Increased Chymase in Livers with Autoimmune Disease: Colocalization with Fibrosis

Katsuaki Satomura1, Mingshi Yin1, Shuzi Shimizu1, Yoshihito Kato1,
Takamichi Nagano2, Hirokazu Komeichi2, Masaru Ohsuga2,
Yasumi Katsuta3, Takumi Aramaki3 and Yasukazu Omoto4

1First Department of Internal Medicine, Nippon Medical School
2Biologic Research Institute, Otsuka Pharmaceutical Co., Ltd.

Abstract
Chymase, one of the proteases contained in human mast cells, promotes myocardial and renal interstitial fibrosis by converting angiotensin I to II (AII). We previously established a method for measuring chymase in liver tissue and examined the relationship between chymase and fibrosis in chronic hepatitis. In the present study, chymase was determined in liver specimens affected by autoimmune hepatitis (AIH, n = 10) or primary biliary cirrhosis (PBC, n = 12). To investigate spatial relationships between hepatic fibrosis and human chymase, mast cell distribution in the specimens was determined immunohistochemically using anti-chymase antibody. The mean amounts of chymase in livers with AIH and PBC were 11.56 ± 10.64 and 11.67 ± 9.96 ng/mg respectively. Hepatic chymase in AIH and PBC was significantly more abundant than in acute hepatitis (AH, 2.72 ± 2.23 ng/mg, n = 10; p < 0.05). When sections from patients with AIH and PBC were immunostained for chymase, immunoreactive mast cells were detected in portal areas and sinusoidal walls, coinciding with zones of fibrosis. Thus chymase appears to be involved in hepatic fibrosis in AIH and PBC.

(J Nippon Med Sch 2003; 70: 490–495)

Key words: chymase, mast cells, hepatic fibrosis, immunohistochemistry, enzyme-linked immunosorbent assay

Introduction
Chymase, one of the proteases contained in secretory granules of human mast cells, produces angiotensin II (AII) by conversion of angiotensin I. In recent years, considerable investigation focusing on interactions between mast cells and fibroblasts has indicated that in the presence of fibroblasts, secretion of chymase by mast cells promotes differentiation of interstitial connective tissue. This secretion has been implicated in myocardial and renal interstitial fibrosis1-4. AII produced by the action of chymase promotes both cell proliferation and extracellular matrix production5-6. Involvement of mast cells in hepatic fibrosis has been investigated so far from a histologic viewpoint7-12. We previously established a method for determination of chymase in liver tissue to study fibrosis in chronic hepatitis11,14. Chymase was significantly more abundant in liver specimens

Correspondence to Katsuaki Satomura, MD, First Department of Internal Medicine, Nippon Medical School, Sendagi, 1-1-5, Bunkyo-ku, Tokyo 113-8603, Japan
E-mail: satomura@nms.ac.jp
Journal Website (http://www.nms.ac.jp/jnms/)
from chronic hepatitis patients with advanced hepatic fibrosis than in those from patients with incipient fibrosis. Thus chymase appears to be involved in hepatic fibrosis in chronic hepatitis. Autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC) are autoimmune liver diseases that both cause fibrosis leading to cirrhosis. In the present study of AIH and PBC, we investigated the association between amounts of chymase in liver specimens and severity of fibrosis. We also immunohistochemically localized chymase (and therefore mast cells) in AIH and PBC liver specimens.

**Materials and Methods**

**Patients**

Subjects included 10 patients with AIH (1 man, 9 women; age, 56.0 ± 11.2 years) and 12 patients with PBC (2 men, 10 women; age, 60.0 ± 12.6 years). AIH was diagnosed according to the 1993 International Diagnostic Criteria; PBC was diagnosed based on biochemical, serologic, and histologic findings. Subjects selected as controls were 10 patients with acute hepatitis (AH) (5 men, 5 women; age, 48.2 ± 10.3 years). Types of AH were hepatitis A in 2 patients; hepatitis B in 4; non-A non-B types in 2; and drug-induced hepatitis in 2. Liver biopsy was performed in all subjects to collect liver tissue specimens. Patients were told before the procedure that a portion of the specimen would be used for investigational purposes, and written consent was obtained from all patients. This study was approved by the ethics committee at our institution.

**Preparation of human chymase**

For amplification of prochymase cDNA, the restriction enzyme site for a cloning vector was attached to the prochymase cDNA and subjected to polymerase chain reaction (PCR). The PCR product was inserted into the vector for cloning. The prochymase cDNA then was inserted into the expression vector (pCI-neo mammalian expression vector; Promega, San Luis Obispo, CA). The expression vector was used to transform Chinese hamster ovary (CHO) cells to produce recombinant human prochymase. Expression of prochymase protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Preparation of anti-human chymase antibodies**

Together with Freund’s complete adjuvant, CHO-cell-derived prochymase was administered subcutaneously three times to mice. Splenic lymphoid cells from the mice were made to fuse with mouse P3UI tumor cells. Positive clones were identified by screening with a microtiter plate coated with recombinant human prochymase. Among the hybridomas, three clones were selected (ANOC10801 to 3). Western analysis indicated that all three monoclonal antibodies reacted with CHO cells in manner specific for recombinant human prochymase; as opposed to CHO cell-derived proteins.

**Measurement of chymase in liver**

A portion of the liver biopsy specimen was frozen until chymase in the tissue was measured by an enzyme-linked immunosorbent assay (ELISA) as previously described, using the ANOC10803 monoclonal antibody.

**Immunohistochemical identification of mast cells**

A portion of the liver biopsy specimen was embedded in paraffin. Thin sections were cut, affixed to slides, and deparaffinized. Antigen availability was enhanced by immersing sections in 10 m M citrate buffer (pH 6.0) for 40 min at 95°C. Endogenous peroxidase then was inactivated by treatment with 0.3 % H2O2 in methanol for 20 min. ANOC10803 monoclonal antibody was diluted 1:100 with an antibody diluent (DAKO) and allowed to react with sections for 1 hr at room temperature. The section was washed with phosphate buffered saline (PBS), and then exposed to peroxidase-labeled anti-mouse IgG antibody (EmVision) for 30 min at room temperature. After repeated, washing with PBS, a color reaction was carried out with diaminobenzidine (DAB). After termination of the reaction, mounting, and coverslipping, localization of staining was compared by light microscopy with histologic patterns in hematoxylin and eosin (H and E)-stained sections.
Results

Chymase content of liver tissue
Mean quantities of chymase in liver tissue were 11.56 ± 10.64 ng/mg in patients with AIH and 11.67 ± 9.96 ng/mg in patients with PBC. Chymase was significantly more abundant in specimens with AIH and PBC than in specimens with various forms of AH (2.27 ± 2.23 ng/mg, p<0.05; Table 1).

Intrahepatic distribution of mast cells immunoreactive for chymase
Liver tissue sections from patients with AIH and PBC were stained with monoclonal antibody to human prochymase and compared with H and E staining patterns in adjacent sections. Mast cells immunoreactive for chymase, staining yellowish-brown, were present at sites of fibrosis in the portal areas (Figs. 1A and 2A).

Immunoreactive mast cells also were seen in intralobular sinusoidal walls involved by fibrosis (Fig. 1B) and fibrotic areas surrounding central veins (Fig. 2B). Immunoreactive mast cells were numerous in specimens with increased chymase content. In AH, very few mast cell immunoreactive for chymase could be seen in the portal areas and lobules (Fig. 3).

Discussion

Mast cells are present in the connective tissues of nearly all organs. Major inflammatory and profibrogenic mediators of mast cells are stored in their cytoplasmic granules. Degranulation of mast cells may occur in response to various immunologically nonspecific agents. Accordingly, mast cells are considered to participate importantly in development and persistence of inflammation and fibrosis.

Table 1 Chymase level in liver tissue in patients with AIH and PBC

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Chymase (ng/mg)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIH</td>
<td>10</td>
<td>11.56 ± 10.64*</td>
<td>*</td>
</tr>
<tr>
<td>PBC</td>
<td>12</td>
<td>11.67 ± 9.96</td>
<td></td>
</tr>
<tr>
<td>AH</td>
<td>10</td>
<td>2.72 ± 2.23</td>
<td></td>
</tr>
</tbody>
</table>

*Results are expressed as mean ± S.D. *: P<0.05.
AIH: Autoimmune hepatitis.
PBC: Primary biliary cirrhosis.
AH: Acute hepatitis.

Fig. 1 Microscopic findings in a liver biopsy specimen showing autoimmune hepatitis.
A: Mast cells immunoreactive for chymase are seen in the portal area. Left, hematoxylin and eosin; original magnification, ×40. Right, chymase immunostaining; original magnification, ×40. B: Mast cells immunoreactive for chymase also are seen in a fibrotic intralobular sinusoidal wall. chymase immunostaining; original magnification, ×100.
Interactions between mast cell and fibroblasts involving direct intercellular contact or dispersion of mediators by degranulation of mast cells promotes proliferation of fibroblasts and collagen formation\textsuperscript{23}. Indeed, when mast cells and fibroblasts are cocultured, fibroblast proliferation and collagen formation are enhanced\textsuperscript{24}. In longstanding fibrotic reactions, mast cells appear to interact with other cells such as fibroblasts, macrophages, and T cells\textsuperscript{25}. Hepatic fibrosis, which represents a form of healing associated with longstanding liver inflammation and necrosis, is considered a physiologic defense mechanism that can be deleterious when exaggerated.

The relationship between mast cells and hepatic fibrosis in liver disease has been investigated histologically. Murata et al.\textsuperscript{26} demonstrated that mast cell numbers increase as liver cirrhosis progresses. By immunohistologically identifying mast cells, Armbrust et al.\textsuperscript{27} demonstrated the presence of numerous mast cells in the portal region and walls of fibrous septa in cirrhosis. Yamashiro et al.\textsuperscript{28} identified mast cells by immunofluorescence in patients with acute, chronic, and cholestatic liver disease, finding significantly increase a numbers of mast cells in patients
with alcoholic hepatic fibrosis, PBC, and intrahepatic cholelithiasis. Farrell et al. found that mast cell numbers increased as fibrosis progressed in patients with PBC or alcoholic liver disease, becoming particularly high in PBC. Other reports including immunohistochemical studies, have shown increasingly numerous mast cells with increasing severity of fibrosis in chronic liver disease.

Chymase, stored in secretory granules of mast cells and released extracellularly when mast cells degranulate, converts AI to AII with only minor cleavage elsewhere in the molecule. In addition to acting on vascular smooth muscle, AII stimulates proliferation, hypertrophy, and migration of smooth muscle cells and fibroblasts. Extracellularly, chymase is stabilized by binding to extracellular matrix constituents such as heparin and glycosaminoglycan, extending enzyme activity over time. Chymase initiates degranulation of surrounding mast cells by binding to them, representing a pro-inflammatory chain reaction.

We previously hypothesized that chymase secreted by mast cells was involved in liver fibrosis and developed a method for determining amounts of chymase in liver tissue, finding more chymase in livers involved by more severe fibrosis in chronic hepatitis (F3 and F4 by the International Classification) than in F1 and F2 livers. We therefore concluded that chymase contributed to fibrosis in chronic hepatitis.

In addition to tissue chymase assay, the present study of AIH and PBC included immunohistochemical determination of chymase (i.e., mast cell) distribution in the liver. Immunoreactive mast cells were found in portal areas and sinusoidal walls in patients with AIH and PBC, particularly in livers with increased chymase. The presence of mast cells in sinusoid with fibrosis suggested a relationship between these cells and hepatic stellate cells in the space of Disse. The stellate cells are the main cells producing extracellular matrix constituents in hepatic fibrosis. Hepatic stellate cells, which normally represent a stationary cell population, proliferate in liver disease and ultimately become fibroblasts producing increased extracellular matrix constituents. Type 1 AI receptors (AT1) are expressed by human hepatic stellate cells; acting through the receptors, AII contributes to hepatic fibrosis in response to liver injury. Schneider et al. recently demonstrated that the AT1 antagonist losartan decreased elevated portal vein pressure in patients with cirrhosis. In an analogous manner, chymase inhibitors may be able to limit hepatic fibrosis in patients with AIH and PBC.

In our present study chymase was significantly more abundant in livers with AIH or PBC than in specimens with AH and it colocalized with fibrosis. This suggests that as previously demonstrated for chronic hepatitis, chymase is related to hepatic fibrosis in AIH and PBC.

Numbers of patients in our study were too limited to conclusively relate severity of hepatic fibrosis to amounts of chymase detectable in livers with AIH and PBC. This will require investigation in larger groups of patients.

References

7. Farrell DJ, Hines JE, Walls AF, Kelly PJ, Bennett...

(Received, February 17, 2003)
(Accepted, July 8, 2003)