Different Mechanisms of Increased Luminal Stenosis After Arterial Injury in Mice Deficient for Urokinase- or Tissue-Type Plasminogen Activator

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Background—Tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) are thought to play critical roles in vascular remodeling after injury, with tPA mediating intravascular clot lysis and uPA modulating cell migration within the vessel wall. In human vascular disease, however, thrombus organization and neointimal formation are closely interrelated processes. This study examines the differential roles of tPA and uPA in these processes in mice.

Methods and Results—Carotid artery injury and thrombosis were induced in wild-type (WT), uPA-deficient (uPA−/−), and tPA-deficient (tPA−/−) mice with the use of ferric chloride. The expression of uPA and tPA was significantly upregulated in the vessel wall of WT mice 1 week after injury, and compared with WT mice, uPA−/− and tPA−/− mice had lower carotid patency rates after injury. At 3 weeks, only 55% of uPA−/− mouse vessels were patent compared with 81% in tPA−/− mice and 100% in WT mice (P=0.014). Morphometric analysis of injured arterial segments revealed severe luminal stenosis (62±28%) in uPA−/− mice compared with their tPA−/− (16±12%) and WT (6.3±3.6%, P<0.001) counterparts. Moreover, although the vascular walls of WT mice and, particularly, tPA−/− mice developed a cell-rich multilayered neointima and media, the lumen of uPA−/− vessels remained obstructed with acellular unorganized thrombotic material, and their medial areas did not expand.

Conclusions—These results indicate that the roles of uPA and tPA in the arterial response to injury are different and more complex than previously assumed and emphasize the critical role of thrombus organization and resolution in neointimal formation and vascular pathology. (Circulation. 2002;106:1847-1852.)

Key Words: atherosclerosis ■ carotid arteries ■ genes ■ plasminogen activators ■ thrombosis

The fibrinolytic system mediates the resolution of vascular thrombi, thus maintaining hemostatic balance and restoring vascular patency. Recent studies in tissue-type plasminogen activator (tPA)−/− deficient mice (the tPA−/− group) and urokinase-type plasminogen activator (uPA)−/− deficient mice (uPA−/− group) demonstrated that these fibrinolytic factors play a critical role in vascular remodeling, particularly in the pathogenesis of atherosclerosis and restenosis. It has been suggested that the role of tPA is confined to intravascular clot lysis, whereas uPA mediates cell migration within the vessel wall. However, in human vascular disease, thrombus organization and neointimal formation are closely interrelated and frequently coexistent processes. Thus, to clarify the differential role and the overall effect of tPA and uPA on arterial remodeling and luminal narrowing, it is essential to use an in vivo model in which both thrombosis and cell migration can be taken into account.

We previously showed that ferric chloride–induced injury of mouse carotid vessels is a sensitive model for defining the role of specific fibrinolytic factors in the acute thrombotic response to injury and that it also can be used for systematic gene expression studies during the ensuing wound-healing process. Therefore, in the present study, we used this model to compare vascular remodeling in wild-type (WT), tPA−/−, and uPA−/− mice. Our results extend previous reports by showing that tPA and uPA are both important for intraluminal fibrinolysis and that the loss of uPA leads to a marked reduction of cell migration in the vessel wall. Unexpectedly, the most dramatic effect of uPA deficiency was the persistence of large amounts of unresolved acellular thrombus, which resulted in significantly increased intimal area and severe luminal stenosis. Luminal narrowing was also more prominent in the tPA−/− mice than in the WT control mice, but in this case, it resulted from the development of a cell-rich multilayered media and neointima. These observations indicate that the contributions of uPA and tPA to the arterial response to injury are far more complex than previously
assumed and emphasize the critical role of thrombus organization and resolution in vascular pathology.

Methods

Experimental Animals
tPA−/− and uPA−/− mice were from Jackson Laboratories (Bar Harbor, Me) and had been backcrossed to the C57BL/6J genetic background for at least 7 generations. C57BL/6J WT mice were used as controls. All animal care and experimental procedures were approved by the Animal Research Committee of The Scripps Research Institute and by Georg August University of Goettingen and complied with national guidelines for the care and use of laboratory animals.

Carotid Injury and Thrombosis
WT (n=43), tPA−/− (n=49), and uPA−/− (n=26) mice (aged 6 to 8 weeks) were anesthetized by inhalation of methoxyflurane (Metofane, Janssen). The left carotid artery was carefully dissected and then injured by placing a 0.5×1.0-mm strip of filter paper soaked in 10% FeCl3 solution onto the adventitia for 3 minutes. Carotid blood flow was monitored before and after injury by using an ultrasound flow probe (0.5VB) interfaced with a flowmeter (model T106, Transonic Systems) and a computerized data acquisition program (WinDaq Lite, DATAQ Instruments). Thrombotic occlusion (non-patent) was considered to occur when flow decreased to 0.0–0.2 mL/min, a range corresponding to the accuracy of the system (zero offset) as specified by the manufacturer.

Tissue Harvest and Processing
For histological studies, anesthetized animals were perfusion-fixed with 4% zinc formalin for 5 to 10 minutes through the left ventricle. The injured vessel segment was excised and processed for paraffin embedding. For gene expression studies, the animals were gently perfused with normal saline, and then the arteries were removed, snap-frozen, and stored in liquid nitrogen for preparation of total RNA.

Histological, Morphometric, and Immunohistochemical Studies
Carotid artery sections (5 μm) were stained with Masson’s trichrome reagent or with hematoxylin for the determination of the average cell nuclei count in the intima and the media. For morphometric analysis, sections were stained with Verhoeff’s elastic stain and analyzed by image analysis software (ImagePro Plus, Media Cybernetics) as described elsewhere. For all cell counting and morphometric studies, 5 sections equally spaced throughout the injured segment were measured per artery and then averaged. Mean±SD values were calculated from 9 or 10 arteries per genotype.

Endothelial cells were detected by using a rabbit anti-human von Willebrand factor antibody (Dako, dilution 1:100), fibrinogen was detected with the use of a rabbit anti-human fibrinogen antibody (Dako, dilution 1:200), and goat antisera to mouse proteins were used to detect uPA (Santa Cruz, dilution 1:20) and tPA (Santa Cruz, dilution 1:20). Macrophages were detected with the use of rat
antisera to Mac-3 (Pharmingen, dilution 1:200). Smooth muscle cells (SMCs) were stained by use of an anti-human α-actin antibody (peroxidase-labeled, Dako). Oxidized LDL (oxLDL) was detected with the use of an antiserum against murine malondialdehyde-conjugated LDL (kind gift from Dr W. Palinski, University of California at San Diego; dilution 1:50) and an avidin-biotin phosphatase detection system (Universal APAAP kit, Dako). All incubations, blocking, and antigen unmasking steps were carried out as described.10

Gene Expression Studies
Total RNA was extracted from pooled pairs of mouse carotid arteries by using Ultraspec RNA (Biotex) and chloroform, and quantitative reverse transcription (RT)-polymerase chain reaction (PCR) was performed to determine tPA, uPA, and β-actin mRNA levels.11 Aliquots of the PCR reaction were fractionated by electrophoresis through a 1.8% agarose gel, and specific RNAs were quantified by measuring the optical density of the bands corresponding to either the target or the control (β-actin) RNA with the use of computer-based image analyzer (Alpha Imager 2000, Alpha Innotech). For in situ hybridization, radiolabeled antisense and sense riboprobes for uPA and tPA were prepared and used essentially as described.12–14 Slides were exposed in the dark as indicated, developed, and counterstained with hematoxylin and eosin. Parallel sections were hybridized by using the sense probe to control for nonspecific hybridization.

Statistical Analysis
For continuous variables, differences between WT, tPA−/−, and uPA−/− mice or between various time points within the same genotype were tested by ANOVA followed by the Bonferroni test for pairs of means. Qualitative variables were tested by the Fisher exact test. All statistical tests were 2-sided.

Results
Recanalization of Injured Arteries Is Impaired in tPA−/− and uPA−/− Mice
Carotid artery injury was induced in 43 WT, 49 tPA−/−, and 26 uPA−/− mice. Complete thrombotic occlusion occurred in 82% of the WT mice compared with 86% of the tPA−/− mice and 93% of the uPA−/− mice (P=NS). Figure 1A shows that in those vessels that occluded, the mean time to thrombotic occlusion was 11.1±0.4 minutes in WT mice and slightly shorter in tPA−/− mice (10.3±0.4 minutes, P=NS). Interestingly, uPA−/− mice exhibited even shorter times to occlusion than did tPA−/− mice (9.1±0.5 minutes for uPA−/− mice, P<0.01 vs WT mice). Doppler flow studies revealed lower rates of recanalization and restoration of vascular patency in mice deficient for either tPA or uPA at all times (Figure 1B). Vascular patency was restored in all (100%) injured vessels of the WT mice by 3 weeks compared with 81% of the tPA−/− mice (P=NS versus WT mice) and only 55% of the uPA−/− mice (P=0.0104 versus WT mice).

Increased Luminal Stenosis in Absence of tPA and uPA
Figure 2 shows representative examples of arterial cross sections of the 3 mouse genotypes before and 3 weeks after

Figure 3. Morphometric analysis of vascular remodeling in WT, tPA−/−, and uPA−/− mice. Cross-sectional area of neointima (A) and media (B), intima-to-media (I/M) ratio (C), and percentage of luminal stenosis (D) were determined in WT mice (n=10, open bars), tPA−/− mice (n=10, solid bars), and uPA−/− mice (n=9, stippled bars) 3 weeks after FeCl3-induced arterial injury. In panel B, area of media is compared with that of uninjured (no inj) mice (n=3) in each group. Values represent mean±SEM of measurements of 5 cross sections per mouse. *P<0.05 for values 3 weeks after injury vs no inj (B) and for tPA−/− vs WT mice (D). **P=0.001 for uPA−/− vs tPA−/− or WT mice.

Figure 4. Histochemical analysis of vascular wound-healing response in tPA−/− and uPA−/− mice. Characterization of thrombus and vessel wall 1 week (A through D) and 3 weeks (E through H) after injury. Masson’s trichrome staining (A and B) and immunohistochemical staining for fibrinogen (C and D) for tPA−/− (A and C) and uPA−/− (B and D) mice. Panel E shows immunostaining for α-actin (red); panel F, for Mac-3 (dark brown); and panel G, for oxLDL (pink) in WT mice 3 weeks after injury. Panel H (staining for α-actin) shows almost acellular vessel wall without detectable α-actin immunosignal and with occlusive thrombotic material in uPA−/− mice 3 weeks after injury. Original magnification ×400 (A, B, and D) and ×1000 (all other panels). Bar=20 μm.
stenosis was increased almost 3-fold (Figure 2E and 2F and Figure 3A), and the severity of luminal injury (Figure 1B). However, in contrast to WT and tPA mice, the adventitia of uPA/H11002 mice (Figure 4B and 4D) exhibited amorphous, acellular, fibrin-rich thrombus accumulation, and it partially or totally occluded the vascular lumen in uPA mice (Figures 3C) and only 6.3±3.6% luminal stenosis (Figure 3D). In tPA−/− mice, thickening of the intima was more pronounced (Figure 2E and 2F and Figure 3A), and the severity of luminal stenosis was increased almost 3-fold (P=0.034 versus WT mice, Figure 3D). Unexpectedly, the uPA−/− mice demonstrated a significantly greater increase in intimal area after injury (Figure 2G and 2H) compared with that in WT and tPA−/− mice (P<0.001, Figure 3A). As a result, uPA−/− mice exhibited severe luminal stenosis (62±28%, P<0.001 versus WT and tPA−/− mice; Figure 3D), which probably explains the decreased patency rates in these animals at this time (Figure 1B). However, in contrast to WT and tPA−/− mice, the medial area of injured vessels from uPA−/− mice did not expand over the 3-week interval (Figure 3B), and this failure resulted in a particularly high intima-to-media ratio (Figure 3C).

### Differential Roles of tPA and uPA in Wound-Healing Response

No histological differences were apparent when the carotid vessels of the 3 mouse groups were compared before or 30 minutes after injury (not shown). As expected, there was marked cellular infiltration into the adventitial and the medial layer of the vessel wall and into the intraluminal fibrin-rich thrombotic material in WT (not shown) and tPA−/− mice by 1 week after injury (Figure 4A and 4C). In contrast, only amorphous, acellular, fibrin-rich thrombus accumulated, and it partially or totally occluded the vascular lumen in uPA−/− mice (Figure 4B and 4D). The adventitia of uPA−/−, tPA−/−, and WT (not shown) mouse vessels was similarly infiltrated with cells. However, these cells did not appear to migrate into the media of uPA−/− vessels, which remained very thin and acellular.

Three weeks after injury, WT and tPA−/− (not shown) mouse vessels exhibited a SMC-rich multilayered neointima (Figure 4E) and a macrophage-rich media (Figure 4F), which stained strongly for oxLDL cholesterol (Figure 4G). Although oxLDL staining was more prominent in WT mouse vessels, it was also detectable in the arteries of tPA−/− mice (not shown). However, the intima and media of injured vessels from uPA−/− mice were still relatively devoid of infiltrating cells at this time (Table), and immunostaining for α-actin (Figure 4H) and oxLDL-containing macrophages (not shown) remained undetectable. Plasma cholesterol levels were similar for the 3 mouse genotypes (mean values 73 to 78 mg/dL).

### Analysis of tPA and uPA Expression in Vessel Wall After Injury

RT-PCR analysis revealed relatively low levels of tPA (Figure 5A) and uPA (Figure 5B) mRNA in uninjured WT mouse arteries and in arteries at 30 minutes and 24 hours after injury. The expression of both genes was significantly up-regulated 1 week after injury and returned to the low baseline levels by the end of the third week. In situ hybridization and immunohistochemistry studies (Figure 6) confirmed the very low levels of tPA (not shown) and uPA (panel A) expression in uninjured WT mouse carotid arteries. In accordance with the RT-PCR findings, strong uPA mRNA (Figure 6B) and antigen (Figure 6C) signals were detected 1 week after injury and were localized to numerous cells in the adventitia, the media, and the organizing thrombus. As expected from the RT-PCR results, uPA expression returned to baseline levels by 3 weeks after injury and was detected only occasionally in isolated cells of the media or the adjacent adventitia (Figure 6D and 6E). The temporal and spatial patterns of tPA mRNA and protein induction were similar to, but less prominent than, those for the induction of uPA expression (Figure 6; compare panel F with panel B). Comparison of the expression of uPA protein and mRNA in WT and tPA−/− mice did not reveal significant differences. Similarly, tPA expression was not significantly different between the WT and the uPA−/− mice (data not shown).

### Discussion

In the present study, we showed that tPA and uPA were both essential for early thrombus resolution and restoration of vascular patency (Figures 1 through 3). This observation is consistent with the original report showing that extensive spontaneous fibrin deposition is observed in the mouse only...
when both genes are deleted or inactivated.12 We also showed that vascular wound healing was associated with transient induction of both genes in the vessel wall (Figure 5). Although deletion of the uPA gene resulted in marked impairment of cell migration into the media and neointima (Figures 2 and 3 and Table), the uPA−/− mice were found to develop more severe luminal stenosis than either the WT or tPA−/− mice. This unexpected observation reflected the persistence and endothelialization of amorphous unorganized thrombotic material in the uPA−/− vessels (Figures 2 and 4). Although tPA−/− mice also exhibited reduced thrombolysis after injury (30-minute patency rate, 33% versus 51% in WT mice), they showed no significant impairment of cell migration. Luminal stenosis was more pronounced in tPA−/− mice than in WT mice, but in contrast to the uPA−/− mice, it resulted from thrombus organization and formation of a multilayered cell-rich neointima (Figures 2 and 4 and Table). Three weeks after injury, the neointimal area in tPA−/− vessels was 9205±2780 μm² compared with 4380±1155 μm² in WT vessels.

Thrombosis is a critical event in the pathophysiology of human atherosclerosis,5,6 and the complex interactions among thrombocytes, coagulation and fibrinolytic factors, and cells of the vessel wall appear to play a crucial role in atherosclerosis progression.6,13 Besides removing intraluminal and/or intramural thrombi, the plasminogen activators, particularly uPA, may facilitate cell migration, neointimal formation, and possibly plaque destabilization.14–17 Previous studies in which mouse vessels were subjected to mechanical or electrical injury showed that uPA deficiency resulted in less neointimal and luminal stenosis2,17 and that vessels from tPA−/− mice did not appear to differ from those of WT control mice.2 However, thrombosis did not appear to be a consistent finding in these experiments, and the thrombi that formed initially in WT mouse vessels were generally no longer detectable beyond the first few days after injury.3 Thus, it is possible that the overall effect of uPA and tPA on vascular remodeling would have been different in the presence of a more prominent thrombotic response.

The ferric chloride model used in the present study is characterized by injury to the endothelium and medial layer of the arterial wall and, importantly, by the reproducible formation of occluding platelet-rich thrombi, which gradually dissolve or organize over a period of 3 weeks.7,18 Rather than contradicting previous observations in other injury models, our findings emphasize the importance of considering the kinetics of intravascular thrombus formation, organization, and dissolution as part of the vascular remodeling process. Our results also support the notion that it is uPA, rather than tPA, that modulates cell migration during the wound-healing process in the vessel wall.2 Unexpectedly, in the absence of uPA, the impairment of cell migration is more than offset by the presence of unresolved mural thrombus (Figure 2). In contrast, the vascular phenotype of the tPA−/− mice is characterized by increased cellularity and thickness of the intima, which are possibly due to the release of growth factors from the persisting cell-rich thrombus.

Our observation that uPA contributes to vascular patency agrees with previous observations that uPA deficiency leads to increased fibrin deposition.11,12 However, these observations disagree with a recent study that implicated only tPA in this process.4 These differences may reflect the different models examined, inasmuch as FeCl₃, compared with rose bengal, induces more severe injury to the vessel wall and a more intense thrombotic reaction.7,19 Thus, the FeCl₃ model may be more sensitive for the detection of differences between WT and uPA−/− mice regarding arterial clot lysis.

Neither WT mice nor those deficient in uPA or tPA spontaneously developed atherosclerotic lesions. However, the present study demonstrated that the neointima formed in the injured carotid arteries of WT mice at 3 weeks was rich in fibrin and SMCs and, importantly, in macrophages, which stained strongly for oxLDL. These results, together with a recent report,20 indicate that in the carotid arteries of mice, it is possible to induce lesions that share important histological features with human atherosclerotic plaques.21 Preliminary data22,23 suggest that these changes are much more pronounced in the presence of severe hypercholesterolemia, as occurs in the apoE-knockout mouse. Thus, the results of the present study may prove useful for future studies designed to dissect the roles of uPA and tPA in the development and progression of atherothrombosis.

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References

Figure 6. Patterns of uPA and tPA mRNA and protein expression after arterial injury. In situ hybridization (A, B, D, and F) and immunohistochemistry (C and E) for uPA and tPA in WT mouse carotid vessels. uPA mRNA (A) and tPA mRNA (not shown) were almost undetectable in sections of uninjured carotid arteries. Expression of uPA increased markedly 1 week after injury (B and C) and returned to low levels by end of third week (D and E). Compared with increase in uPA mRNA, increase in tPA mRNA (panel F) and protein (not shown) expression at 1 week after injury was much less prominent. Original magnification ×1000 (E) and ×400 (all other panels). In panels D through F, arrows indicate examples of positive signals.


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