A Specific Chymase Inhibitor, 2-(5-Formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[(3,4-dioxo-1-phenyl-7-(2-pyridyloxy))-2-heptyl]acetamide (NK3201), Suppresses Development of Abdominal Aortic Aneurysm in Hamsters

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ABSTRACT

In this study, we investigated the effect of a specific chymase inhibitor, 2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[(3,4-dioxo-1-phenyl-7-(2-pyridyloxy))-2-heptyl]acetamide (NK3201), in the development of abdominal aortic aneurysm in a hamster experimental model. The abdominal aortic aneurysm was induced by application of elastase onto the abdominal aorta in hamster. Each hamster was administered NK3201 (30 mg/kg/day p.o.) or placebo beginning 4 days before application of elastase and continuing through the experiments. Sham-operated hamsters received saline application onto the abdominal aorta. Two weeks after application of elastase, the aortic diameter in the placebo-treated group was significantly increased to 1.6-fold compared with the value for the sham-operated group, whereas that in the NK3201-treated group was significantly reduced. The chymase activities in the sham-operated and the placebo-treated groups were 0.35 ± 0.01 and 3.44 ± 0.62 mU/mg protein, respectively, and this difference was significant. NK3201 significantly reduced the chymase activity in the placebo-treated group. Here, we demonstrated for the first time that a chymase inhibitor prevented the development of abdominal aortic aneurysm in a hamster experimental model.

Aneurysmal aorta which represents a chronic degenerative condition associated with atherosclerosis is characterized by segmental weakening and dilatation of the aortic wall (Thompson, 1996; Grange et al., 1997). Although the immediate risk associated with small symptomatic aneurysmal aorta is very low, the natural history of these lesions involves gradual expansion over a period of years with a substantial number of aneurysmal aorta eventually rupturing (Delin et al., 1985; Sterpetti et al., 1987; Nevitt et al., 1989). The pathophysiology of aneurysmal aorta includes aortic atherosclerosis, chronic inflammation within the outer aortic wall, and an imbalance between the production and degradation of structural extracellular matrix proteins (White et al., 1993).

Angiotensin II has been found to play an important role in the activation of inflammatory reactions in atherosclerosis (Miyazaki et al., 1999; Berk et al., 2000; Daugherty et al., 2000). Nishijo et al. (1998) suggested that angiotensin II is related to aortic aneurysm, because angiotensin II infusions into mice promote aortic aneurysm, and salt-loaded hypertensive mice are characterized by higher angiotensin II levels occurring in hemorrhage before the development of aneurysm. Angiotensin II is generated from angiotensin I by two distinct types of angiotensin II-forming enzymes, angiotensin-converting enzyme (ACE) and chymase in human cardiovascular tissues (Takai et al., 1997b, 1999), and ACE and chymase are associated with the development of atherosclerosis (Takai et al., 1997a; Miyazaki et al., 1999). Ihara et al. (1999) reported that ratio of chymase-dependent angiotensin II-forming activity to total angiotensin II-forming activity was significantly higher in aneurysmal aorta than in normal aorta. We also reported that in human abdominal aortic aneurysm, chymase activity was significantly increased and that accumulated chymase-positive mast cells were observed in the media and adventitia (Nishimoto et al., 2002; Tsunemi et al., 2002). However, it has been unclear whether the in-

ABBREVIATIONS: ACE, angiotensin converting enzyme; MCP-1, monocyte chemoattractant protein; NK3201, 2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[(3,4-dioxo-1-phenyl-7-(2-pyridyloxy))-2-heptyl]acetamide.
crease in chymase in abdominal aortic aneurysm is related to the development of abdominal aortic aneurysm.

In this study, we measured chymase activity and aortic diameter in a hamster experimental model of abdominal aortic aneurysm induced by application of elastase, and we investigated the effect of a specific chymase inhibitor in the development of abdominal aortic aneurysm.

Materials and Methods

Drugs. 2-(5-Formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[(3,4-dioxo-1-phenyl-7-(2-pyridyloxy))-2-heptyl]acetamide (NK3201) was synthesized as a specific chymase inhibitor (Nippon Kayaku Co. Ltd., Tokyo, Japan) (Takai et al., 2001, 2003c).

Animal Treatment. Male hamsters weighing 180 to 210 g were obtained from Japan SLC (Shizuoka, Japan). The hamsters were fed with regular hamster chow, had free access to tap water, and were housed in a temperature-controlled, humidity-controlled, and light-controlled room. A hamster experimental model of abdominal aortic aneurysm induced by application of elastase was modification of a previous report (Origuchi et al., 1998). Under pentobarbital anesthesia (50 mg/kg i.p.), the abdominal aorta from the infrarenal aorta to the bifurcation of the aorta was isolated, and the exposed abdominal aorta was circumferentially wrapped with gauze, onto which 200 units/ml porcine pancreatic elastase (type I; Serva, Heidelberg, Germany) in saline was applied for 1 h. The control hamsters were treated with the application of saline without elastase. To investigate the effect of a chymase inhibitor, each hamster was orally administered NK3201 (30 mg/kg/once/day) or placebo beginning 4 days before application of elastase and continuing through the experiments. In the placebo-treated group, hamsters were administered the same volume of 0.5% carboxymethyl cellulose as the placebo. We assessed the diameter and the chymase activity in the abdominal aorta after the operation. The experimental procedures for the animals were conducted in accordance with guidelines of Osaka Medical College.

Preparation of Vascular Tissue. After anesthetizing with sodium pentobarbital (50 mg/kg i.p.), maximal transverse diameter of the abdominal aortas was directly measured, and the aortas were removed. The abdominal aorta was homogenized in 20 mM Na-phosphate buffer, pH 7.4 (Takai et al., 2003a). The homogenate was centrifuged at 20,000 rpm for 30 min. The supernatant was used for measurement of the chymase activity.

Measurement of Chymase Activity. Chymase activities were measured by incubating the tissue extracts for 1 h at 37°C with 4 mM angiotensin I in 150 mM borax-borate buffer, pH 8.5, containing 5 mM EDTA, 8 mM dipyridyl, and 0.77 mM diisopropylfluorophosphate (Takai et al., 2003a). The enzyme reaction was terminated by addition of 15% trichloroacetic acid. For fluorometric quantitation of His-Leu formed from an angiotensin I substrate, 10% o-phthaldialdehyde was incubated for 10 min at room temperature. Reaction was terminated by addition of 6 M HCl, and the fluorescence was measured at 340-nm excitation and 455-nm emission. One unit of chymase activity was defined as the amount of enzyme that cleaved 1 µmol angiotensin II/min. Protein concentration was assayed with BCA protein assay reagents (Pierce Chemical, Rockford, IL), using bovine serum albumin as a standard.

Histological Analysis. The aortic segments were fixed in 10% methanol-Carnoy’s fixative overnight and embedded in paraffin. The sections were cut from each block at 3 µm in thickness. The sections were stained with hematoxylin-eosin and van Gieson’s elastin stain, respectively. The ratio of medial area to total area in the aorta was measured by using a computerized morphometry system, MACSCOPE version 2.2 (Mitani Co., Fukui, Japan). The mast cells in every section from the four slices were stained with toluidine blue, and the number of mast cells was quantified, using the computerized morphometry system, and expressed as the number of stained mast cells per square millimeter.

Statistical Analysis. Statistical analysis was performed using a parametric test with Fisher’s protected least significant difference. Values were considered significant at P < 0.05. Data are expressed as mean ± standard error of the mean.

Results

Aortic Diameter after Application of Elastase. The aortic diameter was increased time dependently, and it was 1.12 ± 0.03, 1.52 ± 0.14, 1.70 ± 0.16, and 1.72 ± 0.18 mm before and 0.5, 1, and 2 h after the application of elastase (each point, n = 5). The aortic diameter was significantly increased 1 h after application of elastase compared with the value of age-matched control hamsters, and this increased aortic diameter was continued even 4 weeks later (Fig. 1).

Effects of NK3201 on Aortic Diameter. Figure 1B shows the effect of NK3201 on the aortic diameter before and 2 weeks after the application of elastase. One hour after the application of elastase, the aortic diameters in the placebo-treated groups were significantly increased compared with...
that in the control group, and the increased aortic diameter was not affected by treatment with NK3201. Two weeks after the operation, the aortic diameters in the control and the placebo-treated groups were 1.34 ± 0.03 and 2.18 ± 0.04 mm, respectively, and this difference was also significant. However, the aortic diameter in the NK3201-treated group was 1.66 ± 0.06 mm, and this was significantly smaller than the placebo-treated group.

**Effects of NK3201 on Vascular Chymase Activity.** In the placebo-treated group, the chymase activity was significantly increased about 10-fold compared with the control group 2 weeks after the application of elastase (control group, 0.35 ± 0.10 mU/mg protein; placebo-treated group, 3.44 ± 0.62 mU/mg protein; Fig. 2A). On the other hand, the chymase activity in the NK3201-treated group was 2.03 ± 0.31 mU/mg protein, and this was significantly lower than the placebo-treated group.

**Effects of NK3201 on Medial Area to Total Area.** Two weeks after the application of elastase, typical photographs of aortas in the control, placebo- and, NK3201-treated groups stained with van Gieson’s elastin stain are shown in Fig. 3. The medial area was clearly decreased in the placebo-treated group, whereas NK3201 suppressed this decrease of medial area. The ratio of medial area to total area in the placebo-treated group was significantly decreased compared with the control group (control group, 91.2 ± 3.5%; placebo-treated group, 41.3 ± 6.7%; Fig. 2B). However, the ratio in the NK3201-treated group was 68.4 ± 7.5%, and this was significantly higher than the placebo-treated group.

**Effects of NK3201 on Number of Mast Cells.** In the placebo-treated group, the number of mast cells per square milliliter was significantly increased compared with the number in the control group (control group, 2.78 ± 0.2; placebo-treated group, 14.3 ± 1.3; Fig. 2C). In the NK3201-treated group, the number of mast cells per square millimeter was 8.58 ± 0.9, and this number was significantly decreased compared with that in the placebo-treated group.

**Discussion**

In this study, using a hamster aneurysm model, we demonstrated for the first time that a specific chymase inhibitor, NK3201, could suppress both the increase in chymase activity and the expansion of the aortic diameter that is induced by the application of elastase onto abdominal aorta. This finding clearly suggests that chymase may play an important role in the development of abdominal aortic aneurysm. It is widely recognized that aortic aneurysm is closely associated with chronic inflammation in the aortic wall (White et al., 1993; Freestone et al., 1995). Chymase is known to be stored in mast cells, which play an important role in inflammation. On the other hand, chymase in vascular tissues is also known to convert angiotensin I to angiotensin II (Takai et al., 1997b, 1999). In patients, the numbers of mast cells and macrophages are increased in abdominal aortic aneurysm, and recent research suggests that angiotensin II activates inflammatory cells such as macrophages and mast cells via activation of chemokines and cytokines (Hernandez-Presa et al., 1997; Schieffer et al., 2000). The activated macrophages induce nuclear factor-κB, and this in turn induces an inflammatory cytokine, interleukin-1, and a chemokine, monocyte chemoattractant protein (MCP)-1 (Collins et al., 1995). Interleukin-1 produced by activated macrophages induces tissue damage, and MCP-1 induces the activation and migration of monocytes, resulting in an accumulation of macrophages (Chen et al., 1998; Mabuchi et al., 2000). An angiotensin II type 1 receptor antagonist was found to reduce gene expression of MCP-1, and this drug reduced the accumulation of macrophages (Kato et al., 1999; Hilgers et al., 2000). Daugherty et al. (2000) demonstrated that infusion of angiotensin II leads to development of aortic aneurysm in apolipoprotein E-deficient mice. The activation of inflammatory cells via angiotensin II-induced by chymase...
may play an important role in the development of aortic aneurysm.

In the present study, the number of mast cells per square millimeter of the aortas in the placebo-treated hamsters was significantly higher than in the normal hamsters, whereas it was significantly decreased in the NK3201-treated hamsters. It is known that chymase plays an important role in the accumulation of mast cells by activating stem cell factor (Zhang et al., 1998). In our previous study, the number of mast cells per square millimeter of the hearts in the placebo-treated cardiomyopathic hamsters was significantly higher than in the normal hamsters, whereas it was decreased by treatment with a chymase inhibitor, 4-[1-[(bis-(4-methyl-phenyl)-methyl]-carbamoyl]-3-(2-ethoxy-benzyl)-4-oxo-azetidine-2-yloxy]-benzoic acid (Takai et al., 2003a). NK3201 also reduced the number of mast cells increased in the grafted veins 28 days after the operation in a dog model (Takai et al., 2003b). In human abdominal aortic aneurysm, chymase activity was significantly increased, and accumulated chymase-positive mast cells were observed in the media and adventitia (Nishimoto et al., 2002; Tsunemi et al., 2002). Therefore, decreases in chymase activity by NK3201 might be associated with decreases in mast cells, in addition to direct suppression.

In the present study, we used NK3201, which was developed recently as an orally active specific chymase inhibitor (Takai et al., 2001, 2003b). NK3201 is a strong competitive inhibitor, and this inhibitor shows inhibition to chymase in the mode of the formation of acyl-intermediate between active serine residue of the enzyme and di-ketone structure of NK3201 (Sukenaga et al., 2002). NK3201 inhibited human, dog, and hamster chymases by IC50 at concentrations of 2.5, 1.2, and 28 nM, respectively (Takai et al., 2001). These findings suggest that 30 mg/kg NK3201 used in the present study is expected to inhibit chymase activity. In fact, in our recent study, oral administration of NK3201 at the dose (30 mg/kg per day) same to that used in the present experiments, 3 days before the operation significantly improved cardiac function and reduced the mortality rate after myocardial infarction in hamster (Jin et al., 2003). On the other hand, NK3201 has no inhibitory activity to other types of serine proteases, trypsin, thrombin, elastase, plasmin, urokinase, and plasminogen activator, even at concentration of 1 nM (Takai et al., 2001). Plasma concentrations of NK3201 are 500, 850, and 1100 nM even at 2 h after a single oral administration, at doses of 10, 100, and 1000 mg/kg, respectively (Takai and Miyazaki, 2003). Therefore, in the present study, NK3201 was thought not to inhibit elastase activity.

Atherosclerosis has been reported to be closely associated with abdominal aortic aneurysm (Thompson 1996; Grange et al., 1997). Although we did not consider the correlation between atherosclerosis and aneurysmal aorta in the hamster aneurysmal model used in the present study, chymase has been reported to correlate with the development of atherosclerosis (Takai et al., 1997a). For example, chymase can degrade the apolipoprotein B-100 component of low-density lipoprotein particles and the apolipoprotein A component of high-density lipoprotein particles (Kokkonen et al., 1986; Lindstedt et al., 1996). These actions of chymase on apolipoproteins may increase the cholesterol content of macrophages and convert them into the foam cells that are typical of early atherosclerotic lesions. These findings suggest that chymase may be related to atherosclerosis, which is an important factor in the development of abdominal aortic aneurysm.

In conclusion, we demonstrated that chymase may be an important contributor to the development of abdominal aortic aneurysm and suggest the novel possibility of using a specific chymase inhibitor as a new therapeutic strategy for the aortic aneurysm.

References


Kokkonen JO, Vartiainen M, and Kovanen PT (1986) Low density lipoprotein degradation by secretary granules of rat mast cells. Sequential degradation of apop-}

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