Effect of Chymase Inhibition on the Arteriovenous Fistula Stenosis in Dogs

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It was hypothesized that chymase may participate in hemodialysis vascular access dysfunction, as chymase has been known to be an effective enzyme in the conversion of angiotensin I (Ang I) to Ang II and in the latent TGF-β1 to the active form. An arteriovenous (AV) fistula was created between the brachial artery and vein in dogs. In the AV anastomosis, when the walls of the venous and arterial sides were compared, the eccentric neointimal formation was most evident in the venous wall. Compared with the venous side downstream of the AV anastomosis, a severe neointimal hyperplasia was found in the venous side upstream of the AV anastomosis (intima/media, 153 ± 25%). The chymase- and TGF-β-positive mast cells were markedly accumulated in the proliferous neointima and media. In association with the reduction of chymase expression, a marked decrease in Ang II-, AT1 receptor-, and TGF-β-positive areas was achieved by NK3201 (a chymase inhibitor) treatment, and the neointima formation (intima/media: region A, 53 ± 9%, P < 0.001; region B, 54 ± 14%, P < 0.001) was also significantly suppressed in this group. Although lisinopril treatment also provided some beneficial effects with regard to the prevention of neointimal formation, the degree was less than that seen with chymase inhibition. These findings indicate that mast cell–derived chymase plays an essential role in the pathogenesis of the AV fistula access failure and that chymase inhibition may be a therapeutic target for the treatment of hemodialysis vascular access dysfunction in clinic settings.


Materials and Methods

Agents and Animal Treatments

Fifteen beagle dogs that weighed 9 to 12 kg were obtained from Japan SLC (Shizuoka, Japan), and the animals were divided into placebo-, NK3201-, and lisinopril-treated groups. Each group contained five animals. Nippon Kayaku Co., Ltd. (Tokyo, Japan) synthesized the NK3201 for use as the orally active and specific chymase inhibitor. It has been reported that the vascular proliferation of vein grafts in dogs can be suppressed by oral administration of NK3201 at a dose of 5 mg/kg per d (8). Therefore, the same dose of NK3201 was chosen in this study, and the treatment was initiated 3 d before surgery. Lisinopril was administered orally at 20 mg/kg body wt per day 3 d before the construction of the AV fistula. Under anesthesia, the mean arterial BP (MABP) was determined 28 d after construction of the AV fistula by using a Polygraph System (Nihon Kohden, Tokyo, Japan). The experimental procedures used in the animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Animal Research Laboratory, Osaka Medical College).
Surgery
Anesthesia was induced in the dogs by an intravenous injection of pentobarbital sodium (35 mg/kg) and was followed by an intravenous injection of 1000 U of heparin. After isolation of the brachial artery and vein, a 1.2- to 1.5-cm longitudinal incision was made in each blood vessel and a side-to-side AV fistula was created. Finally, the skin was closed and the animals were allowed to recover. All animals received antibiotic therapy through an intramuscular injection of cefminox sodium (0.5 g).

Preparation of Vascular Tissue
Animals were killed 28 d after the construction of the AV fistula by administering an overdose of pentobarbital sodium. The region of the AV fistula together with the upstream and downstream sections of the brachial artery and vein then was isolated and carefully removed. These samples were cut into three regions as indicated in Figure 1. Region A (upstream) contained both a perfect artery and vein and was the region where most of the arterial blood flowed into the vein. Region B was the AV anastomosis and contained portions of the arterial and venous walls of one duct. Region C (downstream) contained both a perfect artery and vein and was the region where the arterial blood did not flow into the vein. These regions were fixed in methanol-Carnoy’s fixative and embedded in paraffin.

Histologic Examination
From each region, we cut 10 serial sections that were 3 μm in size. The first and second sections were stained with hematoxylin and eosin and azan Mallory stain, respectively. In the third section, expression of fibronectin in the hyperplastic intima was determined using the IgG fraction of mouse antihuman fibronectin (Wako, Osaka, Japan). The fourth section was used to examine the intima and media areas and was evaluated by van Gieson’s elastic staining. The fifth section was stained with toluidine blue to identify mast cells. To determine the distribution of chymase, we performed immunohistochemical staining on the sixth section using anti-chymase antibody, as has been previously described (6,7). In the seventh section, monoclonal anti–TGF-β1, -β2, and -β3 antibody (Techne Co., Minneapolis, MN) was used to determine the location of the TGF-β family. Expression of ACE was examined in the eighth section by using mouse anti-ACE mAb (Chemicon, Temecula, CA). A TdT-FragEL DNA Fragmentation Detection Kit (Oncogene Research Products, San Diego, CA) was used in the ninth section to determine apoptotic cells. Mouse monoclonal anti–proliferating cell nuclear antigen (PCNA; Dako, Glostrup, Denmark) was used in the final section to determine the proliferative cells. To examine the expression of the AT1 and AT2 receptors as well as the distribution of their ligand Ang II, we also performed immunohistochemical studies in another three serial sections by using antihuman AT1 (sc-1173; Santa Cruz Biotechnology, Santa Cruz, CA), AT2 (sc-9040; Santa Cruz Biotechnology), and Ang II (IgG Corp., Nashville, TN) polyclonal antibody.

Statistical Analyses
All numerical data shown in the text are expressed as the mean ± SEM. Significant differences among the mean values of multiple groups were evaluated by a one-way ANOVA followed by a post hoc analysis (Fisher test). P < 0.05 was the threshold for significance.

Results
MABP was 127 ± 13 mmHg in the placebo-treated dogs, and it did not differ significantly from that observed in the NK3201-treated dogs (129 ± 20 mmHg) 28 d after surgery. However, the MABP was found to be significantly lower in the lisinopril-treated dogs (112 ± 11 mmHg; P < 0.01 versus placebo, P < 0.05 versus NK3201) when compared with the placebo- and NK3201-treated dogs.

Figure 2, A and B, shows representative van Gieson’s elastic staining from the contralateral normal brachial artery and vein. As can be seen in the figure, no remarkable intima could be observed in the normal artery and vein. Figure 2C shows a representative cross-section from downstream (Figure 1, region C) of the AV fistula. As mentioned in the Materials and Methods section, in this physiologic section, the arterial blood did not flow into the venous side. We found that there was no obvious neointimal proliferation in either the arterial or the venous side. A similar pattern was also observed in the NK3201- and lisinopril-treated dogs (Figure 2, D and E).

Figure 3A shows a cross-section from the portion of the AV anastomosis (Figure 1, region B) that was stained by van Gieson’s staining in the placebo-treated dogs. Figure 3, B and C, shows representative sections from NK3201- and lisinopril-treated dogs 28 d after surgery, respectively. This region contains a portion of the arterial and venous wall from one duct. There was no significant neointimal formation ob-

Figure 1. Tissue preparation. Diagrammatic representation of the arteriovenous (AV) fistula anastomosis, which shows the location where the tissue samples were cut within the AV fistula anastomosis. Region A (upstream) contains both a perfect artery and vein, and this is the point within the region where most of the arterial blood will flow into the vein. Region B of the AV anastomosis contains a portion of both arterial and venous walls in one duct. Region C (downstream) contains both a perfect artery and vein and is the point where the arterial blood does not flow into the vein.
served in the arterial wall side; however, an eccentric neo-intimal formation was evident in the venous wall side. When the magnitude of the neointimal hyperplasia that was found in the venous wall was expressed as a percentage of the intima area to the media area, this ratio was approximately 180 ± 8% in the placebo-treated dogs. NK3201 treatment starting 3 d before construction of the fistula suppressed this neointimal formation markedly (54 ± 15%; \(P < 0.001;\) Figure 3, B and D). Compared with the placebo-treated dogs, the neointimal formation was also significantly suppressed by the treatment with lisinopril (73 ± 14%; \(P < 0.001;\) Figure 3, C and D). To evaluate the degree of stenosis in the A and B regions, we also analyzed the lumen sizes for the areas surrounded by both the arterial and venous walls in the region of the fistula. \(n = 5. \#\#\# P < 0.001\) versus placebo. A, artery; V, vein. Bar indicates intima.

Figure 2. (A and B) Representative van Gieson’s elastic staining from the contralateral normal brachial artery and vein. (C through E) Representative cross-sections from region C (downstream) for the placebo-, NK3201-, and lisinopril-treated dogs. A, artery; V, vein. Bar indicates intima.

Figure 3. (A through C) Cross-sections from the portion of the AV anastomosis (region B) that was subjected to van Gieson’s staining in dogs that were treated with placebo, NK3201, or lisinopril. In this region, a part of the arterial wall in conjunction with a part of the venous wall forms a circle. There was no significant neointimal formation observed in the arterial wall side; however, eccentric neointimal formation was evident in the venous wall side. (D) Magnitude of the neointimal hyperplasia when expressed as a percentage of the area of intima to media of the venous wall. (E) Lumen sizes for the areas surrounded by the arterial and venous walls in the region of the fistula. \(n = 5. \#\#\# P < 0.001\) versus placebo. A, artery; V, vein. Bar indicates intima.
sizes of the AV anastomosis were found among the placebo-, NK3201-, or lisinopril-treated dogs.

Figure 4A shows a representative cross-section from the region of the upstream vein (Figure 1, region A) in the placebo-treated dogs. As most of the arterial blood flows into this vein, this region is a main exit for arterial blood after the AV fistula construction. In the placebo-treated dogs, stenosis induced by proliferation of the neointima in the upstream venous side region after construction of the AV fistula was extremely evident, with a high ratio of intima to media being found in the region (153 \( \pm \) 25\%; Figure 4, A and D). NK3201 treatment starting 3 d before construction of the AV fistula resulted in marked suppression of the ratio of intima to media (53 \( \pm \) 9\%; \( P < 0.001 \); Figure 4, B and D). In addition, the lumen of the venous side was also significantly larger in these animals as compared with the placebo-treated dogs (placebo, 1.02 \( \pm \) 0.35 mm\(^2\); NK3201, 3.71 \( \pm \) 1.14 mm\(^2\); \( P < 0.01 \); Figure 4E). However, the ratio of the intima to media was not significantly suppressed by lisinopril treatment (117 \( \pm \) 7\%), although the value of the ratio of the intima to media was somewhat decreased in this group (Figure 4, C and D). Compared with lisinopril treatment, a more suppressive effect on the intimal formation was observed after NK3201 treatment (\( P < 0.05 \); Figure 4D). When compared with the lisinopril-treated dogs, the lumen size also tended to increase additively in the NK3201-treated dogs (lisinopril, 2.51 \( \pm \) 1.01 mm\(^2\); Figure 4E).

The expression of chymase-positive mast cells was essentially absent in the contralateral artery or vein (Figure 5, A and E). However, a large number of chymase-positive cells were expressed in the media and proliferative neointima after construction of the AV fistula (Figure 5, B, E, and F). These expressions were inhibited significantly by treatment with NK3201 (Figure 5, C, E, and F). Lisinopril treatment also significantly suppressed the accumulation of chymase-positive cells in the media and proliferative neointima (Figure 5, D, E, and F), although the degree of suppression was somewhat weaker than that seen for the NK3201 treatment. Histologic studies of the serial sections demonstrated that mast cells stained by toluidine blue were almost entirely consistent with the expression of chymase-positive cells, indicating that mast cells are the main source of chymase (Figure 6, A and B). TGF-\( \beta \) expression was also examined in this study. As can be seen in Figure 6C, a positive immunoreaction against monoclonal anti–TGF-\( \beta \)1, -\( \beta \)2, and \( \beta \)3 antibody was found in an adjacent section that was in close proximity to the area for the expression of the chymase-positive mast cells, although the quantity was markedly less than that seen for the chymase-positive cells. This observation supports the previous findings that mast cells are the main source of the TGF-\( \beta \) family (12,13). In a comparison with the contralateral control vein, the TGF-\( \beta \)-positive cells were found to be significantly increased both in the media and in the intima at region A in the placebo-treated dogs (Figure 6G). The ex-

![Figure 4.](image-url) (A through C) Representative cross-sections from region A (upstream) for the placebo-, NK3201-, and lisinopril-treated dogs. Most of the arterial blood flows into this vein; thus, this region is the main exit of the arterial blood after the AV fistula construction. (D) Magnitude of the neointimal hyperplasia when it is expressed as a percentage of the area of intima to media of the venous wall. (E) Lumen sizes for the areas surrounded by the venous tissue. \( n = 5 \). *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) versus placebo; †\( P < 0.05 \) versus lisinopril. A, artery; V, vein. Bar indicates intima.
pression of TGF-β in both region A and region B was markedly suppressed by NK3201 treatment but was found to be less affected by the lisinopril treatment (Figure 6, G and H). As per previous reports, ACE was expressed mainly in the vascular endothelium and in the media of the smooth muscle cells (Figure 6D). When compared with the contralateral control vein, the amount of ACE staining tended to increase in the placebo-treated dogs at region A of the intima and media, although the difference was not statistically significant. Neither NK3201 nor lisinopril treatment significantly affected the expression of ACE in both regions A and B when compared with the placebo-treated dogs (Figure 6, E and F).

We also performed immunohistochemical staining to evaluate the tissue distribution of Ang II as well as the expression of Ang II receptors 28 d after the construction of the AV fistula (Figures 7 and 8). As shown in Figure 7A, in placebo-treated dogs, there was a wide immunoreactive staining for Ang II seen in the proliferative neointima and media 28 d after construction of the AV fistula. No staining was found when the primary antibody was substituted with nonimmune rabbit serum, thereby confirming the specificity of the immunoreaction (data not shown). In comparison with the contralateral vein, the amount of Ang II–positive staining was significantly higher in the placebo-treated dogs (Figures 7 and 8A). NK3201 treatment significantly decreased the Ang II–positive areas in both the intima and the media, although it was less affected by lisinopril treatment (Figures 7 and 8, A and B). The expression of AT1 receptors was also significantly increased in the placebo-treated dogs (Figures 7 and 8C). NK3201 and lisinopril treatments both were found to decrease the AT1–positive areas to some extent in the intima or the media for regions A and B (Figures 7 and 8, C and D). No obvious positive immunoreaction for AT2 was found in the other three experimental groups (Figure 7).

Figure 9A shows a representative azan Mallory staining from a placebo-treated dog. Dark blue staining could be found in the area of the hyperplastic intima, indicating that collagen deposition was present during the neointimal formation after construction of the AV fistula. Lisinopril treatment did not significantly decrease collagen deposition (Figure 9C), although collagen deposition was markedly inhibited by NK3201 treatment. Because of a weak blue staining can be found in the NK3201-treated group (Figure 8B). In this study, we also examined the expression of fibronectin 28 d after construction of the AV fistula (Figure 8D). However, no obvious differences in the expression of fibronectin were found among the three experimental groups (Figure 9E).

In this study, apoptosis was detected by in situ terminal deoxynucleotidyl transferase (TdT)–mediated digoxigenindUTP nick end labeling (TUNEL), and cell proliferating activity was estimated using an antibody to PCNA. As can be seen in
Figure 10. TUNEL- and PCNA-positive cells in the contralateral vein were rare. However, both TUNEL- and PCNA-positive cells significantly increased after construction of the AV fistula. Compared with the placebo-treated dogs, the number of proliferating cells in the hyperplastic intima was significantly lower in the NK3201- and lisinopril-treated dogs, although the number of the apoptotic cells did not differ significantly among the three groups (Figure 10, A and B).

Discussion

There are two main conclusions that can be drawn from this study. First, in association with the expression of chymase-positive mast cells, stenosis of the upstream venous wall by the proliferation of the neointima was observed 28 d after construction of the AV fistula. Second, when the chymase-specific inhibitor NK3201 was administered to dogs starting 3 d before construction of the AV fistula, there was an antiproliferative effect with regard to the neointimal formation, which was achieved in accordance with a reduction of chymase-positive mast cell accumulation. Although the ACE inhibitor lisinopril treatment also showed an antiproliferative effect in this model, the degree was significantly lower than that observed for the chymase inhibitor. These findings demonstrate for the first time that mast cell–derived chymase rather than the traditional ACE plays a major role in the pathogenesis of the AV fistula access failure by accelerating exit stenosis via promotion of neointima formation in the upstream venous wall.

There are several reasons that have been reported for the failure of vascular access in hemodialysis patients. First, thrombosis is reported to be a major cause for failure either soon after construction of the AV fistula or later, as a secondary event to the venous neointimal hyperplasia at the AV anastomotic region or in the proximal vein (14–16). Second, failure secondary to infection or other complications has been noted to occur in
approximately 15 to 20% of cases (1). In our study, no animals were omitted as a result of thrombotic episode. The avoidance of thrombosis during the acute phase after construction of the AV fistula may have been due to the single intravenous injection of 1000 U of heparin that we used in our study. As mentioned above, a thrombotic episode is accelerated by lumen stenosis. After construction of the AV fistula, lumen stenosis usually occurs in the venous circulation, especially in the region of the arterial blood exit in humans. In our canine AV fistula model, stenosis induced by proliferation of the neointima is most evident in the upstream venous side region, as was indicated by the high ratio of the intima to media and the diminished lumen area. Therefore, the pathologic properties of stenosis that occur after construction of the AV fistula in dogs may closely resemble the stenosis observed in humans.

Why does chymase inhibition prevent the aggressive stenosis after AV fistula? It is well known that neointimal hyperplasia largely contributes to the development of such stenosis. Neointimal hyperplasia is essentially related to the proliferation of vascular smooth muscle cells (VSMC) in the media and migration of the VSMC from the media into the intima (17,18). Exuberant synthesis of extracellular matrix protein by the phenotypically changed VSMC is mainly responsible for the neointimal expansion (17,18). Recent studies have demonstrated the close participation of Ang II or TGF-β1 in neointimal hyperplasia. For example, the continuous infusion of Ang II enhances intima proliferation markedly in injured arterial walls (19). In the neointima of experimental vein grafts, the AT₁ receptor is expressed focally and the degree of intimal hyperplasia is reduced significantly by the treatment with the AT₁ receptor antagonist losartan (20). In vitro studies confirm that Ang II directly promotes the growth of VSMC and the migration of the VSMC from the media into the intima (21–23). In the hyperplastic intima and media of the placebo-treated dogs, our immunohistochemical studies showed that the proliferative procedure but not the apoptotic procedure was evident and that there was a marked increase in the amount of the Ang II and AT₁ receptors. In association with the reduction of the Ang II– and AT₁–positive areas, a strong suppression in the number of proliferative cells as well as neointimal formation occurred with NK3201 treatment. This indicates that the accumulation of chymase-positive mast cells in the hyperplastic intima and media results in an increase in the generation of Ang II, which via stimulation of the AT₁ receptors plays an important role in the pathogenesis of the fistula stenosis in our experimental dog AV-fistula model. The expression of AT₂ receptors may not be so important during the development or regression of neointimal formation after construction of the AV fistula in dogs, as no significant change in the expression of AT₂ receptors was found in this study. TGF-β1 may also play a crucial role in the pathogenesis of neointimal formation. In vivo studies show that the administration of TGF-β1 enhances intimal thickening, and the neointimal formation is suppressed markedly by the administration of the specific antibodies against TGF-β1 in balloon-injured arteries (24,25). TGF-β1 expression is also increased in the luminal and abluminal neointima of stenosed AV fistulas and correlates to the neointimal cell number (26–28). An important finding that was recently reported for hemodialysis...
patients is that the AV fistula patency is determined by TGF-β1 genotype polymorphisms (29). TGF-β1 not only has a bimodal effect on VSMC proliferation (30) but also stimulates extracellular matrix protein production and inhibits the degradation of matrix protein in vitro (31). In addition, TGF-β1 accelerates the expression of PDGF (32) and fibroblast growth factor (33), the important mediators for VSMC proliferation and migration, as well as the deposition of extracellular matrix proteins (34–36). Chymase itself promotes collagen biosynthesis (37) and fibronectin degradation (38), and these effects may also influence the deposition of extracellular matrix protein in the intima and migration of VSMC from the media into the intima. In this study, the positive immunoreaction against anti-chymase antibody was found in an adjacent section that was in close proximity to the distribution of the mast cells, indicating that mast cells seem to be the main source for chymase. Recently, the expression of chymase in VSMC in pathologic conditions such as hypertrophied pulmonary rat artery has been found 21 d after injection of the toxin monocrotaline (39). Unlike rat mast cell chymase I and II, this enzyme is capable of converting Ang II from Ang I. Whether such chymase is also expressed in the hyperplastic region of the VSMC after construction of the AV fistula was not determined in this study. To the best of our knowledge, no expression of chymase in the normal dog VSMC has been reported; thus, this needs to be investigated in the future under both normal and pathophysiologic conditions. This study confirmed that mast cells were the main source for the TGF-β1 family and it to be benefit for chymase cleavage latent TGF-β1 to active form. The amount of the TGF-β1–positive cells also certainly must be increased in placebo-treated dogs. In accordance with a reduction of the TGF-β1–positive mast cells, the neointimal formation and the deposition of collagen in the hyperplastic intima were inhibited significantly by the NK3201 treatment. In contrast to a previous report (38), no significant changes in fibronectin expression were found with the NK3201 treatment. Taken together, chymase via acceleration in the generation of Ang II, which through the stimulation of the AT1 receptors and via acceleration of the generation of the active form of TGF-β, may participate in the formation of aggressive intima hyperplasia in this model. ACE expression may also participate in the creation of the neointimal formation, as the inhibition of ACE by treatment with lisinopril also reduced the neointima formation in the AV anastomosis. However, the degree of suppression was clearly less than that seen for chymase inhibition, indicating that the role of chymase in the formation of the neointima may be more
Figure 10. In situ terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-dUTP nick end labeling (TUNEL) method was used to detect apoptosis, and a proliferating cell nuclear antigen (PCNA) antibody was used to estimate the cell proliferating activity. TUNEL- and PCNA-positive cells in the contralateral vein were rare. However, both TUNEL- and PCNA-positive cells increased significantly after construction of the AV fistula. As indicated by the bar graphs A and B, compared with the placebo-treated dogs, the number of proliferating cells in the hyperplastic intima were significantly lower in the NK3201- and lisinopril-treated dogs, although the number of the apoptotic cells did not differ significantly among the three experimental groups.

$n = 5$. ***$P < 0.001$ versus placebo. P, placebo; N, NK3201; L, lisinopril.
important than that for ACE. Compared with ACE inhibition, the more superior effect of the chymase inhibition on the inhibition of Ang II formation (Figure 8) may be a foregone conclusion to interpretation our present this phenomenon. However, as mentioned above, unlike ACE, in addition to the Ang II generation property, chymase possess other pathophysiologic actions. Therefore, in this study, we speculated that the greater beneficial effects that are seen with chymase inhibition might also be due to results linked to the suppression of these other pathophysiologic actions. Unfortunately, in our study, we could not determine the effects of a chymase inhibitor combined with an ACE inhibitor or an Ang II receptor antagonist. Theoretically, these combinations might be ideal for the prevention of neointimal formation after construction of the AV fistula, as there is not only an increase in the amount of chymase but also a significant increase in the number of AT1 receptors. The amount of ACE also tended to increase in the hyperplastic region of the placebo-treated dogs, and, as was theorized, the lisinopril treatment did indeed suppress the neointimal formation to some extent. Therefore, in the future, it will be interesting to investigate whether combination therapies that use a chymase inhibitor, ACE inhibitor, and Ang II receptor antagonist will be able to provide a more superior effect than monotherapy.

A recent study showed that the upregulation of chymase correlates significantly with the severity of collagen matrix deposition in the renal glomeruli, tubulointerstitium, and arterial walls in patients with end-stage diabetic nephropathy. This finding indicates that mast cell–derived chymase may play a pivotal role in the development and progression of diabetic nephropathy in patients (40). Patients with end-stage diabetic nephropathy usually undergo hemodialysis; therefore, chymase inhibitors may become a useful agent for treating this disease in the near future.

In conclusion, stenosis of the blood flow exit after construction of an AV fistula by the proliferation of the neointima upstream of the venous region is associated with the expression of chymase-positive mast cells. This neointima formation is suppressed significantly by treatment with a specific chymase inhibitor, demonstrating that mast cell–derived chymase plays an important role in the pathogenesis of the AV fistula access failure. Although ACE inhibition may also be of benefit in preventing formation of the neointima after construction of the AV fistula, it may be less effective than methods that are designed to provide chymase inhibition. Therefore, within the clinic setting, chymase inhibition may need to be considered as the primary therapeutic target for the treatment of hemodialysis vascular access dysfunction.

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