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Analysis of non-alcoholic steatohepatitis in children : pathological characteristics, cytokine/chemokine profiling, and single nucleotide polymorphism

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Abstract

Objective To elucidate the clinical, pathological, and inflammatory characteristics of pediatric non-alcoholic steatohepatitis (NASH).

Methods We studied 12 male pediatric patients with a histological diagnosis of NASH. Demographic and clinical characteristics and histological criteria were investigated in each patient. Data were obtained on Matteoni's criteria, non-alcoholic fatty liver disease activity score (NAS), Brunt's inflammatory grading, and fibrosis staging. Liver immunostaining with intercellular adhesion molecule-1 (ICAM-1) and profiling of hepatic expression of cytokines/chemokines was also performed. Sanger sequencing was used to detect a previously reported single nucleotide polymorphism (SNP) in the tumor necrosis factor alpha (*TNF*α) gene (c.308G>A) believed to indicate genetic predisposition.

Results The median age and Ponderal Index of the patients was 14 years and 35.6%, respectively. The laboratory data revealed a mild increase in transaminase, mild impairment of lipid metabolism, and increased insulin resistance. The median histological scores were 4 (Matteoni's criteria), 6 (NAS), 2 (Brunt's inflammatory grading), and 3 (Brunt's fibrosis staging). No relationship was recognized between ICAM-1 immunostaining and any other type of score investigated. Cytokine/chemokine profiling was performed on liver samples from 4 of the patients. These patients were classified into a high or low TNFα-expressing group. No mutation was found in either of the patients in whom the SNP of the *TNF*α gene was investigated.

Conclusions Liver fibrosis develops in pediatric NASH patients as well as in adults, leading to liver failure at a young age. Two hypotheses are suggested to explain the observed variation in cytokine/chemokine expression : 1) it changes dynamically as NASH progresses ; and 2) there are subtypes with different immunologic mechanisms. Further investigation is needed to elucidate cytokine/chemokine dynamics and genetic predisposition in this disease.

Introduction

More and more patients are presenting with metabolic syndrome (MtS), a serious health problem, and the number of children with obesity is continuing to increase in Asia. Non-alcoholic steatohepatitis (NASH), a severe sub-type of non-alcoholic liver disease (NAFLD), is characterized by infiltration of inflammatory cells into the liver, ballooning of hepatocytes, the appearance of Mallory-Denk bodies, and fibrosis of the liver, leading to liver cirrhosis and carcinoma[1]. Non-alcoholic steatohepatitis is also recognized as a hepatic manifestation of MtS[1]. The "two-hit theory" is the generally accepted pathophysiology of NASH. According to this theory, the accumulation of triglycerides is considered to be the first hit, while cytokines, adipokines, oxidative stress, and inherited predisposition are considered to contribute to the course of the second hit[2], although reports on this

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stage are few. Additionally, the histological characteristics of pediatric NASH are reported to differ to some extent to those in adults[3].

The purpose of this study was to investigate demographic characteristics, histological findings, including by immunostaining, cytokine/chemokine expression, and a single nucleotide polymorphism (SNP) in the tumor necrosis factor alpha gene (*TNFα*) in pediatric NASH patients to elucidate the pathophysiological characteristics of this disease.

Expression of cytokines/chemokines and its role in inflammation in the liver was examined to investigate inherited predisposition in pediatric NASH patients.

Materials and Methods

Pediatric patients with NASH as diagnosed by percutaneous liver biopsy at Tokyo Medical University Hospital between 2008 and 2012 were enrolled in the study. Liver biopsy was considered applicable in patients with obesity in whom liver dysfunction was diagnosed based on laboratory data. The guidelines of the American Association for the Study of Liver Disease, the American College of Gastroenterology, and the American Gastroenterological Association were used as the diagnostic criteria for NASH[4]. Patients with a history of alcohol consumption or any disease or medication that would induce the secondary accumulation of fat were excluded. This study was approved by the Institutional Review Board of Tokyo Medical University.

Twelve male patients were included in the study. The median age was 14 (range : 6-16) years and the median Standard Deviation (SD) score for body height and body weight was +0.98 (−1.12-2.04) and +2.81 (0.46-5.54), respectively. The median Body Mass Index (BMI) and Ponderal Index (PI) was 26.3 (22.8-37.7) and 35.6% (8.1%-81.3%), respectively. The median collected laboratory data were as follows : aspartate aminotransferase (AST), 40.5 U/l (23-145) ; alanine aminotransferase (ALT), 81.5 U/l (16-212) ; γ-glutamyltranspeptidase (γGTP), 42.5 U/l (1-81.3) ; total bilirubin (T-bil), 0.59 mg/dl (0.37-2.18) ; total protein (TP), 7.3 g/dl (6.8-8.0) ; albumin (ALB), 4.6 g/dl (3.9-5.0) ; platelet count (PLT), 273,500/ml (160,000-379,000) ; prothrombin time-international normalized ratio (PT-INR), 0.97 (0.8-1.16) ; low-density lipoprotein (LDL), 129 mg/dl (95-202) ; high-density lipoprotein (HDL), 44.5 mg/dl (29-64) ; triglyceride (TG), 159 mg/dl (62-533) ; fasting blood sugar (FBS), 96 mg/dl (82-133) ; insulin, 23.5 μU/ml (11.7-272.2) ; and homeostasis model assessment-insulin resistance (HOMA-R), 5.36 (2.07-67.7) (Table 1).

Body height (BH), body weight (BW), and abdominal circumference were also retrospectively investigated. The BMI and PI were calculated by the calculation formulas (BW)/(BH)^2 and (BW−standard body weight)/(standard body weight)×100, respectively. Laboratory data on AST, ALT, γGTP, TP, ALB, PT-INR, T-bil, PLT, LDL, HDL, TG, FBS, and HOMA-R were collected as indicators of liver and other metabolic functions. Diastolic/systolic blood pressure at rest was investigated.

Table 1 Clinical characteristics of patients

	Median (range)	Normal range	
Age	14 (6-16)		years
Body Height	0.98(−1.12-2.04)		SD
Body Weight	2.81 (0.46-5.54)		SD
BMI	26.3 (22.8-37.7)	18.5-25	
Ponderal Index	35.6 (8.1-81.3)	≤20	%
AST	40.5 (23-145)	14-30	U/l
ALT	81.5 (16-212)	9-35	U/l
GGTP	42.5 (14-124)	9-48	U/l
TP	7.3 (6.8-8.0)	6.3-7.8	g/dl
ALB	4.6 (3.9-5.0)	3.8-4.8	g/dl
PT-INR	0.97 (0.8-1.16)	1	
T-bil	0.59 (0.37-2.18)	0.3-1.3	mg/dl
PLT	273,500 (160,000-379,000)	150,000-400,000	/μl
LDL	129 (95-202)	≤110	mg/dl
HDL	44.5 (29-64)	>40	mg/dl
TG	159 (62-533)	24-227	mg/dl
FBS	96 (82-133)	60-110	mg/dl
Insulin	23.5 (11.7-272.2)	2-10	μU/ml
HOMA-R	5.36 (2.07-67.7)	<2	

Table 2 Criteria for metabolic syndrome

Patient number	Age (years)	Abdominal circumference (cm)	TG (mg/dl)	HDL (mg/dl)	SBP (mmHg)	DBP (mmHg)	FBS (mg/dl)
4	11	*87.4*	114	49	*130*	*90*	*104*
8	14	*108*	*318*	44	118	*81*	89
12	16	*78.5*	*167*	45	*130*	68	*103*
2	9	*78.1*	*146*	56	112	50	94
14	14	*107.7*	*205*	*29*	116	65	87

TG : triglyceride ; SBP : systolic blood pressure ; DBP : diastolic blood pressure
Upper 3 patients (denoted in bold characters) met criteria for MtS. Italics indicate where data met criteria for MtS.

When adequate data (abdominal circumference, TG, HDL, systolic and diastolic blood pressure, and FBS) were available, we also retrospectively investigated whether the patient met the diagnostic criteria for pediatric MtS as advocated by the Japanese Ministry of Health, Welfare and Labor in 2010[5]. The global criteria recommended by the International Diabetes Federation, which require the same dataset described above, were applied in one 16 year-old patient[6]. An adequate dataset was available in 5 patients, and 3 of these also met the criteria for MtS (Table 2).

Liver samples were collected by percutaneous liver biopsy. Hematoxylin-Eosin (HE) and Azan staining were performed. The diagnosis of NASH was performed by two pathologists. Scoring of Matteoni's criteria (type 1-4)[7], non-alcoholic fatty liver disease activity (NAS, 0-8) (which is subdivided into degree of steatosis, lobular inflammation, and hepatocyte ballooning[8]), inflammatory grading (grade 1-3), and fibrosis staging (stage 1-4) (in reference to Brunt *et al*.[9]) was performed by two pediatricians. Correlations between each score (Matteoni's criteria, NAS, Brunt's inflammatory grading, and fibrosis staging) and the laboratory data (AST, ALT, FBS, insulin, and HOMA-R) were evaluated by the Spearman's rank correlation coefficient test. The same liver samples were also stained with human intercellular adhesion molecule-1 (ICAM-1)/CD54 antibody (OriGene Technologies TA307840, Maryland, USA) diluted 100-fold. Two pediatricians classified them into 3 grades by extent of staining (grade 1, weak ; grade 2, moderate ; and grade 3, strong) (Fig. 1). Correlations between the ICAM-1 staining grade and above-mentioned other scores were analyzed by the Spearman's rank correlation coefficient test.

We performed cytokine/chemokine profiling of the liver from 4 patients whose sample volumes were adequate for profiling. Each sample (1 mm×1 mm×1 mm) was lysed with 200 µl cell lysis buffer. Interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFNγ), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), and TNFα were measured with the Bio-Plex Suspension Array System and 17-Plex Panel (Bio-Rad Laboratories, Tokyo, Japan).

For the assay of the SNP (c.308G>A of *TNF*α), peripheral blood samples were collected from 2 patients after obtaining written informed consent. DNA was extracted from blood leukocytes using Quiagen DNA Mini (QUIAGEN, Dusseldorf, Germany). PCR primers were purchased from Tsukuba Oligo Service (Ibaraki, Japan). The primers used were as follows : 5′-CTGAAGCCC CTCCCAGTTCT-3′ (sense) and 5′-CGGTTTCTTCTC-CATCGCG-3′ (anti-sense). The preparation consisted of 0.5 µl polymerase, 2.5 µl dNTP, 5 µl buffer, 2 µl DMSO, 37 µl diluted water, 0.5 µl primer, and 0.5 µl DNA sample. Amplifications were performed for 35 cycles at 95˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 1 min. The *TNF*α gene mutation c.308G>A was detected by Sanger sequencing using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, California, USA).

Results

All the requisite information for identification of MtS was collected from the medical records of 5 out of 12 patients. Three of them (patients 4, 8, and 12) met the criteria (Table 2). Metabolic syndrome was not diagnosed in patients 2 and 14 due to the data on HDL, systolic and diastolic blood pressure, and FBS.

According to the histological analysis, all except one were compatible with type 4 according to the Matteoni criteria. The remaining case was type 3. The median NAS score was 6 (3-8). The median scores were as follows : steatosis, 2.5 (1-3) ; lobular inflammation, 2 (1-3) ; and hepatocellular ballooning, 2 (1-3). The median Brunt's inflammatory grade was 2 (1-3). The median Brunt's fibrosis stage was 3 (1-3) (Table 3). No significant correlation between each score and the laboratory data was recognized. Five cases out of 12 were classified as grade 1, 2 as grade 2, and 5 as grade 3 with respect to ICAM-1 staining (Table 3). There were no

ICAM-1 Grade	Grade 1	Grade 2	Grade 3
Patient	3	11	1
Matteoni	4	4	4
NAS	6	7	7
Degree of steatosis	3	3	3
Lobular inflammation	1	2	2
Ballooning	2	2	2
Brunt's grade	2	2	3
Brunt's stage	3	3	2

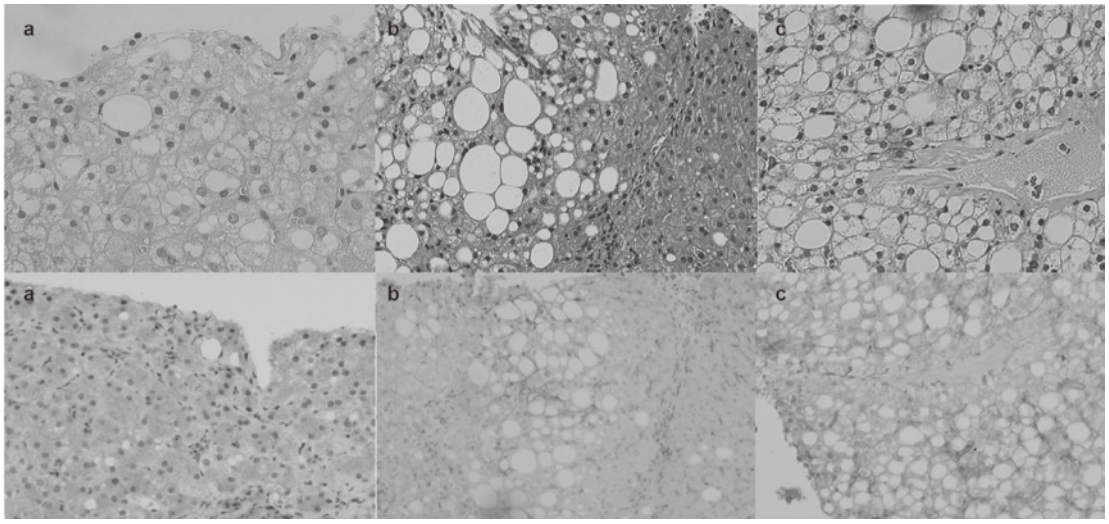

Fig 1 Grading of ICAM-1 staining (a : weak, b : medium, c : strong). Corresponding images of H.E. staining and each score are also shown.

Table 3 Comparison between ICAM-1 immunostaining grade and other scores

Patient number	Age	Matteoni	NAS				Brunt's grade	Brunt's stage	ICAM-1
			Degree of steatosis	Lobular inflammation	Ballooning	Total			
1	6	4	3	2	2	7	3	2	3
2	9	4	2	1	1	4	2	2	1
3	11	4	3	1	2	6	2	3	1
4	11	4	2	2	2	6	2	3	2
5	13	4	2	1	2	5	2	3	3
6	14	4	2	1	2	5	2	2	1
7	14	4	3	2	2	7	2	2	1
8	14	4	3	3	2	8	2	3	1
9	14	4	1	1	1	3	1	3	3
10	15	4	2	2	2	6	2	2	3
11	15	4	3	2	2	7	2	3	2
12	16	3	3	2	2	7	2	1	3
Median (range)	11 (6-16)	4 (3-4)	2.5 (1-3)	2 (1-3)	2 (1-2)	6 (3-8)	2 (1-3)	3 (1-3)	2 (1-3)

NAS : non-alcoholic fatty liver disease activity score ; ICAM-1 : intracellular adhesion molecule 1

significant correlation between the ICAM-1 staining grade and any other score. The correlation coefficients and *P*-values were −0.3303 and 0.587 (Matteoni's criteria), −0.09508 and 0.587 (NAS), 0 and 0.587 (Brunt's inflammatory grade), and −0.08827 and 0.587 (Brunt's fibrosis stage), respectively.

Cytokine/chemokine profiling was performed in the 4 cases shown in Table 4 (cases 3, 6, 10, and 11). The results are shown in Table 5. TNFα expression in patients 3 and 6 was 18.31 pg/ml and 16.91 pg/ml,

Table 4 Clinico-pathological characteristics of patient who underwent liver cytokine/chemokine profiling

Pt	Age (yo)	BH (SD)	BW (SD)	BMI	PI (%)	HOMA-R	Matteoni	NAS				Brunt's grade	Brunt's stage
								Degree of steatosis	Lobular inflammation	Ballooning	Total		
3	11	+0.35	+1.79	25.7	36.5	4.6	4	3	1	2	6	2	3
6	14	+2.04	+5.48	34.9	66.8	58.5	4	2	1	2	5	2	2
10	15	+1.68	+2.93	28.7	34.6	67.7	4	2	2	2	6	2	2
11	15	+1.12	+5.25	37.7	75	4.89	4	3	2	2	7	2	3

Pt : patient ; yo : years old ; BH : body height ; BW : body weight ; PI : Ponderal Index.

Table 5 Cytokine/chemokine profiling of liver

Pt	IL-1β	**IL-2**	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	**IL-12**	IL-13	IL-17	**G-CSF**	**GM-CSF**	IFN-γ	MCP-1	MIP-1β	**TNFα**
3	4.32	**N.D.**	0.17	0.03	0.59	1.53	0.85	0.56	**15.47**	1.45	3.39	**132.49**	**2.92**	5.71	8.38	34.06	**18.31**
6	0.51	**0.27**	0.19	0.04	2.27	1.73	0.9	1.54	**25.59**	3.37	7.61	**63.07**	**4.38**	6.67	13.62	45.05	**16.91**
10	0.58	**3.76**	0.11	0.16	0.67	1.46	0.61	0.45	**2.73**	0.95	5.08	**6.97**	**66.39**	6.45	3.57	39.51	**1.11**
11	0.44	**2.08**	0.05	N.D.	0.83	0.32	1.18	0.96	**0.46**	1.29	1.85	**26.22**	**34.57**	1.75	3.91	71.97	1.44

(pg/ml)

Pt : patient.

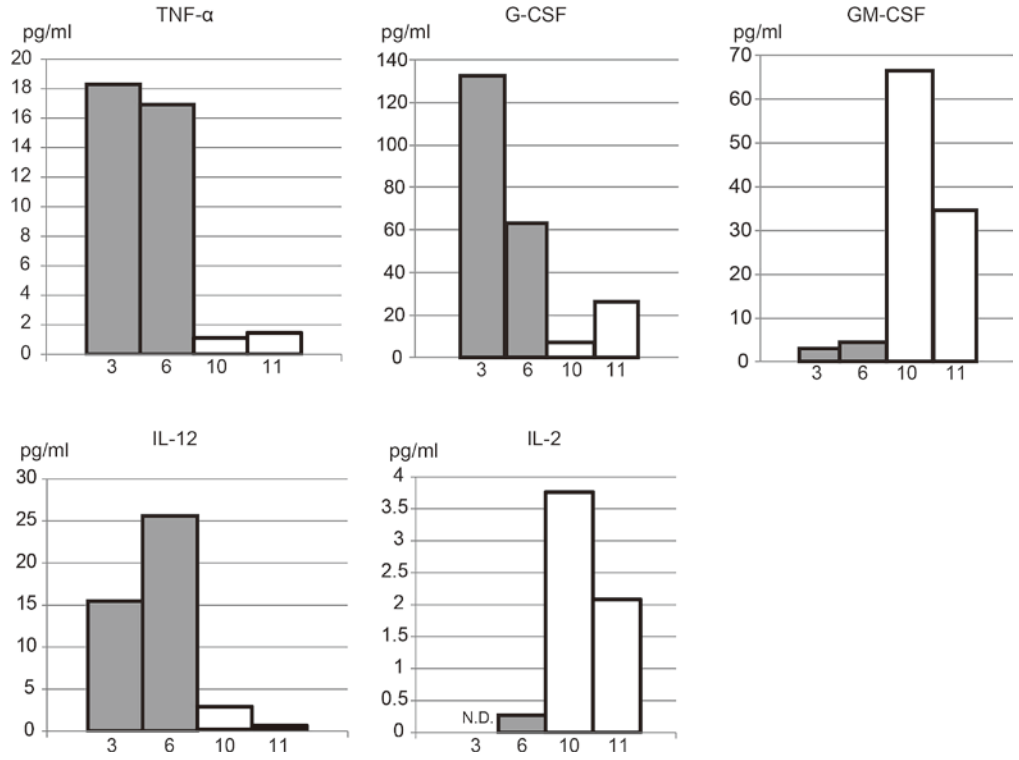

Fig 2 Cytokine/chemokine profiling between two groups (high TNFα group and low TNFα group). Black bar designates high TNFα group, whereas white bar designates low TNFα group. N.D., designated data not detected.

whereas that in patients 10 and 11 was 1.11 pg/ml and 1.44 pg/ml, respectively. Patients 3 and 6 were classified into a high TNFα group, and patients 10 and 11 into a low TNFα group (Fig. 2). Expression of G-CSF and IL-12 tended to be higher in the high TNFα group. In contrast, expression of GM-CSF and IL-2 was higher in the low-TNFα group.

No mutation was detected in the two patients assayed for the SNP (patients 11 and 12).

Discussion

Between 1980 and 2000, obesity rose among children in Japan ; the obese pediatric population increased two- to four-fold in these 20 years and accounted for 5-10% of children in each age group[10]. Metabolic syndrome, as identified by the World Health Organization in 1999, is a disorder that is induced by obesity, and it carries the risk of insulin-dependent diabetes mellitus and cardiac death[11]. Recently, MtS has been recognized as a chronic systemic inflammatory disorder characterized by persistent activation of the innate immune system[12)13]. The liver contains a large number of macrophages (Kupffer cells) and natural killer (NK) cells, and is the main innate immunocompetent organ[14]. Based on this function, steatohepatitis is to be regarded as a hepatic manifestation of MtS-related systemic inflammation.

In the present demographic study, NASH was observed in two patients that did not meet the criteria for MtS. There have been several reports investigating the relationship between the development of NAFLD and MtS criteria[15-17]. Fu *et al.* reported that among 861 Chinese obese children, only 37.64% of NAFLD cases met the MtS criteria, whereas NAFLD was diagnosed in 84.61% of MtS cases[17]. Tominaga *et al.* and Kelishadi *et al.* reported that the number of items which met the MtS criteria correlated with the odds ratio of NAFLD development[15)16]. The present results suggest that inflammation and fibrosis in pediatric NASH progress before the development of MtS. This indicates the need to perform a follow-up of liver function in obese children from an early stage.

Fibrosis has been reported to be recognized in 71%-87% of biopsies of pediatric NASH cases[3)18-21]. Carter-Kent *et al.* reported that the mean NAS was 4 and Brunt's fibrosis stage 20% in pediatric NASH cases[20)21]. In the present study, fibrosis was recognized in all cases and bridging fibrosis in 50%, while the other 50% of cases showed perisinusoidal fibrosis. All of our patients were classified into type 3 or 4 based on Matteoni's criteria, and the median NAS was 6, suggesting that histological changes and functional impairment occur earlier than in adults.

Chronic inflammation is a pathogenic feature of atherosclerosis. A pro-inflammatory state results in activation of NF-kappaB. NF-kappaB regulates the transcription of ICAM-1. Expression of NF-kappaB followed by ICAM-1 is increased by pro-inflammatory cytokines such as TNFα[22]. Therefore, we investigated expression of ICAM-1 on the basis that ICAM-1 can act as a marker of NASH liver. Contrary to our expectations, however, ICAM-1 expression did not correlate with the other histological scores.

The innate immune system, which is mainly considered to consist of Kupffer cells and TNFα, is considered an important factor in the "second hit" in NASH liver[23-25]. In studies using obese animal models, expression of TNFα was reported to increase in both serum and the liver. Increased TNFα contributes to the development of inflammation via activation of the Jun N-kinase and inhibitor kappa beta kinase beta/NF-κB pathway[26-29]. In the present study, expression of TNFα in two cases was relatively high, whereas in the other two cases it was low. The relationship between severity of NAFLD and expression of TNFα is controversial. There have been several reports both denying and confirming this relationship[23)30]. This discrepancy and the present findings may be explained by the following concepts. First, the half-life of TNFα is short *in vivo* ; second, the amount of circulating TNFα *in vivo* is exceedingly low ; third, differences in sensitivity of measurement methods ; and lastly, differences in selection of subject population[30]. IL-12 expression was also higher in the high-TNFα group. IL-12 is a pro-inflammatory cytokine produced by macrophages and dendritic cells, inducing functional differentiation of Th1 cells and NK cells[31]. On the other hand, IL-2 expression was higher in the low-TNFα group. IL-2 is mainly produced by Th0 cells and Th1 cells, activating cytotoxic T cells (CTLs), NK cells, and macrophages[32]. Solari *et al*. reported that CTL and Th1 cells mainly invaded immune cells in NASH liver[33], and the present findings are consistent with this report. In addition, the comparison here of the clinical characteristics and NASH scoring systems between the two groups revealed no apparent differences, except in patient age. High TNF group patients tended to be younger than the others (Table 5). These observations may be explained by the following hypothesis : 1) there are two unidentified immunological phenotypes in NASH inflammation related to patient age ; 2) we observed different stages of the disease, and expression of cytokine/chemokine varies with progression of stage. Because we could assay only 4 patients, a larger cohort is needed for a more precise assessment of these hypotheses. G-CSF expression was higher in the high TNFα group, whereas GM-CSF expression was lower than in the low TNFα group. Although both are hematopoietic factors, they have contrary effects on the functions of neutrophils and

monocytes to produce TNFα. G-CSF suppresses the production of TNFα, whereas GM-CSF increases it[34]. In addition, G-CSF stimulates the regeneration of liver tissue[35]. In the high-TNFα group, increasing expression of G-CSF may have resulted in feedback against higher expression of TNFα and liver injury. On the other hand, GM-CSF induces Th1 cell differentiation in addition to neutrophils and macrophages[36], suggesting that increased expression of GM-CSF might have contributed to Th1 cell-dominant inflammation in the low-TNFα group.

The human *TNF* gene is located on chromosome 6. An SNP, c.308G>A, has been reported previously[37]. Abraham *et al.* reported that the homozygote of this mutation induced an increase in TNFα expression[38]. Although we investigated this mutation in two of the present patients on the basis of these previous reports, no such mutation was detected. A larger population-based investigation is necessary to further elucidate the genetic factors involved in NASH.

Conclusions

Both pediatric NASH and fibrosis emerge before MtS has fully developed, suggesting that early onset NASH patients may develop liver failure and subsequent liver cell carcinoma earlier than adult onset cases. Variation in liver expression of cytokines/chemokines, including TNFα, IL-2, IL-12, G-CSF, and GM-CSF, suggests that this is extensively involved in the inflammation mechanism of NASH.

This study was limited by the small sample size. Further investigation and detailed statistical processing with a larger cohort are needed to further corroborate the present findings.

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小児期非アルコール性脂肪性肝炎の組織学的検討と サイトカイン・ケモカインプロファイルおよび一塩基多型の検討

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【要旨】

【目的】小児期 non-alcoholic steatohepatits（NASH）の臨床像、病理組織学的特徴、炎症の病態および遺伝学的背景を解明する。【対象と方法】当科で病理学的に診断した小児期 NASH12 例を対象とした。臨床所見や背景因子は診療録から後方視的に検討し、病理組織学的に Matteoni 分類、Non-alcoholic fatty liver disease activity score（NAS）、Brunt 分類により各症例を検討した。炎症の動態の検討として肝組織に対する ICAM-1 による免疫染色およびサイトカイン／ケモカインプロファイルを行った。さらにサンガー法により、NASH と関連が報告のある *TNF* 遺伝子上の単一遺伝子変異（c.308G>A）の検索も行った。【結果】症例は全例男児で、年齢の中央値は 14 歳だった。肥満度の中央値は 35.6% だった。血液検査上は肝逸脱酵素の上昇、脂質代謝異常、インスリン抵抗性の異常を認めた。病理組織学的分類の中央値はそれぞれ Matteoni 分類が 4、NAS は 6、Brunt 分類は grading が 2、staging が 3 だった。ICAM-1 の免疫染色による発現の程度と各分類との対応は乏しかった。サイトカイン／ケモカインプロファイルでは TNF-α が高値の群と低値の群に分かれ、TNF-α 高値群では低値群に比べ G-CSF、IL-12 が高値を示し、GM-CSF、IL-2 が低い傾向があった。今回遺伝子解析を行った 2 例では *TNF* 遺伝子上の対象とした変異は同定されなかった。【結語】今回検討した例は肥満と肝機能障害を認めた時点で肝生検を行った。その多くで線維化の進行が見られ、サイトカイン／ケモカインの動態は複雑に変化しながら NASH の進行に寄与している可能性が示唆された。また必ずしもメタボリックシンドロームの基準を満たさずに病変が進行している例もあり、未だ他の臨床データとの関連は不明のため、今後更なる症例の蓄積が望まれる。

〈キーワード〉ケモカイン、サイトカイン、小児、非アルコール性脂肪肝炎、病理診断