Fibroblast Growth Factor-5 Participates in the Progression of Hepatic Fibrosis

Hiromi HANAKA1), Tsuyoshi HAMADA2), Masataka ITO1), Hiroyuki NAKASHIMA3), Kenjo TOMITA4), Shuhji SEKI3), Yasushi KOBAYASHI2), and Junko IMAKI1)

1) Department of Developmental Anatomy and Regenerative Biology, National Defense Medical College, 3–2 Namiki, Tokorozawa, Saitama 359-8513, Japan
2) Department of Anatomy and Neurobiology, National Defense Medical College, 3–2 Namiki, Tokorozawa, Saitama 359-8513, Japan
3) Department of Immunology and Microbiology, National Defense Medical College, 3–2 Namiki, Tokorozawa, Saitama 359-8513, Japan
4) Division of Gastroenterology and Hepatology, Department of Internal Medicine, National Defense Medical College, 3–2 Namiki, Tokorozawa, Saitama 359-8513, Japan

Abstract: Non-alcoholic steatohepatitis (NASH) is characterized by the presence of steatosis, inflammation, and fibrosis and is believed to develop via a “two-hit process”; however, its pathophysiology remains unclear. Fibroblast growth factors (FGFs) are heparin-binding polypeptides with diverse biological activities in many developmental and metabolic processes. In particular, FGF5 is associated with high blood pressure. We investigated the function of FGF5 in vivo using spontaneously Fgf5 null mice and explored the role of diet in the development of NASH. Mice fed a high-fat diet gained little weight and had higher serum alanine transaminase, aspartate amino transferase, and non–high-density lipoprotein-cholesterol levels. Liver histology indicated marked inflammation, focal necrosis, fat deposition, and fibrosis, similar to the characteristics of NASH. FGF5 and a high-fat diet play significant roles in the pathophysiology of hepatic fibrosis and Fgf5 null mice may provide a suitable model for liver fibrosis or NASH.

Key words: FGF5, hepatic fibrosis, NAFLD, NASH, two-hit theory

Introduction

One of the most common liver pathologies, hepatic steatosis, has been traditionally believed to result from obesity and alcohol abuse; however, focus is now on non-alcoholic steatohepatitis (NASH), which does not carry the same associations [3]. NASH is characterized by steatosis, inflammation, and fibrosis [4, 6, 7, 22]. The development of NASH requires a “double hit,” meaning that steatosis first develops in the liver, followed by the development of sufficient oxidative stress to initiate significant lipid peroxidation [9]. A recent study also reported that LDL-oxidizing antibodies play an important role in NASH pathogenesis [5], although the mechanisms of NASH development are unknown.

Fibroblast growth factors (FGFs) are heparin-binding polypeptides with diverse biological activities in many developmental and metabolic processes. The FGF gene family comprises 22 members [14]. Originally identified by Zhan et al. [24], FGF5 regulates hair growth [11, 19, 20, 21], and subsequent studies have found that Fgf5 knockout [11, 18] and null mice [15] have an angora phenotype. Although the role of FGF5 in pathology is not clear, recent studies have shown that FGF5 influ-
ences blood pressure [17, 23] and human glioblastomas [2]. We used Fgf5 null mice to investigate the in vivo function of FGF5 and the role of diet in the development of NASH.

**Methods**

**Animals and diets**

This study was approved by the National Defense Medical College Animal Care and Use Committee and conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” of the National Academy of Sciences (USA). Male ICR (4 weeks old, wild-type, WT) mice were used as the control in all experiments (CLEA Japan, Tokyo, Japan). The mice were fed a high-fat (TD.88137, Harlan Laboratories, Madison, WI, USA) or control diet (CE-7, CLEA Japan) for 4 weeks. The control diet contained 3.8% fat, 17.7% protein, 59.4% carbohydrate, and 4.5 kcal/g. The high-fat diet contained 21.2% fat, 0.2% cholesterol, 17.3% protein, 48.5% carbohydrate, and 4.5 kcal/g. The mice had unlimited access to water and food during the study period.

Body weights were measured weekly. Serum high density lipoprotein (HDL), triacylglycerol (TG), total cholesterol (TCHO), serum alanine transaminase, and aspartate amino transferase were measured at 8 weeks of age using a DRI-CHEM 4000V analyzer (Fuji Film, Tokyo, Japan).

**Polymerase chain reaction**

Purified tail skin genomic DNA from Fgf5 null mice was amplified using primer sets for exon 1 (5′-TACCG-GCGTGAGTACACA-3′, 5′-TCTAAGGAAACCGTGTGC-3′), exon 2 (5′-CTGAGAACAGTTGAGTAAGT-3′, 5′-CTATCTGGTAAACGGATTCC-3′), and exon 3 (5′-ATCTTGCCATTCTGAGTCAA-3′, 5′-TG-TATACAAAATACCCGGGT-3′). The amplified fragments were cloned into pGEM-T-easy (Promega, Madison, WI, USA). Transformants were screened by PCR and sequenced using the T7 and S6 primers (Promega) on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

**Morphological study**

Mice were anesthetized with isoflurane (Mylan, Canonsburg, PA, USA) and sacrificed. Liver tissues were harvested and histology was performed. The livers were fixed with 10% formalin and slides were stained with hematoxylin and eosin to evaluate steatosis and steatohepatitis. Additional liver sections were stained with Masson trichrome.

**Statistical analyses**

All values are expressed as the mean ±SD. Statistical significance was determined using Excel Ver. 14.3.5 (Microsoft, WS, USA) by Student’s *t*-test.

**Results**

**Morphology and genome structure of the FGF5 null mouse**

Figure 1 shows examples of the WT ICR (Fig. 1A) and Fgf5 null mice (Fig. 1B). ICR mice were obtained from CLEA Japan Co., Ltd. in the early 1980s, and have been maintained in our laboratory. The long hair (LH) mouse type found by our laboratory and has been maintained for over 20 years. PCR analyses were performed to determine the presence or absence of Fgf5 exons 1, 2, and 3. A 10-kb sequence, including exon 3, was deleted in the Fgf5 allele of the LH mice and a 0.5-kb sequence was inserted at the same site (Fig. 1C). This sequence shared more than 98% identity with an early transposon long terminal repeat (LTR). These LH mice may be genetically identical to mice recently reported by Mizuno *et al*. [15].

**Body weight**

At 8 weeks, the WT and LH mice fed the high-fat diet (WT-H, LH-H) weighed more than those fed the control diet (WT-C, LH-C). As shown in Fig. 2, the LH-H mice (28.6 ± 1.0 g) weighed slightly more than the LH-C mice (26.8 ± 0.7 g, *P* = 0.023), but the WT-H mice (34.0 ± 1.7 g, *P* = 0.002) demonstrated a marked increase in weight compared with the WT-C mice (27.2 ± 2.9 g).

**Laboratory assessment**

The LH-H mice had higher levels of alanine transaminase (150 ± 81 mg/dL) than the WT-C mice (28.6 ± 1.0 g; *P* = 0.055), WT-H (42 ± 15 mg/dL; *P* = 0.074), and LH-C (27 ± 4 mg/dL, *P* = 0.055) mice. In addition, serum aspartate aminotransferase in the WT-H (226 ± 91 mg/dL, *P* = 0.061) and LH-H (248 ± 94 mg/dL, *P* = 0.046) mice was higher than in the control animals (WT-C, 107 ± 56 mg/dL, *P* = 0.057; LH-C, 102 ± 38 mg/dL, *P* = 0.046) (Fig. 3A). TG in the LH-H mice (80 ± 32 mg/dL) was
lower than in the WT-C (151 ± 18 mg/dL, P=0.015), WT-H (145 ± 50 mg/dL, P=0.013), and LH-C (179 ± 83 mg/dL, P=0.095) mice. Finally, TCHO was higher in LH-H mice (272 ± 76 mg/dL) than in the other groups (WT-C, 94 ± 3.0 mg/dL, P=0.013; WT-H, 203 ± 24.5 mg/dL, P=0.094; LH-C, 137 ± 12.5 mg/dL, P=0.09) (Fig. 3B). Non–high-density lipoprotein-cholesterol (non–HDL-C) was calculated as the difference between HDL and total cholesterol. Non–HDL-C in LH-H mice (54 ± 15.9 mg/dL) was also much higher than in the other groups (WT-C, 4 ± 4 mg/dL, P=0.03; WT-H, 2 ± 25 mg/dL, P=0.01; LH-C, −3 ± 10.6 mg/dL, P=0.01) (Fig. 3C).

**Fig. 2.** Body weight in Fgf5 null mice fed a high-fat diet. Body weights at ages 4, 5, 6, 7, and 8 weeks. Wild type (WT) control, WT mice fed a normal diet (n=5); WT High fat, WT mice fed a high fat diet (n=5); Long hair (LH) control, Fgf5 null mice fed a normal diet (n=4); LH High fat, FGF5 null mice fed a high fat diet (n=4). *P<0.01 and **P<0.05 compared to the LH High fat mice.
Liver histology

Histology of LH-H mice revealed marked infiltration of inflammatory cells in the periportal area (Zone 1) and mid-zonal parenchyma (zone 2) associated with massive piecemeal necrosis and focal necrosis (Figs. 4A and C). Swollen and vacuolated hepatocytes were observed around the central vein (Zone 3) (Fig. 4A, gray arrowhead). Higher magnification of this area revealed that these hepatocytes represented microvesicular steatosis and hepatocyte ballooning (Figs. 5A and B). Additionally, Masson-trichrome stain revealed severe fibrosis specifically in the inflamed areas (Figs. 4B and D). LH-C mice exhibited normal liver histology, without steatosis, inflammation (Figs. 4E, 5C and 5D), fibrosis (Figs. 4F). Although enhanced hepatocyte steatosis and ballooning was observed in WT-H mice (Figs. 5E and F), they exhibited neither inflammation nor fibrosis (Figs. 4G and H). In human cases, NASH is diagnosed histopathologically using the NAFLD Activity Score (NAS) [1, 12]. In this experimental model, LH-H mice demonstrated moderate steatosis (score 2), prominent ballooning (score 2), and severe lobular inflammation (score 3),
Fig. 4. Histology. Livers were collected from each mouse and fixed with 10% formalin for hematoxylin-eosin (HE) and Masson-Trichrome (Masson) staining. Livers from LH mice fed with high fat diet were examined at 200× magnification (A), 400× (C) after HE staining, and 200× (B), 400× (D) after Masson staining. White arrowheads indicate piecemeal and focal necrosis. Gray arrowheads indicate microvesicular steatosis and ballooning of hepatocytes. Black arrowheads indicate fibrosis. Livers from LH mice fed with control diet (E) (F) and WT mice fed with high fat diet (G) (H) were also compared. The white line in each picture indicates 100 µm.
for a total NAS score of 7. Emergence of severe fibrosis is consistent with NASH. Although WT-H mice demonstrated severe steatosis (score 3) and prominent ballooning (score 2), the absence of lobular inflammation and fibrosis indicated fatty liver.

**Discussion**

FGF5 null mice fed a high-fat diet (LH-H) demonstrated clinical, laboratory, and hepatic histological characteristics similar to those observed in animals with NASH. Histology indicated marked inflammation, focal necrosis, fat deposition, and fibrosis in the livers of LH-H animals. NASH is also characterized by varying degrees of progressive steatosis, lobular inflammation, and fibrosis of the liver [16]. In this study, the LH-H mice demonstrated lower body weights, lower TG levels, and higher non–HDL-C levels, suggestive of a lipid metabolism disorder. In comparison, the WT-H mice had fatty livers and higher TG levels. The decreased fat deposition in LH vs. WT mice indicated possible impaired TG uptake in Fgf5 null mice. Thus, lipid balance

**Fig. 5.** Higher magnification of hepatic steatosis in LH mice and WT mice. Liver from high-fat diet-fed LH mouse at 400× (A) and 1,000× (B) after HE staining. Livers from Control Diet-fed LH mice at 400× (C) and 1,000× magnification (D) and high-fat diet-fed WT mice at 400× (E) and 1,000× (F) were compared. The white line in each image indicates 50 µm.
in the liver may be critical in the pathophysiology of NASH.

A recent study found that in patients who were not taking lipid-lowering agents, high levels of non–HDL-C were more closely associated with NASH than with steatosis. In that study, non-HDL-C was used to differentiate between NASH and steatosis [8]. The total cholesterol level was high in LH-H mice and non-HDL-C was significantly higher than in other groups. Hence, LH-H mice may have a metabolism disorder involving total cholesterol, and FGF5 may be involved in cholesterol metabolism, which may explain the association between FGF5 and the risk of hypertension observed in a recent genome-wide association study [13, 17].

NASH results from 2 distinct events, evolving from steatosis to fibrosis, known as a “two-hit process” [9, 10]. In our study, the WT-H livers exhibited steatosis but no fibrosis, and the LH-H livers exhibited both conditions. Thus, FGF5 is implicated in the progression from steatosis (first hit) to NASH (second hit). A high-fat diet also precipitated NASH in Fgf5 null mice. With 4.7 times more fat and 1.3 times more calories than the control diet, the high-fat diet likely has a role in the “two-hit process.”

In conclusion, we demonstrated associations between hepatic fibrosis, steatosis, FGF5, and a high-fat diet; however, the mechanism underlying these associations is unclear, and a comprehensive analysis of the livers of WT and LH mice with microarrays and omics studies is needed. We believe that the Fgf5 null mouse with a high-fat diet can be a suitable animal model for liver fibrosis or NASH and suggest FGF5 may be a therapeutic target for hepatic fibrosis in NASH.

Acknowledgment

This work was supported by a grant from the National Defense Medical College.

References

1. Afshal, N.H. 2012. Management of nonalcoholic fatty liver disease: a 60-year-old man with probable nonalcoholic fatty liver disease: weight reduction, liver biopsy, or both? JAMA. 308: 608–616. [Medline] [CrossRef]
2. Allerstorfer, S., Sonvila, G., Fischer, H., Spieg-Kreinecker, S., Gauglhofer, C., Setinek, U., Czech, T., Marosi, C., Buchroithner, J., Pichler, J., Silve, R., Mohr, T., Holzmann, K., Grasl-Kraupp, B., Marian, B., Grusch, M., Fischer, J., Mick-sche, M., and Berger, W. 2008. FGF5 as an oncogenic factor in human glioblastoma multiforme: autocrine and paracrine activities. Oncogene 27: 4180–4190. [Medline] [CrossRef]
3. Angulo, P., Keach, J.C., Batts, K.P., and Lindor, K.D. 1999. Independent predictors of liver fibrosis in patients with non-alcoholic steatohepatitis. Hepatology 30: 1356–1362. [Medline] [CrossRef]
4. Bacon, B.R., Farahvash, M.J., Janney, C.G., and Neuschwander-Tetri, B.A. 1994. Nonalcoholic steatohepatitis: an expanded clinical entity. Gastroenterology 107: 1103–1109. [Medline]
5. Biegls, V., van Gorp, P.J., Waleenbergh, S.M., Gijbels, M.J., Verheyen, F., Buurman, W.A., Briles, D.E., Hofker, M.H., Binder, C.J., and Shiri-Sverdlov, R. 2012. Specific immunization strategies against oxidized low-density lipoprotein: A novel way to reduce nonalcoholic steatohepatitis in mice. Hepatology 56: 894–903. [Medline] [CrossRef]
6. Caldwell, S.H., Oelsner, D.H., Iezzoni, J.C., Hespenheide, E.E., Battle, E.H., and Driscoll, C.J. 1999. Cryptogenic cirrhosis: clinical characterization and risk factors for underlying disease. Hepatology 29: 664–669. [Medline] [CrossRef]
7. Castellano Tortajada, G. 1999. Nonalcoholic steatohepatitis. Gastroenterol. Hepatol. 22:(Suppl 1): 13–19. [Medline]
8. Corev, K.E., Lai, M., Gerlrd, L.G., Misdraji, J., Barlow, L.L., Zheng, H., Andersson, K.I., Thimm, M., Pratt, D.S., and Chung, R.T. 2012. Non-high-density lipoprotein cholesterol as a biomarker for nonalcoholic steatohepatitis. Clin. Gastroenterol. Hepatol. 10: 651–656. [Medline] [CrossRef]
9. Day, C.P. 2002. NASH-related liver failure: one hit too many? Am. J. Gastroenterol. 97: 1872–1874. [Medline] [CrossRef]
10. Day, C.P. and James, O.F. 1998. Steatohepatitis: a tale of two “hits”? Gastroenterology 114: 842–845. [Medline] [CrossRef]
11. Hebert, J.M., Rosenquist, T., Gotz, J., and Martin, G.R. 1994. FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. Cell 78: 1017–1025. [Medline] [CrossRef]
12. Kleiner, D.E., Brunt, E.M., Van Natta, M., Behling, C., Contos, M.J., Cummings, O.W., Ferrell, L.D., Liu, Y.C., Torbensen, M.S., Unalp-Arida, A., Yeh, M., McCullough, A.J., Sanyal, A.J., and Nonalcoholic Steatohepatitis Clinical Research, N. 2005. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 41: 1313–1321. [Medline] [CrossRef]
13. Liu, C., Li, H., Qi, Q., Lu, L., Gan, W., Loos, R.J., and Lin, X. 2011. Common variants in or near FGF5, CYP17A1 and MTHFR genes are associated with blood pressure and hypertension in Chinese Hans. J. Hypertens. 29: 70–75. [Medline] [CrossRef]
14. McKeenhan, W.L., Wang, F., and Kan, M. 1998. The heparan sulfate-fibroblast growth factor family: diversity of structure and function. Prog. Nuclease. Acid Res. Mol. Biol. 59: 135–176. [Medline] [CrossRef]
15. Miyano, S., Iijima, S., Okano, T., Kajiwara, N., Kunita, S., Sugiyama, F., and Yagami, K. 2011. Retrotransposon-mediated Fgf5(g0-Utr) mutant mice with long pelage hair. Exp. Anim. 60: 161–167. [Medline] [CrossRef]
16. Nakagami, H., Kiomy Osako, M., Nakagami, F., Shimosato, T., Minobe, N., Moritani, T., Shimamura, M., Miyake, T., Shimizu, H., Takeya, Y., and Morishita, R. 2010. Prevention and regression of non-alcoholic steatohepatitis (NASH) in a rat model by metabosartan, telmisartan. *Int. J. Mol. Med.* 26: 477–481. [Medline]

17. Newton-Cheh, C., Johnson, T., Gateva, V., Tobin, M.D., Bochud, M., Coin, L., Najjar, S.S., Zhao, J.H., Heath, S.C., Eyheramendy, S., Papadakis, K., Voight, B.F., Scott, L.J., Zhang, F., Farrall, M., Tanaka, T., Wallace, C., Chambers, J.C., Khat, K.T., Nilsson, P., van der Harst, P., Polidoro, S., Grobbee, D.E., Onland-Moret, N.C., Bots, M.L., Wain, L.V., Elliott, K.S., Teuner, A., Luan, J., Lucas, G., Kuusisto, J., Burton, P.R., Hadley, D., Mcardle, W.L., Brown, M., Dominiczak, A., Newhouse, S.J., Samani, N.J., Webster, J., Zeggini, E., Beckmann, J.S., Bergmann, S., Lim, N., Song, K., Vollenweider, P., Waeber, G., Waterworth, D.M., Yuan, X., Groop, L., Orho-Melander, M., Allione, A., Di Gregorio, A., Guarerra, S., Panico, S., Ricceri, F., Romanazzi, V., Sacerdote, C., Vineis, P., Barroso, I., Sandhu, M.S., Luben, R.N., Crawford, G.J., Joshi, P., Perola, M., Boehnke, M., Bonnycastle, L.L., Collins, F.S., Jackson, A.U., Mohlke, K.L., Stringham, H.M., Valle, T.T., Willer, C.J., Bergman, R.N., Morken, M.A., Doring, A., Gieger, C., Illig, T., Meitinger, T., Org, E., Pfeufer, A., Wichmann, H.E., Kathiresan, S., Marroquin, J., O’Donnell, C.J., Schwartz, S.M., Siscovick, D.S., Subirana, I., Freimer, N.B., Hartikainen, A.L., McCarthy, M.I., O’Reilly, P.F., Peltonen, L., Pouta, A., de Jong, P.E., Snieder, H., van Gilst, W.H., Clarke, R., Goel, A., Hamsten, A., Peden, J.F., Seedorf, U., Syvanen, A.C., Tognoni, G., Lakatta, E.G., Sanna, S., Scheet, P., Schlessinger, D., Sutcli, A., Doyt, M., Ernst, F., Felix, S.B., Homuth, G., Lorbeer, R., Reffelmann, T., Retig, R., Volker, U., Galan, P., Gut, I.G., Hereberg, S., Lathrop, G.M., Zelenika, D., Deloukas, P., Soranzo, N., Williams, F.M., Zhai, G., Salomaa, V., Laakso, M., Elova, R., Forouhi, N.G., Volke, H., Uitterlinden, A.C., van der Schouw, Y.T., Numans, M.E., Matullo, G., Navis, G., Berglund, G., Bingham, S.A., Kooper, J.S., Connel, J.M., Bandinelli, S., Ferrucci, L., Watkins, H., Spec-tor, T.D., Tuomilehto, J., Altshuler, D., Strachan, D.P., Laan, M., Meneton, P., Wareham, N.J., Uda, M., Jarvelin, M.R., Mooser, V., Melander, O., Loos, R.J., Elliott, P., Abecasis, G.R., Caulfield, M., and Munroe, P.B. 2009. Genome-wide association study identifies eight loci associated with blood pressure. *Nat. Genet.* 41: 666–676. [Medline] [CrossRef]

18. Sundberg, J.P., Rourk, M.H., Boggess, D., Hogan, M.E., Sundberg, B.A., and Bertolino, A.P. 1997. Angora mouse mutation: altered hair cycle, follicular dystrophy, phenotypic maintenance of skin grafts, and changes in keratin expression. *Vet. Pathol.* 34: 171–179. [Medline] [CrossRef]

19. Suzuki, S., Kato, T., Takimoto, H., Masui, S., Oshima, H., Ozawa, K., Suzuki, S., and Imamura, T. 1998. Localization of rat FGF-5 protein in skin macrophage-like cells and FGF-5S protein in hair follicle: possible involvement of two Fgf-5 gene products in hair growth cycle regulation. *J. Invest. Dermatol.* 111: 963–972. [Medline] [CrossRef]

20. Suzuki, S., Ota, Y., Ozawa, K., and Imamura, T. 2000. Dermal-mode regulation of hair growth cycle by two Fgf-5 gene products. *J. Invest. Dermatol.* 114: 456–463. [Medline] [CrossRef]

21. Taniguchi, F., Harada, T., Sakamoto, Y., Yamaguchi, N., Yoshiba, S., Iwabe, T., and Terakawa, N. 2003. Activation of mitogen-activated protein kinase pathway by keratinocyte growth factor or fibroblast growth factor-10 promotes cell proliferation in human endometrial carcinoma cells. *J. Clin. Endocrinol. Metab.* 88: 773–780. [Medline] [CrossRef]

22. Teli, M.R., James, O.F., Burt, A.D., Bennett, M.K., and Day, C.P. 1995. The natural history of nonalcoholic fatty liver: a follow-up study. *Hepatology* 22: 1714–1719. [Medline] [CrossRef]

23. Xi, B., Shen, Y., Reilly, K.H., Wang, X., and Mi, J. 2013. Recapitulation of four hypertension susceptibility genes (Csk, CYP17A1, MTHFR, and FGF5) in East Asians. *Metabolism* 62: 196–203. [Medline] [CrossRef]

24. Zhan, X., Bates, B., Hu, X.G., and Goldfarb, M. 1988. The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. *Mol. Cell Biol.* 8: 3487–3495. [Medline]