LIVER DISEASE

Chitotriosidase gene expression in Kupffer cells from patients with non-alcoholic fatty liver disease

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Background and aims: Non-alcoholic steatohepatitis (NASH) is a clinicopathological condition characterised by a necroinflammatory disorder with fatty infiltration of the hepatocytes. The molecular mechanisms involved in the anomalous behaviour of liver cells have only partially been determined. Human chitotriosidase (Chit) is a chitinolytic enzyme mainly produced by activated macrophages. The aim of this study was to investigate the expression of the chitinase-like gene in Kupffer cells, to determine how chitotriosidase may be implicated in the progression from uncomplicated steatosis to steatohepatitis with progressive fibrosis.

Methods: 75 subjects were studied: 40 with NASH, 20 with simple steatosis, and 15 normal controls. Kupffer cells obtained from liver biopsies were used to detect CHIT expression, superoxide anion (O_2^-), lipid peroxidation, and tumour necrosis factor α (TNF α) and ferritin levels.

Results: CHIT expression differed markedly in livers from normal controls and in those from patients with simple steatosis or non-alcoholic steatohepatitis. A significant correlation between mRNA CHIT and O_2^- , lipid peroxidation, TNF α , and ferritin levels was observed in both NASH and simple steatosis.

Conclusions: Human Kupffer cells in NASH patients overproduce chitotriosidase. At the highest levels of production, this enzyme may play a role in increasing the risk for a poor outcome in steatohepatitis.

Liver injury resulting from metabolic disorders is common and may lead to non-alcoholic steatohepatitis (NASH) in individuals with obesity, type 2 diabetes, dyslipidaemias, and other kinds of the insulin resistance syndrome.^{1–3} The term NASH refers to a histological pattern of injury which resembles alcoholic liver disease but occurs in the absence of alcohol abuse. The probability of developing advanced hepatic fibrosis is significantly greater in individuals with NASH than in those with simple steatosis.⁴ Two pathways of injury are implicated in the pathophysiological events: increased oxidative stress and lipid peroxidation associated with increased fat deposition in the liver,^{5,6} and tumour necrosis factor endotoxin mediated injury.⁷

Chitotriosidase (Chit) belongs to the family of glycosylhydrolases and is highly homologous to chitinases from lower organisms.^{8,9} The CHIT gene is located in chromosome 1q31–q32,¹⁰ consists of 12 exons, and spans about 20 kilobases of genomic DNA.⁸ This enzyme is selectively expressed in chronically activated tissue macrophages, like the lipid laden storage cells that accumulate in large quantities in the spleen, liver, and other tissues of Gaucher patients.¹¹ In some other inherited lysosomal storage disorders—especially sphingolipidoses such as Niemann Pick, GM1-gangliosidosis, and Krabbe disease, which involve accumulation of different lipids—more modest elevations in plasma Chit have been noted.¹² Tissue macrophages largely secrete newly synthesised 50 kDa Chit, but about one third is directly routed to lysosomes and proteolytically processed to the 39 kDa unit that remains catalytically active.¹³ Interestingly, one in every three individuals from various ethnic groups carries one abnormal CHIT gene with a 24 base pair (bp) duplication that prevents production of the enzyme.^{14,15} About 6% of the population are homozygous for this mutant allele and consequently completely lack Chit activity.¹⁵

Pathological tissue macrophages in several disease conditions massively express CHIT. A shared feature of such cells in the different conditions is the accumulation of lipid material in the lysosomal apparatus. Serum Chit activity is

significantly increased in individuals suffering from atherosclerotic disease and is related to the severity of the atherosclerotic lesion, suggesting a possible role as a marker of atherosclerosis.^{16–18} Increased Chit activities are also detectable in the plasma of patients with sarcoidosis,¹² thalassaemia,¹⁹ and malaria.²⁰

The liver is the major organ responsible for the uptake of oxidised low density lipoproteins (Ox-LDL) from the blood circulation, with Kupffer cells as the major cellular uptake site. It has been established that, when injected in vivo, acetyl lipoprotein (Ac-LDL) is rapidly cleared from the blood circulation by the liver.^{21–23} Upon injection into rats endothelial cells clear Ac-LDL preferentially, whereas Ox-LDL is concentrated mainly in Kupffer cells.²⁴ It has been observed that various scavenger receptors are involved in the avid interaction of Ox-LDL with the liver, and that a highly active receptor specific to Ox-LDL and responsible for the Kupffer cell mediated uptake is present in these cells.^{24,25} In all forms of steatohepatitis, lipid accumulation is associated with oxidative stress and lipid peroxidation, processes that cause hepatocellular injury.⁶

An improved understanding of NASH requires more precise characterisation of the proteins secreted in Kupffer cells that are involved in this pathological process following lipid accumulation. For this reason we examined the occurrence of mRNA levels of CHIT and its activity in Kupffer cells from patients with simple steatosis and NASH, and the relation between CHIT levels and superoxide anion (O_2^-) production, lipid peroxidation, and tumour necrosis factor α (TNF α) levels to determine whether induction of CHIT is involved in the inflammatory reactions occurring in NASH.

Abbreviations: Ac-LDL, acetyl lipoprotein; BMI, body mass index; Chit, chitotriosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, human hepatic stellate cell; NASH, non-alcoholic steatohepatitis; Ox-LDL, oxidised low density lipoproteins; O_2^- , superoxide anion; ROS, reactive oxygen species; TNF α , tumour necrosis factor α .

METHODS

Patients

Forty patients with NASH (19 male, 21 female, median age 53 years, range 28 to 75) and 20 with simple steatosis (12 male, 8 female, median age 45 years, range 30 to 63) belonging to the same ethnic group were entered into the study. As control, 15 specimens were obtained by resection during unrelated surgical procedures from subjects with normal liver function (8 male, 7 female, median age 45 years, range 35 to 60). The liver biopsies were obtained following intestinal by pass surgery, surgery for diverticular disease, and laparotomy for abdominal trauma.

The local ethics committee approved the study and informed written consent was obtained from all patients at the time of their liver biopsy. This study was conducted in conformance with the Helsinki Declaration.

Patients consuming more than 15 g of alcohol a day and those with evidence of liver cirrhosis or hepatocellular carcinoma were excluded. None of the patients had ingested drugs known to produce hepatic steatosis (including corticosteroids, oestrogens, methotrexate, tetracycline, calcium channel blockers, or amiodarone) in the previous 12 months. Five patients with NASH had a history of gastric bypass surgery; 15 other NASH patients had type 2 diabetes and 12 were obese. None of the patients with simple steatosis had received drug treatment.

A diagnosis of NASH was established on the basis of the following clinical and histopathological features:

- abnormal liver biochemistry for more than three months;
- liver biopsy showing steatosis (10%) in the presence of lobular or portal inflammation, or both, with or without Mallory bodies or fibrosis;
- exclusion of other diseases (viral hepatitis, autoimmune hepatitis, drug induced liver disease, primary biliary cirrhosis, biliary obstruction, haemochromatosis, Wilson's disease, and α 1-antitrypsin deficiency associated liver disease).

Simple steatosis was diagnosed by liver biopsy. Body mass index (BMI) was calculated using the formula: weight (kg)/height² (m). Diabetes mellitus was diagnosed if the patient was receiving oral hypoglycaemic drugs or insulin, had a random glucose level of <11 mmol/l (200 mg/dl), or a fasting

glucose level of greater than 7 mmol/l (126 mg/dl). Hyperlipidaemia was diagnosed if the cholesterol level was <5.7 mmol/l (220 mg/dl) or if the triglyceride level was <1.9 mmol/l (170 mg/dl), or both. Hypertension was diagnosed if the patient was on antihypertensive drug treatment or had a resting recumbent blood pressure of $\leq$ 140/90 mm Hg, or both.

The main demographic and clinical laboratory features of the patients with NASH and simple steatosis are compared in table 1. In the control subjects all the clinical variables were in the normal range. The tests were carried out the morning after a 12 hour overnight fast and assayed using the standard techniques. DNA analysis to select the subjects homozygous for the wild CHIT allele was carried out in all patients.

Histological analysis

Liver biopsies were divided and used for both routine histological examination and the determination of CHIT and TNF α mRNA levels, ferritin, O⁻₂, and lipid peroxidation. For the histological examination, sections were fixed in 10% buffered formalin, embedded in paraffin, and stained with haematoxylin and eosin, Masson's trichrome, reticulin silver stain, and Perls' Prussian Blue. A semiquantitative histological grading was used for the histological assessment of hepatic iron accumulation as follows: grade 0, no detectable iron; grade 1, iron granules seen at a magnification of 400 $\times$; grade 2, discrete iron granules seen at a magnification of 100 $\times$; grade 3, iron granules seen at a magnification of 25 $\times$; grade 4, masses of iron visible to the naked eye or at a magnification of 10 $\times$. The histological findings of NASH were interpreted using the classification proposed by Brunt *et al.*^{26,27} The activity of hepatitis (necroinflammatory grade) was determined by the presence of hepatocellular steatosis, ballooning, and inflammation (acinar and portal) features as follows: grade 1, mild; grade 2, moderate; grade 3, severe. The severity of hepatic fibrosis (stage) was defined as follows: stage 1, zone 3 perisinusoidal fibrosis; stage 2, zone 3 perisinusoidal fibrosis with portal fibrosis; stage 3, zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis. A single pathologist without knowledge of the patients' clinical or laboratory data carried out all grading and staging. A comparison of histological iron staining patterns between patients with NASH and those with simple steatosis is shown in table 2.

Table 1 Baseline characteristics of the population

Variable	Control (n = 15)	Simple steatosis (n = 20)	NASH (n = 40)
Age (years)	45 (35 to 60)	45 (30 to 63)	53 (28 to 75)
BMI (kg/m ²)	23.9 (20.4 to 26.2)	23.9 (20.8 to 29.6)	27.2 (22.4 to 36.6)
Obesity	None	7	12
Type II diabetes	None	1	15
Gastrointestinal surgery	15	1	5
Hyperlipidaemia	None	16	35
Hypertension	None	2	8
Haemoglobin (g/dl)	13.5 (11.0 to 14.8)	13.4 (11.2 to 15.6)	14.2 (10.5 to 15.6)
Serum ferritin (ng/ml)	157 (51 to 215)	178 (38 to 346)	351 (76 to 590)
Glucose (mmol/l)		5.38 (3.94 to 7.99)	6.38 (4.83 to 10.43)
AST (IU/l)	32 (15 to 34)	35 (21 to 81)	86 (20 to 169)
ALT (IU/l)	21 (8 to 33)	36 (19 to 141)	69 (28 to 250)
GGT (IU/l)	34 (3 to 52)	95 (22 to 367)	109 (30 to 536)
ChE (IU/l)	221 (208 to 425)	227 (127 to 489)	397 (63 to 567)
γ Globulin (g/dl)	1.41 (0.79 to 1.56)	1.12 (0.85 to 1.67)	1.27 (0.77 to 1.81)
Cholesterol (mmol/l)	4.69 (3.44 to 5.34)	5.49 (4.48 to 6.76)	6.24 (4.43 to 7.69)
Triglyceride (mmol/l)	1.53 (0.68 to 2.12)	1.90 (0.64 to 4.95)	3.37 (0.87 to 5.79)

Results are given as n (%) for qualitative data and median (range) for quantitative data.

Abbreviations and normal values: ALT, alanine aminotransferase, 6 to 33 IU/l; AST, aspartate aminotransferase, 12 to 35 IU/l; BMI, body mass index, 25 kg/m²; ChE, cholinesterase, 206 to 476 IU/l; cholesterol, 3.24 to 5.70 mmol/l; ferritin, 21 to 247 ng/ml; GGT, γ -glutamyl transpeptidase, 3 to 54 IU/l; γ globulin, 0.77–1.86 g/dl; glucose, 3.61 to 6.11 mg/dl; haemoglobin, 10.8 to 16.9 g/dl; triglyceride, 0.35 to 1.81 mmol/l.

Table 2 Comparison of histological iron staining patterns, NASH v simple steatosis

Iron staining	NASH (n = 40)	Simple steatosis (n = 20)
Grade 0	6	12
Grade 1	9	8
Grade 2	8	–
Grade 3	10	–
Grade 4	7	–

NASH, non-alcoholic steatohepatitis.

Isolation and culture of Kupffer cells, hepatocytes, and human hepatic stellate cells

Liver biopsies were incubated with pronase during continuous monitoring of pH. Human Kupffer cells (1.5×10^6 per gram liver) were separated from other non-parenchymal cells by Nycodenz gradient centrifugation and purified as described previously.²⁸ The purity of the cell cultures obtained was at least 95%, as estimated by the staining of Kupffer cells for endogenous peroxidase. Identification of Kupffer cells was achieved by immunophenotyping using monoclonal antibodies reacting with CD68, CD72, and myeloperoxidase (Sigma Chemical Co, Milan, Italy). Hepatocytes were isolated using a modified two step EGTA/collagenase perfusion procedure as described.²⁹ Human hepatic stellate cells (HSCs) were isolated by collagenase/pronase digestion according to published procedures.³⁰

Superoxide anion (O_2^-) production

The O_2^- production was assayed by the spectrophotometric measurement of ferricytochrome *c* reduction, as previously described,³¹ in Kupffer cells (3×10^5) plated on 24-well tissue culture plates.

Lipid peroxidation

Lipid peroxidation was determined by measuring the rate of production of TBARS (thiobarbituric acid reactive substances, expressed as malondialdehyde equivalents). One volume of lysate was mixed with 0.5 volume trichloroacetic acid (15% wt/vol) and centrifuged at $1000 \times g$ for 15 minutes at room temperature. Supernatant (1 ml) was mixed with 0.5 ml thiobarbituric acid (0.7% wt/vol) and boiled for 10 minutes.

After cooling, sample absorbance was read spectrophotometrically at 535 nm. The malondialdehyde levels were assayed for products of lipid peroxidation.³²

Quantitative real time polymerase chain reaction

Total RNA was extracted from Kupffer and hepatic cells using Trizol reagent (Invitrogen, San Diego, California, USA) according to the manufacturer's instructions. Real time fluorescence polymerase chain reaction (PCR), based on SYBR Green, was carried out in a 30 μ l final volume containing $1 \times$ SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA), 200 nM forward and 200 nM reverse

primers (table 3) and 20 ng of cDNA. Thermal cycling for each gene CHIT and TNF α was carried out in triplicate on cDNA samples in MicroAmp Optical 96-well reaction plate (Applied Biosystems) with MicroAmp optical caps (Applied Biosystems), using the ABI PRISM 7700 sequence detection system (Applied Biosystems). Amplification was carried out with the following conditions: 50°C for two minutes, 95°C for 10 minutes, and 50 cycles each of 95°C for 15 seconds and 60°C for one minute. The levels of CHIT and TNF α mRNA were normalised to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All data were captured using sequence detector software (Applied Biosystems).

Chit activity determination

For Chit assay, cells were harvested using cell lysis buffer (10 mM HEPES pH 7.9, 1 mM EDTA pH 8, 60 mM KCl, 1 mM PMSF, 0.5% NP40). The lysates were used for determination by a fluorimetric method.¹⁶ Chit activity was measured as previously described.³¹

Ferritin assay

For the ferritin assay cells were harvested using cell lysis buffer (10 mM HEPES pH 7.9, 1 mM EDTA pH 8, 60 mM KCl, 1 mM PMSF, 0.5% NP40). The lysates were used for determination of ferritin content with the use of the ferritin enzyme radioimmunoassay (ICN Biomedicals, Costa Mesa, California, USA). The results are expressed as ng ferritin/No of cells.

Statistical analysis

Data are expressed as mean (SD). Significance was assessed by one way analysis of variance (ANOVA) and Student's *t* test. For correlations we employed the SPSS-10 program.

RESULTS**Superoxide anion production**

O_2^- released by Kupffer cells was significantly raised in NASH patients (mean (SD), 25.2 (16.12) $mMO_2^-/60'$ /mg proteins; range 6.1 to 66.1; median 23.3) compared with those of the simple steatosis patients (2.91 (1.09); range 1.6 to 5.4; median 2.85) and with those of control subjects (1.24 (0.24); range 1.0 to 1.8; median 1.21) (table 4).

Lipid peroxidation levels

Lipid peroxidation levels were significantly raised in NASH patients (80.00 (26.53) μ mol/ml; range 26.9 to 137; median 86.5) compared with the values in the patients with simple steatosis (18.82 (7.05); range 9.2 to 36.9; median 18.45). The levels of lipid peroxidation were significantly raised in both NASH and steatosis compared with control (10.78 (3.39); range 5.3 to 17.6; median 10) (table 4). Lipid peroxidation levels in patients with NASH did not differ, regardless of history of obesity or the presence of type II diabetes or hyperlipidaemia. Examination of the association between lipid peroxidation and the extent of NASH showed that lipid

Table 3 Synthetic primers for reverse transcriptase polymerase chain reaction for gene verification

Target gene	Primer	Sequence 5'–3'	Fragment length
CHIT	Forward	CTGCATCAT GGTGCGGTC	210
	Reverse	CAAGGCAAGGCTGAGAGC	
TNF α	Forward	CCCCAGGGACCTCTCTAATC	97
	Reverse	GGTTTGCTACAACATGGGCTACA	
GAPDH	Forward	ACTCCCATTCCTCCACCTTT	144
	Reverse	TTACTCCTGGAGGCCATGT	

CHIT, chitotriosidase gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF α , tumour necrosis factor α .

Table 4 CHIT expression, O ⁻ ₂ , lipid peroxidation, TNFα, ferritin, and Chit activity in normal subjects and in simple steatosis and non-alcoholic liver disease						
Groups	CHIT (2 ^{-ΔΔct})	O ⁻ ₂ (mMO ⁻ ₂ /60' /mg proteins)	TBARS (μmol/ml)	TNFα (2 ^{-ΔΔct})	Ferritin (ng/ml)	Chit activity nmol/ml/h
Normal	1.08 (0.16)	1.24 (0.24)	10.78 (3.39)	1.11 (0.06)	16.76 (2.50)	12.17 (43.7)
Simple steatosis	2.22 (0.91)*	2.91 (1.09)*	18.82 (7.05)*	4.47 (1.78)*	30.53 (9.16)*	19.12 (9.54)
NASH	2.81 (24.71)*	25.2 (16.12)*	80.00 (26.53)*	75.19 (42.03)*	253.66 (156.94)*	12.17 (1.21)

Patients v healthy controls: *p<0.001.
Chit, chitotriosidase; CHIT, chitotriosidase gene; NASH, non-alcoholic steatohepatitis; O⁻₂, superoxide anion; TBARS, thiobarbituric acid reactive substances; TNFα, tumour necrosis factor α.

peroxidation levels differed significantly between the different grades of severity (fig 3C).

TNFα gene expression

To validate our results regarding Chit enzyme as an index of Kupffer cell activation, we determined the expression levels of TNFα released from activated Kupffer cells in all NASH patients, simple steatosis patients, and control subjects by quantitative real time PCR. The levels of TNFα expression were significantly higher in NASH patients (75.19 (42.03); range 19.4 to 171.9; median 70.45) than in steatosis patients (4.47 (1.78); range 2.00 to 8.05; median 4.3) and in the control group (1.11 (0.06); range 1.00 to 1.24; median 1.1) (table 4).

Ferritin levels

Ferritin levels in Kupffer cells lysates were significantly raised in NASH patients (253.66 (156.94) ng/ml; range 57 to 667; median 228.5), compared with simple steatosis (30.53 (9.16); range 18 to 47; median 27) and with those of the control subjects (16.76 (2.58); range 12.9 to 24.00; median 16.7) (table 4).

Chitotriosidase activity determination

We also examined the Chit activity in lysates of Kupffer cells and in plasma collected from NASH and steatosis patients and from control subjects. The levels of Chit activity in cell lysates were significantly higher in NASH patients (137.22 (57.64) nmol/ml/h; range 47 to 256; median 132.5) than in

Figure 1 Quantification of CHIT mRNA levels by real time polymerase chain reaction in (A) normal subjects and NASH patients, and (B) normal subjects and simple steatosis patients. mRNA levels are expressed as 2^{-ΔΔct} value.

steatosis patients (34.03 (13.61); range 19 to 58; median 27.45) or in the control group (18.24 (1.78); range 15.8 to 21.0; median 18.6). The plasma level of Chit activity was higher in NASH patients (118 (43.7) nmol/ml/h; range 33 to 197; median 126) than in steatosis patients (19.12 (9.54); range 19 to 27; median 15.8) or in the control group (12.17 (1.21); range 8.81 to 19.00; median 15.7) (table 4).

Correlation between CHIT expression liver histological findings, ferritin, O_2^- , lipid peroxidation, and TNF α levels in simple steatosis and NASH patients

The levels of CHIT expression were significantly higher in NASH patients (42.81 (24.71); range 6.45 to 92.1; median 41.05) than in steatosis patients (2.08 (0.81); range 1.05 to 3.70; median 2) or controls (1.08 (0.16); range 0.87 to 1.50; median 1.12) (fig 1, panels A and B) (table 4).

Positive associations were observed with CHIT and the degree of NASH as the CHIT expression tended to increase in parallel with the severity of NASH. In fact, the levels of CHIT were low in samples in which histological analysis indicated no detectable iron, and gradually increased as the iron granules became much more visible (fig 1A). NASH patients with iron grades of 0 or 1 tended to have higher CHIT expression levels than simple steatosis patients with the same iron grades (fig 1B).

A significant correlation between mRNA CHIT and O_2^- levels was observed in both NASH patients ($r = 0.964$,

$p < 0.0001$) (fig 2 C) and simple steatosis ($r = 0.93$, $p < 0.0001$) (fig 2B), whereas in control subjects correlation between mRNA CHIT and O_2^- levels was lower ($r = 0.088$; NS) (fig 2A).

The increase in lipid peroxidation correlates with CHIT induction in NASH ($r = 0.873$; $p < 0.0001$) and steatosis ($r = 0.812$; $p < 0.0001$) patients (fig 3, panels B and C). In control subjects correlation between mRNA CHIT and lipid peroxidation was $r = 0.130$ (NS) (fig 3A).

The expression of TNF α correlated with CHIT induction in NASH ($r = 0.978$; $p < 0.0001$) and simple steatosis ($r = 0.894$; $p < 0.0001$) (fig 4, panels B and C). In control subjects the correlation between CHIT and TNF α mRNA levels was not significant ($r = 0.36$) (fig 4A).

A significant correlation between CHIT mRNA and ferritin levels from cells lysates was observed in both NASH ($r = 0.963$, $p < 0.0001$) (fig 5C) and simple steatosis ($r = 0.872$, $p < 0.0001$) (fig 5B); in control subjects the correlation between mRNA CHIT and ferritin levels was not significant ($r = 0.034$) (fig 5A).

Correlations between Chit activity and O_2^- , lipid peroxidation, TNF α levels, and ferritin were similar to those observed between CHIT mRNA and the same variables in all groups examined (table 5). A significant correlation was also observed between plasma levels of ferritin and Chit activity in the NASH patients (table 6).

Figure 2 Correlations between CHIT and O_2^- levels: (A) Normal subjects. (B) Subjects with simple steatosis: grade 0 (grey triangles), grade 1 (grey squares). (C) NASH patients: grade 0 (grey triangles), grade 1 (grey squares), grade 2 (grey diamonds), grade 3 (grey circles), grade 4 (black triangles). Results in the different groups are expressed as mM O_2^- /60' / mg proteins.

Figure 3 Correlations between CHIT and lipid peroxidation levels: (A) Normal subjects. (B) Simple steatosis: grade 0 (grey triangles), grade 1 (grey squares). (C) NASH patients: grade 0 (grey triangles), grade 1 (grey squares), grade 2 (grey diamonds), grade 3 (grey circles), grade 4 (black triangles). Results in the different groups are expressed as TBARS (thiobarbituric acid reactive substances) formation in μ mol per No of cells.

Figure 4 Correlations between CHIT and TNF α mRNA expression levels. (A) Normal subjects. (B) Simple steatosis: grade 0 (grey triangles), grade 1 (grey squares). (C) NASH patients: grade 0 (grey triangles), grade 1 (grey squares), grade 2 (grey diamonds), grade 3 (grey circles), grade 4 (black triangles). Results in the different groups are expressed as a $2^{-\Delta\Delta Ct}$ value.

CHIT expression in hepatocytes and HSCs

To assess whether Kupffer cells were indeed the only hepatic cell type responsible for CHIT overexpression in diseased livers, we compared the CHIT gene expression profiles in hepatocytes and HSCs from NASH patients, simple steatosis patients, and control subjects. Figure 6 confirms that CHIT was expressed exclusively by Kupffer cells.

DISCUSSION

Our study provided evidence, for the first time, that CHIT expression in human Kupffer cells increases significantly in NASH steatohepatitis compared with simple steatosis. The increased levels of CHIT expression in Kupffer cells were related to O $_2^-$ production and to lipid peroxide levels.

In the evolution of steatohepatitis, three vicious cycles may impair the flow of electrons along the respiratory chain and thus increase the formation of mitochondrial reactive oxygen species (ROS). First, ROS may oxidise fat deposits, releasing

Figure 5 Correlation between CHIT and ferritin levels. (A) Normal subjects. grade 0 (grey triangles), grade 1 (grey squares). (C) NASH patients: grade 0 (grey triangles), grade 1 (grey squares), grade 2 (grey diamonds), grade 3 (grey circles), grade 4 (black triangles). Results in the different groups are expressed as nmol ferritin/No of cells.

lipid peroxidation products that damage mitochondrial DNA and proteins to partially block the flow of electrons along the respiratory chain. The increased mitochondrial ROS formation may further oxidise fat deposits to cause a positive feedback loop; this represents an important amplifying mechanism of the inflammatory response with more lipid peroxidation, more mitochondrial damage, and more ROS formation.³³ An added vicious cycle could involve the ROS mediated release of TNF α by hepatocytes and Kupffer cells.

A crucial event in the initiation of NASH seems to involve early lipid accumulation and lipid peroxidation in the hepatocytes, followed by Kupffer and HSC activation, liver cell injury, inflammation, and eventual evolution to hepatic fibrosis. These changes are identical to those seen in lipid associated disorders. Two interrelated processes that could be promoted as a result of liver injury are oxidative stress and the liberation of cytokines. Our findings suggest that Kupffer cells are important in the progression of NASH. Several mechanisms initiate liver cell damage and aggravate the

Table 5 Correlation between Chit activity and O $_2^-$, lipid peroxidation TNF α levels and Ferritin in cells lysates of normal subjects and of either simple steatosis and non-alcoholic liver disease				
Groups	Chit activity v O $_2^-$	Chit activity v lipid peroxidation	Chit activity v TNF α	Chit activity v ferritin
Normal	$r=0.261$ (NS)	$r=0.162$ (NS)	$r=0.521$ (NS)	$r=0.109$ (NS)
Simple steatosis	$r=0.906$ ($p<0.0001$)	$r=0.809$ ($p<0.0001$)	$r=0.862$ ($p<0.0001$)	$r=0.783$ ($p<0.0001$)
NASH	$r=0.932$ ($p<0.0001$)	$r=0.908$ ($p<0.0001$)	$r=0.936$ ($p<0.0001$)	$r=0.913$ ($p<0.0001$)

NASH, non-alcoholic steatohepatitis; TNF α , tumour necrosis factor α .

Table 6 Correlation between Chit activity and Ferritin in the plasma of normal subjects and of either simple steatosis and non-alcoholic liver disease

Groups	Chit activity v ferritin
Normal	$r=0.065$ (NS)
Simple steatosis	$r=0.472$ ($p<0.0001$)
NASH	$r=0.703$ ($p<0.0001$)

NASH, non-alcoholic steatohepatitis.

Figure 6 CHIT gene expression. Lane 1: Kupffer cells from control subject; lane 2: Kupffer cells from simple steatosis patient; lane 3: Kupffer cells from NASH patient; lane 4: hepatocytes from control subject; lane 5: hepatocytes from simple steatosis patient; lane 6: hepatocytes from NASH patient; lane 7, HSCs from control subjects; lane 8, HSCs from simple steatosis patients; lane 9: HSCs from NASH patients. NASH, non-alcoholic steatohepatitis; HSC, human hepatic stellate cell.

ongoing injury processes which activate Kupffer cells. Although responsible for removal of cell debris and part of the host defence system, under certain circumstances these inflammatory cells initiate additional liver injury. However, cell injury and death are also determined by factors such as an individual's gene expression profile, antioxidant status, and capacity for regeneration. As Kupffer cells possess scavenger receptors, they can be activated by exposure to products of lipid peroxidation.²⁴⁻³⁴ Furthermore, exposure to pro-oxidants or end products of lipid peroxidation activates the transcription of collagen genes.³⁵⁻³⁶

Recently, a vertebrate chitin synthase has been identified, which is supposed to create short chitin stretches that are essential to initiate hyaluronan synthesis.³⁷⁻³⁹ The glucosaminoglycan hyaluronan level is higher in the liver of NASH patients, and seems to be associated with liver fibrosis.⁴⁰ It has been hypothesised that Chit recognises hyaluronan (precursor) as a substrate and interferes with its synthesis, which could affect local hyaluronan concentrations and consequently influence the extent of cell migration in the injured hepatic tissue. We propose that chitotriosidase could be involved in the modulation of the extracellular matrix in hepatic tissue affecting cell adhesion and migration during the tissue remodelling processes that take place in fibrogenesis.

Kupffer cells are a source of proinflammatory cytokines, chemokines, and reactive oxygen species and could activate HSCs to synthesise collagen. Overproduction of collagen then leads to hepatic fibrosis and cirrhosis. Furthermore, Kupffer cells are the only source of CHIT expression, as we observed when carrying out RT-PCR analysis in hepatocytes and HSCs. Thus our results do not exclude a contribution of Chit release to activation by non-parenchymal cells, particularly as the biological effects of chitotriosidase are regulated by the release of cytokines. These findings are consistent with our earlier demonstration that the enzyme is selectively expressed and released upon specific immunological stimuli, namely by interferon γ , TNF α , and lipopolysaccharide by human macrophages, suggesting that Chit is not only a biochemical marker of macrophage activation but can also be

regarded as an important player in the inflammatory process.⁴¹ This evidence was confirmed by the finding that the augmentation of Chit activity followed the induction of O $_2^-$ release in human macrophages.³¹ On the other hand, ROS formation, lipid peroxidation, and TNF α production alone does not appear to be sufficient to bring about changes in CHIT level. In fact, the highest levels of CHIT were observed in patients with the highest degree of hepatic iron accumulation, as confirmed by the high correlation observed between CHIT and ferritin levels in both Kupffer cell lysates and plasma of NASH patients. Moreover, it is noteworthy that iron is a potent catalyst of lipid peroxidation and thus participates in hyperlipidaemia. It appears that the increase of CHIT in Kupffer cells may reflect iron mediated damage to lysosomes, suggesting that the physiological function of CHIT probably lies in regulating the acute phase response as an adaptive metabolic response to iron.

A preliminary study carried out in only a few patients with alcoholic steatohepatitis showed an increase in CHIT expression (data not shown). In view of the small number of specimens examined up to now, will be necessary to carry out further studies in other patient populations before this marker can be considered specific for NASH or other forms of chronic liver disease in which oxidative stress plays a major role.

Conclusions

It is conceivable that the Chit activity is also enhanced in other liver diseases, in which stimulation of Kupffer cells by portal vein endotoxin may cause the release of cytokines and chemokines, hepatocyte hypermetabolism, and activation of HSCs.⁴² In addition, it has been suggested that different genetic and environmental factors may influence the ability of individual subjects to overproduce CHIT in certain situations.¹⁴⁻¹⁵ Improved knowledge of the molecular mechanisms regulating CHIT gene transcription, including studies of gene promoter polymorphisms, could provide insight into the significance of CHIT overproduction in the Kupffer cells in NASH patients.

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