Chymase inhibition suppresses high-cholesterol diet-induced lipid accumulation in the hamster aorta

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Abstract

Objective: The role of chymase (a mast cell-derived angiotensin II-forming serine proteinase) in aortic lipid deposition was investigated using an orally active, non-peptide chymase inhibitor, SUN-C8257. Methods: Male golden Syrian hamsters, 8 weeks old, were fed with a standard rodent meal supplemented with or without 0.5% cholesterol and 10% coconut oil for 12 weeks. The hamsters fed high cholesterol diet were further separated into two groups treated with or without SUN-C8257 for 12 weeks. The aortic lipid deposition was visualized by Oil red O staining and planimetrically measured. Immunohistochemical staining for angiotensin II (Ang II) of the aortic root region was performed. Aortic Ang II-forming activity was measured using Ang I as a substrate. Plasma total-, low-density lipoprotein (LDL)-, and high-density lipoprotein (HDL)-cholesterol and triglyceride were quantified by enzymatic methods. Plasma Ang I and Ang II were measured by radioimmunoassay. Results: After 12 weeks of high cholesterol diet, aortic chymase activity in the untreated group increased significantly and showed a positive correlation with plasma total- and LDL-cholesterol. This group of hamsters developed marked lipid deposits in the aortic intima. However, treatment with SUN-C8257 significantly suppressed aortic lipid deposition without changing body weight, blood pressure, plasma LDL-cholesterol and Ang II levels. The level of the adventitial Ang II-immunoreactivity was markedly inhibited in the group treated with SUN-C8257. Conclusion: Our results suggest that arterial chymase may participate in the acceleration of lipid deposition in arterial walls exposed to high plasma cholesterol and that inhibition of arterial chymase may retard the progression of atherosclerosis.

Keywords: ACE inhibitors; Angiotensin; Arteries; Atherosclerosis; Cholesterol; Lipoproteins; Renin angiotensin system

1. Introduction

Activation of the tissue renin-angiotensin system (RAS) appears to be involved in progression of atherosclerosis in experimental animals [1] as well as in humans [2]. This concept is substantiated by the finding that the activity of aortic angiotensin converting enzyme (ACE) and density of angiotensin II (Ang II) type 1 receptor (AT₁-R) are both significantly higher in rabbits fed a high cholesterol diet than in controls [3]. Furthermore, various studies have demonstrated the existence of alternative Ang II-forming pathways involving several serine proteinases, such as kallikrein, cathepsin G and chymase, which are probably responsible for ACE-independent Ang II-forming pathways [4–6]. In humans, the tissue content of chymase is quite high in several organs [7]. Our recent clinical study has shown that human atherosclerotic aortas contain significantly higher levels of chymase-dependent Ang II-forming activity (dAIIFA) compared to non-atherosclerotic aortas [8]. In addition, the chymase dAIIFA of internal thoracic arteries obtained from coronary bypass operations correlated positively with serum low-density lipoprotein (LDL)-cholesterol levels [9]. This indicates that arterial chymase could be upregulated by hypercholesterolemia.
and that it may play an important role in atherogenic development. Therefore, the anti-atherogenic effect of orally active chymase-specific inhibitor was challenged in the hamsters fed a high cholesterol diet.

2. Methods

2.1. Diet and drug control for hamsters

Male golden Syrian hamsters (KBT Oriental Co.), 8 weeks old and weighing 100–130 g, were selected because they possess human-like Ang II-forming chymase. The hamsters were housed in a rodent cage on wood-chip bedding and allowed free access to food and water (4–6 housed per cage). The standard chow group (n = 6) received standard rodent meal. The high-cholesterol and high-fat chow group received a standard rodent meal supplemented with 0.5% cholesterol and 10% coconut oil (KBT Oriental Co). The hamsters fed on high-cholesterol and high-fat diet were treated with (n = 6) or without SUN-C8257 (n = 6) added to the drinking water (100 mg kg\(^{-1}\) per day, continuously) for 12 weeks. The investigations conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal experiments were approved by our institutional committee and were performed according to its guidelines.

The chymase inhibitor, (3-[((3-amino-4-carboxy)phenylsulfonyl]-7-chloroquinazoline-2,4(1H,3H)-dione, compound number #44 in Ref. [10], SUN-C8257) was a kind gift from Suntory Institute for Biomedical Research, Osaka, Japan and highly specific for human chymase. The IC\(_{50}\) values for human chymase, hamster chymase, human cathepsin G and bovine pancreas chymotrypsin are 0.31, 0.68, 5.5 and >10 \(\mu\)mol/l, respectively [10].

2.2. Blood pressure measurement

Hamsters were anesthetized with an intraperitoneal injection of 5% sodium thiobutabarbitral (final dose: 100 mg/kg body weight, Sankyo, Tokyo, Japan). Steady state heart rate and systolic blood pressure were measured by the standard hind-limb cuff method with an MK-1100 (Muromachi Kikai, Tokyo, Japan).

2.3. Biochemical assays

At the end of the study, aortic tissue was quickly removed from each hamster and rapidly washed in ice-cold saline, immediately frozen in liquid nitrogen and subsequently analyzed for chymase, ACE and non-ACE non-chymase dAIIFA. This was performed according to a previously described method [8,9]. Briefly, aortic homogenate suspensions were incubated with authentic substrate Ang I and the formed Ang II was analyzed by reversed-phase high performance liquid chromatography (HPLC, Shimazu, Kyoto, Japan) using a C\(_{18}\) reversed-phase HPLC column (2.2×25 cm; Vydac, Hesperia, CA, USA) with a 15-min linear acetonitrile gradient (3–13%) in 25 mM triethylamine-phosphate buffer, pH 3, at a flow rate of 2 ml/min. To determine the captopril or chymostatin-inhibitable Ang II formation, incubations were performed with or without 100 \(\mu\)mol/l captopril or 10 \(\mu\)mol/l chymostatin, respectively. Ang II-forming activities were expressed as nmol of Ang II formed/min/mg protein. All analyses for each sample were performed in duplicate and the reproducibility and quality of all of the data were confirmed before the statistical analysis. The inter- and intra-assay coefficients of variation of this assay were 8.6% (n = 12) and 5.1% (n = 10), respectively.

Plasma total-, LDL-, high density lipoprotein (HDL)-cholesterol and triglyceride were quantified by enzymatic methods [11]. Plasma and aortic Ang I and Ang II levels were measured by the method previously described [12]. In brief, peptides in hamster plasma were extracted by ethanol precipitation and Sep-Pak purification. The residues containing Ang II were separated by a C\(_{18}\) reversed-phase HPLC column and the peak corresponding to authentic standard Ang II was collected to measure Ang II concentration by radioimmunoassay.

2.4. Histological studies

The aortas from their junction with the heart to its mid portion of the ascending aorta was removed, and rinsed in ice-cold saline. Segments 3–5 mm in length, containing the aortic cusp region, were cryopreserved in Tissue-Tek (Muromachi Kikai, Tokyo, Japan) and is highly specific for human chymase. The IC\(_{50}\) values for human chymase, hamster chymase, human cathepsin G and bovine pancreas chymotrypsin are 0.31, 0.68, 5.5 and >10 \(\mu\)mol/l, respectively [10].

The aorta from its junction with the heart to its mid portion of the ascending aorta was removed, and rinsed in ice-cold saline. Segments 3–5 mm in length, containing the aortic cusp region, were cryopreserved in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA) at −80°C. Transverse frozen sections of 6 \(\mu\)m were cut and placed on glass slides. To determine lipid deposition, sections were fixed in 10% formalin for 10 min, rinsed in distilled water, and stained with Oil red O solution (Muto Pure Chemicals, Tokyo, Japan) which was preheated to 60°C, for 5 min. Sections were rinsed with 60% isopropanol for 2 min and distilled water and counterstained with hematoxylin for 2 min with rinsing in quarter saturated LiCO\(_3\). These were subsequently used to assess the area of lipid deposition. The positive area at the level of coronary ostia was planimetrically measured using NIH Image software v1.61. The area of each lesion was expressed relative to the cross-sectional area of the total intima and media.

Immunohistochemical staining for Ang II in frozen sections from the hamster aortic root region was performed by a previously described method [8]. Rabbit anti-human Ang II polyclonal antibody (CHEMICON International, Temecula, CA, USA) was used at 500-fold dilution.
Sections were mounted without counterstaining to obtain a clearer Ang II immunoreactivity.

2.5. Measurement of chymase and ACE expressions

Aortic tissue was quickly removed from each hamster and rapidly washed in ice-cold saline and then immediately frozen in liquid nitrogen. Aortic chymase and ACE mRNA levels were determined by a method of semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR). The PCR primer for hamster chymase, ACE and β-actin were selected according to the hamster cDNA sequence (chymase: sense primer, 5′-AAT CGC TCA CCC AAA CTA CAG C-3′, antisense, 5′-GCA GAC CTG GAA GTC ATA ACT G-3′, ACE: sense primer, 5′-GTC TGC CCA ACA AGA CTG CCA-3′, antisense, 5′-CCA CAT GTG TCC CAG CAG ATG-3′, β-actin: sense primer, 5′-TGG TGA CCG AGC GTG GCT ACA AGA CTG CCA-3′, antisense, 5′-CTG CTA GAA GGT GTA GGA CAG TGA GG-3′). The PCR conditions for hamster chymase, ACE, and β-actin consisted of denaturation at 94 °C for 1 min, annealing at 56, 68, or 64 °C for 1 min and extension at 72 °C for 1 min for a total of 40, 35, or 30 cycles, respectively. Amplification of chymase, ACE and β-actin mRNA by the specific primers, gave an expected 277, 388 and 488 bp product, respectively. The inter- and intra-assay coefficients of variation of this assay were 9.2% (n=8) and 7.2% (n=7), respectively.

2.6. Statistical analysis

Values are expressed as the mean±standard deviation. A standard linear regression analysis was used to compare tissue Ang II-forming activity to plasma cholesterol levels. Multiple comparisons among the groups were carried out by both one-way ANOVA with Fisher’s exact test for post hoc analysis and non-parametric analysis with Mann–Whitney U-test. A probability value of less than 0.05 was considered significant.

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>High cholesterol diet</th>
<th>High cholesterol diet + SUN-C8257</th>
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<tbody>
<tr>
<td>Animal number (n)</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>166±8 (154–176)</td>
<td>153±10 (138–173)</td>
<td>160±10 (150–178)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>134±19 (103–159)</td>
<td>139±18 (121–179)</td>
<td>133±18 (100–154)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.00±0.54 (4.11–5.56)</td>
<td>12.10±1.11* (10.40–13.11)</td>
<td>9.93±3.20* (7.73–12.54)</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>1.17±0.15 (0.98–1.80)</td>
<td>5.07±0.55* (4.01–5.74)</td>
<td>5.22±0.87* (4.34–6.67)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>2.94±0.37 (2.22–3.32)</td>
<td>3.80±0.56* (2.72–4.78)</td>
<td>3.03±0.50* (2.51–3.62)</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>3.74±1.06 (2.20–3.23)</td>
<td>10.91±1.62* (8.56–14.10)</td>
<td>10.54±2.41* (7.27–13.42)</td>
</tr>
<tr>
<td>ACE dAIIFA (nmol/min/mg)</td>
<td>0.28±0.19 (0.09–0.48)</td>
<td>0.52±0.29 (0.18–0.85)</td>
<td>0.30±0.22 (0.06–0.62)</td>
</tr>
<tr>
<td>Plasma Ang II (pg/ml)</td>
<td>22±6 (15–34)</td>
<td>20±7 (10–40)</td>
<td>19±5 (13–26)</td>
</tr>
<tr>
<td>Aortic Ang II concentration (pg/g)</td>
<td>29±9 (18–42)</td>
<td>30±12 (18–58)</td>
<td>38±16 (23–69)</td>
</tr>
</tbody>
</table>

LDL, low density lipoprotein; HDL, high density lipoprotein; dAIIFA, dependent angiotensin II-forming activity; Ang II, angiotensin II. Values in the parentheses show the minimum and maximum, respectively. *P<0.01 vs. control; †P<0.01 vs. high cholesterol diet group.

### 3. Results

3.1. Effects of high cholesterol diet and co-treatment with chymase inhibitor

After taking the high cholesterol diet, plasma total, LDL-, and HDL-cholesterol levels significantly increased (Table 1). Hamsters receiving a high cholesterol diet exhibited significantly higher aortic chymase dAIIFA than the control group (Fig. 1a). Aortic ACE dAIIFA increased, but this was not statistically significant (Table 1). There was a significant positive correlation between aortic chymase dAIIFA and plasma total cholesterol or LDL-cholesterol levels of the hamsters fed with or without a high cholesterol diet (Fig. 1a). In addition, the hamsters fed a high cholesterol diet developed lipid deposition in the ascending aortas (Fig. 2d). In a separate group, a specific non-peptide chymase inhibitor (SUN-C8257) was administered to hamsters receiving the high cholesterol diet. Such treatment significantly inhibited lipid deposition in the ascending aortas (Figs. 1b and 2a and c). The following parameters were not significantly different between the SUN-C8257-treated and untreated groups (Table 1): body weight, right hind-limb cuff blood pressure at week 11, plasma total-cholesterol, LDL-cholesterol, plasma triglyceride, plasma and aortic Ang II levels. SUN-C8257 significantly decreased plasma HDL-cholesterol levels (Table 1). The aortic chymase dAIIFA significantly decreased by treatment with chymase inhibitor for 12 weeks (Fig. 1c), but the aortic ACE dAIIFA did not (Table 1). Both cardiac chymase and ACE expressions significantly increased in the cholesterol diet-fed group and treatment with the chymase inhibitor significantly suppressed the elevated aortic chymase and ACE expressions (Fig. 1d).

3.2. Aortic immunohistochemistry for Ang II

The intensity of aortic Ang II immunoreactivity slightly increased in hamsters fed high cholesterol diet (Fig. 2f and g). In addition, the increased aortic Ang II immuno-
reactivity was markedly suppressed by treatment with SUN-C8257; this suppression was limited to the adventitia (Fig. 2h).

4. Discussion

Our results indicate that aortic chymase is involved in lipid deposition because a high cholesterol diet induced atherogenic lesion formation and increased aortic chymase expression and activity. These changes were significantly inhibited by treatment with the orally active chymase inhibitor, SUN-C8257. In addition, the chymase inhibitor did not affect blood pressure, body weight, and plasma LDL-cholesterol levels. These facts indicated that the inhibitory action of SUN-C8257 on aortic lipid deposition was due to the inhibition of the proteolytic activity of chymase and was not due to the changes in LDL-cholesterol metabolism or appetite.

The reduction in the aortic Ang II seems to provide a potent impact on the reversal of atherosclerotic changes, since recent studies have shown direct evidence that Ang II is involved in atherogenesis in vivo [13,14]. For example, an AT receptor antagonist decreases lipid peroxidation and mean atherosclerotic lesion area by 80% in apo-E deficient mice [13]. Similarly, in the monkey, AT receptor antagonists reverse the development of atherosclerotic lesion by ~50% together with the decrease in monocyte chemoattractant protein-1 and in vitro oxidation of LDL [14]. These data clearly demonstrate the proatherogenic effects of Ang II and support our conclusion that chymase-mediated arterial Ang II formation is also an accelerator of atherogenic lesion formation.

The increased adventitial Ang II immunoreactivity of the hamster aorta was suppressed by treatment with SUN-C8257. This may reduce Ang II-dependent atherogenic stimulatory signals, which include monocyte migration [15], oxidized LDL uptake to macrophage [16] and production of superoxide [17]. Although the location of chymase-dependent Ang II formation in the adventitia is distant from the intimal lesion area, Wilcox et al. have shown that the arterial adventitia is involved in the...
development of neointimal lesions in atherosclerosis [18]. Experimental hypercholesterolemia in pigs and non-human primates stimulates the accumulation of inflammatory cells in arterial adventitia. Furthermore, the extent of the inflammatory process in the adventitia of atherosclerotic plaques correlates with the severity of intimal disease [18]. These experimental observations were also confirmed with the clinical data [8,19,20]. Those studies showed that a number of various inflammatory cells including mast cells were localized in the adventitia of the human atherosclerotic aorta. In addition, an experimental study provided direct evidence that chronic adventitial stimulation with interleukin (IL)-1β, a cytokine known to be involved in the development of atherosclerosis, induced intimal lesions and vasospastic responses in the coronary artery of pigs [21]. Since human chymase is known to convert the IL-1β...
precursor to active IL-1β [22], we hypothesize that adventitial chymase is involved in the development of intimal atherosclerosis [23]. Although the plasma and aortic levels of Ang II did not change significantly after SUN-C8257 treatment, a reduction of Ang II limited in the adventitia may not have been detected in the assay for total measurement of aortic Ang II. Similarly, total measurement of Ang II levels by radioimmunoassay could not detect a small change in plasma or aortic Ang II levels. In turn, our data suggested that chymase inhibition did not cause an elevation in plasma and/or aortic Ang II concentrations.

There was a significant positive correlation between serum LDL-cholesterol levels and aortic chymase dAIIFA. A similar significant positive association was shown between serum LDL-cholesterol levels and chymase dAIIFA in the internal thoracic artery derived from patients undergoing coronary bypass surgery [9]. These results indirectly suggested that higher LDL-cholesterol levels might stimulate chymase expression. In addition, the present study showed that the treatment of chymase inhibitor suppressed both the increased chymase and ACE expression and reduced atherogenic lesion. This may further indicate the existence of an autocrine or paracrine regulatory mechanism of Ang II on the expression of Ang II-forming enzyme especially for chymase.

It has been reported that mammalian chymase has various proteolytic actions on different substrates other than Ang I. Human chymase can activate precursor IL-1β to the active form which is known to be a potent proatherogenic cytokine [22]. Furthermore, chymase not only activates procollagenase but also degrades fibronectin, which may facilitate the process of atherogenesis [24,25]. However, IL-1β or procollagenase activity was not determined in the present study, therefore, the effects of these chymase-related cytokines and extracellular matrix degradation were not clear in this study. These possibilities should be determined in a future study.

It is known that high cholesterol feeding increases plasma HDL- and LDL-cholesterol in the hamster, but does not increase the rate of reverse cholesterol transport [26], indicating that the increased HDL-cholesterol may not be beneficial in preventing atherogenic changes in hamsters. A similar increase in HDL-cholesterol was observed in the present study using hamsters. Furthermore, treatment with SUN-C8257 significantly lowered the HDL-cholesterol level. This indicates that treatment with the SUN-C8257, which suppressed aortic lipid deposition, may alter the metabolism of HDL and reverse cholesterol transport activity. This might be another mechanism for the reduction of atherogenic lesion in addition to the decreased adventitial Ang II levels. However, it is known that the lipid profile of the hamster is different from that of humans. In the hamster, the LDL level and the HDL level are equivalent [26], whereas in man the LDL fraction is dominant compared to the HDL fraction. This fact suggests that our present results obtained in hamsters may not be directly applicable to humans.

The present study describes for the first time the antiatherogenic effect of a newly developed orally active chymase inhibitor and suggests that inhibition of arterial chymase may retard the progression of atherosclerosis in the hamster.

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References


