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Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Increased Hepatic Myeloperoxidase Activity in Obese Subjects with Nonalcoholic Steatohepatitis

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Inflammation and oxidative stress are considered critical factors in the progression of nonalcoholic fatty liver disease. Myeloperoxidase (MPO) is an important neutrophil enzyme that can generate aggressive oxidants; therefore, we studied the association between MPO and nonalcoholic fatty liver disease. The distribution of inflammatory cells containing MPO in liver biopsies of 40 severely obese subjects with either nonalcoholic steatohepatitis (NASH) (n = 22) or simple steatosis (n = 18) was investigated by immunohistochemistry. MPO-derived oxidative protein modifications were identified by immunohistochemistry and correlated to hepatic gene expression of CXC chemokines and M1/M2 macrophage markers as determined by quantitative PCR. MPO plasma levels were determined by ELISA. The number of hepatic neutrophils and MPO-positive Kupffer cells was increased in NASH and was accompanied by accumulation of hypochlorite-modified and nitrated proteins, which can be generated by the MPO-H_2O_2 system. Liver CXC chemokine expression was higher in patients with accumulation of MPO-mediated oxidation products and correlated with hepatic neutrophil sequestration. Plasma MPO levels were elevated in NASH patients. Interestingly, neutrophils frequently surrounded steatotic hepatocytes, resembling the crown-like structures found in obese adipose tissue. Furthermore, hepatic M2 macrophage marker gene expression was increased in NASH. Our data indicate that accumulation of MPO-mediated oxidation products, partly derived from Kupffer cell MPO, is associated with induction of CXC chemokines and hepatic neutrophil infiltration and may contribute to the development of NASH. ***(Am J Pathol 2009, 175:1473–1482; DOI: 10.2353/ajpath.2009.080999)***

The obesity-associated infiltration of neutrophils and macrophages into liver and adipose tissue is increasingly appreciated to be central to the development of chronic diseases such as nonalcoholic fatty liver disease (NAFLD) and insulin resistance.[1] In both liver and adipose tissue, increased levels of free fatty acids are thought to initiate the activation of inflammatory pathways that are responsible for attraction and activation of immune cells.[2,3] Furthermore, recruitment of neutrophils to the liver can be mediated by secretion of the CXC chemokines interleukin (IL)–8 and growth-related oncogene (GRO)-α.[4,5] The inflammatory processes in the liver have been shown to play a particularly important role in the progression of the benign steatotic stage of NAFLD to the more advanced stages of nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis.[6,7]

Next to inflammation, oxidative stress has been put forward as an important contributor to the progression of NAFLD.[8,9] For example, oxidative stress leading to lipid peroxidation induces the progression of steatosis toward necroinflammation or fibrosis in both animal models of steatohepatitis and humans with steatotic liver disorders.[10–13] Oxidative stress in the fatty liver can result from cytochrome P450 activity, peroxisomal β-oxidation, mitochondrial electron leak, as well as activities of recruited inflammatory cells.[14]

One of the principal molecules released after recruitment and activation of phagocytes is myeloperoxidase (MPO), an important enzyme involved in the generation of reactive

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oxygen species.[15] MPO is highly expressed by neutrophils and as such widely used as a neutrophil marker. In the presence of physiological chloride concentrations, MPO reacts with hydrogen peroxide (H_2O_2, formed by the respiratory burst) to catalyze formation of hypochlorous acid/hypochlorite ($HOCl/OCl^-$) and other oxidizing species.[15] These oxidants may contribute to host tissue damage at sites of inflammation through reactions with a wide range of biological substrates, including DNA, lipids, and protein amino groups.[16] In the absence of physiological chloride concentrations, the MPO-H_2O_2 system can also form reactive nitrogen species[17] that may initiate lipid peroxidation or form protein tyrosine residues, another posttranslational modification found in many pathological conditions.[18]

Besides its abundant presence in neutrophils, where MPO makes up ~5% of the total cell protein content, lower levels of MPO have been found in monocytes, certain macrophages,[19–21] and in a subpopulation of Kupffer cells.[22] The presence of MPO in these cells may reflect their inflammatory status, which was recently shown to be modulated in obesity. For example, anti-inflammatory M2 macrophages with tissue-remodeling capacities were largely absent in obese adipose tissue in mice, whereas accumulation of proinflammatory M1 macrophages was associated with obesity and insulin resistance.[23] Moreover, attenuation of the M2 differentiation program of Kupffer cells was associated with hepatic steatosis.[24] Interestingly, M1 macrophages are known to generate high amounts of reactive oxygen and nitrogen species.[25] Therefore, MPO-containing M1 macrophages/Kupffer cells could play a role in NAFLD.

To study the potential contribution of increased MPO activity to the progression of human NAFLD, we quantified inflammatory cells that express MPO and assessed hepatic accumulation of HOCl-modified proteins and nitrated proteins. Furthermore, we determined hepatic gene expression of IL-8 and GRO-α in relation to MPO-derived tissue damage and hepatic neutrophil sequestration to investigate a potential link between MPO activity and neutrophil accumulation. In addition, we analyzed for the first time hepatic gene expression of M1 and M2 macrophage phenotype markers in human NAFLD. Our results support an important role for Kupffer cell MPO-induced oxidative stress in the progression of NAFLD.

Materials and Methods

Liver Specimens

Human liver specimens were obtained during bariatric surgery from 40 severely obese patients (body mass index > 40). None of the patients had suffered from viral hepatitis or autoimmune-related disorders or reported excessive alcohol consumption (>20 g/day). Each liver specimen was divided into three pieces. One part was used for RNA isolation, another part was fixed in formalin, and a third part was embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and snap-frozen in 2-methylbutane. Biopsies were stained with H&E, periodic acid-Schiff with diastase, and Masson's trichrome and contained a sufficient number of portal tracts to allow correct evaluation of histological features by an experienced pathologist who was blinded to the clinical context of the biopsy and laboratory parameters. Subjects were divided into two groups. The steatosis group comprised patients showing >5% steatotic hepatocytes without significant inflammation as observed after H&E staining (no intra-acinar inflammatory foci per ×20 with a ×20 ocular and no portal tract inflammation). The NASH group showed >5% steatotic hepatocytes together with lobular inflammation and hepatocellular ballooning. Grading and staging of NASH severity were performed according to the criteria of Brunt.[26] Important patient characteristics are summarized in Table 1. This study was approved by the Medical Ethical Board of the Maastricht University Medical Centre in line with the ethical guidelines of the 1975 Declaration of Helsinki, and informed consent in writing was obtained from each subject.

Table 1. Clinical Characteristics of Patients Studied

	Steatosis	NASH
No. of patients	18	22
Sex (female/male)	13/5	16/6
Age (years)	42.8 ± 2.3	43.6 ± 2.0 ($P = 0.55$)
BMI (kg/m^2)	46.7 ± 1.4	50.0 ± 2.2 ($P = 0.68$)
Fasting glucose (mmol/L)	6.1 ± 0.3	6.2 ± 0.4 ($P = 0.95$)
ALT (IU)	22.5 ± 3.0	50.3 ± 11.0 ($P < 0.01$)
AST (IU)	18.3 ± 2.2	40.4 ± 6.4 ($P < 0.01$)

NASH, nonalcoholic steatohepatitis; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Immunohistochemistry

Formalin-fixed, paraffin-embedded liver biopsies were cut into 5-μm-thick tissue sections, deparaffinized, and rehydrated. Endogenous peroxidase activity was blocked by incubation in 0.6% H_2O_2 for 15 minutes. Primary antibodies were applied for 1 hour after blocking with 10% serum. The following murine monoclonal antibodies were used as primary antibodies: (i) anti-CD68 (clone Kp1, dilution 1/400; Dako, Glostrup, Denmark) specific for monocytes/macrophages; (ii) anti-human neutrophil peptide 1-3 (HNP1-3, clone D21, dilution 1:500; Hycult Biotechnology, Uden, The Netherlands) specific for neutrophils; (iii) anti-α-smooth muscle actin (clone 1A4, dilution 1:200; Sigma-Aldrich, Zwijndrecht, The Netherlands) specific for activated stellate cells; (iv) anti-HOCl-modified proteins (clone 2D10G9,[27] dilution 1:10); and (v) anti-nitrotyrosine (clone HM11,[28] dilution 1:50; Hycult Biotechnology). Polyclonal rabbit anti-human MPO antiserum (dilution 1:1000; Dako) was used as primary antibody to identify MPO. Horseradish peroxidase-labeled goat-anti-rabbit IgG (1:500; Jackson ImmunoResearch, Suffolk, UK) was applied as secondary antibody in the MPO staining and horseradish peroxidase-labeled goat-anti-mouse IgG (1:1000; Invitrogen, Carlsbad, CA) was applied during the HNP1-3, α-smooth muscle actin, and nitrotyrosine staining procedure. For CD68 and HOCl-modified protein stainings, endogenous biotin was blocked before primary antibody incubation with a biotin blocking system (Dako),

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