REVIEW

The Role of Cytokines in Non-Alcoholic Steatohepatitis. A Systematic Review

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Abstract

The pathogenesis of NASH is being unraveled by studies of animal models and humans with this disorder. The necro-inflammatory component of NASH appears to be modulated by interactions among various factors (for example cytokines, hormones, neurotransmitters) that regulate the biological activity of TNF-α and other proinflammatory (Th-1) cytokines. Hepatic necroinflammation is necessary, but not sufficient, for progression to cirrhosis. Factors such as leptin inducible factors (for example, noradrenaline), that regulate the activity of profibrogenic cytokines, such as IL-10 and TGF-β, dictate the extent of fibrosis that occurs during liver injury. A better understanding of how these and other soluble and cell associated factors regulate the phenotypes of different types of liver cells should help us to develop rationale treatments for NASH and other disorders in the metabolic syndrome.

Key words

NASH - cytokines - TNFα - liver fibrosis - metabolic syndrome

Introduction

Non-alcoholic fatty liver disease (NAFLD) and its more severe form non-alcoholic steatohepatitis (NASH) are associated with several diseases (obesity, type 2 diabetes, dyslipidaemia and hypertension), having insulin resistance as the common factor. These conditions cluster to form the 'insulin resistance syndrome' or, according to a recent proposal, the 'metabolic syndrome', carrying a high risk for cardiovascular complications. NASH itself, as well as pure fatty liver, is an insulin-resistant state, not only in subjects with additional metabolic disorders, but also in lean normoglycaemic patients. The prevalence of the metabolic syndrome, according to well-defined criteria, is higher in NAFLD patients compared with the general population. NAFLD patients with metabolic syndrome have a higher prevalence and severity of fibrosis and necroinflammatory activity, compared to subjects with pure fatty liver. The presence of the metabolic syndrome is associated with a high risk of NASH among NAFLD subjects, after correction for gender, age and body mass. In particular, it is associated with a high risk of severe fibrosis. The increasing prevalence of obesity, coupled with diabetes, dyslipidaemia, hypertension and metabolic syndrome puts a very large population at risk for succumbing to liver failure in the next decades. Due to the fact that treatment with lifestyle interventions has proven effective in the metabolic syndrome, studies with similar lifestyle interventions in NASH are eagerly awaited.

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Definition of the metabolic syndrome

The clustering of metabolic disorders had been known for a long time before Avogaro (1) first reported the association of obesity, hyperlipidaemia and diabetes in 1967. Hypertension is also frequently present. These features are independently related to cardiovascular mortality, which has given rise to the name of 'deadly quartet' for this syndrome.

In 1988, Gerald Reaven proposed the term 'X syndrome' (2) to define the contemporary presence of diabetes and/or impaired glucose tolerance, hypertriglyceridaemia, low HDL-cholesterol and hypertension. He pointed out the role of hyperinsulinaemia and insulin resistance in the pathogenesis of the disease. The metabolic disorder is probably much wider and other features might be added. Most subjects have evidence of additional metabolic disorders (elevated urate concentrations, impaired fibrinolysis and endothelial dysfunction).

The primary role of hyperinsulinemia is supported by several cross-sectional and longitudinal studies. Central obesity, type 2 diabetes, hyperlipidemia and hypertension are all characterized by raised insulin levels, that predict the

development of the metabolic disorder. Accordingly, DeFronzo and Ferrannini proposed the term 'insulin resistance' to define this clustering of diseases.

The borders of the syndrome are still difficult to define. The critical number of metabolic disorders to define the syndrome has not been specified; the disorders may progressively develop over the course of time, with obesity usually occurring first, followed by hyperlipidemia and diabetes. Hypertension may frequently be present independently from other components. In addition, the 'normal' limits for the individual disorders have been repeatedly changed in the last few years, so as to prevent a clear-cut assessment.

The first attempt to define the metabolic syndrome came from the World Health Organization (WHO) (3). The expert committee setting new criteria for the definition of diabetes proposed a classification based on the presence of one out of two necessary conditions (altered glucose regulation and insulin resistance), coupled with two additional features (Table I). These criteria may be easily applied to diabetic populations, but are not useful in a general setting. The assessment of insulin resistance requires complex techniques. Surrogate markers (fasting insulin, HOMA values), although validated by correlation analysis, have no defined 'normal' limits (4).

Table I Comparison of different diagnostic criteria for the metabolic syndrome

WHO proposal (1998, revised 1999)
Altered glucose regulation
or
insulin resistance
plus
two of the following:
1 Obesity (BMI ≥ 30 kg/m^2 or WHR > 1.0 [M] or > 0.9 [F]
2 High triglycerides (> 150 mg/dL) or low HDL-cholesterol (< 35 mg/dL [M] or < 39 mg/dL [F])
3 Hypertension (≥ 140/90 mmHg)
4 Microalbuminuria (≥ 30 μg/min)

ATP III proposal (2001)
Three of the following:
1 Waist girth (> 102 cm [M] or > 88 cm [F])
2 Arterial pressure (≥ 130/85 mmHg)
3 Triglycerides (≥ 150 mg/dL)
4 HDL-cholesterol (< 40 mg/dL [M] or <50 mg/dL [F])
5 Glucose (> 110 mg/dL)

AHA/NHLBI Scientific Statement (2005)
Any 3 of 5 constitute diagnosis of metabolic syndrome:
1. Elevated waist circumference
   - ≥102 cm (≥40 inches) in men
   - ≥88 cm (≥35 inches) in women
2. Elevated triglycerides
   - ≥150 mg/dl (1.7 mmol/l), or
   - on drug treatment for elevated triglycerides
3. Reduced HDL-C
   - <40 mg/dl (1.03 mmol/l) in men
   - <50 mg/dl (1.3 mmol/l) in women, or
   - on drug treatment for reduced HDL-C
4. Elevated blood pressure
   - ≥130 mmHg systolic blood pressure, or
   - ≥85 mmHg diastolic blood pressure, or
   - on antihypertensive drug treatment in a patient with a history of hypertension
5. Elevated fasting glucose
   - ≥100 mg/dl, or
   - on drug treatment for elevated glucose

BMI: body mass index; F: female; HDL: high-density lipoprotein; M: male; WHR: waist:hip ratio

New criteria were defined by the European Group for Insulin Resistance in 1999, limiting the syndrome to non-diabetic subjects, but the critical problem of insulin resistance was not set.

In 2001 a new proposal by the Third Report of The National Cholesterol Education Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III, ATPIII) provided a working definition of the metabolic syndrome, based on a combination of five categories and discrete risk factors, which can easily be measured in clinical practice, and are suitable for epidemiological purposes. The limits for individual components (central obesity, hypertension, hypertriglyceridemia, low HDL-cholesterol and hyperglycemia) are derived from the guidelines of the international societies or the statements of WHO. It is important to note that the anthropometric criteria vary between ethnic groups, with values being substantially lower among Asians (5).

NASH as part of the metabolic syndrome

A very recent study including a large number of NAFLD patients was specifically aimed at assessing the prevalence of the metabolic syndrome in relation to liver histology. In 304 consecutive NAFLD patients without overt diabetes, Marchesini (5) defined the metabolic syndrome according to the ATPIII proposal. The population had a mean age of 41 years and a BMI of 27.5, but nearly 80% were overweight or obese. Over 80% were males. At least one criterion for the metabolic syndrome was present in 96% of females and 83% of males, and three criteria were fulfilled in 60% of females and 30% of males. The prevalence of the metabolic syndrome increased with increasing BMI, from 18% in normal-weight subjects to 67% in obese subjects (6).

The presence of the metabolic syndrome was significantly associated with female gender (OR, 3.08; 95% CI, 1.57-6.02) and age (OR, 1.54; 1.23-1.93 per 10 years) after adjustment for BMI class. The presence of impaired fasting glucose (blood glucose = 110 mg/dL) was the most predictive criterion for the metabolic syndrome (OR, 18.9; 6.8-52.7) also in the non-diabetic population. Insulin resistance (HOMA method) was significantly associated with the metabolic syndrome (OR, 2.5; 1.5-4.2; $p<0.001$) (7,8).

Recently, the threshold for IFG was reduced from 110 to 100 mg/dl; this adjustment corresponds to the recently modified American Diabetes Association criteria for IFG.

Obesity involves the accumulation of fat not only in adipocytes, but also in muscle cells, and this accumulation can cause insulin resistance in adipocytes and muscles.

After meals insulin acts on its receptor on the surface of adipocytes and miocytes to trigger the phosphorylation of

insulin receptor substrates (IRS) which activate the translocation of GLUT-4 glucose transporters from intracellular storage vesicles to the plasma membrane. In obese people, adipocytes may produce less GLUT-4 transporter. Both fat-engorged adipocytes and fat-laden miocytes are resistant to the signalling effects of the insulin receptor. The mechanism could involve the activation of Jun-N-terminal Kinase (JNK) and, hence, the serine phosphorylation and thus inactivation of IRS and activation of IKKb (9).

The adipocyte as a source and target for inflammation

The adipocyte is a remarkable cell type in several respects. It stores excess energy in the form of lipids and is thus able to dramatically change its size in accordance with changing metabolic needs. This ability gives adipose tissue an almost unlimited capacity for growth, making it perhaps the only tissue in the body with the ability to so drastically increase its size without an underlying transformed cellular phenotype. Adipose tissue is responsive to both central and peripheral metabolic signals and is itself capable of secreting a number of proteins. These adipocyte-specific or enriched proteins, termed adipokines, have been shown to have a variety of local, peripheral, and central effects that will be discussed below. Adipose tissue is therefore able to integrate signals from other organs and respond by regulating secretion of multiple proteins. As an active participant in whole body energy homeostasis, adipose tissue can negatively influence other systems when dysregulated. Although adipocytes are capable of increasing in size, the cellular homeostasis and the secretory profile of larger adipocytes become altered and increasingly dysregulated compared with adipocytes of smaller size (10).

Leptin

Leptin is a highly conserved 16-kDa hormone that is predominantly expressed in adipose tissue and is found both in circulation and cerebrospinal fluid. Circulating leptin levels are positively correlated with body mass index (BMI) with concentrations in human serum at approximately 1-10 ng/ml (11).

Centrally, it is capable of altering food intake, body weight, energy expenditure, and neuroendocrine function, whereas it also has peripheral effects on skeletal muscle, liver, pancreas, adipose tissue, and numerous other cell types (12).

A recently reported mechanism is based on the observation that leptin is capable of activating 5'-AMP-activated protein kinase (AMPK) in muscle and liver both by acting directly on these tissues and by acting centrally through the central nervous system (13).

Adiponectin

Adiponectin is a 30-kDa adipose-specific secreted protein that circulates in human serum at 5-30 nm concentrations, with circulating levels approximately two to three times higher in females than in males.

An analysis of obesity-prone rhesus monkeys that often progress to type 2 diabetes examined the plasma concentration of adiponectin longitudinally (14). A decrease in circulating FL-Ad was seen with increasing BMI (15). This decrease in FL-Ad strongly correlated with the concomitant decrease in insulin sensitivity. Although the relationship between insulin action and adiponectin plasma levels is independent of body adiposity, the levels of FL-Ad are almost always decreased in obesity (16,17). Spranger et al have recently shown that baseline plasma adiponectin levels in apparently healthy individuals are independently associated with future risk for the development of type 2 diabetes (18).

Further genetic studies examined whether polymorphisms in the locus for adiponectin, 3q27, could affect the circulating levels of adiponectin and whether these polymorphisms were associated with increased risk for the development of type 2 diabetes. The results of one study showed evidence of linkage with metabolic syndrome (19), whereas another showed evidence of a type 2 diabetes susceptibility locus at 3q27-qter in a French population with early-onset diabetes. Polymorphisms within the adiponectin locus were also linked with increased risk for type 2 diabetes in a Japanese cohort (20).

Studies link hypoadiponectinemia with NAFLD, in particular with necro-inflammatory NASH. Additional studies indicate that in NASH (but not in steatosis) local effects of adiponectin are limited through two different mechanisms: decreased adiponectin mRNA and decreased hepatic adipoR2 mRNA expression (21). Furthermore, hypoadiponectinemia is present before overt diabetes and obesity appear and correlates with the severity of liver histology in NASH, thus indicating its pathogenic role in beta-cell dysfunction and hepatic necro-inflammation and fibrosis, independent of IR, visceral fat accumulation, TNFα and dietary habits (22).

Resistin

Resistin is a 10-kDa adipose tissue-specific hormone that was recently identified in a screen designed to enrich for transcripts that were up-regulated during adipogenesis but decreased with PPARγ agonist treatment (23). Injection of resistin into wild-type mice resulted in reduced glucose tolerance and insulin action, whereas injection of neutralizing antibodies into diabetic obese mice improved insulin action (23). When resistin was infused at near physiological levels in the presence of physiologically high circulating insulin, lower rates of glucose infusion were necessary to maintain basal glucose levels. The insulin resistance caused by resistin infusion was completely attributed to an increase in the rate of glucose production and not to an increase in glucose uptake. This indicates that resistin has a clear and rapid effect on hepatic, but not peripheral, insulin sensitivity (24).

Acylation-stimulating protein (ASP)

ASP is produced by a two-step process involving three proteins of the alternate complement system: C3, factor B, and adipsin. All three of these precursor proteins are produced and secreted by adipocytes (25). ASP increases lipogenesis locally in adipocytes and inhibits hormone-sensitive lipase-mediated lipolysis. Mice lacking complement factor C3 (and therefore deficient in ASP) display greater caloric intake with normal fat absorption but are significantly leaner (26). These mice are therefore resistant to diet-induced weight gain and display increased postprandial free fatty acid levels. ASP levels are elevated in obese humans and decrease after fasting or weight loss (27). The rationale for studying this adipokine in NASH is supported by many facts: the stimulatory effects of ASP on TG synthesis are independent of and complementary to those of insulin; insulin increases the production of ASP precursor C3 in adipocytes; all factors needed for ASP production are present in the liver and the production of two of them may be increased by another cytokine, IL6, which is also increased in patients with NASH (28).

Obesity is associated with an increase in TNFα production in adipose tissue. The locally elevated TNFα directly interferes with proper insulin signal transduction through specific phosphorylation of critical serine residues in the insulin receptor and insulin receptor substrate-1, thereby leading to a local desensitization to insulin signaling (29). In addition to local increases in TNFα, a systemic increase in inflammatory markers has been shown to be associated with obesity. C-reactive protein (CRP) is an unspecific acute phase reactant that serves as an excellent indicator of systemic inflammation. Insulin resistance is not only associated with a significant increase in CRP, but a whole host of additional acute phase reactants that are elevated as well. Many of these additional factors including IL-6, α1 acid glycoprotein, and serum amyloid A (SAA) are expressed in adipose tissue (30). All of these proteins (with the exception of CRP) are up-regulated in adipose tissue in the insulin-resistant state. Increased serum IL-6 is predictive of future cardiovascular problems. SAA can effectively compete for binding of apolipoprotein A-I on high-density lipoprotein particles, thereby altering trafficking of these particles.

Additional acute phase reactants produced in adipocytes include the pentraxin family member PTX-3, which is closely related to CRP, as well as the lipocalin 24p3, whose roles in the innate immune response and as an iron binding protein have recently been established (31). Additionally, ceruloplasmin and macrophage migration inhibitory factor have also been identified as secretory products of adipocytes, albeit it is not known whether expression of these proteins is altered with the development of insulin resistance (32). Interestingly, the antiinflammatory factor IL-1 receptor antagonist (IL-1Ra) is also expressed in adipose tissue where it is significantly up-regulated in obesity, concomitant with an increase in systemic IL-1 receptor antagonist levels (33).

It is technically difficult to gauge the relative contribution of adipocytes to the systemic levels of these proteins in any given metabolic state. However, in a direct comparison, adipocytes have a proinflammatory potential equal or superior to that of macrophages with respect to a subset of inflammatory markers (34). Combined with the highly significant biomass that adipocytes can contribute to whole body weight, particularly in obese individuals, there is little doubt that the systemic contribution of adipose tissue is significant. As an example, increased systemic TNFα was seen in the recently described adiponectin knockout mouse with adipose tissue being the only tissue examined with a significant increase in TNFα expression. This demonstrates that an adipose-specific increase in an inflammatory cytokine was capable of translating into a significant systemic increase in concentration.

Recent reports described the existence of individuals with normal body weight but with a cluster of obesity-related characteristics. They are characterized by excess visceral fat, insulin resistance, and hyperinsulinemia and have been called metabolically obese, normal weight (MONW) subjects. Subjects with BMI < 25 kg/m^2 and increased visceral fat areas > 100 cm^2 fulfill the criteria for categorizing them in the MONW group (35). Regarding the association of the MONW state with diabetes, higher prevalence of hyperglycemia has been observed in MONW subjects than in normal individuals.

Previous studies have demonstrated that visceral fat areas are associated with insulin resistance in Japanese subjects with normal glucose tolerance and impaired glucose tolerance and in nonobese Japanese patients with type 2 diabetes. Visceral fat accumulation is also associated with serum triglyceride (TG) levels, and the disturbance of TG metabolism precedes the development of insulin resistance in nonobese type 2 diabetic patients (36).

Cytokines and NASH

Because the histopathology of NASH resembles that of alcohol-induced steatohepatitis (ASH), common pathogenic mechanisms may mediate both of these diseases. Immunological mechanisms have a pivotal role in the pathogenesis of ASH. This has been remarkably well demonstrated by studies of patients and experimental animals. In hospitalized patients with severe ASH, serum levels of several pro-inflammatory cytokines, including TNF-α, are increased significantly (37,38). Cytokine levels correlate well with liver disease severity, generally decreasing in those who recover but remaining elevated in those who do not. These seminal observations stimulated subsequent studies in small animal models for ASH to determine if inflammatory cytokines directly mediate alcohol-related hepatotoxicity. The results strongly support this concept. For example, various therapies that inhibit gut-derived lipopolysaccharide (LPS) endotoxaemia or that block the activity of TNF-α, an LPS-induced cytokine, provide mice and rats nearly complete protection from ASH (39).

This chapter reviews human and animal data concerning potential involvement of inflammatory cytokines and intestinal endotoxins in the pathogenesis of NASH.

NASH is strongly associated with obesity in humans, mice and rats (40,41). Once considered to be a relatively inert storage depot for fat, adipose tissue is now known to produce many different hormones and cytokines, including TNF-α (42). Thus, the increased adipose tissue mass of obese individuals provides a major source of serum TNF-α. Recent evidence suggests that resident immune cells in organs such as the liver may also contribute to obesity-related increases in proinflammatory cytokine production. Table II summarizes the human data supporting the role of TNF in the pathogenesis of NAFLD.

Table II Evidence supporting the pathophysiological role of tumour necrosis factor (TNF) in non-alcoholic fatty liver disease (NAFLD)

Serum TNF levels increased in obese and NASH patients
TNF gene expression is increased in adipose and liver tissue of obese patients
TNF mRNA expression higher in NASH patients with hepatic fibrosis
Increased prevalence of TNF polymorphisms in NAFLD patients
Liver histology similar between NASH and alcoholic steatohepatitis (ASH), a disease in which TNF is known to be an important pathophysiological factor.

Increased TNF-α in obese patients with NASH

While it is widely acknowledged that TNF-α expression increases in obesity (42), the mechanisms driving chronic overproduction of TNF-α in obese humans remain obscure. However, the resultant chronic inflammatory state has been implicated in the pathogenesis of the metabolic syndrome that often accompanies obesity. The robustness of the relationship between chronic inflammation and end-organ damage in individuals with the metabolic syndrome is illustrated by recent recommendations that serum C-reactive protein levels should be tested to assess risk for adverse cardiovascular events. Increased TNF-α production has also been described in obese patients with NASH. A recent Spanish study correlated liver disease severity with TNF-α gene expression in adipose and liver tissues of 52 obese patients (43). TNF-α mRNA was overexpressed in adipose tissues and livers of patients with NASH, and was higher in patients with than in those without significant hepatic fibrosis. Another study of 99 Italian patients with NAFLD noted an increased prevalence of TNF-α polymorphism in subjects with NAFLD compared with controls (44). In that study, subjects who had the TNF-α polymorphism had greater insulin resistance, and were more likely to exhibit glucose intolerance. In addition, they were less likely to have other risk factors for hepatic steatosis. Thus, the authors concluded that TNF-α polymorphism represents a susceptibility genotype for insulin resistance, NAFLD and NASH.

In mice and rats with ASH, products of intestinal bacteria, particularly LPS endotoxin, are key inducers of hepatic TNF-α expression. Similar mechanisms appear to contribute to hepatic insulin resistance and NASH in at least one murine model for NAFLD. However, whether or not intestinal flora promotes pro-inflammatory cytokine production, insulin resistance and/or NASH in obese patients is uncertain. Wigg (45) investigated the prevalence of small intestinal bacterial overgrowth, increased intestinal permeability, elevated endotoxin and TNF-α levels in 22 patients with NASH and 23 control subjects. Patients with NASH were more than twice as likely as controls to have small intestinal bacterial overgrowth. Serum TNF-α levels were also approximately two-fold greater in the NASH group than controls. However, intestinal permeability (as measured by a dual lactulose-rhamnose sugar test) and serum endotoxin levels (as measured by the limulus lysate assay) were similar in the two groups. Hence, the authors concluded that intestinally derived endotoxin is not likely to be the factor that increases TNF-α production in patients with NASH. However, because endotoxin levels were not assessed in portal blood and gut-derived endotoxin is efficiently cleared during its first-pass through the liver, differences in hepatic endotoxin exposure may have been underestimated. Therefore, the possibility that intestinal bacterial products contribute to hepatic TNF-α induction in human NASH has not been excluded. Indeed, a role for the gut bacteria in human NASH pathogenesis is supported by the results of a recent small study that demonstrated improved liver enzymes in NASH patients who were treated with oral probiotics (mixtures of 'non-pathogenic' bacterial strains) to modify their intestinal flora. Potentially pertinent to this debate is recent evidence that in Scandinavian alcoholics, promoter polymorphisms of the CD14 endotoxin receptor gene significantly increase the risk of developing steatohepatitis and cirrhosis.

Increased TNF-α in animal models of obesity-related NAFLD

Inbred strains of mice and rats provide convenient tools to study the pathogenesis of NAFLD because they provide opportunities to control genetic and environmental factors that might influence the natural history of NAFLD. A number of small animal models of NAFLD have emerged. The fact that various genetic alterations or environmental stresses produce a similar phenotype proves that many different immunological, neuronal and hormonal factors are involved in the pathogenesis of NAFLD. Therefore, any one of these animal models could be used to clarify how altered cross-talk among immune cells, neurons and endocrine cells promotes NAFLD. Unfortunately, an in-depth analysis of the inter-relationships among these systems has been lacking in all but a few of these models. The immuno-pathogenesis of obesity-related NASH has been studied most extensively in *ob/ob* mice. Like obese humans with

NASH, *ob/ob* mice overexpress TNF-α in adipose tissue and liver. The ensuing discussion focuses on the mechanisms that promote abnormal cytokine production in this model. Additional work is needed to determine if results in genetically obese *ob/ob* mice have general relevance to NASH pathogenesis in other animal models and humans.

Immunological mechanisms for NASH in leptin-deficient mice

Ob/ob mice have a naturally occurring mutation in the *ob* gene that prevents the synthesis of leptin, the *ob* gene product (46). Leptin regulates feeding behavior and energy homoeostasis. Congenital leptin deficiency results in obesity, insulin resistance, hyperinsulinemia, glucose intolerance, dyslipidemia and hepatic steatosis. Similar to obese or alcoholic patients with fatty livers, leptin-deficient *ob/ob* mice are unusually sensitive to liver injury induced by a secondary inflammatory stress, such as bacterial LPS endotoxin (47). LPS-induced liver injury is generally mediated by Th-1, pro-inflammatory cytokines, while Th-2, anti-inflammatory cytokines, protect the liver from LPS-toxicity. Therefore, *ob/ob* mice have been evaluated before and after LPS exposure to determine how leptin deficiency enhances sensitivity to cytokine-mediated hepatotoxicity. Surprisingly, as discussed in subsequent sections, work with the *ob/ob* mouse model has uncovered conserved mechanisms that may regulate hepatic cytokine production in both leptin-deficient and leptin-sufficient states.

Immune cells express leptin and leptin receptors

It has gradually become apparent that leptin is a potent immunomodulator. Lymphocytes and macrophages express leptin receptors and some immune cells can produce leptin (48). Interactions between leptin and its receptors on immune cells regulate immune cell functions, including phagocytosis and cytokine production. Leptin deficiency also sensitizes T cells to corticosteroid-induced apoptosis and this promotes thymic atrophy in *ob/ob* mice. Defects in the innate immune system, including selective reductions in hepatic NKT cell populations, also develop during leptin deficiency (49). The authors have suggested that the latter may be particularly relevant to LPS hepatotoxicity, because NKT cells regulate local pro-inflammatory and anti-inflammatory cytokine production by other liver mononuclear cells. Interestingly, infection with *Propionibacterium acnes* is thought to sensitize normal livers to subsequent LPS-induced injury by reducing hepatic NKT cells and causing Th-1 polarization of other cytokine-producing cells in the liver (50).

Leptin deficiency alters the hypothalamic-pituitary-adrenal axis

Leptin deficiency also profoundly alters the hypothalamic-pituitary-adrenal (HPA) axis, increasing certain stress-related factors (e.g. corticosteroids), while decreasing others (e.g. norepinephrine). Recent seminal studies in *ob/ob* mice demonstrate that many of the effects of leptin deficiency are mediated by changes in the activity of neurohumoral factors that are regulated by leptin. Indeed, Dr Friedman's group recently proved that leptin-regulated neuronal factors have a role in the pathogenesis of NAFLD. They generated mice with a neuronal-specific deletion of *Ob/Rb* (51). These mice produce leptin and are capable of receiving the signals that result when leptin interacts with its receptors on all other types of cells. Amazingly, mice with neuronal-specific deletion of *Ob/Rb* resemble *ob/ob* mice. These mice (which cannot receive leptin signals in neurons) develop insulin-resistance and NAFLD. Therefore, although leptin is clearly a key inhibitory factor for NAFLD pathogenesis, this does not mean that other factors are the proximal mediators of obesity-related diseases in both leptin-deficient and leptin-sufficient states.

Reduced neurotransmitters and immune dysfunction

This, in turn, suggests novel, generally applicable mechanisms for obesity-related steatohepatitis because neurotransmitters can regulate the immune system. The regulation may be mediated indirectly, as when neurotransmitters influence the release of corticosteroids and other immunomodulatory hormones from the hypothalamus, pituitary and adrenal glands. In addition, direct regulation of immune cells by neuronal factors is possible because some immune cells express neurotransmitter receptors. For example, Kupffer cells and certain types of liver lymphocytes, including NKT cells, express adrenergic receptors and respond to norepinephrine (NE) by producing various cytokines. Neurotransmitters may also regulate the hepatic accumulation of certain lymphocyte subpopulations. Minagawa reported that pretreatment with adrenergic receptor antagonists virtually abolished the accumulation of NKT cell populations in the livers of mice that were subjected to partial hepatectomy (52).

The hypothesis that *ob/ob* mice are sensitized to LPS hepatotoxicity was evaluated, because reduced NE inhibits the hepatic accumulation of NKT cells and results in Th-1 polarization of hepatic cytokine production in leptin-deficient mice. If NE proves to be a major proximal regulator of hepatic NKT cell populations, then changes in NE activity may alter hepatic NKT cell numbers and influence hepatic cytokine production independently of leptin. This, in turn, suggests a mechanism for sensitization to LPS hepatotoxicity that may have general relevance to the pathogenesis of steatohepatitis.

TNF-α, hepatic resistance and NASH in ob/ob mice

The studies clearly demonstrate that cytokine-producing cells in ob/ob livers are Th1 polarized. This microenvironment favours the perpetuation of inflammatory signals. Sustained activation of inflammatory kinases, including Jun-N-terminal kinase and inhibitor of K-Kinase b (IKK-b) was recently found to cause cellular insulin resistance. Both kinases are targets for TNFα initiated activation (53).

To evaluate the role of TNFα in hepatic insulin resistance, Li Z et al treated obese adult ob/ob mice with vehicle

or neutralizing anti-TNF antibodies for 1 month and compared hepatic activities of JNK and IKKb in the two groups. Inhibiting TNFα significantly reduced the hepatic activities of both kinases, thereby supporting the concept that excessive TNFα activity contributes to hepatic insulin resistance in leptin-deficient mice (54).

To determine if products of intestinal flora might trigger hepatic cytokine production, insulin resistance and NASH, the same authors administered probiotics (a mixture of live lactobacillus and bifidobacteria) to another group of ob/ob mice. Probiotics did not inhibit hepatic expression of TNFα mRNA, but significantly down-regulated JNK and IKKb activities, improved histological and biochemical evidence of steatohepatitis.

A strong positive correlation has been noted between hepatic insulin resistance and NASH in many experimental animals and humans (55).

Norepinephrine and its relation with hepatic NKT and Th1

Because leptin deficiency induces multiple neuronal, hormonal, metabolic and immunological abnormalities, including relative deficiency of NE, it is difficult to predict which factors are predominantly responsible for decreasing NKT cells in the livers of leptin-deficient mice.

Minipumps containing NE or saline vehicle were implanted subcutaneously into ob/ob mice. Three weeks later, hepatic mononuclear cells were isolated and fluorescent analysis was performed to determine if NE altered hepatic mononuclear cell populations. NE significantly increased hepatic NKT cells in the leptin-deficient mice (56). The same authors isolated hepatic mononuclear cells from NE-treated ob/ob mice and vehicle-treated ob/ob mice and measured the levels of apoptotic cells using Annexin V. The hepatic NKT cells apoptosis was increased significantly in ob/ob mice. Moreover, three weeks of NE treatment decreased hepatic NKT cell apoptotic activity to normal levels.

The livers of leptin-deficient mice are unusually sensitive to LPS-induced injury, a process that is mediated by Th1 cytokines, such as TNFα and IFNγ. Studies with TNFα neutralizing antibodies demonstrate that TNFα is required for LPS liver injury. NKT cell populations produce both IFNγ and IL4. While the former exacerbates TNFα toxicity, the latter is a key inducer of anti-inflammatory (Th2) cytokines, which generally attenuate the toxic effects of TNFα. ELLISPOT assays of mononuclear cells harvested from ob/ob livers demonstrate significantly reduced production of IL4 (49). This suggested that in the liver, reducing NKT cell populations promotes unbalanced overproduction of Th1 cytokines. Treatment with doses of NE that restore hepatic NKT cell numbers reduced Th1 cytokine production.

Pathogenesis and progression in early stage of NASH

Exposure to excessive levels of adipocite-derived TNF-α relative to its antagonist, adiponectin, favors increased biologic activity of TNF, which further inhibits the actions of adiponectin. Reduced adiponectin activity promotes hepatocyte steatosis by enhancing fatty acid uptake, inhibiting fatty acid oxidation and reducing lipid export. Faced with excessive TNF-α and fatty acids but little adiponectin, hepatocytes store lipids. The retention of fatty acids, in turn, unleashes signals that activate NF-κB within hepatocytes, inducing NF-κB-sensitive genes, and thereby increases the generation of various mediators, including IL-6, TNF-α, and IL-8. The pro-inflammatory hepatic milieu is perpetuated by the relative death of local anti-inflammatory (Th-2) cytokines that results from liver NKT cell depletion. Increased sustained release of IL-6 from the liver causes systemic insulin resistance. Local increases in TNF-α and IL-8 promote hepatocyte oxidative stress and eventual apoptosis, and recruit inflammatory cells into the liver. As antioxidant and antiapoptotic defenses are overwhelmed hepatocyte death increases and inflammatory cells accumulate, signifying the emergence of NASH. Animal studies have proved that the unbalanced production of fat-derived cytokines promotes the early stages of NAFLD (57,58). Xu demonstrated high ratios of serum TNF to adiponectin in *ob/ob* mice (59). Treating *ob/ob* mice with adiponectin improved their hyperglycemia and insulin resistance while reducing hepatomegaly, hepatic steatosis, serum alanine aminotransferase, and TNF-α levels. Adiponectin therapy also lowered TNF-α and improved alcohol-induced steatohepatitis. Yamauchi demonstrated similar benefits of adiponectin treatment in KK-Ay mice, another model of obesity that has increased TNF-α and is prone to liver disease (60).

Given that reduced TNF-α activity was a consistent consequence of adiponectin treatment in these studies, it is not surprising that treating *ob/ob* mice with neutralizing TNF-α antibodies improves their NASH. Abrogating TNF activity also prevents the NAFLD that accompanies diet-induced obesity. Mice genetically deficient in TNF receptor-type 1 are protected from NASH despite the development of obesity, insulin resistance, and hyperleptinemia when these mice consume high sucrose diets. Thus, the ratio of TNF to adiponectin is high in different mouse models of steatohepatitis, and steatohepatitis is improved by treatments that normalize the ratio of TNF to adiponectin, either by increasing adiponectin or by reducing TNF activity. Studies confirm that increases in TNFα and decreases in adiponectin exert similar effects in humans. Multiple logistic regression analysis demonstrates that the odds ratio for having NASH (as opposed to simple hepatic steatosis) increases six-fold for each 5 μg/ml decline in serum adiponectin levels, whereas each picogram per mililiter increase in serum TNFα concentration doubles the risk of NASH (61,62). Analysis of other patients demonstrated reduced hepatic expression of adiponectin and its receptor in NASH compared with simple steatosis. Notably, various treatments that restore the balance of TNF to adiponectin improve NASH in humans. Weight loss induced by lifestyle modification or bariatric surgery decreases TNF, increases

adiponectin, and improves NASH (63,64). Treatment with metformin or thiazolidinediones exerts similar effects (65). Two recent studies of the TNF antagonist, pentoxifylline, also suggest that this drug improves NASH.

To elucidate the role of systemic inflammation in nonalcoholic fatty liver disease (NAFLD), serum samples of 47 patients with histologically verified NAFLD (22 with simple steatosis and 25 with NASH) and 30 age, sex and ethnicity-matched healthy controls, were assessed for general markers of inflammation (C reactive protein, TNFα and IL-6), chemokines (CC – chemokine ligand 2 [CCL]/ monocyte chemoattractant protein 1 [MCP], CCL19 and CCL 21), adipocytokines related to insulin resistance and inflammation (adiponectin and leptin) and a marker of oxidative stress (8 isoprostane F2a) (66). Serum levels of several inflammatory cytokines (IL6, CCL2/MPC1 and CCL19 but not CRP) were increased in NAFLD as compared to controls. Comparing NASH with simple steatosis, levels of TNFα and CCL2/MCP1 were elevated and adiponectin were decreased also after the adjustment for sex, BMI and presence of the metabolic syndrome.

Like TNFα, IL6 may interfere with insulin signalling (67) thereby favoring steatosis and inflammation, although somewhat discrepant results have been reported.

A main finding in the present study is the gradual rise in serum levels of CCL2/MCP1, showing increasing levels from healthy controls to simple steatosis, reaching the highest levels in NASH. CCL2/MCP1 appears to play an early and important role in several inflammatory disorders such as atherosclerosis, rheumatoid arthritis, inflammatory bowel disease and autoimmune kidney disorders. Thus, CCL2/ MCP1 is not only of major importance for monocyte recruitment into inflamed tissue, but is also a potent activator of leukocytes and other cell types at the site of inflammation, for example, through its ability to induce oxidative stress and matrix degradation (68).

Interestingly, and with relevance to steatosis, CCL2/ MCP1 has been found to induce macrophage recruitment into adipose tissue in obese subjects (69).

Recently, Neisberg et al gave further support to this finding reporting reduced inflammation profile and macrophage content in adipose tissue, improved insulin sensitivity and even marked reduction of hepatic steatosis in obese mice deficient of CCR2 (receptor for CCL2/MCP1) (70).

On immunohistochemical analysis the strong CCL2/ MCP1 expression in liver from NASH patients reflects increased infiltration of CCsL2/MCP1 leukocytes. It is tempting to hypothesize that CCL2/MCP1 could be of importance for the persistent inflammation in NASH, possibly playing a role in the progression from simple steatosis to NASH, at least partly by promoting infiltration of leukocytes into the liver.

Pathogenesis and progression in late stage of NASH

As in the early stages of NAFLD, both fat-derived and liver-derived factors may be involved in the later stages of disease. Fat is an important source of several factors such as leptin, TGF-β, angiotensinogen, and plasminogen activator inhibitor-1, which promote fibrosis. TNF-α induces adipose production of many of these pro-fibrogenic factors while inhibiting the expression and activity of adiponectin, an important fibrosis inhibitor. This may help to explain the association between hepatic inflammation and fibrosis; however, it does not clarify why liver fibrosis is relatively unusual in individuals with visceral adiposity and metabolic syndrome, because the latter is a chronic inflammatory condition.

Studies in *ob/ob* mice suggest that the answer resides within the hepatic innate immune system. *Ob/ob* mice have a naturally occurring mutation in the leptin gene and are leptin deficient. In these mice obesity, type 2 diabetes, dyslipidemia and NASH develop spontaneously; yet, these mice are protected from cirrhosis despite having chronic liver damage and metabolic syndrome. Leptin treatment corrects leptin deficiency and normalizes fibrotic response to various exogenous hepatotoxins. Interestingly, however, because supplemental leptin corrects obesity and other aspects of the metabolic syndrome in *ob/ob* mice, it also eliminates NASH, raising the possibility that the propensity for hepatic inflammation and fibrosis may, in fact, be independent phenomena (71,72).

Ob/ob mice have reduced numbers of hepatic $CD4^+$ NKT cells. In other animal models of fibrosis and in humans with various types of chronic liver disease, hepatic $CD4^+$ NKT cells are the predominant source of pro-fibrogenic cytokines, IL-4 and IL-13. During progression of chronic viral hepatitis to cirrhosis for example, human livers accumulate invariant NKT cells, and NKT cell effector function shifts to favor the production of Th-2 cytokines, such as interleukin-4 and IL-13 (73). Conditioned medium from $CD4^+$ NKT cells contains IL-4 and IL-13 and induces collagen mRNA and protein production by cultured HSCs because IL-13 has also been shown to activate rodent fibroblastic cells (74). Experiments in mice that are genetically deficient in IL-13 prove that IL-13 is the primary mediator of liver fibrosis by demonstrating that these mice are protected from cirrhosis despite having appropriate injury-related induction of other fibrogenic factors, such as TGF-β. Additional research is necessary to clarify how IL-13 influences the actions of other profibrogenic and antifibrogenic factors that are dysregulated in the metabolic syndrome. Some of these mediators are likely to be involved in the progression of NASH to cirrhosis; a recent study in patients with NASH suggests that treatment with losartan, an angiotensin receptor-1 antagonist, reduces hepatic fibrosis (75).

Both leptin (which increases noradrenaline) and noradrenaline itself enhance the activity of Th2 cytokines. The Th2 cytokines are necessary to antagonise the activity of proinflammatory cytokines, such as TNFα. However, as demonstrated by studies of schistosoma infected mice with targeted disruption of Th2 cytokine genes, Th2 cytokine activity is also necessary for hepatic fibrosis to develop during liver injury (76,77).

Administration of NE to ob/ob mice (which have mild NASH) for 2-4 weeks, generated increased numbers of activated stellate cells, induced TGFβ mRNA, upregulated collagen gene expression and led to the pericellular and perisinusoidal accumulation of fibrosis tissue. Enhancement of hepatic fibrogenesis occurred despite reductions in proinflammatory cytokine production by liver mononuclear cells and decreases of serum ALT (78).

Adrenergic activity is necessary for leptin to activate cultured stellate cells because prazosin, an alpha adrenergic antagonist, partially blocks leptin mediated increases in the growth of stellate cells (79).

Conclusion

Steatosis and NASH develop as a result of excessive pro-inflammatory factors. Thus early-stage NAFLD is very common in individuals with metabolic syndrome. However, although sustained exposure to these inflammatory mediators generally promotes the generation of various pro-fibrogenic factors, progression from NASH to cirrhosis is actually relatively uncommon. This paradox may reflect the requirement for additional factors, such as certain Th-2 cytokines, that are more selectively induced in subpopulations of individuals who have particular hepatic innate immune system defects.

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