—Original—

Increased Chymase in Livers with Autoimmune Disease: Colocalization with Fibrosis

Katsuaki Satomura[1], Mingshi Yin[1], Shuzi Shimizu[1], Yoshihito Kato[1], Takamichi Nagano[1], Hirokazu Komeichi[1], Masaru Ohsuga[1], Yasumi Katsuta[1], Takumi Aramaki[1] and Yasukazu Omoto[2]

[1]First Department of Internal Medicine, Nippon Medical School
[2]Biologic 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 fibrosis[1–3]. AII produced by the action of chymase promotes both cell proliferation and extracellular matrix production[4,5]. Involvement of mast cells in hepatic fibrosis has been investigated so far from a histologic viewpoint[6–12]. We previously established a method for determination of chymase in liver tissue to study fibrosis in chronic hepatitis[13,14]. 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[15]; 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 vecter 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 measuured by an enzyme-linked immunosorbent assay (ELISA) as previously described[13], 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℃. Endogenous peroxidase then was inactivated by treatment with 0.3 % H_2O_2 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 hematoxilin 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**).

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

	Cases	Chymase (ng/mg)	P value
AIH	10	11.56 ± 10.64[a]	
PBC	12	11.67 ± 9.96	*
AH	10	2.72 ± 2.23	*

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

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[16]. Degranulation of mast cells may occur in response to various immunologically nonspecific agents[17]. Accordingly, mast cells are considered to participate importantly in development and persistence of inflammation and fibrosis[18,19].

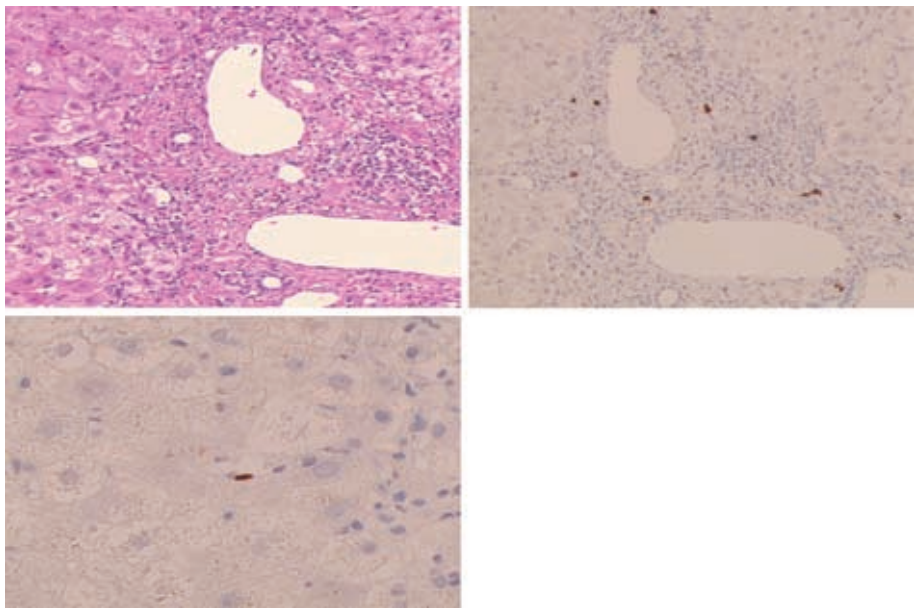

Fig. 1 Microscopic findings in a liver biopsy specimen showing autoimmune hepatitis.
A: Mast cells immunoreactive for chymase are seen in the portal area. Left, hematoxylin and eosin; original magnification, ×40. Right, chymase immunostaining; original magnification, ×40. B: Mast cells immunoreactive for chymase also are seen in a fibrotic intralobular sinusoidal wall. chymase immunostaining; original magnification, ×100.

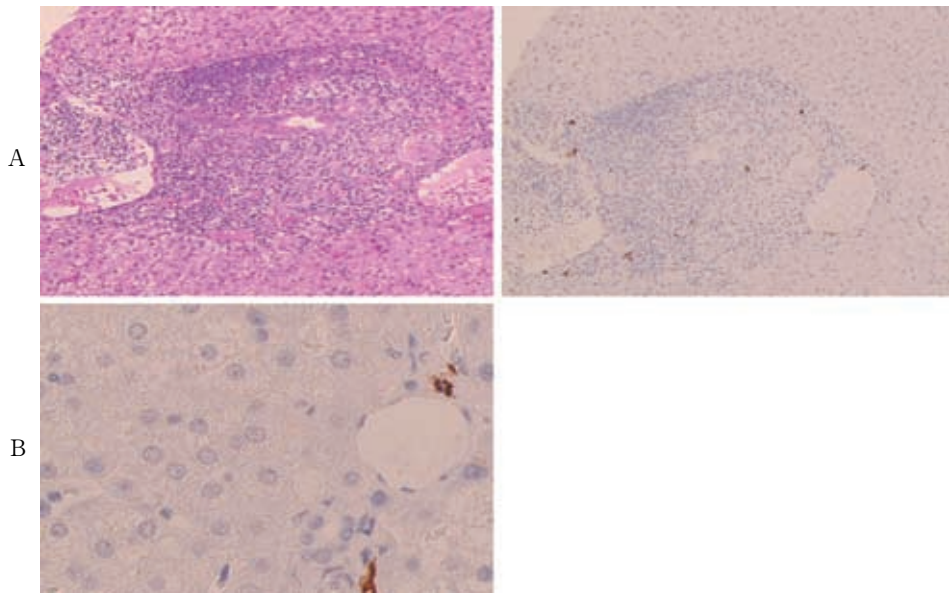

Fig. 2 Microscopic findings in a liver biopsy specimen showing primary biliary cirrhosis.
A: Mast cells immunoreactive for chymase are seen in the portal area. Left, hematoxilin and eosin; original magnification, ×40. Right, chymase immunostaining; original magnification, ×40. B: Mast cells immunoreactive for chymase are seen in fibrotic zones surrounding central veins. chymase immunostaining; original magnification, ×100.

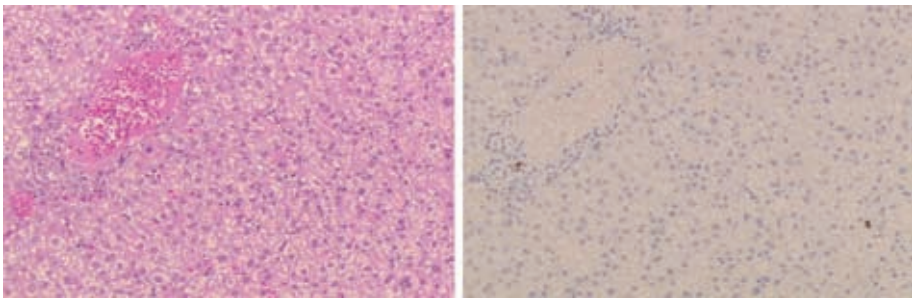

Fig. 3 Microscopic findings in a liver biopsy specimen showing acute hepatitis. Few mast cells immunoreactive for chymase can be seen in the portal area or lobules. Left, hematoxilin and eosin; original magnification ; ×40. Right, chymase immunostaining; original magnification, ×40.

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[20,21]. Indeed, when mast cells and fibroblasts are cocultured, fibroblast proliferation and collagen formation are enhanced[22]. In longstanding fibrotic reactions, mast cells appear to interact with other cells such as fibroblasts, macrophages, and T cells[23]. 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[6]. demonstrated that mast cell numbers increase as liver cirrhosis progresses. By immunohistologically identifying mast cells, Armbrust et al.[9] demonstrated the presence of numerous mast cells in the portal region and walls of fibrous septa in cirrhosis. Yamashiro et al.[10] 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 choledocholithiasis. Farrell et al.[7] found that mast cell numbers increased as fibrosis progressed in patients with PBC or alcoholic liver disease, becoming particularly high in PBC. Other reports[8,10-12], 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[24]. In addition to acting on vascular smooth muscle, AII stimulates proliferation, hypertrophy, and migration of smooth muscle cells and fibroblasts[25]. Extracellularly, chymase is stabilized by binding to extracellular matrix constituents such as heparin and glycosaminoglycan, extending enzyme activity over time[26]. Chymase initiates degranulation of surrounding mast cells by binding to them, representing a pro-inflammatory chain reaction[27].

We previously hypothesized that chymase secretd by mast cells was involved in liver fibrosis and developed a method for determining amounts of chymase in liver tissue[13], finding more chymase in livers involved by more severe fibrosis in chronic hepatitis (F3 and F4 by the International Classification[28]) than in F1 and F2 livers[14]. 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 AII receptors (AT1) are expressed by human hepatic stellate cells; acting through the receptors, AII contributes to hepatic fibrosis in response to liver injury[29]. Schneider et al.[30] recently demonstrated that the AT1 antigonist 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

1. Marone G, de Crescenzo G, Florio G, Granata F, Dente V, Genovese A: Immunological modulation of human cardiac mast cells. Neurochem Res 1999; 24: 1195–1202.
2. Urata H, Boehn KD, Phillip A, Kinoshita A, Gabrovsek J, Bumpus FM, Husain A: Cellular localization and regional distribution of an angiotensin II-forming chymase in the heart. J Clin Invest 1993; 91: 1269–1281.
3. Murakami M, Matsuda H, Kubota E, Wakino S, Honda M, Hayashi K, Saruta T: Role of angiotensin II generated by angiotensin converting enzyme-independent pathways in canine kidney. Kidney Int (Suppl) 1997; 52: S 132–135.
4. Algermissen B, Hermes B, Feldmann-Boeddeker I, Bauer F, Henz BM: Mast cell chymase and tryptase during tissue turnover: analysis on in vitro mitogenesis of fibroblasts and keratinocytes and alterations in cutaneous scars. Exp Dermatol 1999; 8 : 193–198.
5. Kofford MW, Schwartz LB, Schechter NM, Yager DR, Diegelnn RF, Graham MF: Cleavage of type I procollagen by human mast cell chymase initiates collagen fibril formation and generates a unique carboxyl-terminal propeptide. J Biol Chem 1997; 272: 7127–7131.
6. Murata K, Okudaira M, Akashio K: Mast cells in human liver tissue. Increased mast cell number in relation to the components of connective tissue in the cirrhotic process. Acta Derm Venereol (Suppl) 1973; 73: 157–165.
7. Farrell DJ, Hines JE, Walls AF, Kelly PJ, Bennett

MK, Burt AD: Intrahepatic mast cells in chronic liver disease. Hepatology 1995; 22: 1175–1181.
8. Nakamura A, Yamazaki K, Suzuki K, Sato S: Increased portal tract infiltration of mast cells and eosinophils in primary biliary cirrhosis. Am J Gastroenterol 1997; 92: 2245–2249.
9. Armbrust T, Batusic D, Ringe B, Ramadori G: Mast cells distribution in human liver disease and experimental rat liver fibrosis. Indications for mast cell participation in development of liver fibrosis. J Hepatol 1997; 26: 1042–1054.
10. Yamashiro M, Kouda W, Kono N, Tsuneyama K, Matsui O, Nakanuma Y: Distribution of intrahepatic mast cells in various hepatobiliary disorders: An immunohistochemical study. Virchows Arch 1998; 433: 471–479.
11. Matsunaga Y, Kawasaki H, Terada T: Stromal mast cells and nerve fibers in various chronic liver diseases: Relevance to hepatic fibrosis. Am J Gastroenterol 1999; 94: 1923–1932.
12. Matsunaga Y, Terada T: Mast cell subpopulations in chronic inflammatory hepatobiliary diseases. Liver 2000; 20: 152–156.
13. Satomura K, Shimizu S, Nagato T, Komeichi H, Osuga M, Katsuta Y, Aramaki T, Omoto Y: Establishiment of an assay method for human mast cell chymase. Hepatol Res 2002; 24: 361–367.
14. Shimizu S, Satomura K, Aramaki T, Katsuta Y, Takano T, Omoto Y: Hepatic chymase level in chronic hepatitis and co-localization of chymase with fibrosis. Hepatol Res 2003; 27: 57–61.
15. Johnson PJ, Mcfarlane IJ, Meeting report: International autoimmune hepatitis group. Hepatology 1993; 18: 998–1005.
16. Galli SJ: New concepts about the mast cells. N Engl J Med 1993; 11: 458–464.
17. Galli SJ: New insights "the riddle of the mast cells" microenvironmental regulation of mast cell development and phenotypic heterogeneity. Lab Invest 1990; 62: 5–33.
18. Yoshikawa H, Tasaka K: Suppression of mast cell activation by glucocorticoid. Arch Immunol Ther Exp (Warsz) 2000; 48: 487–495.
19. Terada T, Matsunaga Y: Increased mast cells in hepatocellular carcinoma and intrahepatic cholangiocarcinoma. J Hepatol 2000; 33: 961–966.
20. Ginsburg H, Ben-Shahar D, Ben-David E: Mast cell growth on fibroblast monolayers: two-cell entities. Immunology 1982; 45: 371–380.
21. Atkins FM, Friedman MM, Subba Rao PV, Metcalfe DD: Interaction between mast cells, fibroblasts and connective tissue components. Int Arch Allergy Appl Immunol 1985; 77: 96–102.
22. Levi-Schaffer F, Austen KF, Gravallese PM, Stevens RL: Coculture of interleukin 3-dependent mouse mast cells with fibroblasts results in a phenotypic change of the mast cells. Proc Natl Acad Sci USA 1986; 83: 6485–6488.
23. Enerbac L, Norrby K: The mast cells. Curr Top Pathol 1989; 79: 169–204.
24. Reilly CF, Tewksbury DA, Soechter NM, Travis J: Rapid conversion of angiotensin I to angiotensin II by neutrophil and mast cell proteinases. J Biol Chem 1982; 257: 8619–8622.
25. Tani K, Ogushi F, Kido H, Kawano T, Kunori Y, Kamimura T, Cui P, Sone S: Chymase is a potent chemoattractant for human monocytes and neutrophils. J Leukoc Biol 2000; 67: 585–589.
26. Sayama S, Iozzo RV, Lazarus GS, Schechter NM: Human skin chymotrypsin-like proteinase chymase. Subcellular localization to mast cell granules and interaction with heparin and other glycosaminoglycans. J Biol Chem 1987; 262: 6808–6815.
27. Fukusen N, Kato Y, Kido H, Katunuma N: Kinetic studies on the inhibitions of mast cell chymase by natural serine protease inhibitors: indications for potential biological functions of these inhibitors. Biochem Med Metab Biol 1987; 38: 165–169.
28. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ: Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994; 19: 1513–1520.
29. Bataller R, Gines P, Nicolas JM, Gorbig MN, Garcia-Ramallo E, Gasull X, Bosch J, Arroya V, Rodes J: Angiotensin II induces contraction and proliferation of human hepatic stellate cells. Gastroenterology 2000; 118: 1149–1156.
30. Schneider AW, Kalk JF, Klein CP: Effect of losartan, an angiotensin II receptor antagonist, on portal pressure in cirrhosis. Hepatology 1999; 29: 334–335.

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