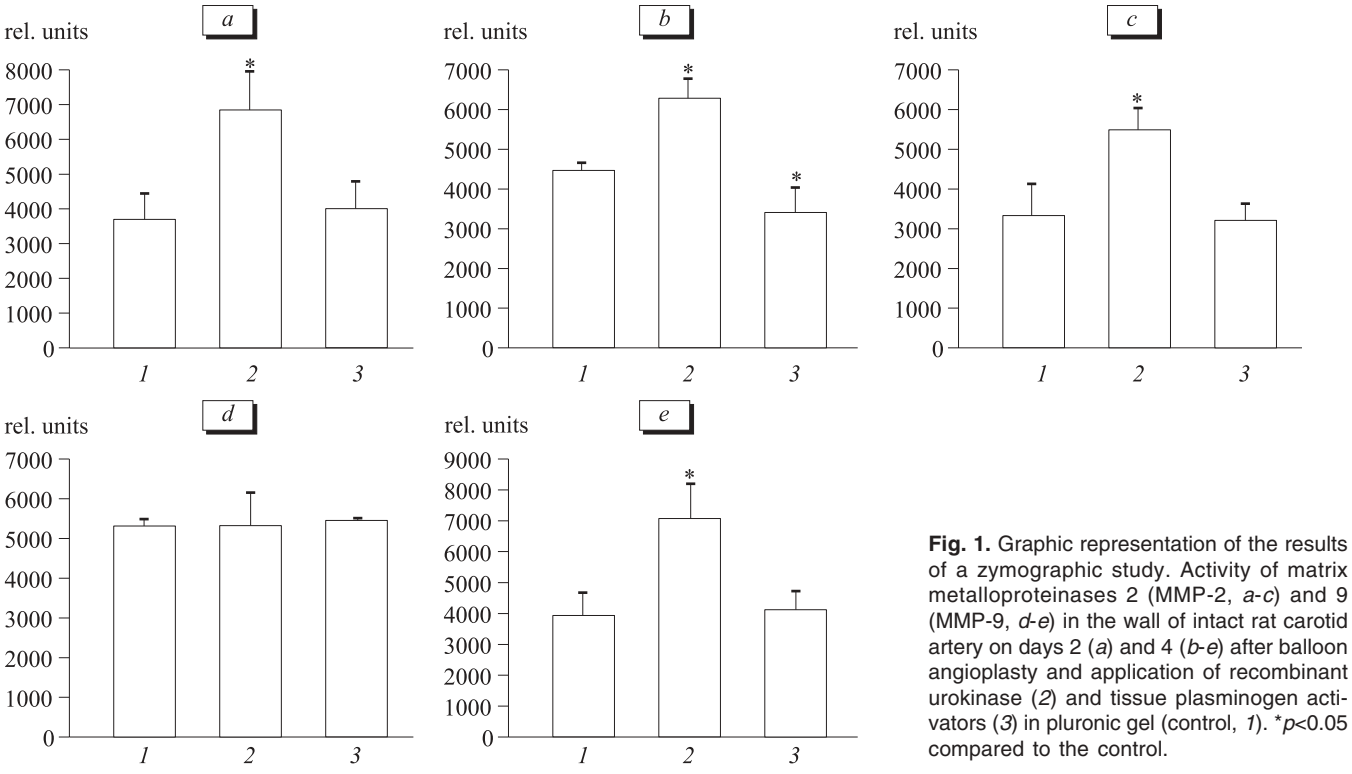

Fig. 1. Graphic representation of the results of a zymographic study. Activity of matrix metalloproteinases 2 (MMP-2, *a-c*) and 9 (MMP-9, *d-e*) in the wall of intact rat carotid artery on days 2 (*a*) and 4 (*b-e*) after balloon angioplasty and application of recombinant urokinase (*2*) and tissue plasminogen activators (*3*) in pluronic gel (control, *1*). *p<0.05 compared to the control.

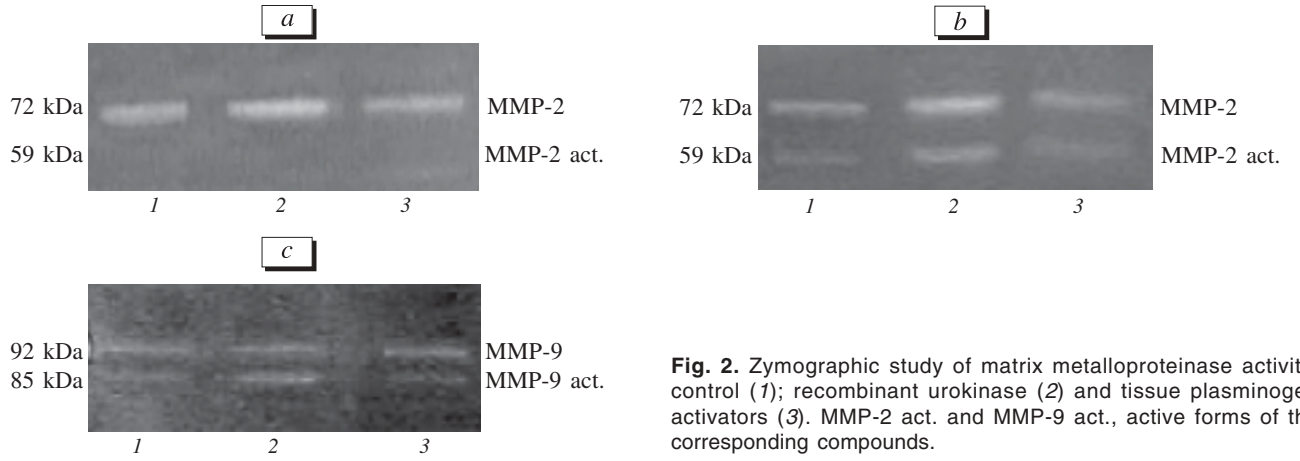

Fig. 2. Zymographic study of matrix metalloproteinase activity: control (*1*); recombinant urokinase (*2*) and tissue plasminogen activators (*3*). MMP-2 act. and MMP-9 act., active forms of the corresponding compounds.

inhibition of cell migration, formation of neointima, and prevention of negative arterial remodeling after balloon injury. The content and activity of MMP-9 remained unchanged under these conditions ($p>0.05$; Table 1, Figs. 1, *d*, 2, *c*).

Our results show that urokinase plays a role in stimulation of expression and activation of MMP after arterial injury. This effect is a characteristic property of urokinase. Tissue plasminogen activator decreases the content and activity of MMP-2 on day 4 after injury, but has no effect on the content and activity of MMP-9. Urokinase-mediated activation of the proteolytic cascade probably contributes to directed degradation of proteins in the extracellular matrix, stimulation of cell migration, and constrictive arterial remodeling in response to injury [1,2,4,13]. The data suggest that well-balanced control over expression of plasminogen activators and MMP in the site of vascular wall injury can contribute to the regulation of undesirable remodeling in the vascular wall.

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