Prevention of Neointimal Formation by a Serine Protease Inhibitor, FUT-175, After Carotid Balloon Injury in Rats

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Background and Purpose—In vivo and vitro studies revealed the activation of thrombin and the complement system in vascular lesion formation during the process of atherosclerosis, along with pathological proliferation of smooth muscle cells. We examined the effect of the synthetic serine protease inhibitor FUT-175 (developed as a potent inhibitor of thrombin and the complement system) on vascular lesions using balloon dilatation–induced neointimal formation in the carotid artery of rats.

Methods—Sprague-Dawley (SD) rats underwent balloon dilatation injury of the left carotid artery to induce neointimal formation. Three groups of these rats (n=8, each) were treated with daily intraperitoneal injections of 1 of the following doses of FUT-175: 0.5, 1.0, or 2.0 mg/d in 1 mL of saline for 7 consecutive days. The control group (n=8) was similarly treated with 1 mL of saline for 7 days. The injections were started immediately after balloon injury. Two weeks after the injury, the left carotid arteries were perfusion-fixed, and the areas of the neointimal and medial layer were analyzed under a microscope.

Results—A morphometric analysis revealed that there were significant differences in the intima-media ratio between the 4 groups treated with vehicle (saline) or a low, medium, or high dose of FUT-175 (1.45±0.11, 1.08±0.06, 0.71±0.04, or 0.32±0.04, respectively). This suppression was achieved in a dose-dependent manner by the administration of FUT-175 after balloon injury. In the histological study, it was demonstrated that FUT-175 suppresses the production of platelet-derived growth factor (PDGF)-BB in the neointima and the medial smooth muscle cell layer.

Conclusions—After balloon injury activated proteases that were inhibited by FUT-175 were demonstrated to have an essential role in the development of the pathological thickening of the arterial wall. (Stroke. 1999;30:644-650.)

Key Words: carotid stenosis ■ growth factors ■ muscle, smooth ■ thrombin ■ rats

The thickening of the arterial wall in developing atherosclerosis or in the healing process after vascular injury generates serious stenosis and ultimately causes ischemic organ dysfunction such as a brain or heart attack.1 The pathological thickening of the arterial wall has been a target for the prevention of ischemic circulatory disorders. The pathophysiology of this thickening is thought to be a remodeling failure of the arterial wall along with excessive and disordered proliferation of migrated smooth muscle cells.1-3 In the process of the thickening of the vascular wall, evidence of thrombin activation in the coagulation system is accumulating, and the participation of the complement system is implicated.4,5

Thrombin, a serine protease cleaved from prothrombin in plasma, is a potent stimulator of platelet aggregation and is known to act as a growth factor for vascular smooth muscle cells (VSMCs) via a specific thrombin receptor.4,6 An increased expression of the thrombin receptor was observed in lesions of atherosclerosis and in VSMCs after balloon injury.7,8 The complement system is a self-defense mechanism that destroys injured cells or acts as a chemoattractant for inflammatory cells; this mechanism has also been reported to be activated in the process of atherosclerosis.5,9-11

It is unknown whether activation of thrombin and of the complement system is an essential trigger of intimal hyperplasia or an epiphenomenon in the remodeling failure. The sequence of events giving rise to neointimal formation has been studied extensively in experimental animals.12,13 To test the hypothesis that the activation of thrombin in the coagulation system, the activation of the complement system, or both are necessary for neointimal formation, we used a synthetic serine protease inhibitor, FUT-175, in a carotid balloon-injury model in rats.
In addition, the distribution of platelet-derived growth factor (PDGF)-BB in the vascular wall after balloon injury was analyzed in FUT-175–treated and nontreated groups using an immunohistochemical technique.

Materials and Methods

Carotid Balloon-Injury Model

Fifty-seven male Sprague-Dawley rats, weighing from 350 to 400 g (SLC, Kyoto, Japan), were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg for the surgical operation or 100 mg/kg for euthanasia). Balloon injury to the carotid artery was induced based on the model described by Clowes et al and Morishita et al. Under a surgical microscope, the left common, internal, and external carotid arteries (CCA, ICA, and ECA, respectively) were exposed using a midline linear skin incision in the neck. The left CCA, 15 mm proximal to the carotid bifurcation, and the left ICA at the orifice were temporarily occluded. The left ECA was ligated at the exposed distal end. A 2F balloon catheter (Fogarty, E-060-2F; Baxter) was used to induce the denudation and mechanical stretching injury of the left CCA. The catheter was introduced into the CCA through a small window opened in the ECA, which is proximal to the ligation site. After the clip of the CCA was removed, the deflated catheter was passed through the CCA into the aortic arch. An inflated balloon with 0.01 to 0.02 mL of saline in the aortic arch was slowly pulled back toward the ECA. After 3 repetitions of the inflation-pull-deflation procedure, the catheter was removed. After the ligation of the ECA, the blood flow to the CCA and ICA was restored by releasing the clips, and the wound was closed.

The experimental protocols were approved by the animal research committee at the National Cardio-Vascular Center Research Institute. All efforts were made to minimize suffering and to minimize the number of animals used.

Inhibitory Drug Treatment Protocol

The synthetic serine protease inhibitor FUT-175 (nafamostat mesilate; 6-amidino-2-naphthyl p-guanidinobenzenonate; molecular weight, 539.58) was purchased from Torii Pharmaceutical Co. The protocol design of FUT-175 administration was established after taking into consideration reported pharmacological properties and kinetics (specific inhibition of serine proteases) of this drug studied in vitro and in vivo.

Thirty-two rats were randomly assigned to 4 groups (from groups A through C, n = 8 each). In group A, 0.5 mg; in group B, 1 mg; and in group C, 2 mg of FUT-175 was injected intraperitoneally every day for 7 days, starting immediately after the surgical wound closure. Regarding the period of the 7-day administration starting soon after the balloon injury, it was considered that VSMCs are most active in proliferation, because it has been reported that the smooth muscle cell proliferation in the neointima reaches a maximum 4 days after injury and that the number of such cells does not increase after 2 weeks in this model. FUT-175 was dissolved in saline and administered at a concentration of 1 mg/mL. Eight rats of the vehicle-control group received an intraperitoneal injection of 1 mL saline every day for 7 days. In an additional group (n = 5), the left CCA was exposed but not balloon-dilated (sham control). Because the safety of this drug has been confirmed in a clinical phase 1 study, we did not monitor physiological parameters during and after the treatment.

Morphometric Study

Two weeks after the balloon injury, all animals were deeply anesthetized and perfused intracardially with 200 mL of 10% (wt/vol) formaldehyde in 10 mmol/L sodium phosphate–buffered saline with heparin at approximately 100 to 130 mm Hg. After perfusion fixation, the right and left CCAs were retrieved en bloc, including the aortic arch, innominate artery, and carotid bifurcation. The arteries were further fixed by immersion in the same fixative. Five cross sections (4 mm in length) from the single left CCA were stained with hematoxylin and eosin or Masson’s trichrome. Neointimal formation was analyzed with a computerized analysis system (in a blind manner by the analyzer; SD-510C, WACOM). Cross-sectional areas of the medial smooth muscle cell layer and neointimal layer were calculated by tracing the exact border of each area under constant magnification with the use of a microscope and the computerized analysis system. For the calculation of the average thickness of each layer, the analyzed area was divided by the mean of the outer and inner circumferences of the intimal layer.

Immunohistochemical Study of Rat Carotid Artery

To examine the expression of platelet-derived growth factor-BB (PDGF-BB) after balloon injury and the effect of the treatment on this expression, a separate group of rats was euthanized at the end of treatment to determine the regional and cellular distributions of PDGF-BB, with (n = 5) or without (n = 5) 2 mg/d (for 7 days) of FUT-175 treatment. In an additional group of 5 rats, the left CCA was exposed under the same anesthesia but not balloon-dilated to study the effect of the sham operation on the expression of PDGF-BB. Rats were deeply anesthetized and perfused with ice-cold sodium phosphate–buffered saline with heparin 7 days after the balloon injury. The cervical arteries were removed en bloc and immersed and fixed by methyl Carnoy’s solution (60% methanol, 30% chloroform, 10% acetic acid); 5 segments (4-mm thick) from the left CCA were embedded in paraffin. All segments were stained immunohistochemically using the murine monoclonal antibody PGF-007 (generously donated by Mochida Pharmaceutical Co) for PDGF-BB as described elsewhere. The antibody PGF-007 was generated in response to a 25-amino acid peptide located near the COOH terminus of the PDGF B-chain and does not cross-react with PDGF A-chain. The specificity of PGF-007 has been characterized in fixed tissue sections by studies of cell types known to express, or not to express, PDGF A-chain and of its ability to block the immunostaining by incubation of the antibody with synthetic PDGF-BB but not PDGF-AA.

Data Analysis

Statistical analysis was performed by 1-way ANOVA. If multiple comparisons were indicated, the Student-Newman-Keuls test was applied. The results are presented in the text as mean ± SEM. A value of P < 0.05 was considered significant.

Results

The formation of the neointimal layer was confirmed 2 weeks after carotid balloon injury. Figure 1 shows cross sections of rat carotid arteries stained with Masson’s trichrome, treated with vehicle (Figures 1A and 1B) or with 1 of the 3 dosages of FUT-175 (Figures 1C through 1H). In the vehicle-treated group, intimal thickening with smooth muscle cells in a malarranged inner layer was prominent in every rat, accompanied by a normal-appearing medial smooth muscle layer and adventitia (smooth muscle cells, red; collagen fibers, blue), whereas in all of the rats treated with FUT-175, the neointimal thickening was suppressed, and the degree of suppression was more pronounced as the administered dose increased (Figures 1C through 1H). In the sham-operated control group, there was no neointimal formation 14 days after the CCA exposure (data not shown).

The neointimal areas, the thickness of the neointimal layer, and the ratios of the neointima/medial area of the rats treated with vehicle or with 0.5, 1.0, or 2.0 mg/d of FUT-175 are shown in the Table. In the FUT-175–treated groups, each of these values was significantly smaller than those in the vehicle-treated group (P < 0.05).

The ratios of the neointima/medial area are illustrated in Figure 2. The ratios of the groups treated with FUT-175 were
all significantly smaller than those in the vehicle-treated group. In addition, there were significant differences when the FUT-175–treated groups were compared to each other.

Figure 3 shows the results of immunostaining with polyclonal antibody to rat PDGF-BB on the cross sections of balloon-injured rat carotid arteries. Strong immunoreactivity for PDGF-BB was observed in the neointimal layer and in the vehicle-treated group 7 days after balloon injury (Figure 3A). Under higher magnification, immunoreactivity was observed mainly in the cytoplasm of the neointimal cells. The smooth muscle cells in the medial layer were also immunopositive for PDGF-BB after balloon injury (Figures 3A and 3B). In contrast, in the group treated with 2 mg/d of FUT-175, the cells in the neointimal layer showed positive but reduced immunoreactivity compared with that of the vehicle-treated group both in the neointimal layer and in the medial smooth muscle cell layer (Figures 3C and 3D). In the sham-operated group (Figures 3E and 3F), there was no neointimal formation or immunoreactivity for PDGF-BB in the medial smooth muscle cell layer. The immunoreactivity for PDGF-BB was constantly observed in the adventitial layer, probably because of existing fibroblasts24 (Figures 3A, 3C, and 3E).

Discussion

We examined the inhibitory effects of FUT-175 on neointimal formation after balloon injury of the rat carotid wall. The expression of PDGF-BB after arterial injury was suppressed, and the neointimal formation after balloon injury was significantly prevented by the FUT-175 administration. The synthetic protease inhibitor FUT-175 was developed by Fuji and Hitomi.25 The pharmacological properties of this drug have been thoroughly investigated in vivo, and it is now recognized as an inhibitor of thrombin, XII, Xa, and the activated Hageman factor in the coagulation system.8,19,26–28 FUT-175 also inhibits factors B and D in the alternative complement pathway and Clr and Cls in the classic complement pathway. Intravenous administration of FUT-175 was reported to inhibit thrombin and the complement activation in a competitive fashion in experimental animals and humans; the inhibition constant ($K_i$) values were in the order of $10^{-7}$ to $10^{-6}$.

![Figure 1. Cross sections of rat carotid artery 14 days after balloon injury and treated with vehicle (A, B), or low (C, D), medium (E, F) or high (G, H) dose of FUT-175. Thick neointimal formations, composed of proliferated smooth muscle cells, were seen in the inner layer in all sections. The development of the neointima generated in the inner layer was suppressed by the systemic intraperitoneal administration of FUT-175. The thickness of the neointimal layer was suppressed in the FUT-175–treated groups (B through H) compared with the vehicle-treated group (A, B) in a dose-dependent manner (magnification ×40 for A, C, E, and G; ×100 for B, D, F, and H; Masson’s trichrome).](http://stroke.ahajournals.org/)

![Figure 2. The ratio of neointima/medial area measured from cross sections in balloon-injured rat carotid arteries. The administration of FUT-175 inhibited neointimal formation analyzed 14 days after the injury in a dose-dependent manner. There was a significant difference between each group, ie, control (vehicle) and low-dose, low-dose and medium-dose, medium-dose and high-dose FUT-175 groups ($^*P<0.05$).](http://stroke.ahajournals.org/)
Thrombin, a multifunctional serine protease that is generated by activated factor, and its plasminogen-activator inhibitor. Thrombin is chemotactic for monocytes and known as a most potent stimulator of platelet aggregation, exhibits a host of activating effects for VSMCs.4,6,7,30 Regarding the composition of PDGF, the heterodimer and homodimer of 2 distinct sequences termed the A and B chains have been reported.48–50 In advanced human atherosclerotic plaques, PDGF B–chain mRNA was detected in the endothelial cells and mesenchymal-like intimal cells. A pronounced expression of PDGF B–type receptors was seen in VSMCs in human atherosclerotic plaques. In our preliminary study, an elevated expression of PDGF-BB immunoreactivity was observed in the intimal layer of carotid atherosclerotic lesions from human carotid lesion (unpublished data). The functional role of the elevated PDGF-BB production in the neointima is unknown, as is the inhibitory mechanism of the synthetic serine protease inhibitor FUT-175. However, the reduced production of PDGF-BB in the neointima and in the medial smooth muscle cell layer seen in the FUT-175–treated group, which accompanies significant inhibition of the neointimal formation, agrees with findings that inhibition of the PDGF receptor tyrosine kinase inhibited VSMC migration and proliferation in vitro.53,54 Regarding receptor expression, PDGF-β receptor mRNA was elevated between 2 and 14 days after rat carotid injury in vivo. In addition, PDGF and other growth factors have been reported to be produced from endothelial cells or VSMCs after tissue injury. After injury, macrophages or endothelial cells in atherosclerotic lesion and VSMCs and fibroblasts in the connective tissue have been reported to produce PDGF.12,45–47 It has been postulated that the expression and release of PDGF from VSMCs, endothelial cells, and fibroblasts have the potential to generate an atherosclerotic lesion or intimal hyperplasia after injury. An antibody to PDGF inhibited the neointimal formation in a rat balloon-injury model.20

Studies of PDGF biology revealed that many cell types (including macrophages, cultured endothelial cells, and cultured arterial smooth muscle cells) produce PDGF-like molecules. PDGF is expressed at low or undetectable concentrations in normal adult tissues, but its expression is increased after tissue injury. After injury, macrophages or endothelial cells in atherosclerotic lesion and VSMCs and fibroblasts in the connective tissue have been reported to produce PDGF,12,45–47 and in advanced human atherosclerotic plaques, PDGF B–chain mRNA was detected in the endothelial cells and mesenchymal-like intimal cells. A pronounced expression of PDGF B–type receptors was seen in VSMCs in human atherosclerotic plaques. In our preliminary study, an elevated expression of PDGF-BB immunoreactivity was observed in the intimal layer of carotid atherosclerotic lesions from human carotid lesion (unpublished data). The functional role of the elevated PDGF-BB production in the neointima is unknown, as is the inhibitory mechanism of the synthetic serine protease inhibitor FUT-175. However, the reduced production of PDGF-BB in the neointima and in the medial smooth muscle cell layer seen in the FUT-175–treated group, which accompanies significant inhibition of the neointimal formation, agrees with findings that inhibition of the PDGF receptor tyrosine kinase inhibited VSMC migration and proliferation in vitro.3,38 The thrombin receptor is expressed in platelets and arterial endothelium under normal conditions. In a study of atherosclerotic plaque in humans, the thrombin receptor was found to be widely expressed within atheroma lesions, including macrophages and VSMCs.7,40 In the mechanisms of restenosis after balloon injury, thrombin and its receptor may play a central role in mediating the disordered proliferative actions for VSMCs.4,6,7,30

Figure 3. Immunostaining with the monoclonal antibody (PGF-007) to PDGF-BB on cross sections of balloon-injured rat carotid artery (7 days after the injury). Strong immunoreactivity for PDGF-BB was demonstrated in the neointimal layer, the medial smooth muscle cell layer, and the adventitial layer (A). Under higher magnification (B), the immunoreactivity was observed primarily in the cytoplasm of the neointimal and the medial smooth muscle cells. In contrast, in the group treated with FUT-175 (2 mg/d for 7 days), immunoreactivity in the neointima and the medial layer was decreased (C, D). In the sham-operated group (E, F), immunoreactivity for PDGF-BB was hardly detected except for the adventitial layer (magnification for A, C, and E) ×200; magnification for B, D, and F; ×600.

and 10⁻⁸ to 10⁻⁷ mol/L in serum, respectively. In the present study, it was indicated that the inhibitory effects of FUT-175 on the complement system, thrombin activation, or both are essential to the prevention of the migration and proliferation of the VSMCs seen as neointimal formation after balloon injury.

Thrombin, a multifunctional serine protease that is generated at the site of vascular injury and known as a potent stimulator of platelet aggregation, exhibits a host of activating effects on vascular endothelial cells, VSMCs, and macrophages. The effects of thrombin on vascular endothelial cells include the production of prostacyclin, platelet-activating factor, and its plasminogen-activator inhibitor. Thrombin is chemotactic for monocytes and known as a mitogen for mesenchymal cells, including VSMCs. Teleologically, these multiple cell-activating functions of thrombin may be viewed as orchestrating normal remodeling responses to vascular injury, potentially mediating hemostatic, inflammatory, and proliferative processes in the healing responses.3,38

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crease platelet prothrombinase activity. It has been postulated that the complement system plays a role in persistent tissue injury in the development of atherosclerosis or restenosis. We confirmed regional distributions of C5b-9 antigen in the atherosclerotic lesions of human carotid artery (unpublished data), which supported the hypothesis that regional complement activation is involved in the formation of the lesion. However, the etiologic significance of the complement system in the disordered VSMC proliferation observed in vivo remains to be elucidated.

At present, it is unknown whether and, if so, how the complement system, the thrombin activation, or both operate in the process of the disordered proliferation of VSMCs, which leads to the pathological thickening seen in an atherosclerotic lesion or remodeling failure after stretching injury. However, the significant and striking reduction rate of the neointimal formation, ie, 78% reduction compared with the control seen in the highest dosage group using a relatively large number of animals should be noted compared with the results obtained with a variety of treatments of carotid balloon injury in rats. The previously reported reduction rates are 46±3.0% on average, ranging between 34% and 50%. FUT-175 is now used to treat acute pancreatitis and disseminated intravascular coagulation by intermittent or continuous intravenous administration in humans.

The optimal FUT-175 dosage for acute pancreatitis has been shown to be between 0.4 and 0.8 mg/kg per day, and the optimal dosage for disseminated intravascular coagulation is between 3.8 and 4.8 mg/kg per day. In a rabbit subarachnoid hemorrhage model, a dosage of 2 mg/kg per day of FUT-175 has been demonstrated to be optimal in preventing the development of experimental cerebral vasospasm (delayed pathologic narrowing of cerebral arteries). In another clinical trial targeted to prevent cerebral vasospasm, a dosage of 1 to 3 mg/kg per day for 4 days was demonstrated to be effective in humans. The dosage of FUT-175 used in the present study was similar to those reported optimal dosages. Although the half-life of FUT-175 in plasma is only several minutes, an unchanged chemical form has been found to remain for 24 hours in vascular walls, which is beneficial for the treatment of vascular lesion formation. In this study, FUT-175 was administered for only 7 days in the acute phase after injury. The obtained good outcome is in line with the finding that cellular responses to balloon injury, including reactive DNA synthesis and proliferation of VSMCs, are acute events in the development of neointimal formation.

However, the molecular mechanisms involved in the preventive effect of FUT-175 on neointimal formation remain obscure. Trypsin, kallikrein, and plasmin are also serine proteases, which are specifically inhibited by FUT-175. Plasmin is thought to lyse cell substrate attachments and to release the cell to allow it to migrate. It is possible that these serine proteases, separately or in combination, are suppressing multiple essential cascades to allow the potent inhibitory action of FUT-175 against neointimal formation.

In conclusion, it was demonstrated that activated serine proteases, which are inhibited by FUT-175, are involved in the development of pathological thickening of the rat arterial wall after balloon injury. A low incidence of side effects of FUT-175 in humans has been reported: skin eruption, 1.7%; liver dysfunction, 0.5%; and diarrhea, 0.3%. Systemic FUT-175 administration may be a safe and useful treatment for proliferative disorders of VSMCs such as atherosclerosis, or restenosis formation after percutaneous transluminal coronary angioplasty or carotid endarterectomy. Further studies are required to clarify the precise molecular mechanisms underlying the inhibitory effects of FUT-175 on disordered proliferation of VSMCs. The optimal timing and protocol for this treatment must also be determined.

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References


Prevention of Neointimal Formation by a Serine Protease Inhibitor

The authors present new evidence that a serine protease inhibitor, FUT-175, causes dose-dependent reductions in neointimal thickening after balloon injury of the rat carotid artery. They noted that there was a correlation between the inhibition of intimal proliferation and suppression of immune-reactive PDGF-BB in the neointima and smooth muscle layers of the arteries. These experiments do not specifically prove that FUT-175 directly inhibits this response nor do they implicate PDGF-BB in the restenosis. Other evidence reviewed in the discussion, however, suggests that PDGF is an important mediator of the intimal proliferation that characterizes restenosis and that the increase in PDGF in arteries after balloon injury or angioplasty is not just an associated abnormality or epiphenomenon.

Questions can always be raised about the specificity of FUT-175 as a serine protease inhibitor. It may be a relatively nonspecific inhibitor of many serine proteases. In addition, it could have other unknown mechanisms of action to inhibit intimal proliferation after balloon injury. Demonstrating similar effects with another structurally different serine protease inhibitor would strengthen the conclusions, although such an agent is not available at this time. However, this does not mitigate against trying this agent clinically for restenosis, because it is already in clinical use according to the authors and presumably has a reasonable safety profile.

Schwartz et al. reviewed the role of the intima in restenosis and noted that there may be important differences between intimal proliferation after balloon injury of the rat carotid artery and that seen after angioplasty or during atherogenesis in human arteries. Drugs that have shown efficacy in the rat model did not have an effect in some human trials of restenosis after coronary angioplasty. However, FUT-175 has already been used clinically, and based on the new results of Sawada et al., FUT-175 would seem to be worthy of investigation in humans as an anti-restenosis agent.

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