Clinical Study of Liver Fibrosis Biomarkers in Patients with Histologically Proven Nonalcoholic Steatohepatitis

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Contents

Abstract

Background/Aims

Nonalcoholic steatohepatitis (NASH) is prevalent in both economically developed and developing countries. Twenty percent of patients with NASH progress to cirrhosis with/without hepatocellular carcinoma, and there is an urgent need to identify biomarkers to facilitate early diagnosis and monitor disease progression. Approximately 30% of patients with NASH have diabetes mellitus or insulin resistance. According to the nonalcoholic fatty liver disease and NASH guidelines published by the Japan Society of Hepatology, pioglitazone is used for treatment in patients with concomitant diabetes mellitus or insulin resistance. Liver fibrosis is denoted by an increase in collagen production around hepatic parenchymal cells and stromal cells by activated hepatic stellate cells due to steatosis. In addition, various cytokines, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) promote fibrosis. The present study investigated whether serum cytokine, MMP, and TIMP levels reflect disease activity to permit early detection and treatment in patients with NASH.

Methods

This study was conducted at the Kitasato University Medical Center and International University of Health and Welfare Hospital between April 1, 2013 and December 31, 2016. Prior to implementation, the study protocol was approved by the ethics committee of both institutions. All patients provided written informed consent. Thirty-three patients with histologically proven NASH (24 men and 9 women; mean age, 50.6 ± 12.3 years) were enrolled in addition to 14 health controls (9 men and 5 women; mean age, 51.9 ± 13.2 years). Serum levels of MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2, stromal cell-derived factor 1α, stem cell factor-1, stem cell growth factor-β, monocyte chemotactic protein-1 (MCP-1), granulocyte-colony stimulating factor, leptin, and ghrelin were measured via a fluorescent beads-based immunoassay. Next, changes in their levels in response to lifestyle modification (diet/exercise) and pioglitazone treatment were examined.

Results

Serum leptin and MCP-1 levels were significantly increased in the fibrosis stage 1 (F1) group than healthy controls and the F2 group. In addition, serum MMP-1 levels were significantly higher in the F1 group than in healthy controls, although this finding did not

hold for more advanced fibrosis stages. TIMP-1 and TIMP-2 levels were not elevated in the F1 group. Serum MMP-1 levels in patients with NASH reflected disease activity estimated using serum aminotransferase values during the follow-up period. Conversely, MMP-2, MMP-9, and TIMP levels did not change with changes in disease activity. In a comparison between the F1 and F2 groups, pioglitazone treatment tended to decrease MMP-1 levels in the F1 group after 2 months.

Discussion

Serum levels of cytokines related to macrophage and hepatic stellate cell activation were not elevated in the F1 group. In addition, as TIMP levels increased in the same stage, it was considered that MMP-1 serum concentrations had increased. Histologically, MMP-1 expression has been reported to be increased in monocytes, Kupffer cells, and hepatic stellate cells in early-stage NASH. Relationships between hepatic stem and progenitor cells have also been suggested for histological MMP-1 level elevation. The decrease of MMP-1 levels was considered the result of reduced collagen production following improvement of ALT levels, inflammation, and steatosis due to lifestyle modification and pioglitazone treatment. These

results suggest that serum MMP-1 levels reflect disease activity and that they may serve as a potential biomarker for monitoring the progression of NASH.

Conclusions

MMP-1 was considered a biomarker for the early detection and treatment evaluation of NASH in the early stage, in which serum leptin and MCP-1 levels are increasing.

Abbreviations

ALT, alanine transaminase

AST, aspartate transaminase

BS, blood sugar

BMI, body mass index

F, fibrosis stage

γ-GTP, gamma-glutamyl transpeptidase

G-CSF, granulocyte-colony stimulating factor

HbA1c, hemoglobin A1c

HCC, hepatocellular carcinoma

HPCs, hepatic stem/progenitor cells

HSCs, hepatic stellate cells

HDL, high-density lipoprotein

IEM, immunoelectron microscopy

IHC, immunohistochemistry

KCs, Kupffer cells

LDL, low-density lipoprotein

MCP-1, monocyte chemotactic protein-1

MCs, monocytes

MMP, matrix metalloproteinase

NAFL, nonalcoholic fatty liver

NAFLD, nonalcoholic fatty liver disease

NAS, NASH activity score

NASH, nonalcoholic steatohepatitis

SCF-1, stem cell factor-1

SCGF-β, stem cell growth factor-β

SDF-1α, stromal cell-derived factor-1α

TC, total cholesterol

TG, triglyceride

TIMP, tissue inhibitor of metalloproteinase

Introduction

Nonalcoholic steatohepatitis (NASH) is prevalent in both economically developed and developing countries. (1) In Japan, the number of patients with NASH has been increasing in recent years. (2) Most patients with NASH exhibit increased body weight due to overeating and low physical activity, and they frequently present with metabolic syndrome and diabetes mellitus. (3, 4) Triglyceride (TG) deposits in hepatocytes cause oxidative stress in mitochondria, leading to the death of hepatocytes and inflammation with infiltration of monocytes (MCs) into the liver (3-6) as well as hepatocellular carcinoma (HCC) with or without cirrhosis. (7) If inflammation is not observed, then the term nonalcoholic fatty liver (NAFL) is generally used in clinical practice, and the term nonalcoholic fatty liver disease (NAFLD) includes both NASH and NAFL. (4) According to the NAFLD and NASH guidelines published by the Japan Society of Hepatology, pioglitazone is used for treatment when patients have concomitant diabetes mellitus or insulin resistance. (8)

There are two clinical problems associated with NASH. One is that liver biopsy is necessary to diagnose simple fatty liver and NASH. The invasiveness of liver biopsy results in bleeding, in addition to the time and cost required for examination. Therefore, liver biopsy is not performed in all patients with suspected NASH. The other issue is that NASH progresses

without subjective symptoms. In general, the subjective symptoms of liver diseases such as liver cirrhosis include fatigue, jaundice, and ascites, but these are difficult to detect in earlier stages because of the compensatory mechanisms of the liver. In addition, if a person with abnormal liver function data in an annual medical examination does not receive further scrutiny, the diagnosis of NASH will be delayed.

NASH increases the risk of liver cancer. Globally, the carcinogenesis rate of NALFD is assumed to be approximately 0.5% (9), versus 2–5% for NASH. (10, 11) In addition, as patients with NASH develop cirrhosis over a period of 10–15 years, early detection and treatment before cirrhosis and liver cancer appear are important.

Various cytokines are involved in the development of NASH. Cytokines secreted from adipocytes are called adipocytokines. Leptin, which has an appetite-lowering effect, is an adipocytokine, and leptin resistance leads to high levels of this adipocytokine in patients with NAFLD. Leptin resistance enhances overeating and obesity. (12) Ghrelin is an adipocytokine that promotes appetite, and its blood concentration is decreased in obese patients. (12) In patients with inflammation due to adipocytes and fatty liver, the concentration of MC chemotactic factor 1 (MCP-1) is increased. MCP-1 promotes hepatitis and hepatic fibrosis by activating macrophages. (13) Conversely, in the presence of cytotoxicity due to inflammation,

hepatic parenchymal and stromal cells are regenerated without mitosis via stem or progenitor cell recruitment. (14) Stem cell factor 1 (SCF-1), stem cell growth factor β (SCGF-β), and stromal cell-derived factor-1α (SDF-1α) have been reported as factors for liver regeneration. (15-17) SDF-1α activates hepatic stellate cells (HSCs) and promotes hepatic fibrosis. (18) Matrix metalloproteinase (MMPs), which are extracellular matrix (ECM)-degrading enzymes, facilitate the improvement of hepatic fibrosis. (4) MMP-1, MMP-2, and MMP-9 are involved in the degradation of collagen, which causes fibrosis in the liver. TIMPs inhibit MMP activation. Various cytokines, MMPs, and TIMPs have been inferred to play important roles in fibrosis formation in early NASH.

Therefore, in this study, we aimed to clarify whether NASH can be detected early or whether therapeutic effects can be assessed by measuring the blood concentrations of biomarkers involved in NASH.

Chapter 1

This chapter discusses the histological structure of the liver and the mechanism of liver fibrosis. In addition, this chapter explains the pathology and treatment of NAFLD and NASH in detail.

Overview of liver structure

The liver is generally composed of two cell populations, namely the parenchyma and mesenchyme. The parenchyma is a tissue that performs liver functions such as metabolism and detoxification, and it is composed of hepatocytes. Contrarily, mesenchyme tissue supports the structures and functions of parenchyma tissue, and it is composed of nonparenchymal cells (HSCs, sinusoidal endothelial cells, Kupffer cells [KCs], and cholangiocytes) (Fig. 1). (19) The liver is composed of a large number of structures called “hepatic lobules.” A hepatic lobule, which has a hexagonal shape, is the smallest structural unit of the liver. Two blood vessels, namely the hepatic artery and portal vein, are wrapped within a membrane of connective tissue in the bile duct. In particular, the border of the leaflets is called Glisson’s sheath. (19)

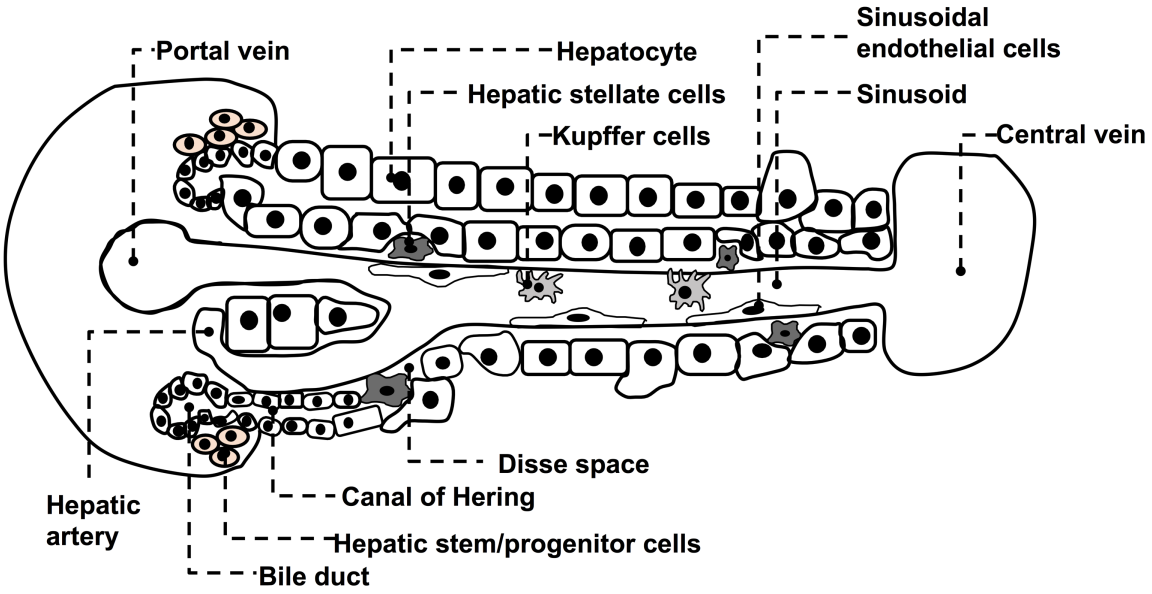

Fig. 1 Cell structure of the liver

HSCs

HSCs have an important role in relation to hepatic tissue injury-induced fibrosis. As one of the processes to repair disordered tissues, HSCs produce and secrete an ECM including collagen. (19, 20) When the liver is injured by inflammation via viral infection or drug allergy, various cytokines such as interleukin-1 (IL-1), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF) are produced. (4) These cytokines stimulate quiescent HSCs, which become activated HSCs (aHSCs), also termed myofibroblast-like cells. aHSCs strongly contribute to fibrosis. (21)

ECM

ECM plays an important role in regulating function in cell and tissue networks via cell-cell interaction signals using cytokines. (22) ECM regulates a variety of cellular functions such as adhesion, migration, differentiation, proliferation, and survival, in addition to supporting physical scaffolds and structures for cells. The cellular response is dependent on the constitution of ECM; therefore, dysregulation of ECM production and degradation is often associated with the development of liver lesions. (23) ECM in normal liver primarily consists of collagen, fibronectin, laminin, proteoglycan, and matrix cell proteins. (24) These are mainly detected in endothelial cells of Glisson's sheath, the portal vein area, the central vein, and the space of Disse. (25)

Collagen

Collagen is a polymeric peptide molecule composed of 18 types belonging to the collagen family and 30 or more types of polypeptide chains. Collagen is secreted as pro-collagen from collagen-producing cells to the extracellular space. Cleavage of three peptides from the C- and N-termini by extracellular peptidase converts pro-collagen into collagen. These collagen molecules associate via crosslinking at cysteine residues. (26)

It is known that types I, III, IV, V, and VI collagen exist in the liver. (27) Type I collagen is mainly located in Glisson's sheath in the liver. Type III collagen is distributed around the vasculature and sinusoids. Type IV collagen binds to laminin to form the basement membrane, which is distributed in the sinusoid, perivascular, and pericapillary bile ducts and around nerve fibers in the liver. (28) Type V collagen is interposed between type III or I collagen on the cell surface, basement membrane, or fibrotic areas, and it is responsible for maintaining the tissue structure. Type VI collagen is linked to fibrous connective tissue.

Collagen is degraded via two pathways: extracellular and intracellular. (26, 28) In the extracellular pathway, depolymerase removes matrix components such as proteoglycan from collagen. Subsequently, the collagen residue is decomposed into TC^A (three-quarter length) and TC^B (one-quarter length) by collagenase, thus becoming gelatin. Gelatin is degraded to peptides by gelatinase. Meanwhile, in the intracellular pathway, collagenolytic cathepsin (a member of the protease family) is secreted around the cell, and collagen degradation occurs in an acidic environment. The decomposed peptides are taken into the cell by the phagocytic action of macrophages.

Fibrosis

Overview of fibrosis

Liver fibrosis occurs as part of the tissue repair mechanism in the liver. Regardless of the causative disease, its mechanism plays a central role in ECM overproduction via the activation of HSCs. (29) Conversely, excessive ECM production and deposition by activated HSCs exacerbate the liver function by decreasing hepatic blood and bile flow. This phenomenon causes necrosis of the hepatic parenchyma (hepatocytes), the production of regenerative nodules, sinusoidal capillarization, and the progression of fibrous septa due to chronic inflammation. Finally, liver cirrhosis arises because of hepatic lobular structure remodeling and hemodynamic abnormality. (30)

Mechanism of liver fibrosis

The mechanism of hepatic fibrosis is currently being elucidated, and HSCs and KCs play a central role. When hepatocyte inflammation is prolonged, the ECM environment changes, and KCs are activated. Activated KCs produce MMPs, TIMPs, PDGF, and TGF-β. Then, HSCs activated by PDGF and TGF-β release collagen, TIMPs, and retinoids. (31) TGF-β and PDGF promote fibrotic proliferation and ECM accumulation. (32) In other words, in the liver, KCs

and HSCs are deeply involved in the production of substances that promote hepatic fibrosis. Additionally, levels of the proteoglycan hyaluronic acid increase over the course of tissue inflammation, leading to healing tissue production. TGF-β is involved in the increased production of hyaluronic acid. (33)

As ECM, the production of which is increased by liver fibrosis, accounts for 85% of types I and III collagen and it is a structurally stable molecule, collagenases are important for improving fibrosis. (28) In addition, gelatinases, which mainly degrade basement membrane types IV and V collagen and gelatin, are also important in relation to liver fibrosis. In liver fibrosis, the appearance of basement membrane-like structures is observed in contact with sinusoidal endothelial cells and hepatocytes. This is called the capillary formation of sinusoid, and it causes further hepatocellular injury by decreasing blood flow and substance exchange between hepatocytes. The basement membranes are mainly composed of type IV collagen. (26)

Active oxygen/nitrogen species and pro-inflammatory mediators are released in response to direct damage to hepatocytes and organelles, and these species activate innate immune cells. (34) Next, activated KCs and infiltrating leukocytes exacerbate the early stage of liver injury. (35) In the damaged acute stage liver, ECM proteins such as fibronectin and tenascin are synthesized. (36-38) ECM accumulation, which is increased by chronic inflammation,

gradually results in the replacement of hepatic parenchyma with scar fibrous tissue. (39) In chronic liver injury, hepatic fibrosis occurs due to prolonged activation cycles of HSCs. Liver fibrosis causes widespread disability formation, disturbances of liver structure, and disruption of liver function. To ameliorate fibrosis, in addition to the removal of myofibroblast-like cells that generate scarring, ECM degradation is a necessary. (40)

NAFLD and NASH

NAFLD is defined as a fatty liver similar to alcoholic liver disease in people who are not heavy alcohol drinkers (less than 20 g/day). (41) Many cases of NAFLD are simple steatosis (NAFL), and its symptoms are mostly mild. However, 20% of cases of NAFLD are NASH. (19, 41) As a historical background of fatty liver disease, Ludwig et al. (42) in 1980 reported a case of hepatitis that was histologically similar to alcoholic liver disease that progressed to liver cirrhosis despite the absence of alcohol consumption (less than once a week), and this case was defined as NASH. In addition, in 1986, Schaffner et al. reported a group of diseases with similar pathological findings as alcoholic liver disorder despite the absence of heavy alcohol drinking (20 g/day or less) that were defined as NAFLD. (43)

Origin of fatty liver disease

The main etiologies of fatty liver are obesity and insulin resistance. Both etiologies are linked to the abnormal secretion of adipocytokines and free fatty acids. Therefore, metabolic syndrome and related diseases such as type 2 diabetes, dyslipidemia, and hypertension as background diseases are related to insulin resistance, and the relevance between background disease and insulin resistance is strongly is strongly considered. (44) Therefore, the increased fatty liver is often regarded as a metabolic syndrome phenotype in the liver. NAFL is caused by TG accumulation. The balance of two factors, namely increasing (inflow of TGs into the hepatocytes and synthesis in hepatocytes) and decreasing factors (consumption and release in hepatocytes), is important. Meanwhile, a critical factor for defining NAFL or NASH has not been elucidated. In the past, the two-hit theory leading to NASH via NAFL due to secondary stress next to fatty deposition in hepatocytes was widely endorsed. Recently, a multifactor hit theory that comprises multiple factors such as liver inflammation, oxidative stress, genetic factors, and environmental factors was proposed. (45)

Epidemiology of NAFLD

As the obese population in Japan is growing, the number of patients with metabolic

syndrome and NAFLD is increasing steadily. (46) Currently, 20–30% of adults have NAFLD. (46) In total, 70–80% of such people are men, although the frequency of NAFLD in women has increased over the last 50 years. There is no gender difference in the frequency of NASH, and it is estimated to occur at least 1% of adults. (47)

Prognosis of NAFLD

NAFLD consists of NAFL and NASH. The prognosis is different depending on each disease condition. NAFL is less likely to develop into a disease state. (29) Meanwhile, 10–20% of cases of NAFLD are NASH, and 5–20% of these cases progress to cirrhosis. The prognosis of NASH is estimated according to the stage of fibrosis.

Diagnosis of NAFLD

Laboratory examination

Diagnosing NAFLD and NASH is difficult in the laboratory via blood sampling, and liver biopsy tissue is absolutely required for a definitive diagnosis. In NAFLD, aspartate transaminase (AST) and alanine transaminase (ALT) values are normal or mildly elevated, and findings such as diabetes and dyslipidemia based on insulin resistance are commonly found.

(48)

NAFL and NASH differ from alcoholic steatohepatitis (ASH) according to laboratory data. The ALT/AST ratio is high in patients with NAFL and NASH and low in patients with ASH. ALT levels are generally less than 200 IU/L in patients with NAFL patients and higher than 300 IU/L in patients with NASH and severe liver inflammation. (30) The serum levels of gamma-glutamyl transpeptidase (γ-GTP) and ALT are slightly elevated in patients with NASH. Serum cholesterol and TG levels are high in both patients with NAFL and NASH. Therefore, it is difficult to discriminate between NAFL and NASH using only these laboratory data. (30)

Liver fibrosis is extremely important in the progression of NASH, and some liver fibrosis markers are useful in clinical practice. Fibrosis markers such as hyaluronic acid and type IV collagen are elevated in patients with NASH plus fibrosis and liver cirrhosis. (49) The number of platelets decreases, and this decline is often used to predict the reserve of liver function. Recently, *Wisteria floribunda* agglutinin-positive Mac-2 binding protein is a novel fibrosis marker of hepatitis C and B and NAFLD, and high sensitivity, specificity, and diagnostic performance have been demonstrated. (50, 51)

Pathological findings by liver biopsy

Liver biopsy and laparoscopy are useful for diagnosing liver cirrhosis, but due to sampling errors (52) and bias by the evaluator, the accuracy is approximately 80%. (52, 53) Liver biopsy for diagnosis is based on the patient's condition, and it depends on physical findings, laboratory data, organization function, and imaging findings. If the patient is elderly and/or has advanced-stage cirrhosis, liver biopsy should be avoided because of the risk of severe bleeding and complications.

Three indicators are often used for findings of pathological diagnosis. One is the classification of Matteoni. (54) NAFLD is consisted NAFL or NASH, but Matteoni et al. (54) categorized the disease into 14 types based on the pathological findings. Hepatocellular ballooning is observed in types 1 (fatty liver only), 2 (inflammatory fatty liver), 3 (hepatocellular ballooning with inflammation), and 4 (fibrosis and Mallory Denk bodies). Types 3 and 4 are defined as NASH. The second is the classification of Brunt. (55) (Table 1) This classification evaluates the pathological findings in NASH according to the extent ratio of inflammation and fibrosis. The third is the NAFLD activity score (NAS) proposed by Kleiner et al. (56) (Table 2) It scores the degree of steatosis, hepatocyte ballooning, inflammation, and NASH. In clinical practice in Japan, pathologists use these classifications in combination.

Table 1 Staging for Fibrosis by classification of Brunt

Stage	Extent
1	Zone 3 perisinusoidal/pericellular fibrosis; focally or extensively present.
2	Zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis.
3	Zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis.
4	Cirrhosis.

Table 2 NASH activity score

Item	Score	Extent
Steatosis	0	<5%
	1	5-33%
	2	>33-66%
	3	>66%
Lobular Inflammation	0	No foci
	1	<2 foci/200x
	2	2-4 foci/200x
	3	>4 foci/200x
Hepatocyte Ballooning	0	None
	1	Few balloon cells
	2	Many cells/prominent ballooning

Image findings via abdominal ultrasonography

Abdominal ultrasonography has high diagnostic ability for fat deposits in hepatocytes. Therefore, it is a useful test for diagnosing NAFLD. (57) However, it is difficult to evaluate the extent of inflammation and fibrosis, and these findings cannot be used to diagnose early NASH. Therefore, Fibroscan®, which is based on transient elastrography (TE), can distinguish fibrosis in the liver using ultrasound. It is a method for evaluating the hardness of tissue based on differences of the propagation speed of audible vibration generated from its probe .(57, 58) Fibroscan was approved for detecting cirrhosis or suspected cases of cirrhosis in October 2011. However, TE requires expensive equipment, the recruitment of experienced technicians, and extended consulting hours, and patient throughput is low.

Treatment of fatty liver disease

As it is believed that NASH develops from NAFLD, it is important to not leave fatty liver untreated. Therefore, the principle of treatment for NAFLD is treating obesity, diabetes, dyslipidemia, and background hypertension via changes in lifestyle such as diet and exercise therapy. (29) In addition, oxidative stress and insulin resistance are factors of NASH. In any case, there is no established treatment method in Japan at present. To treat NASH, the treatment

for original disease is given priority. For example, if a patient has diabetes mellitus, its treatment is given priority.

Diet therapy

Weight loss achieved using a low-calorie diet improves liver function and hepatic steatosis in patients with NAFLD. To improve NAFLD, it is necessary to optimize calorie and lipid intake. The optimal meal content is 50–60% carbohydrates and 20–25% lipids, but caloric restriction is more important than the carbohydrate/lipid ratio for weight loss.

Exercise therapy

The relationship between exercise and improvements in liver lesions is unclear, but it has been reported that exercise therapy improves serum transaminase level and hepatic steatosis in patients with NAFLD. (59, 60)

Drug therapy

Type 2 diabetes treatment

As insulin resistance is deeply involved in the onset and progression of NASH, the

administration of pioglitazone improves liver function and histology in patients with NASH in a relatively short period. (12) Pioglitazone histologically improves liver steatosis, mesangial inflammation, hepatocyte ballooning, and liver fibrosis. Pioglitazone regulates the transcription of insulin sensitivity genes by stimulating peroxisome proliferator-activated receptor-γ (PPARγ), it improves insulin sensitivity in muscle and adipose tissue, and it reduces insulin resistance in liver and peripheral tissues. (61) On the contrary, the ability of the drug to prevent progression to liver cirrhosis and improve prognosis remains unclear.

Drugs for dyslipidemia

Although HMG-CoA reductase inhibitors can improve serum lipid and ALT levels, there is little evidence that they improve liver histology. (62) However, they represent a treatment option for patients with NAFLD and hypercholesterolemia. Ezetimibe, a cholesterol transporter inhibitor, controls cholesterol absorption from the intestinal tract and generally exerts positive effects on lipid levels, including decreases in LDL-cholesterol levels and cholesterol transport to the liver. In addition, fatty liver, necrotizing inflammation, and hepatocellular balloon-like changes have been reported to be improved by treatment with this drug. (63)

MMPs, TIMPs, and cytokines

Cytokines

Proteinaceous factors released from cells that mediate various cell-cell interactions are called cytokines. (64) Specific receptors have been identified for each cytokine. Cytokines play important roles in immunity, inflammation, and biological defenses. The common properties of cytokines are as follows: (i) protein structure, (ii) exert their effects at extremely low levels, (iii) bind to cell surface receptors, (iv) target cell specificity, (v) primarily function at the site of production, i.e., act in a paracrine or autocrine manner, and (vi) mutually associated effects, i.e., cytokine network. The bioactivity of cytokines includes enhancing and suppressing cellular immunity and enhancing antibody production and the proliferation and differentiation of hematopoietic cells. Cytokines that regulate white blood cell activity are called chemokines. (64)

Leptin

Leptin is an adipocytokine that acts centrally to exert strong appetite-suppressing effects and exhibit various physiological activities such as improving lipid metabolism and increasing

blood pressure. (65) Leptin inhibits fat accumulation in non-adipose tissue such as muscle, the liver, and the pancreas. Serum leptin levels are elevated in obese patients, reflecting leptin resistance. Therefore, the physiological activity of leptin is not sufficient in obese patients. Elevated leptin levels have also been demonstrated to accelerate the onset of liver cancer through promoting hepatic fibrosis and inducing angiogenesis. (66) In particular, leptin suppresses appetite by activating POMC/CART neurons in the arcuate nucleus (ARC) of the hypothalamus. Another effect of leptin is suppressing appetite by inhibiting AgRP/RPY neurons in the ARC. (67)

Ghrelin

Ghrelin regulates eating similarly as leptin. It is mainly secreted by the hypothalamic ARC in the stomach. In addition to potently inducing hyperphagia, ghrelin has various functions such as enhancing gastrointestinal motility, promoting growth hormone secretion, and protecting the cardiovascular system. (12)

Chemokine family

The chemokine family is a generic term for cytokine groups that induce directional cell

migration. The action of inducing directional cell migration is called chemotaxis. (64) Chemotaxis is performed largely in four steps. (i) Leukocytes are temporarily stopped due to gentle binding between selectin and its ligand, rolling on the surface of vascular endothelium in posterior capillary venules. Then, chemokine production occurs by various cells due to inflammation and homeostasis. (ii) The chemokine present on the vascular endothelial cell surface activate leukocytes through its receptor. (iii) Leukocyte integrins are activated, and they firmly bind to vascular endothelial cells. (iv) Chemokines migrate to the tissue through blood vessels according to the concentration gradient.

Chemokines have four cysteine residues, which are classified into four subfamilies of CXC, CC, (X) C, CX3C (X is another amino acid residue) by two motifs formed on the N-terminal side. In these subfamily names, “R” representing the receptor is attached, and likewise, “L” for ligand is also attached. CXC and CC chemokines are mainly inflammatory chemokines targeting neutrophils, MCs, and eosinophils. In humans, 44 chemokines have been identified. In this study, we focused on MCP-1 (also termed CCL 2) and SDF-1 (CXCL 12).

MCP-1

MCP-1 plays an important role in chronic inflammation and allergic inflammation by

modulating the activities of MCs, eosinophils, and memory T cells. MCP-1 is also produced by tumor cells, and it participates in macrophage recruitment and stroma formation. CCR2 is the receptor of MCP-1. In a CCR2 knockout liver injury mouse model using tetrachloride exposure, it was reported that MCP-1 suppresses early fibrosis by reducing the number of hepatic infiltrating macrophages. (68)

SDF-1

Serum SDF-1α serves as a ligand for its receptor CXCR4, and it has been reported to play important roles in endothelial progenitor cell mobilization from bone marrow to reach ischemic tissue/organs to promote repair/regeneration and angiogenesis. (69) CXCR4 is also expressed on sinusoidal endothelial cells and HSCs, and it is believed to contribute to the production and proliferation of cells in the liver by controlling trafficking between cells. (18, 70) SDF-1 is believed to activate HSCs via sinusoidal endothelial cells expressing CXCR4. (71) Through SDF-1/CXCR4 signaling, sinusoidal endothelial cells produce platelet-derived growth factor-β (PDGF-β). It is considered that PDGF-β activates HSCs and contributes to the promotion of fibrosis. In addition, HSCs stimulate hematopoietic stem cell migration to the liver via SDF-1/CXCR4 signaling. It is considered that hematopoietic stem cells are transformed into hepatic

stem/progenitor cells (HPCs), differentiate into bile duct cells, and promote fibrosis in the process of forming the bile ducts. (71) SDF-1 is secreted in two forms, namely α and β. Between them, SDF-1α is secreted in response to hepatic injury, and it stimulates hepatic progenitor cells present around herring canal and bile duct epithelial cells and their controls proliferation and differentiation while simultaneously contributing to the induction of fibrosis. (72)

Stem cell-related cytokines

In the mature liver, HPCs are present near the herring canal, which is the point of contact between hepatocytes and the interlobular bile duct. (73) When these cells are activated during hepatic injury, they are transformed into oval cells, after which they apparently promote liver tissue regeneration. It has been hypothesized that HPCs are derived from hematopoietic stem cells. In this study, the serum concentrations of cytokines involved in hematopoiesis are measured.

SCF-1

SCF-1 plays an important role in the proliferation and initial differentiation of

hematopoietic stem cells. (14) It is secreted as a transmembrane ligand by bone marrow, thymus stromal cells, and osteoblasts, forming a hematopoietic microenvironment. The receptor, KIT (c-kit gene product), is expressed in hematopoietic stem cells and various erythroid, megakaryocytic, myeloid, and lymphoid progenitor cells.

SCGF-β

SCGF is a hematopoietic growth factor that exerts its activity in the early stages of hematopoiesis. SCGF is a non-glycosylated species-specific cytokine capable of supporting the proliferation of primitive hematopoietic cells and, in combination with erythropoietin or granulocyte colony-stimulating factor (G-CSF), promoting the proliferation of red blood cells or bone marrow progenitor cells. (74) It is expressed in skeletal tissues such as bone marrow, chondrocytes, perichondrium, and periosteum, and its expression is low in the spleen, thymus, and fetal liver. (75)

G-CSF

G-CSF is also used as a medicine, and its administration promptly increases neutrophil counts. (76) G-CSF is produced by fibroblasts, vascular endothelial cells, and other cells

stimulated by inflammatory cytokines such as TNF-α and IL-1. (77) Another important function of G-CSF is the mobilization of hematopoietic stem cells. G-CSF acts on neutrophils and hematopoietic stem cells to induce reconstitution of the hematopoietic stem cell niche (microenvironment) in the bone marrow, and indirect actions have been postulated. Meanwhile, attention has been paid to its protective action on the central nervous system, cardiac muscle, and blood vessels. Hematopoietic stem cells mobilized by G-CSF are believed to promote recovery at the lesion site.

ECM-degrading enzyme

MMPs are enzymes that mainly degrade collagen, and their expression differs in individual cells. MMP-1, MMP-2, MMP-3, and MMP-16 are expressed by HSCs. MMP-9 is expressed in KCs, and MMP-13 and MMP-14 are expressed by HSCs and KCs. (4) Conversely, TIMPs are mainly expressed by HSCs, although TIMP-2 is additionally expressed in KCs. In addition, MMP-7 expression has also been reported in hepatocarcinoma cells. As described previously, because HSCs express most MMPs and TIMPs together with ECM production, it is considered that HSCs have a central role in ECM regulation in the liver. (78) There are many unclear points regarding the dynamics of MMPs in vivo. This is due to the multistep involvement of in

vivo enzymatic activity, genetic level production, activation of proMMP, and inhibition of activity by enzyme inhibitors. (26, 79)

In the process of improving fibrosis in cirrhotic livers, TIMP, MMP-2, and MMP-9 expression is slightly decreased, and collagen degradation is also decreased. (80, 81) Conversely, MMP-2 is believed to contribute to the improvement of fibrosis as the expression of TIMPs is further decreased. (82) Both inactive MMPs and active MMPs are inhibited by TIMPs at a 1:1 molar ratio. Therefore, when TIMP levels exceed MMP levels, MMPs do not function as decomposing enzymes. (83, 84) In vitro studies illustrated that MMP-2 and TIMP levels are elevated in activated HSCs, whereas the release of MMP-1 and MMP-9 is decreased.(78)

MMPs

In general, the collagen content in tissues is defined by the balance between synthesis and degradation. If expression is properly maintained, MMPs have important roles in tissue repair and wound healing. Meanwhile, when ECM degradation becomes dominant, MMPs cause tissue destruction, such as synovial destruction in patients with rheumatoid arthritis. Conversely, when the synthetic system becomes excessively activated, tissue fibrosis occurs.

This is the main cause of liver fibrosis. MMPs are mainly involved in the degradation of collagen in ECM. (85) MMPs are capable of collagen degradation, and they are metalloproteases that contain zinc at their active centers. Metalloprotease is a generic term for proteolytic enzymes containing metal ions. According to their ECM substrate specificity, MMPs are classified into five groups: collagenase, gelatinase, stromelysin, matrilysin, and membrane type. (86)

The primary action of MMPs is the degradation of collagen, proteoglycan, and elastin via an extracellular pathway. Regarding MMP gene expression, cytokines such as IL-1 and TNF-α and cell growth factors are potent inducers that are involved in the pathological destruction of connective tissue. MMPs are produced as pro-MMPs and activated via cleavage of the propeptides. Meanwhile, their activity is regulated by TIMPs and plasminogen activator inhibitor. Regulation of MMPs is roughly divided into two types: regulation at the genetic level and regulation of precursor activation. (87)

Tissue destruction occurs in pathological conditions in which MMP levels exceed TIMP levels. Conversely, fibrosis occurs in pathological conditions in which TIMP levels exceed MMP levels. It has actually been confirmed that TIMP levels exceed MMP levels in patients with liver cirrhosis, pulmonary fibrosis, scleroderma, and arteriosclerosis. Thus, the amount of

collagen in tissues is regulated by controlling the expression and activity of MMPs.

There are several types of MMPs. One classification based on substrates encompasses interstitial collagenase, gelatinase, and stromelysin. (88) Interstitial collagenase degrades interstitial types I–III collagen. Gelatinase degrades basement membrane collagen type IV collagen and the degenerating substance (gelatin) of interstitial collagen. MMP-3 is also involved in ECM regulation as a stromelysin that degrades glycoproteins such as proteoglycan and fibronectin. In addition to ECM proteolysis, MMPs also regulate inflammation and immunity by acting on non-ECM substrates such as cytokines and chemokines. (89)

MMP-1

MMP-1 degrades fibrillar types I–III collagen. (82) In addition, expression is suppressed by TGF-β, as a TGF-β inhibitory element exists in the promoter of MMP-1. (80) Decreases of MMP-1 levels and increases of TIMP-1 levels lead to suppression of ECM degradation and promote fibrosis. (21) In addition, carbon tetrachloride was administered chronically to rats, and experimental liver fibrosis was created to examine the expression of MMP-1. (26) As a result, gene expression was strongly observed in the interstitial fibrous septum. In addition, expression tended to be relatively high in the early stage of fibrosis and decreased in later stages.

Furthermore, MMP-1, MMP-8, and MMP-13 are believed to have roles in anti-fibrosis, owing to overexpression, weakened liver fibrosis, and increased hepatocyte proliferation. (79, 90, 91)

MMP-2

MMP-2 expression markedly increases with the progression of liver fibrosis together with that of MMP-14, an upstream activating factor. The substrate of MMP-2 degrades basement membrane constituent type IV collagen, but it also degrades type I collagen and partially degrades gelatin. Alternatively, inhibition of type I collagen expression by HSCs is mainly involved in the remission of hepatic fibrosis. (92-94) Despite the fact that MMP-2 expression is enhanced by liver cirrhosis, accumulation of ECM in liver tissue in patients with liver cirrhosis is believed to be due to the fact that TIMP expression is enhanced in cirrhosis, leading to the complete suppression of MMP-2 activity. (82) Therefore, MMP-2 exerts its action by decreasing TIMP expression, potentially contribute to the improvement of liver fibrosis. (37)

Meanwhile, MMP-2 degrades type IV collagen in the Disse cavity, changing the composition of the matrix. This change suggests activation of HSCs or increased matrix production in myofibroblast-like cells via integrin, which promotes hepatic fibrosis. (95-97) The present findings did not clarify whether MMP-2 improves liver fibrosis or promotes

progression. In addition, it is believed that MMP-2 is responsible for maintaining the homeostasis of liver vessels by participating in the TGF-β activation pathway. (34) Furthermore, in addition to TGF-β activation, activation of IL-1, TNF-α, and MCP-3 can be regulated via proteolytic cleavage. (13, 98)

MMP-9

MMP-9 has been identified from the fraction of $CD11B^{Hi}$ $F4/80^{int}$ $Ly\text{-}6C^{l\circ}$ macrophages associated with the remission of fibrosis. (99) MMP-9 expression has been confirmed in the early stages of hepatic fibrosis, and it activates TGF-β, a major fibrosis-inducible cytokine from the ECM storage site. (40, 100, 101) In addition, in the presence of low TIMP-1 concentrations , MMP-9 can promote HSC apoptosis. (40)

TIMPs

TIMPs are endogenous MMP inhibitors that suppress the overexpression of active MMPs. (78) TIMP-1 is a glycoprotein with a molecular weight of 29 kDa, and TIMP-2 is a non-glycan protein with a molecular weight of 23 kDa. TIMP-1 and TIMP-2 production is similar in cells. Cytokines and growth factors are involved in the expression of TIMP and MMP mRNA. TIMPs

promote TGF-β and IL-6 production while suppressing corticosteroid production. TIMPs bind 1:1 with inactive and active MMPs to regulate their activity. Currently, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 are known, and TIMP-1 and TIMP-2 are important for inhibiting MMPs. Meanwhile, TIMP-1, TIMP-2, and TIMP-3 expression has been reported in the liver.

TIMP-1 and TIMP-2 are expressed at high levels in a mouse liver fibrosis model following carbon tetrachloride administration (102), and TIMP-1 overexpression is due to activation of HSCs after carbon tetrachloride administration. Apoptosis and MMP-2 activation in liver tissue were suppressed, and the improvement of hepatic fibrosis was significantly inhibited. (37) Additionally, upon amelioration of fibrosis, a rapid decline in TIMP levels is observed, and the MMP/TIMP balance is restored, resulting in increased matrix degradation activity and a net reduction in scar tissue. (103, 104)

TIMP-1

Activated HSCs exhibit marked upregulation of TIMP-1 prior to collagen expression and potently inhibit MMP activity. (104, 105) The important roles of TIMP-1 in fibrosis and remission have been confirmed using the genetic recombination system of mice. TIMP-1 overexpression in the liver is believed to both accelerate fibrosis and interfere with the

remission of scar tissue. (106) TIMP-1 also has an anti-apoptotic effect on liver myofibroblast-like cells. (107) In other words, the loss of TIMP-1 in convalescence indicates that it contributes to the reduction of scar-producing cells in the liver.

TIMP-2

Treatment of hepatic fibrosis model mice using synthetic siRNA targeting TIMP-2 has been reported to improve HSC activation and collagen accumulation to reverse fibrosis. (108)

Chapter 2

Background

NASH increases the risk of liver cirrhosis and liver cancer due to the progression of liver inflammation and fibrosis. Therefore, early detection and diagnosis are important. Liver fibrosis is reflected by an increase in collagen production around hepatic parenchymal cells and stromal cells by activated HSCs due to steatosis. In addition, various cytokines, MMPs, and TIMPs promote fibrosis. In this chapter, we focused on serum concentrations of cytokine, MMP, and TIMP in patients with NASH and examined the relationship between their serum concentrations and the hepatic fibrosis stage.

Methods

Patient entry and study criteria

This study was conducted at the Kitasato University Medical Center (Kitamoto City, Saitama, Japan) and the International University of Health and Welfare Hospital (Nasu-Shiobara City, Tochigi, Japan) between April 1, 2013 and December 31, 2016. Prior to implementation, the study protocol was approved by the ethics committee of both institutions.

All patients provided written informed consent for study participation. Thirty-three patients with histologically proven NASH (24 men and 9 women; mean age, 50.6 ± 12.3 years) were entered (Table 3, Supplementary Table 1). Fourteen healthy persons (9 men and 5 women; mean age, 51.9 ± 13.2 years) who visited the Preventive Medical Center of the International University of Health and Welfare Hospital (Nasu-Shiobara City, Tochigi, Japan) for annual health examinations were additionally enrolled.

People who underwent the annual health examination completed questionnaires (present complaint, past medical history, family medical history, drinking and smoking habits, diet health conditions, physical activity, sleeping time, and current drug use), a physical examination (including body weight, waist, and BMI), conventional laboratory tests including urinalysis, peripheral blood examination, clinical chemistry (blood sugar level, hemoglobin A1c [HbA1c], total cholesterol, TG, high-density lipoprotein [HDL]-cholesterol, creatinine, AST, ALT, γ-GTP, CRP), stool occult blood examination, chest X-ray, electrocardiogram, upper GI endoscopy, and abdominal ultrasonography. In total, 53 people visited our institution on July 14–15, 2015, 14 of whom had normal results, including normal ALT levels, the absence of fatty liver on ultrasonography, the absence of diabetes, metabolic syndrome, or cardiovascular disease, and normal BMI (18.5–25).

The exclusion criteria for patients with NASH were as follows: alcohol consumption of more than 20 g/day for men and more than 10 g/day for women; use of a hepatotoxic drug or any agent that alters cytokine levels, e.g., interferon; and the presence of complications, such as alcoholic steatohepatitis, chronic hepatitis B, chronic hepatitis C, primary biliary cholangitis, primary sclerosing cholangitis, chronic pancreatitis, type 1 diabetes, uncontrolled thyroid deficiency, renal failure, or a need for hemodialysis, as reported previously. (109)

Table 3. Clinical and laboratory data of healthy controls and patients with early- and advanced-stage NASH

	Healthy controls	Early NASH	Advanced NASH
Number	14	24	9
Men/Women	9/5	18/6	6/3
Age (years)	51.9 ± 13.2	46.1 ± 9.6	64.1 ± 11.5
BMI (kg/m^2)	23.2 ± 2.95	28.5 ± 4.7**	25.7 ± 2.5†
Diabetes	0	3 (12.5%)	6 (66.7%)
Metabolic syndrome	0	12 (50.0%)	6 (66.7%)
AST (IU/l)	21.0 ± 4.3	54.5 ± 29.5**	60.9 ± 24.9**
ALT (IU/l)	18.7 ± 5.0	89.7 ± 57.2**	71.0 ± 25.4**†
γ-GTP (IU/l)	23.7 ± 10.2	62.0 ± 52.5**	163.5 ± 50.6**††
TC (mg/dl)	208.1 ± 25.6	211.0 ± 43.6	173.3 ± 26.2**††
LDL (mg/dl)	135.3 ± 22.3	132.0 ± 32.2	88.8 ± 13.3*†
HDL (mg/dl)	55.2 ± 16.2	46.4 ± 8.9	53.5 ± 25.4
TG (mg/dl)	92.9 ± 43.0	208.6 ± 155.2**	135.7 ± 79.3†
BS (mg/dl)	94.3 ± 8.3	113.4 ± 44.1**	143.8 ± 42.5*†
HbA1c (%)	5.6 ± 0.4	6.2 ± 1.2**	7.1 ± 1.1*†
Plt ($10^3/\mu l$)	242.6 ± 65.5	213.9 ± 63.0	141.0 ± 37.7*†
Histological characteristics			
Steatosis grade (1/2/3)		4/15/5	4/5/0
Ballooning grade (1/2)		9/15	5/4
Inflammation grade (1/2/3)		11/11/2	2/5/2
Fibrosis stage (1/2/3/4)		14/10/0/0	0/0/3/6

Values are presented as the mean ± standard deviation or number. Abbreviations: *BMI*, body mass index; *AST*, aspartate aminotransferase; *ALT*, alanine aminotransferase; *γ-GTP*, gamma-glutamyl transpeptidase; *TC*, total cholesterol; *LDL*, low-density lipoprotein cholesterol; *HDL*, high-density lipoprotein cholesterol; *TG*, triglyceride. Comparisons between patients with NASH and healthy controls: * $p < 0.05$, ** $p < 0.01$. Comparisons between patients with early- and advanced-stage NASH: † $p < 0.05$, †† $p < 0.01$.

Histological examination of liver biopsy specimens, fibrosis, and NAS

Tissue samples were obtained via liver biopsy and fixed for optical microscopy as described previously (109). Conventional histological examination was performed using hematoxylin and eosin, Azan-Mallory, and silver staining. Histological evaluation was performed using optical microscopy and described according to Brunt's classification. (55) Steatosis and inflammation were graded on a scale of 0–3 (0, none; 1, mild; 2, moderate; 3, severe), and ballooning was graded on a scale of 0–2 (0, none; 1, mild; 2, moderate). Fibrosis was graded on a scale of 0–4 (0, no fibrosis; 1, pericellular or isolated portal fibrosis; 2, combined pericellular and portal fibrosis; 3, bridging fibrosis; and 4, cirrhosis) according to the classification of Brunt. These scales were evaluated according to the NAS. (110)

Patients with NASH were categorized into two groups according to Brunt's staging. (55) Stages 1 and 2 comprised early-stage NASH (n = 24) whereas stages 3 and 4 denoted advanced-stage NASH (n = 9). The early-stage group included 18 men and 6 women, whereas the advanced-stage group included six men and three women. Patients in the early-stage group were younger than those with advanced-stage NASH (46.1 ± 9.6 years vs. 64.1

± 11.5 years), although the difference was not significant (Table 3).

The mean BMI, AST, ALT, γ-GTP, TG, BS, and HbA1c values in the early- and advanced-stage NASH groups were significantly different from those for healthy controls. The mean ALT, γ-GTP, TC, LDL, TG, BS, HbA1c, and platelet values were significantly different between the early- and advanced-stage NASH groups (Table 3). The pharmacological effects of pioglitazone, bezafibrate, ezetimibe, and other medications were investigated in patients with NASH.

Assay methods for serum MMP/TIMP and cytokine levels

Serum MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-2, SDF-1α, SCF-1, SCGF-β, MCP-1, G-CSF, leptin, and ghrelin levels were measured using a fluorescent beads-based immunoassay (Bio-Plex® 200, Bio-Rad Laboratories Inc., Hercules, CA, USA), and kits including antibodies, detection antibodies, and standard controls were used. The samples and standard blanks were diluted 1:4 with sample buffer in a 96-well plate, to which the bead-based capture antibody was added, followed by incubation on a shaker for 60 min at room temperature protected from light and three washes with buffer. The detection antibody was added to each well, followed by incubation on a shaker for 30 min at room temperature

with protection from light. After incubation and three washes with wash buffer, streptavidin was added to each well, followed by incubation for 10 min and three washes with wash buffer. Samples were resuspended with beads in assay buffer. Then, the measured optical densities against beads with the bead system and blank values were subtracted from all readings. Serum MMPs, TIMPs, and cytokine levels were determined using the optical densities. The means of duplicate values were used as the measurement data.

Evaluation of disease activity relative to serum MMP/TIMP and cytokine levels

ALT levels in patients with NASH were used to monitor disease activity. (111) Serum MMP/TIMP and cytokine levels were measured several times throughout the clinical course in 15 of 33 patients with NASH. The 15 patients were divided into two groups (ALT improved and uncontrolled) and analyzed at 0, 21, 35, and 70 weeks. All eight patients in the ALT improved group exhibited ALT levels < 30 IU/l in the final observation at 70 weeks, and all seven patients in the ALT uncontrolled group displayed ALT levels ≥ 30 IU/l at 70 weeks. The ALT levels in the two groups were compared with serum MMP, TIMP, and cytokine levels at the same time points.

Pharmacological effects of pioglitazone on disease activity in patients with NASH

After serum HDL, TG, LDL, and HbA1c levels were stabilized following the administration of lipid-lowering or anti-diabetic agents, we evaluated the effect of pioglitazone on disease activity. Seventeen patients with NASH were treated with 15 mg of pioglitazone once daily for 2 months, and serum MMP, TIMP, and cytokine levels were measured before and after treatment. Clinical manifestations, physical findings, and clinical hematological and biochemical data were additionally compared between before and after the treatment.

Effects of extrahepatic inflammatory conditions on serum MMP-1 levels

Patients with diabetes and/or metabolic syndrome were analyzed to ascertain the relationship between serum MMP-1 levels and HbA1c levels as well as certain clinical findings of metabolic syndrome as markers of the extrahepatic inflammatory conditions observed in patients with NASH.

Statistical analysis

All statistical analyses were performed using EZR version 1.32 (Saitama Medical Center,

Jichi Medical University), which is a graphic user interface for R (The R Foundation for Statistical Computing, version 2.2.0). (112)

Biomarker levels were expressed as the mean ± standard deviation. Clinical data were adjusted post hoc using the Mann Whitney U test for multiple comparisons with Bonferroni's correction after Kruskal–Wallis analysis of variance. Comparisons between controls and grades of cytokines were performed using Steel's test. Spearman's rank correlation was used for correlation analysis of data. A p value of less than 0.05 was regarded as statistically significant for all analyses.

Results

Serum MMP/TIMP levels in patients with early- and advanced-stage NASH

Serum MMP and TIMP levels in patients with early- (n = 24) and advanced-stage NASH (n = 9) were compared with those in healthy controls (n = 14). Serum MMP-1 and MMP-9 levels tended to be higher in patients with early-stage NASH than in those with advanced-stage NASH or healthy controls, albeit without statistical significance (Fig. 2). Serum MMP-2, TIMP-1, and TIMP-2 levels were significantly higher in patients with advanced-stage NASH than in those with early-stage NASH and controls (Fig. 2).

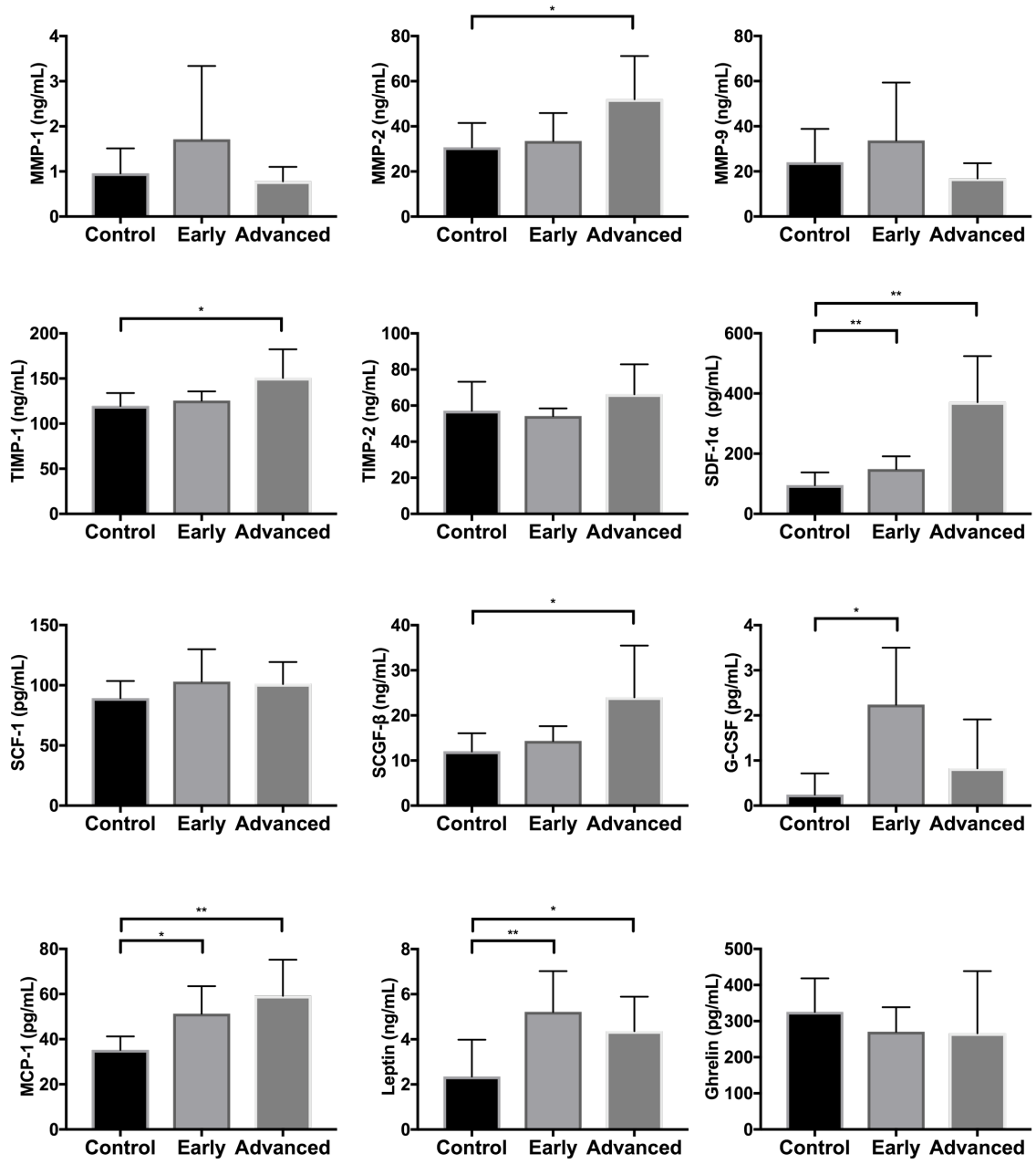

Fig. 2. Comparison of serum MMP, TIMP, and cytokine levels between patients with early- or advanced-stage NASH and healthy controls.

Serum MMP-1 and MMP-9 levels in patients with early-stage NASH tended to be higher than those in patients with advanced-stage NASH and healthy controls without statistical significance. Serum MMP-2 and TIMP-1 levels were significantly higher in patients with advanced-stage NASH than in healthy controls. Serum cytokine levels were significantly higher in patients with NASH. * $p < 0.05$, ** $p < 0.01$.

Relationship between serum MMP/TIMP levels and histological findings in patients with NASH

Serum MMP and TIMP levels in patients were compared with those in healthy controls based on histological characteristics, i.e., steatosis, inflammation, ballooning, and fibrosis.

Serum MMP-1 levels: As shown in Fig. 3, serum MMP-1 levels were significantly higher in patients with stage 1 fibrosis (F1 group) than in healthy controls ($p = 0.019$), but no correlation was observed between MMP-1 levels and fibrosis stage (Table 4). Similarly, serum MMP-1 levels were higher in patients with grade 2 steatosis and grades 1–2 inflammation than in healthy controls, but no correlation between serum MMP-1 levels and histological grades was noted (Table 5).

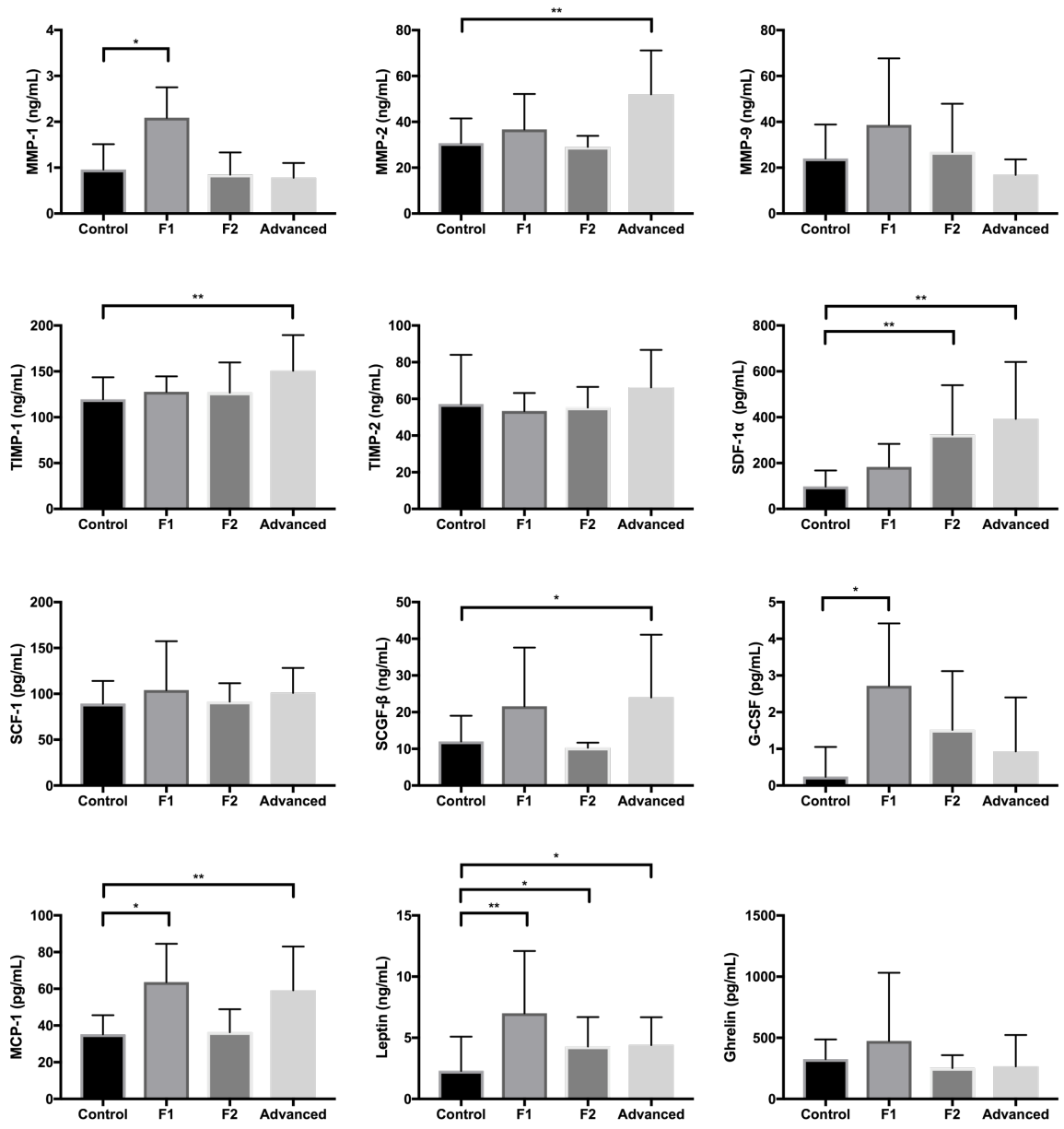

Fig. 3. Serum MMP, TIMP, and cytokine levels in patients with NASH and healthy controls by fibrosis stage.

The numbers of patients were as follows: F1 NASH group (n = 14), F2 NASH group (n = 10), F3+F4 NASH group (n = 9), and healthy controls (n = 14). Compared to healthy controls, significant increases were observed for serum MMP-1 levels in the F1 group ($p < 0.01$), serum MMP-2 levels in the F4 group ($p < 0.01$), and serum TIMP-1 levels in the F4 group ($p < 0.01$). Serum SDF-1α levels were significantly higher in the F2, F3, and F4 groups than in healthy controls. Serum G-CSF levels were significantly higher in the F3 group than in healthy controls. Serum MCP-1 and leptin levels were significantly higher in the F1 group than in healthy controls. * $p < 0.05$, ** $p < 0.01$.

Table 4. Correlations of serum MMP, TIMP, and cytokine levels with histological characteristics

	Steatosis		Inflammation		Ballooning		Fibrosis	
	Correlation coefficient (r)	p value	Correlation coefficient (r)	p value	Correlation coefficient (r)	p value	Correlation coefficient (r)	p value
MMP-1	0.075	0.635	0.068	0.663	0.215	0.167	−0.131	0.402
MMP-2	0.064	0.669	0.188	0.206	0.096	0.522	0.286	0.051
MMP-9	0.182	0.22	0.085	0.572	0.188	0.206	−0.116	0.436
TIMP-1	0.227	0.125	0.256	0.082	**0.359**	**0.013**	**0.304**	**0.038**
TIMP-2	0.069	0.644	0.19	0.201	**0.315**	**0.031**	0.275	0.062
SDF-1α	**0.415**	**0.005**	**0.522**	**<0.001**	**0.433**	**0.002**	**0.649**	**<0.001**
SCF -1	0.047	0.775	0.151	0.358	0.049	0.769	0.233	0.153
SCGF-β	**0.36**	**0.025**	**0.4**	**0.012**	**0.416**	**0.008**	0.295	0.069
MCP-1	**0.472**	**0.002**	**0.443**	**0.005**	**0.529**	**0.001**	**0.321**	**0.046**
G-CSF	0.048	0.861	0.2	0.458	0.139	0.607	0.03	0.912
Leptin	**0.505**	**0.001**	**0.483**	**0.002**	**0.453**	**0.004**	**0.334**	**0.038**
Ghrelin	−0.151	0.371	−0.077	0.649	−0.178	0.293	−0.169	0.317

Values significant at $p < 0.05$ are printed in bold type.

Serum MMP-2 levels: Serum MMP-2 levels in the F4 group were significantly higher than those in healthy controls ($p < 0.01$, Fig. 3). Serum MMP-2 levels tended to be higher in the grade 1 steatosis, grade 3 inflammation, grade 2 ballooning, and F3 groups than in healthy controls (Table 5). The correlation between serum MMP-2 levels and fibrosis stage approached significance ($p = 0.051$) (Table 4).

Table 5. Serum MMP and TIMP levels in patients with NASH and healthy controls by histological grade

		MMP-1 (ng/ml)	MMP-2 (ng/ml)	MMP-9 (ng/ml)	TIMP-1 (ng/ml)	TIMP-2 (ng/ml)
Healthy controls		1.28 ± 1.08	33.2 ± 13.6	28.0 ± 24.6	119.5 ± 24.7	57.1 ± 40.5
Steatosis	1	1.27 ± 0.83	42.2 ± 15.8	22.8 ± 20.2	123.4 ± 22.7	58.6 ± 15.0
	2	1.71 ± 1.93	37.5 ± 18.3	32.0 ± 27.6	135.9 ± 34.3	57.0 ± 15.9
	3	1.24 ± 1.14	37.2 ± 15.9	27.7 ± 9.0	133.1 ± 32.5	57.7 ± 13.7
Inflammation	1	1.52 ± 1.77	39.5 ± 18.3	35.1 ± 33.9	122.0 ± 21.0	56.3 ± 13.8
	2	1.67 ± 1.68	35.8 ± 13.6	23.1 ± 13.6	131.7 ± 22.8	54.4 ± 9.7
	3	0.96 ± 0.44	46.5 ± 26.1	33.7 ± 11.8	169.6 ± 60.8	73.5 ± 27.2
Ballooning	1	1.18 ± 1.53	40.2 ± 16.5	16.5 ± 18.5	116.5 ± 16.3	51.0 ± 11.2
	2	1.39 ± 0.92	43.0 ± 21.7	21.7 ± 15.0	141.2 ± 42.5	63.2 ± 19.5
Fibrosis	1	2.09 ± 0.66*	36.7 ± 15.5	38.6 ± 29.1	124.7 ± 16.8	53.4 ± 9.8
	2	0.85 ± 0.48	29.1 ± 4.8	26.8 ± 21.1	126.9 ± 32.8	55.3 ± 11.3
	3	0.64 ± 0.20	43.2 ± 6.8	14.6 ± 3.7	120.8 ± 14.2	50.5 ± 13.3
	4	0.80 ± 0.40	56.5 ± 23.9**	18.0 ± 8.4	165.6 ± 42.8**	74.1 ± 21.3

Values are presented as the mean ± standard deviation. Comparisons between patients with NASH and healthy controls: * $p < 0.05$, ** $p < 0.01$.

Serum MMP-9 levels: Similar to the findings for MMP-1 levels, serum MMP-9 levels tended to be higher in the grade 2 steatosis, grade 2 inflammation, and F1 groups than in healthy controls, as shown in Table 5 and Fig. 3. There was no correlation between serum MMP-9 levels and histological grades (Table 4).

Serum TIMP levels: Serum TIMP-1 levels were significantly higher in the F4 group than in

healthy controls, as shown in Fig. 3 ($p < 0.01$). Grade 2 steatosis, grade 3 inflammation, and grade 2 ballooning tended to be linked to higher serum TIMP-1 levels (Table 5). Serum TIMP-2 levels exhibited a slight trend toward being higher in the grade 3 inflammation and F4 groups than in healthy controls. A statistically significant correlation was observed between serum TIMP-1 levels and fibrosis stage ($p = 0.038$).

Serum cytokine levels in patients with NASH

It has been reported that HSCs, KCs, and endothelial cells are activated during NASH progression by cytokines via paracrine and autocrine pathways. Serum SDF-1α, SCGF-β, SCF-1, G-CSF, MCP-1, leptin, and ghrelin levels were measured in patients with early- and advanced-stage NASH.

Serum SDF-1α levels: Serum SDF-1α levels were significantly higher in both patients with early- and advanced-stage NASH than in controls ($p < 0.001$, Fig. 2), and its levels were higher in patients with advanced-stage NASH than in those with early-stage NASH ($p = 0.004$).

Patients with grades 1–2 steatosis; grades 2–3 inflammation; grades 1–2 ballooning; and F2, F3, or F4 exhibited significantly higher SDF-1α levels than healthy controls; notably,

patients with F4 and clinical liver cirrhosis had the highest SDF-1α levels, as shown in Table 6. The correlations between serum SDF-1α levels and histological grades (steatosis, inflammation, ballooning, and fibrosis) were all statistically significant ($p = 0.005$, $p < 0.001$, $p = 0.002$, and $p < 0.001$, respectively, Table 4).

Table 6. Serum cytokine levels in patients with NASH and healthy controls by histological grade

		SDF-1α (pg/ml)	SCF-1 (pg/ml)	SCGF-β (ng/ml)	MCP-1 (pg/ml)	G-CSF (pg/ml)	Leptin (ng/ml)	Ghrelin (pg/ml)
Healthy controls		97.8 ± 77.9	89.3 ± 26.8	12 ± 10	35.2 ± 11.6	0.24 ± 0.81	2.35 ± 1.91	325.4 ± 157.2
Steatosis	1	302.0 ± 215.0**	117.9 ± 63	16.2 ± 8.2	52.2 ± 25.3	2.94 ± 6.21	5.42 ± 5.84	478.5 ± 698.7
	2	322.5 ± 206.8**	98.3 ± 26	20.9 ± 15.9	53.2 ± 20.7*	2.00 ± 2.37	4.97 ± 2.53	310.5 ± 191.6
	3	115.2 ± 77.5	88.2 ± 7.3	14.8 ± 3.6	64.9 ± 25.7*	3.25 ± 1.29	6.70 ± 4.01	188.8 ± 126.5
Inflammation	1	233.8 ± 109.5*	113.2 ± 55.2	14.6 ± 7.0	51.5 ± 24.0	1.66 ± 2.29	4.83 ± 3.34	274.4 ± 142.3
	2	319.3 ± 263.7**	89.8 ± 18.7	21.6 ± 16.0	59.5 ± 22.2**	3.44 ± 4.71*	5.82 ± 4.46	430.9 ± 544.5
	3	327.5 ± 192.9	115.3 ± 18.1	20.1 ± 13.4	46.9 ± 18.6	1.24 ± 1.17	5.42 ± 3.24	179.2 ± 120.9
Ballooning	1	254.5 ± 151.8*	108.8 ± 48.7	15.6 ± 7.6	51.2 ± 26.9	2.21 ± 4.57	5.07 ± 3.04	304.2 ± 211.2
	2	237.4 ± 242.6**	93.7 ± 17.8	22.4 ± 16.9*	59.4 ± 14.2**	2.78 ± 2.08	5.76 ± 4.71*	381.2 ± 554.6
Fibrosis	1	182.7 ± 100.7	104.1 ± 54.5	21.6 ± 15.2	63.6 ± 20.6**	2.72 ± 2.53*	6.98 ± 4.84**	474.4 ± 535.4
	2	323.4 ± 216.0**	91.6 ± 19.9	10.3 ± 1.4	36.4 ± 12.5	1.53 ± 1.81	4.28 ± 2.44*	252.5 ± 105.6
	3	344.1 ± 241.9*	103.8 ± 20.7	21.6 ± 12.9	56.6 ± 31.5	1.18 ± 1.09	5.29 ± 2.33*	160.4 ± 278.4
	4	430.7 ± 274.0**	107.2 ± 23.2	18.0 ± 12.3	52.3 ± 18.8	0.79 ± 1.76	3.01 ± 1.43	266.1 ± 197.1

Values are presented as the mean ± standard deviation. Comparisons between patients with NASH and healthy controls: * $p < 0.05$, ** $p < 0.01$.

***Serum SCF-1 levels*:** Serum SCF-1 levels tended to be higher in the grade 1 steatosis; grades 1 and 3 inflammation; grade 1 ballooning; and F1, F3, and F4 groups (Table 6). The grade 2 steatosis, grades 1 and 3 inflammation, and F1, F3, and F4 groups tended to exhibit higher SCF-1 levels. No correlation between serum SCF-1 levels and histological grade was observed (Table 4). Serum SCF-1 levels did not parallel serum SDF-1α and SCGF-β levels.

Serum SCGF-β levels: Serum SCGF-β levels were significantly higher in patients with advanced-stage NASH than in controls ($p = 0.001$, Fig. 2). Grade 2 steatosis, grades 2–3 inflammation, grade 2 ballooning, and F1 and F3 tended to be associated with higher SCGF-β levels (Table 6). Serum SCGF-β levels did not parallel serum SDF-1α levels. Notably, serum SCGF-β levels were not elevated in the F4 group. The correlations of serum SCGF-β levels with steatosis, inflammation, and ballooning grades were significant ($p = 0.025$, $p = 0.012$, and $p = 0.008$, respectively, Table 4). Excluding the F4 group, serum SCGF-β levels were extremely similar to those of SDF-1α.

Serum G-CSF levels: Serum G-CSF levels were significantly higher in patients with early-

stage NASH (Fig. 2). Serum G-CSF levels were significantly higher in the grade 2 inflammation and F3 groups than in healthy controls (Table 6). Serum G-CSF levels were also elevated for all-grade steatosis, grades 1 and 3 inflammation, grades 1–2 ballooning, and F1 and F2, albeit without significance (Table 6). Among these data, it is of special interest that the F3 group displayed the highest G-CSF levels, whereas G-CSF levels were similar between the F4 and healthy control groups. No correlation between serum G-CSF levels and any histological finding was observed (Table 4). Serum G-CSF levels were extremely similar to SCF-1 and MCP-1 levels in all groups excluding the F4 group.

Serum MCP-1 levels: Serum MCP-1 levels were higher in patients with early- and advanced-stage NASH than in healthy controls ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 2). Serum MCP-1 levels were significantly higher in the grades 2–3 steatosis, grade 2 inflammation, grade 2 ballooning, and F1 groups than in healthy controls (Table 6). Statistically significant correlations were observed between serum MCP-1 levels and all histological grades, including those for steatosis, inflammation, ballooning, and fibrosis ($p = 0.002$, $p = 0.005$, $p = 0.001$, and $p = 0.046$, respectively, Table 4). Serum MCP-1 levels were similar to those of SDF-1α and SCGF-β.

Serum leptin levels: Serum leptin levels were significantly higher in both patients with early- and advanced-stage NASH than in healthy controls ($p < 0.01$ and $p < 0.05$, respectively), as shown in Fig. 2. Serum leptin levels were elevated according to the grade of steatosis and inflammation without statistical difference (Table 6). The grade 2 ballooning and F1 groups displayed statistically higher leptin levels than the healthy controls, as shown in Table 6. Statistically significant correlations of serum leptin levels with all histological findings, including steatosis, inflammation, ballooning, and fibrosis, were noted ($p = 0.001$, $p = 0.002$, $p = 0.004$ and $p = 0.038$, respectively, Table 4). Serum leptin levels were similar to those of SDF-1α, SCGF-β, and MCP-1 for all histological findings excluding fibrosis. Serum leptin levels appear to be related to steatosis and inflammation.

Serum ghrelin levels: Serum ghrelin levels were similar between patients with NASH and healthy controls (Fig. 2). Serum ghrelin levels were unique for all histological findings because they were the reverse of those in healthy controls, albeit without statistical difference (Table 6). An inverse but insignificant correlation was noted between serum ghrelin levels and all grades of histological findings (Table 4).

Serum MMP/TIMP and cytokine levels in relation to the clinical course of NASH

Although we could not perform repeated liver biopsies in the same patient, disease activity in patients with NASH was determined using serum ALT levels. Overeating, increases in body weight, and low physical activity can lead to increases in serum ALT levels, and thus, we investigated whether serum MMP, TIMP, and cytokine levels changed in relation to changes in serum ALT levels in individual patients. It is necessary to further clarify whether increases in ALT levels accompany changes in serum cytokine levels due to changes in MMP and/or TIMP levels.

Clinical courses of two patients: We present the clinical courses of two patients, including one patient with early-stage NASH and one patient with advanced-stage NASH, to better understand disease activity. The first case involved a 38-year-old man who developed NAFLD due to metabolic syndrome (BMI = 25.9, waist = 91 cm) in 2009. Liver biopsy performed in March 2014 revealed diffuse steatosis with large droplets in hepatocytes in the pericentral region and intermediate zone with small lymphocyte infiltration, suggesting grade 3 steatosis, grade 1 inflammation, grade 2 ballooning, and stage 1 fibrosis. We categorized this patient as

having early-stage NASH (Fig. 4A, No.13 in Supplementary Table 1). The patient exhibited hyperlipidemia and received ezetimibe, which decreased his LDL (Fig. 4A) and TG levels (data not shown). As his ALT levels remained elevated after 10 months of ezetimibe treatment, the anti-insulin resistance agent pioglitazone (15 mg/day) was prescribed. Fourteen months of treatment with pioglitazone and ezetimibe gradually lowered the patient's ALT level to a normal value of 20 IU/l. His AST and serum γ-GTP levels also improved. Serum levels of MMP-1 and MMP-9 decreased gradually, as shown in Fig. 4A.

Figure 4B shows the clinical course of a 46-year-old man with metabolic syndrome (BMI = 26, waist = 90 cm) who was diagnosed with advanced-stage NASH via liver biopsy in July 2013 (Supplementary Table 1, No. 6). Histological findings revealed remarkable lymphocyte infiltration in the portal region, mild proliferation of bile ductules, and bridging fibrosis. The patient had hyperlipidemia and elevated fasting blood glucose and HbA1c levels. He also exhibited depression, resulting in excessive eating and lowered physical activity as well as altered ALT, AST, and γ-GTP levels. The changes in ALT levels were not correlated with serum MMP-1 levels, but lowered MMP-1 levels preceded the decline in ALT levels in this patient.

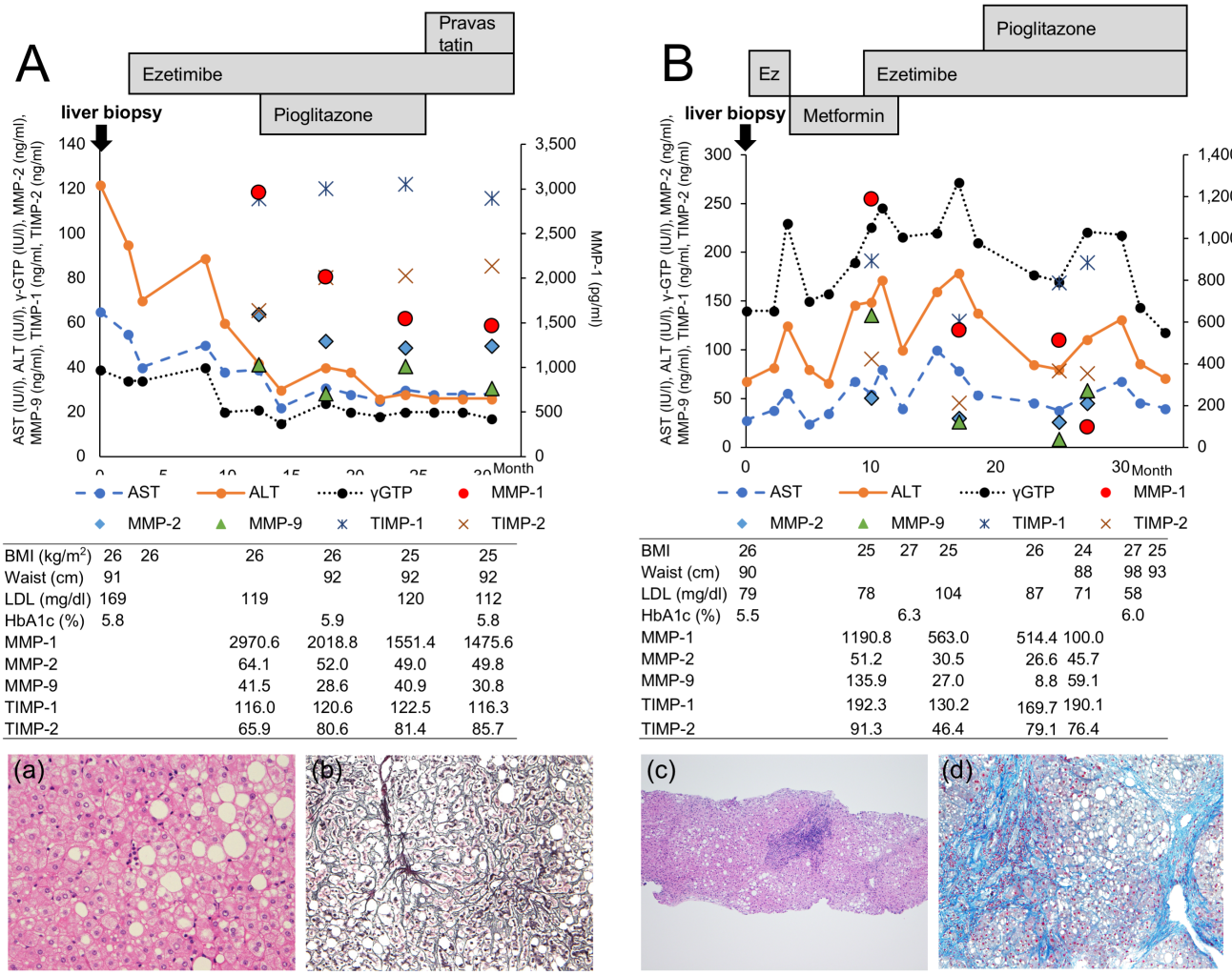

Fig. 4. Clinical courses of one patient with early-stage NASH and one patient with advanced-stage NASH.

A: Patient with early-stage NASH.

(a) HE staining (×100). (b) Silver impregnation (×100).

B: Patient with-stage NASH.

(c) HE staining (×100). (d) Mallory's AZAN staining (×100).

Discussion

Leptin is a well-known cytokine that is related to obesity via its absence or insufficient signaling. It also reduces the development of fibrosis in models of liver injury. (113) KCs have been identified as the cellular targets of the profibrogenic action of leptin. (114) The present study revealed a significant correlation between serum leptin levels and grades of steatosis and inflammation ($r = 0.505$, $p < 0.001$; and $r = 0.483$, $p < 0.002$, respectively). Serum leptin levels were not elevated in the F4 group even though multiple intracellular signaling pathways in HSCs were reported to be activated by leptin. (115) However, rat HSCs exposed to leptin maintained a quiescent state due to the inhibitory activity of PPARγ. (116) This result is consistent with our present findings.

Ghrelin is an appetite-stimulating hormone with an opposite function to leptin. Machado et al. reported that ghrelin is associated with more severe NAFLD. (117) Another report by Gonciarz et al. revealed no significant difference in ghrelin plasma levels in patients with NASH who were treated with melatonin. (118) Ghrelin is an important cytokine in patients with NASH requiring further research.

We previously observed MMP-1 expression in MCs, KCs, and HSCs in patients with early-stage NASH and in HPCs and HSCs in patients with advanced-stage NASH via

immunohistochemistry (IHC) and immunoelectron microscopy (IEM). Our study suggests that MMP-1 can activate KCs, HSCs, endothelial cells, and HPCs and transform these cells into ductular cells, myofibroblasts, and elongated repaired endothelial cells, leading to fibrosis and angiogenesis. (109) We then measured the serum levels of MMP-1 in patients with NASH. As shown in Fig. 4, changes in ALT values paralleled the serum levels of MMP-1. Our intention was to investigate serum MMP-1 levels in patients with NASH relative to ALT values, which to some extent reflect the disease activity of NASH. (6)

The present study uncovered the mechanisms underlying the novel finding that serum MMP-1 levels are higher in patients with F1 NASH than in those with F2–F4 NASH and healthy controls. This increase in MMP-1 levels in the early stage of NASH appears to parallel the intensity of MMP-1 staining in MCs, KCs, and HSCs according to IHC and IEM. TG deposits in hepatocytes cause steatosis and inflammation in early-stage NASH. Thus, oxidative stress and apoptosis of hepatocytes may lead to MC and KC infiltration. (4, 5, 109) MMP-1 in MCs, KCs, and HSCs may be caused by cytokines, as described later in the text, and MMP-1 expression was observed in these cells.

Among MMPs/TIMPs and cytokines, the serum MMP-1 level best reflected disease activity in the clinical course of NASH. Oxidative stress in mitochondria is an essential trigger

of the pathogenesis of NASH, in which serum MMP-1 levels are reflected and serum MMP-9 levels are related to the severity of steatosis and inflammation. The exact localization has not been observed in humans, but the cells responsible for producing MMP-9 may be hematopoietic stem cells and their derivative granulocytes. (119) In the present study, serum MMP-9 levels in patients with NASH did not parallel the disease activity. Serum MMP-2, TIMP-1, and TIMP-2 levels indicate the progression of fibrosis, but they did not reflect steatosis and inflammation in the present study. Miele et al. (120) and Toyoda et al. (121) reported higher serum MMP-2 and TIMP levels, but they did not examine these values in relation to disease activity.

Next, we compared the serum levels of MMPs and TIMPs with those of cytokines, i.e., SDF-1α, SCGF-β, SCF-1, MCP-1, G-CSF, leptin, and ghrelin, in patients with NASH from the viewpoint of histological grading relative to the progression of NASH. Among these cytokines, serum of SDF-1α and SCGF-β levels were extremely similar, and those of SCF-1, MCP-1, G-CSF, and leptin were similar. The former group also displayed levels similar to those of MMP-2 and TIMPs, and the latter value reflected the beginning of steatosis with inflammation. Serum ghrelin levels differed from those of the other cytokines.

We measured the levels of the seven aforementioned cytokines. Each cytokine has a

different physiological function. Marra and Tacke reported that KCs, injured hepatocytes, and activated HSCs secrete high levels of MCP-1, which promote the hepatic accumulation of bone marrow-derived CCR2-expressing MCs that massively expand the local macrophage pool. (122) In the present study, a significant increase in serum MCP-1 levels was observed in the early stage, probably subsequent to the development of steatosis in hepatocytes. G-CSF has been reported to prevent the development of hepatic steatosis in rat NASH models. (123) Moreover, acute liver failure in rats following exposure to D-galactosamine revealed that G-CSF enhanced the SDF-1 gradient between bone marrow and the liver, inducing the chemoattraction of CD34+ cells from bone marrow to the injured liver. (124) Although this model was not a NASH model, G-CSF may increase SDF-1 levels.

SDF-1 is the main factor regulating the trafficking and homing of hematopoietic stem cells to bone marrow. (16) Sinusoidal endothelial cells represent one of the main cellular sources of SDF-1 in the liver, and the SDF-1/CXCR4 axis is involved in the regulation of hematopoietic stem cell migration to the liver. (16) SDF-1α secreted by KCs activates HSCs via CXCR4 on the HSC membrane. (125) This SDF-1α–CXCR4 system directly activates HSCs, which transform into myofibroblasts and express CXCR4 on their membranes. (125) Hong et al. (18) reported that activation of the CXCR4 receptor of SDF-1α causes inflammation and

fibrogenesis in the liver. Autocrine SDF-1α expression by activated HSCs was enhanced with the progression of NASH because activated HSCs in the NAFLD rodent model both increased the number of CXCR4 and enhanced the binding affinity of SDF-1α to CXCR4. (126) The present study revealed that serum SDF-1α levels significantly increased with increasing severity of fibrosis, and a correlation between serum SDF-1α levels and fibrosis was observed ($r = 0.649$, $p < 0.001$). Moreover, serum SDF-1α levels significantly increased with increasing severity of steatosis and inflammation.

Serum SCGF-β and SCF levels were measured in the present study because the progression of NASH involves the proliferation and migration of stem cells and HPCs.(109) No reports were found regarding the serum levels of either SCGF-β or SCF in patients with NASH. Serum SCGF-β and SCF levels appear to be related to inflammation.

In summary, in patients with F1 NASH, HSCs are activated by inflammation of the liver due to MCP-1 and leptin, after which early liver fibrosis and collagen production occur. HSCs activated by inflammation promote hepatic fibrosis and secrete MMP-1. However, TIMP levels were not elevated in the early stage of NASH. Moreover, our previous study revealed that inflammation in hepatocytes promotes the recruitment and growth of HPCs arising from Hering's canal and that MMP-1 is highly expressed. The expression of MMP-1 by HPCs is

observed strongly in early-stage NASH. Therefore, it is believed that MMP-1 contributes to collagen degradation in early-stage NASH, particularly in patients in the F1 group, and it may be involved in the pathogenesis of NASH.

Chapter 3

Background

Chapter 2 described the characteristics of MMP-1 in the early fibrosis stage of NASH. This section discusses the relationship between serum MMP/TIMP levels and the changes in ALT levels following pioglitazone treatment in patients with NASH and diabetes mellitus.

Methods

NASH is generally treated via diet modification and increasing physical activity. If abnormal serum TG and/or low-density lipoprotein cholesterol (LDL) levels were observed in individual laboratory data, lipid-lowering agents such as HMG-CoA reductase inhibitors, bezafibrate, and ezetimibe were prescribed. If the patients had diabetes mellitus, appropriate anti-diabetic drugs were prescribed according to the guidelines of the Japan Diabetes Association (2016). Most patients with NAFL had a good clinical course following these treatments. If patients with NASH did not experience improvement in this study, the anti-insulin resistance agent pioglitazone was administered.

In 15 of 33 patients with NASH, serum MMP/TIMP and cytokine levels were measured 0, 21, 35, and 70 weeks after liver biopsy. ALT levels were compared with serum MMP, TIMP,

and SDF-1α at the same time points.

The numbers of patients with NASH treated with pioglitazone for more than 2 months who underwent measurements of serum MMP, TIMP, and SDF1-α levels were as follows: six patients in the F1 group, nine patients in the F2 group, and one each patient in the F3 and F4 groups. Clinical manifestations, physical findings, and clinical hematological data revealed no changes between before and after 2 months of pioglitazone treatment.

Results

ALT improved group vs. ALT uncontrolled group

The ALT improved group (n = 8) exhibited ALT levels of less than 30 IU/l at 70 weeks, as well as decreased serum MMP-1 levels, although statistical significance was not observed (Fig. 5). On the contrary, serum MMP-1 levels in seven patients in the ALT uncontrolled group were unchanged or elevated during the clinical course, as shown in Fig. 5. Moreover, serum MMP-1 levels at 70 weeks were lower in the ALT improved group than in the ALT uncontrolled group, albeit without significance. This leads us to believe that serum MMP-1 levels in patients with NASH may represent a useful indicator of disease progression.

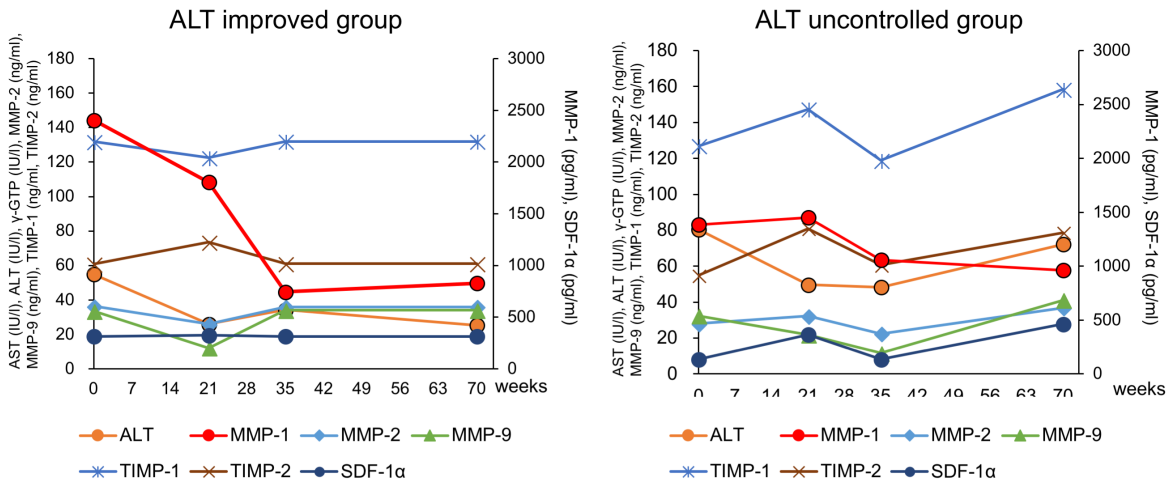

Fig. 5. Clinical courses of the ALT improved and ALT uncontrolled groups: Relationships of ALT changes with serum MMP/TIMP and SDF-1α levels.
The ALT improved and uncontrolled groups included seven and eight patients, respectively. Each plot indicates the mean value of each group.

Pharmacological effects of pioglitazone on serum ALT, MMP, TIMP, and SDF-1α levels

ALT levels tended to decrease in the F1 and F2 groups after pioglitazone administration ($p = 0.225$ and $p = 0.074$, respectively). Serum MMP-1 levels in the F1 group tended to decrease markedly after pioglitazone treatment, whereas those in patients in the F2 group were either unchanged or slightly decreased (Fig. 6). Serum MMP-9 levels in the F2 group declined following pioglitazone administration, whereas those of MMP-2, TIMP-1, and TIMP-2 were not altered by treatment (Fig. 6). Serum SDF-1α levels in the F1 group increased after pioglitazone administration (Fig. 6), albeit without significance ($p = 0.110$). There was no change in SDF-1α levels in the F2 group.

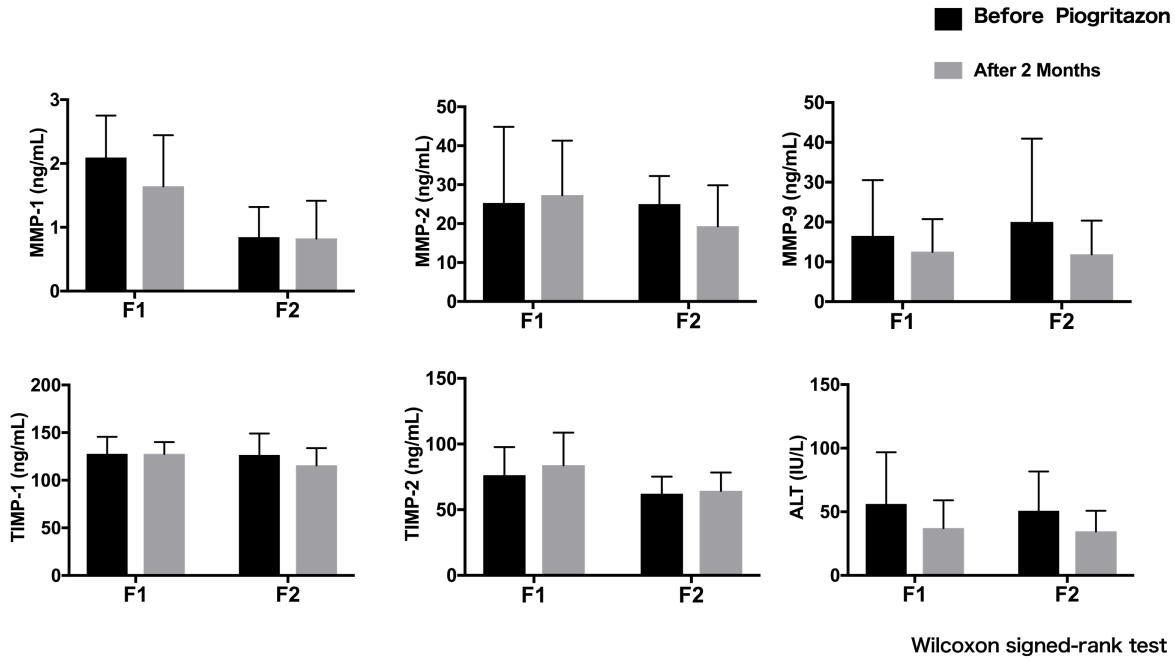

Fig. 6. Effect of pioglitazone administration for 2 months on serum MMP/TIMP and SDF-1α levels in the F1 and F2 groups.
Number of F1 patients, 6; number of F2 patients, 9

Effects of extrahepatic inflammatory conditions on serum MMP-1 levels

NASH is often accompanied by atherosclerosis, diabetes, and cardiovascular disease. In total, 15 patients with NASH underwent repeat serum MMP-1 measurements. Seven patients had neither diabetes nor metabolic syndrome. Five patients had both diabetes and metabolic syndrome, all of whom were in the ALT uncontrolled group. The remaining three patients had only metabolic syndrome.

The relationship between serum MMP-1 and HbA1c levels

Five patients with diabetes in the ALT uncontrolled group exhibited slightly higher HbA1c levels of 5.8–6.6%. Serum MMP-1 levels in the ALT uncontrolled group were not correlated with changes in HbA1c and ALT levels.

Relationship between serum MMP-1 levels and the clinical findings of metabolic syndrome

Three patients with NASH and metabolic syndrome were included in the ALT improved group. In two of these patients, serum MMP-1 levels decreased with decreases in ALT levels and body weight. One patient displayed no changes of serum MMP-1 levels despite decreases in body weight. The remaining five patients with NASH and metabolic syndrome also had diabetes, and all of these patients were in the ALT uncontrolled group. One of these patients exhibited decreases in body weight and serum MMP-1 levels despite increases in ALT levels. Similarly, changes in waist circumference, blood pressure, and serum LDL-cholesterol and TG levels were not consistent with serum MMP-1 levels.

Discussion

The results revealed a decrease in MMP-1 levels in patients with improved ALT levels.

Lifestyle modification (diet/exercise therapy) effectively reduces ALT levels, body weight, and fat levels in patients with NAFLD. In this study, the completion rate for diet/exercise therapy was not obvious, but the success of treatment can be evaluated using ALT changes. However, as diet/exercise therapy and drug therapy were conducted in combination, which treatment was more responsible for reductions of MMP-1 levels is unclear. Furthermore, it was not possible to verify the effect of fibrates, statins, and ezetimibe in combination with pioglitazone, nor was it possible to verify whether histological improvement occurred.

The therapeutic evaluation of NASH is important for improving steatosis, inflammation, and fibrosis in liver tissue. Liver biopsy could not be repeatedly performed in the same patients. Nakayama et al. (127) confirmed via liver biopsy that long-term treatment with pioglitazone for 2 years resulted in decreases in ALT levels and improvements in steatosis, inflammation, and fibrosis in patients with NASH. It is believed that the condition of liver tissue was improved also in this study. Interestingly, the MMP-1 concentration was high in the ALT improved group and conversely low in the ALT uncontrolled group. MMP-1 levels might predict responsiveness to treatment. In other words, in patients with mild fibrosis (e.g., F1), MMP-1 is not suppressed by TGF-β and TIMPs. In fact, in the F2 group, in which fibrosis reached the portal vein, serum MMP-1 levels were low. Therefore, it is believed that liver fibrosis is more

easily improved. Although HPC and HSC activation contributed to the increased MMP-1 concentration, some other factors may also influence treatment reactivity.

Pioglitazone, an adipocyte PPARγ agonist, promotes TG uptake by adipocytes and decreases free fatty acid uptake by hepatocytes. Pioglitazone also improves insulin resistance through AdipoR1/R2 by increasing the blood adiponectin levels via PPARγ activation in muscle, adipocytes, and the liver. Via another mechanism, the drug decreases HSC activation by inhibiting the activity of macrophages similarly as KCs via PPARγ. As a result, pioglitazone decreases MCP-1 concentrations and reduces liver inflammation and fibrosis. From these results, the improvements of fatty liver and inflammation were believed to reduce MMP-1 levels through reductions of collagen production.

Metformin is mainly administered to treat diabetes, whereas pioglitazone is selected for patients with NASH complicated by diabetes. Of course, it is believed that metformin also improves NASH-induced inflammation by reversing insulin resistance and thus improving fatty liver. (128-130) In addition, it was recently reported that SGLT2 inhibitors are effective for treating NASH. (131, 132) It is thus necessary to study changes in serum MMP-1 concentrations during treatment with these drugs.

Overall discussion

NASH is often accompanied by atherosclerosis, diabetes, and cardiovascular disease. (133) The present study demonstrated that HbA1c levels and the clinical findings of metabolic syndrome, as markers of extrahepatic inflammatory conditions in NASH, were not associated with serum MMP-1 levels. The numbers of patients with NASH complicated by diabetes or metabolic syndrome were not sufficient to draw definitive conclusions. Further investigations with larger sample sizes are needed to clarify whether serum MMP-1 and SDF-1α levels are useful biomarkers of disease activity and disease progression in the F1 group of NASH, whether extrahepatic conditions affect serum MMP-1 levels, and whether pioglitazone treatment reduces MMP-1 levels in parallel with serum ALT levels. Belfort et al. (134) reported significant improvements of steatosis, inflammation, and hepatocyte ballooning in patients treated with pioglitazone due to improvements in insulin resistance. In particular, they reported that the rate of steatosis was significantly lower in the pioglitazone treatment group than in the placebo group, whereas plasma adiponectin levels were increased. When pioglitazone was administered for at least 2 years, Nakayama et al. (127) reported significant improvements of ALT, total cholesterol, and HbA1c levels and histologically observed improvements of lipid

droplet levels, intravesicular inflammation, and fibrosis. Pioglitazone decreased MMP-1 levels and increased SDF-1α levels in patients with F1 NASH. These trends were not observed in other fibrosis groups, suggesting that extremely complex cytokine reactions in this particular stage of disease lead to improvement or progression of NASH.

This study excluded patients with findings of atherosclerosis and cardiovascular disease. Indeed, it is an important question whether MMP-1/TIMP-1 affects atherosclerosis and cardiovascular disease. Lehrke et al. reported that serum MMP-1 is associated with coronary artery disease (135), whereas other researchers noted that MMP-1/TIMP-1 is not correlated with the risk of atherosclerosis. (136) Thus, the relationships of MMPs with atherosclerosis and cardiovascular events are unclear. It is considered that liver weight is particularly high in the human body, and fibrotic livers are rich in collagens. Therefore, even if the MMP/TIMP concentration is elevated in patients with atherosclerosis and cardiac disease, the influences of these diseases on MMP levels is expected to be small. In patients with type 1 or 2 diabetes, an increase in MMP levels has been associated with cardiovascular events. (137-139) These reports describe that MMPs are associated with cardiovascular event risk in patients with type 1 diabetes, and it is difficult to provide a similar explanation for patients with NASH but no cardiovascular events in the present study. Although the relationship between diabetes and

MMPs/TIMPs should be elucidated more deeply, when focusing on collagen in the fibrotic liver and MMP as a collagen-degrading enzyme, serum MMP/TIMP levels appear to have a closer association with fibrosis in patients with NASH.

Recently, we published a report on the spatiotemporal expression of MMP-1 in the livers of patients with histologically proven NASH. (109) IHC and IEM revealed that MCs, KCs, and HSCs were positive for MMP-1 in the early stage of NASH, whereas HPCs positive for MMP-1 expression appeared to transform into both bile ductules participating in ductular reactions and elongated capillary endothelial cells involved in angiogenesis in the advanced stage of NASH. MMP-1–positive HPCs were observed in the early stage of NASH. (140) MMP-1, a soluble-type MMP, is a typical interstitial collagenase. Moreover, MMP-1 possesses both the unique function of degrading type I collagen at one-quarter distance from the N-terminus of the collagen molecule (141) and the abilities to initiate cell proliferation (79, 90, 142) and alter cell phenotype. (143)

We then measured serum MMP-1 levels in patients with histologically proven NASH in comparison with those in healthy controls. We measured MMP-1 as well as other MMPs, TIMPs, SDF-1α, and several cytokines to examine disease activity linked with progression to advanced-stage NASH, cirrhosis, and HCC. (109) In the present study, we found that serum

MMP-1 levels uniquely reflect disease activity compared with those of other MMPs, their inhibitors, and cytokines involved in their activation and discuss the possibility of translational research to inhibit exosomes from KCs, MCs, and HSCs, including miRNAs related to MMP-1 expression as novel targets for NASH.

We additionally observed the dual expression of MMP-1 and OV-6/K19 in HPCs, ductular cells, and so-called small hepatocytes, suggesting that MMP-1–positive HPCs differentiated or transformed into hepatocytes, ductular cells, myofibroblasts, and elongated repaired endothelial cells, as described previously. (109) The mechanism of MMP-1 expression in MCs, KCs, and HSCs may involve exposure to exosomes containing miRNAs related to the appearance of MMP-1 secreted from MCs. (144) MMP-1 plays a central role in degrading the extracellular matrix in the recovery phase of experimental liver fibrosis in rats (4, 79, 90, 141, 142, 145), but MMP-1 appears to induce cell dedifferentiation. (109) MMP-1 is considered to be involved in the progression of NASH pathology. (109) MMP-1 may play a kick-starter role as a rapid reaction to oxidative stress; i.e., higher levels of MMP-1 contribute to further progression to NASH.

Immature HSCs/HPCs have been shown to exit from circulation and lodge nearby in the outer wall of the endothelium in experimental animals. (146) We demonstrated the expression

of Angiotensin II receptor-like 1 (APJ) in HSCs/HPCs related to ECs and PCs in human liver. (147) Hypoxia is associated with the development and progress of NAFLD and lipid metabolism in the liver. (148) It has been well established that hypoxia/ischemia triggers HSCs/HPCs to migrate from the bone marrow into peripheral blood. (149) After migration to the site of hypoxic/ischemic tissue, HSCs/HPCs can differentiate into ECs and participate in the formation of novel vessels. HIF 1α is a key determinant of oxygen dependent gene regulation in angiogenesis, which has been shown to be involved in HSC/HPC proliferation and differentiation, through low oxygen tension (hypoxia), which has been termed “hypoxic niche”. (149) HIF-1α, as well as its downstream apelin/APJ signaling, were upregulated in hypoxia-treated HPCs, and might therefore serve as a potential target for the prevention of hypoxic ischemic injury in vitro. (150) In sprouting angiogenesis, angiopoietin-1 and apelin function as important factors that support mature ECs sprouting from pre-existing vessels. (151) APJ was highly expressed in HSCs and hepatocytes in cirrhotic liver, suggesting that hypoxia and inflammatory factors could play major roles in the activation of the hepatic apelin system, which can lead to angiogenic and fibroproliferative responses in chronic liver disease. (152) HSCs/HPCs expressing APJ may contribute to the angiogenesis of liver tissue in early-stage NASH. We expect that APJ and MMP-1 contributes to expression of HPCs and

regeneration of hepatocyte. Currently, relationship of APJ and MMPs have been reported in osteoarthropathy and rheumatoid patients(153, 154), but NASH has not reported yet. These relationships should be clarified in future research.

Rapamycin (sirolimus) has been reported as an agent that inhibits the mRNA transcription of MMP-1. Rapamycin may exert direct antifibrotic effects independent of its immunosuppressive action. It efficiently activates AP-1–driven transcription by rapidly inducing c-Jun/AP-1 phosphorylation with activation of the c-Jun N-terminal kinase cascade, resulting in enhanced binding of AP-1. (155) Although what MMP-1 inhibitor will induce or inhibit early NASH development is unclear, it is expected that inhibitors can be applied to elucidate the relationship between MMP-1 and NASH.

Autotaxin (ATX) is currently under investigation as a new-generation fibrosis maker for patients with chronic hepatitis C (CHC) in a parallel study. ATX is a secreted enzyme originally discovered in conditioned medium from A2058 human melanoma cell cultures. (156) ATX has an important enzymatic function in converting lysophosphatidylcholine to lysophosphatidic acid (LPA), which has various physiological roles in cell migration, neurogenesis, angiogenesis, smooth-muscle contraction, platelet aggregation, and wound healing. (157-160) LPA also stimulates the proliferation and contractility of HSCs. (161)

ATX is present in serum, and it is metabolized by liver sinusoidal endothelial cells. Fibrosis reduces the capacity of the liver to metabolize ATX, resulting in increases in serum ATX levels. (162, 163) ATX is useful as a serum marker for determining the fibrosis stage in patients with CHC. (164, 165) In addition, ATX is suggested to be useful as an indicator of the severity of liver disease and for determining the prognosis of cirrhosis (166) and HCC recurrence in combination with the levels of LPA receptors. (167) Liver fibrosis associated with CHC progression via liver inflammation, and it increases the risk of liver cirrhosis and (HCC). (168) When used to treat CHC, direct-acting antiviral agents (DAAs) achieve high sustained virological response rates. However, despite viral elimination, the risk of progression from liver fibrosis to cirrhosis and carcinogenesis remains. (169, 170) Therefore, liver fibrosis must be monitored after DAA treatment, and the optimal strategy for monitoring liver fibrosis is a similar problem for NASH.

Invasive and noninvasive methods are used to monitor liver fibrosis. Invasive liver biopsies are difficult to undertake regularly because of the risk of bleeding, the length of hospitalization required to manage these risks, and the associated costs. (52) Transient elastography (TE) and blood sampling to determine the fibrosis marker levels are less invasive liver fibrosis monitoring methods. TE determines the degree of hepatic fibrosis, (171, 172); however, it

requires expensive equipment, the recruitment of experienced technicians, and extended consulting hours, and patient throughput is low. Measuring fibrosis marker concentrations in blood can indicate the stage of liver fibrosis, and the related costs are generally lower than those associated with TE. Therefore, biomarkers for early-stage NASH discovery could be applied to patients who cannot undergo liver biopsy and people who complete annual health examinations.

In the future, fibrosis markers can be used for both diagnosis and treatment. This study should contribute to the early detection of NASH and evaluation of drug efficacy. In future research, we will examine the relationships of fibrosis markers with drugs used for the elucidation and treatment of MMP-1–related conditions in NASH in more detail. In addition, I would like to apply these strategies to clinical trials aiming to personalize medicine using hepatic fibrosis markers such as SDF-1α and ATX in combination.

Conclusion

MMP-1 is considered a biomarker for the early detection and treatment evaluation of early-stage NASH, which is typified by increasing serum levels of leptin and MCP-1.

Acknowledgments

I would like to express my greatest appreciation to Prof. Isao Okazaki, whose comments and suggestions were of inestimable value for my study. Prof. Takako Komiyama provided insightful comments and suggestions. I also deeply grateful to Prof. Katsuya Otori, who provided enormous support and advice for this study. Prof. Hiroaki Yokomori provided constructive comments and warm encouragement. Dr. Takayuki Uematsu provided technical assistance and sincere encouragement. I would also like to express my gratitude to my family for their moral support and warm encouragements.

Compliance with ethical standards

Ethical approval

All procedures performed in studies involving human participants were conducted in accordance with the ethical standards of the institutional and/or national research committee and the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all participants enrolled in the study.

Publication of the results

These results were published in the following original papers:

Ando W, Yokomori H, Tsutsui N, Yamanouchi E, Suzuki Y, Oda M, Inagaki Y, Otori K, Okazaki I. Serum Matrix Metalloproteinase-1 Level Represents Disease Activity as Opposed to Fibrosis in Patients with Histologically Proven Nonalcoholic Steatohepatitis. Clinical and Molecular Hepatology. 2018; 24(1):61-76.

Ando W, Yokomori H, Otori K, Oda M. The Apelin Receptor APJ in Hematopoietic Stem Cells/Progenitor Cells in the Early Stage of Non-Alcoholic Steatohepatitis. Journal of Clinical Medicine Research .2017; 9(9):809-811.

Supplementary Table 1. Clinical and laboratory data of NASH patients enrolled in the present study

No.	Sex	Age (years)	BMI (kg/m^2)	AST (IU/l)	ALT (IU/l)	γGTP (IU/l)	TC (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	TG (mg/dl)	FBS (mg/dl)	HbA1c (%)	Plt ($10^3/\mu l$)	Steatosis	Inflammation	Ballooning	Fibrosis	Early (e) or advanced (a) NASH	ALT improved group (i) or ALT uncontrolled group (u)	Enrolled in the pioglitazone effect study (p)	complication
1	M	39	30.7	22	35	20	223	166	46	157	104	5.6	27.5	2	2	1	1	e	u	p	DM, MS
2	F	39	35.3	132	185	86	250	175	36	264	102	6	21.6	3	2	2	1	e	u	p	DM, MS
3	M	45	32.9	40	86	25	203	151	40	101	116	6	24.5	2	3	1	2	e	u	p	DM, MS, HCM
4	M	40	24.3	58	109	38	208	155	37	133	96	5.8	3.07	2	2	2	1	e	u		
5	F	36	28.6	54	105	73	225	156	46	200	115	5.5	21.2	2	2	1	1	e			MS
6	M	46	25.0	29	68	144	141	79	47	113	78	5.5	21.3	2	3	1	4	a		p	
7	M	75	27.6	42	29	132	156	196	24	89	205	9	11.1	2	1	1	4	a			MS
8	M	56	34.0	21	54	113	259	116	38	526	242	8.5	22.8	3	1	2	2	e	u	p	DM, MS
9	M	41	31.4	100	173	79	179	93	40	297	107	5.9	22.3	3	1	1	1	e	u		DM, MS
10	M	49	30.4	26	34	25	174	108	52	96	93	5.4	20.9	1	1	1	2	e		p	MS
11	M	30	37.7	38	71	21	163	82	59	160	101	5.5	21.2	2	1	2	2	e			MS
12	F	59	33.1	20	27	19	183	119	53	68	118	6	14.8	1	2	2	1	e		p	MS
13	M	38	25.9	65	122	39	247	169	53	177	104	5.8	21.5	3	1	2	1	e	i	p	
14	M	61	22.9	33	46	35	180	109	49	113	133	6.1	11.9	2	1	1	2	e	i	p	
15	M	54	28.7	59	97	233	163	84	40	267	173	7.1	11.9	2	2	1	3	a		p	DM, MS
16	M	66	28.4	35	39	141	203	115	79	69	143	6.8	12.5	1	2	2	4	a			DM, MS
17	M	38	32.4	39	63	44	187	123	29	222	97	5.7	30.9	2	1	2	2	e		p	MS
18	M	81	20.3	17	12	28	84	50	31	64	135	6.2	11.5	1	2	1	3	a			
19	M	44	22.6	39	40	34	182	104	48	175	83	5.4	20.4	2	2	2	2	e	i	p	
20	M	46	26.6	29	34	70	198	124	46	167	92	5.6	22.9	2	2	2	2	e	i	p	MS
21	M	58	24.5	34	74	70	200	129	50	154	91	6	18.7	3	1	2	1	e	u	p	
22	M	48	28.1	31	38	40	192	117	59	78	110	6	28.1	2	1	2	1	e	i		MS
23	M	41	23.4	25	57	26	155	79	39	254	78	5.1	17.3	1	2	2	2	e	i	p	
24	M	58	27.6	84	139	308	244	133	41	350	119	6.9	20.2	2	2	2	2	e	i	P	MS
25	M	40	19.8	42	52	84	178	118	43	87	87	5.7	34	2	3	2	1	e	i	p	
26	F	62	25.8	83	87	71	184	117.2	48	94	93	6.1	142	2	1	1	1	e			
27	M	61	28.1	48	46	34	155	86	46	171	97	7.9	24.2	1	1	1	1	e			MS
28	F	49	27.0	55	77	48	213	129	62	148	118	6.8	24.9	2	2	2	1	e			MS
29	M	40	26.5	26	68	64	201	121	40	113	118	6.1	23.7	2	2	1	1	e			MS
30	F	67	24.1	65	85	85	154	86	49.6	92	109	6.6	16.2	1	2	1	3	a			
31	F	65	24.8	66	52	190	188	80	90	42	176	7.4	12	1	1	2	4	a			DM
32	F	59	22.4	105	107	186	208	133	41	171	184	8.9	15.2	2	2	2	4	a			DM
33	M	50	30.1	48	31	320	124	74	41	67	369	10.2	11	2	3	2	4	a			DM, MS

These data were collected before liver biopsy. Abbreviations: *BMI*, body mass index; *AST*, aspartate aminotransferase; *ALT*, alanine aminotransferase; *DM*, diabetes mellitus; *γ-GTP*, gamma-glutamyl transpeptidase; *HCM*, hypertrophic cardiomyopathy; *HDL,* high-density lipoprotein cholesterol; *LDL,* low-density lipoprotein cholesterol; *MS*, Metabolic syndrome; *TC,* total cholesterol; *TG,* triglyceride.

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