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Non-Alcoholic Fatty Steatohepatitis an Inflammatory Disorder Beyond the Liver

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Abstract

Non-alcoholic fatty liver disease (NAFLD) has emerged as the most prevalent chronic liver disease in the United States. Non-alcoholic steatohepatitis (NASH), the most severe form of NAFLD, has an increased risk for progression to cirrhosis and associated comorbidities such as cardiovascular disease. Metabolic syndrome (MS) including insulin resistance and obesity is central to the development of NASH. It is now estimated to affect 30% of adults and about 10% of children in the U.S. Hispanics are disproportionably affected with not only higher rates of NAFLD but also more severe disease. Emerging data indicate that NASH progression results from parallel events originating from the liver as well as from the adipose tissue, the gut and the gastrointestinal tract. Thus, dysfunction of the adipose tissue through enhanced flow of free fatty acids and release of adipocytokines, and alterations in the gut microbiome generate pro-inflammatory signals that increase NASH progression. Additional 'extrahepatic hits' include dietary factors and gastrointestinal hormones. Within the liver, hepatocyte apoptosis, ER stress and oxidative stress are key contributors to hepatocellular injury. In addition, lipotoxic mediators and danger signals activate Kupffer cells which initiate and perpetuate the inflammatory response by releasing inflammatory mediators that contribute to inflammatory cell recruitment and development of fibrosis. Inflammatory and fibrogenic mediators include chemokines, the inflammasome and activation of pattern-recognition receptors.

Keywords: NASH; NAFLD; Steatohepatitis; Inflammatory signaling; Liver; JNK; NF-κB; Liver fibrosis

Definition

NAFLD is an inflammatory chronic liver disease which includes a spectrum of diseases ranging from the simple accumulation of fat or fatty liver to later stages of disease such as cirrhosis, passing through non-alcoholic steatohepatitis (NASH) and liver fibrosis [1-3]. This disease has been recognized in the medical literature in recent decades and is even now considered the hepatic manifestation of metabolic syndrome [4]. In 1980, Ludwig et al. [5] reported a number of patients with this condition who had a liver histology characterized by fat accumulation and the presence of hepatic necroinflammation, also in most of the cases presenting with fibrosis in the absence of a history of excessive alcohol intake. Therefore, he coined the term "non-alcoholic steatohepatitis".

This condition is defined by the following characteristics: a) accumulation of pathological amounts of fat in the liver which is characterized histologically by macrovesicular steatosis, b) ethanol consumption in amounts less than those likely to cause liver damage (considering usually <20 gm of alcohol/day for women and for men 30 gm/day) [6].

The liver histology in patients with NAFLD can be very variable. The common denominator across the spectrum of disease is the presence predominantly of macrovesicular steatosis in the liver. Even this feature is not uniformly observed in those that develop cirrhosis due to this condition. The spectrum of findings includes four major phenotypes:

1) isolated hepatic steatosis (non-alcoholic fatty liver [NAFL]), 2) hepatic steatosis with mild mixed inflammatory infiltrate, 3) hepatic steatosis with ballooning of hepatocyte and various degrees of inflammation, 4) hepatic steatosis, ballooning, Mallory bodies and inflammation [6].

Epidemiology

Epidemiological studies are difficult to perform because there is no evidence in blood, imaging or histological parameter with a sensibility and specificity of 100% for the diagnosis of NASH. The prevalence in Europe and Japan range from 14% to 21% [7,8]. The incidence of NAFLD is underreported and varies widely. In Japan, non-alcoholic hypertransaminasemia has been reported in 31 cases per 1000 person-years.

A recent study by a hepatology outpatient clinic in England reported an incidence rate of 29 cases per 100,000 person-years. However, the overall incidence rates for NAFLD require further study. The prevalence in the general population has been evaluated with a variety of diagnostic tools [9]. The NAFLD has become one of the most common causes of chronic liver disease and impaired liver function in industrialized countries [10-12], where it is estimated between 10 and 23% in the adult population [13]. In the United States, liver biopsies performed to potential donors revealed that 20% of donors were ineligible for organ donation based on the degree of steatosis (>30%) [14]. In Mexico population studies have reported an estimated prevalence of 17% in asymptomatic patients [15].

Furthermore, it was established that NAFLD represents the hepatic manifestation of the metabolic syndrome. Thus it is worth mentioning that in the USA it is estimated that 47 million individuals have metabolic syndrome and about 80% have NAFLD and on the other hand, 90% of patients with NASH have characteristics of metabolic syndrome [6].

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In summary, it can be concluded that most studies have reported a 10-35% prevalence of NAFLD, and about 2-5% have non-alcoholic steatohepatitis (NASH) [9].

Pathogenesis

NASH pathogenesis: a 'two-hit' model

The pathogenesis of NASH is thought to involve a two-step process in which the first 'hit' is excessive triglyceride accumulation in the liver that leads to NAFLD. The second 'hit', which results in NASH, is thought to involve additional pathogenic factors that can eventually induce liver damage, such as inflammatory cytokines, oxidative stress, mitochondrial dysfunction and/or endoplasmic reticulum (ER) stress [16].

Origin of Inflammation: Role of Cytokines

Visceral adipose tissue coexists with other cell types such as macrophages, endothelial cells and other cells involved in immune response. The cytokines secreted by these cells, but primarily by adipocytes, are called adipocytokines and usually in the state of persistent low-grade inflammation present in obese patients with insulin resistance, the total mass of adipose tissue correlates with the amount of cytokines secreted, since weight gain intensifies macrophage infiltration in adipose tissue. The research available on the subject is extensive and every day we provide new related substances, as is the case of visfatin and apelin, two new adipocytokines increased in insulin resistance and related with inflammation and angiogenesis respectively [17-19].

Growing evidence supports a central role for TNFα and other inflammatory cytokines in the progression of NASH [20]. TNFα is an important inflammatory adipocytokine produced by macrophages and other cells including adipocytes and hepatocytes. TNFα is a pleiotropic cytokine that activates signaling mechanisms that can lead to the apoptosis of hepatocytes and in the activation of hepatic stellate cells [21]. An imbalance of cytokine, in particular an increase in the ratio of TNFα/adiponectin can play an important role in the development of NASH [20]. Elevated levels of TNFα have been detected in obese patients with insulin resistance and in patients with NASH [21]. Several groups have investigated circulating levels of these cytokines in liver in patients with NASH and its correlation with disease severity. Gene expression of TNFα receptors and of TNFα is increased in the liver of patients with NASH. Circulating adiponectin levels are significantly lower and TNFα is significantly increased in patients with NAFLD compared to controls. However, the measurement of these cytokines does not appear to have the sensitivity or specificity to distinguish patients with hepatic steatosis vs. those with NASH [20].

IL-6, a proinflammatory cytokine, has been linked in the development of insulin resistance and type 2 diabetes [22]. Serum levels of IL-6 are elevated in animal models and in patients with NASH and alcoholic liver disease [20]. In a study of Nieto et al. IL-6 was found as an important mediator of fibrogenic response in hepatic stellate cells [23]. Leptin causes insulin resistance in hepatocytes. Leptin efficient animal models have massive obesity and do not develop liver fibrosis secondary to necroinflammatory stimuli [24]. In patients with NAFLD and fibrosis, elevated leptin levels exist, but there is not a statistically significant relationship between the degree of fibrosis and leptin levels, ruling out confounding factors such as age, gender, and body mass index, diabetes and insulin resistance [25]. The patients with NASH have high levels of leptin and low soluble leptin receptors, suggesting leptin resistance [26]. Resistin is a protein synthesized in adipose tissue and macrophages that has been linked to insulin resistance and exacerbation of inflammatory response. This is elevated in patients with NAFLD and its levels correlate with histological grade of steatohepatitis [27]. Semba T et al. describe in a recent study the overexpression of the adipokine LCN2 and two chemokines CXCL1 and CXCL9 in the liver of fatty liver Shionogi (FLS) mice as a NASH model, suggesting significant roles of these proteins in the pathogenesis of NASH. This study found that hepatocytes expressing LCN2 were localized around almost all inflammatory cell clusters. Furthermore, there was a positive correlation between the number of LCN2-positive hepatocytes in the specimen and the number of inflammatory foci [28].

Pro-inflammatory Signals

Inflammation is a crucial response to tissue damage or infection in which secreted mediators such as cytokines, chemokines and eicosanoids coordinate cellular defenses and tissue repair. Since this is a systemic body response, it is possible that inflammation affecting the liver in non-alcoholic steatohepatitis (NASH) may originate outside the liver. One of the most sites of interest is the visceral adipose which is expanded in non-alcoholic fatty liver disease (NAFLD) [29,30].

Visceral adipose tissue is inherently pro-inflammatory, but inflammatory also occurred in stressed, de-differentiated subcutaneous adipose tissue in obesity [31]. Inflammation and de-differentiation of adipose also alters release of the key insulin-sensitizing and anti-inflammatory adipokine, adiponectin. Adiponectin blocks elaboration and release of TNF-α. Serum adiponectin levels fall in metabolic syndrome and type 2 diabetes, while low serum adiponectin levels in NAFLD are inversely related to steatosis severity and in some studies to the presence of NASH. Key signaling pathways that explain some of the connections between hepatic inflammation and insulin resistance include IκB kinases (IKK), nuclear factor-kappaB (NF-κB) and JNK [32,33]. In a recent study Van der Poorten et al. suggest that serum adiponectin levels in advance NASH is independently associated with hepatic fat loss and they speculate that adiponectin may in part be responsible for reduction in hepatic fat to the point of complete fat loss (burn-out NASH) [34].

Activation of the endoplasmic reticulum (ER) by stress has been reported in most models of hepatic steatosis in rodents, with lipogenesis being the main metabolic pathway affected. ER stress-related activation, observed in adipose tissue of obese humans [35], could have metabolic consequences and participate in fat deposition in the liver. Activation of ER could directly induce an insulin-resistant state. Indeed, it has been shown that activation of the ER stress sensor kinase/endonuclease inositol-requiring protein 1 (IRE1), a component of the unfolded protein response (UPR) could stimulate c-Jun amino-terminal kinase (JNKs) or SAPKs (stress-activated protein kinases), which, by phosphorylating serine residues of insulin receptor 1, is a key player in the development of insulin resistance [36]. The ER is a crucial organelle for cellular homeostasis; however the ER quality control system can be compromised under a variety of conditions such as accumulation of unfolded proteins, alteration of calcium balance or disturbance of the redox state. The contribution of adipose tissue to metabolic homeostasis has become a focal point of interest. Adipose tissue secrets free fatty acids (FFAs) and hormones, known as adipokines, and thus seems to play a major role in the development of non-alcoholic fatty liver disease (NALFD). Apoptotic cell death is a prominent feature in non alcoholic steatohepatitis; toxic FFAs can activate the intrinsic apoptosis pathway in hepatocytes via c-JNK. JNK activates the proapoptotic protein Bim, resulting in Bax activation and enhanced apoptosis, called "lipoapoptosis" [36].

NF-κB

NF-κB is a transcription factor comprised of five peptides: p50/p105 (NF-jB1), p52/p100 (NF-jB2), p65 (ReIA), ReIB, and c-Rel; the members proteins form homodimeric or heterodimeric complexes; p65 and p50 are highly expressed in liver. NF-κB p65:p50 heterodimers regulate the canonical transcription of several hundred pro-inflammatory molecules, including cytokines, chemokines, adhesion molecules, nitric oxide and cyclooxygenase 2. In resting hepatocytes, NF-κB is sequestered in the cytosol bound to inhibitory proteins (IκB) [37].

NF-κB activation begins with the activation of an IκB kinase (IKK) complex that consist of catalytic subunits IKK-α and IKK-β and the scaffolding subunit IKK-β. Several mitogen-acivated protein (MAP) kinases that also include NF-κB-inducing kinase (NIK) activate IKK through the phosphorylation of IKK-α and IKK-β. IKK-β has higher activity than IKK-α. In the canonical pathway of NF-κB activation, IκB-α is phosphorylated at serine residue (Ser) 32, 36 and/or Tyr42 and separated from the p50/p65 dimer, allowing the dimer to translocation to the nucleus and bind to cognate DNA sequences. IKK is activated directly by oxidative stress and other cellular stressors, such as ER stress, or via liganding of NF-κB- signaling receptors [38].

NF-κB activation is uniformly found in human NASH and in animal models. Aileen de la Peña et al. in an elegant study demonstrated the roles of NF-κB and TNF-α as mediator of inflammation in a nutritional model of steatohepatitis [37]. They employed Wild-type (wt), TNF null and TNF receptor (R)-1 mice, animals were fed with a methionine and choline deficient (MCD) diet for up to 5 weeks. Irrespective of genotype, MCD diet-fed mice developed hepatic lipid peroxidation and serum ALT elevation; at day ten, livers from wt, TNF null and TNFR-1 mice showed equivalent steatohepatitis. To establish whether NF-κB is a primary mediator of inflammation, they overexpressed a mutant, no degradable IκB, delivered by adenovirus in vivo. As expected, hepatic mIκB expression reduced NF-κB/DNA binding induced by MCD dietary feeding, with resultant abrogation of ICAM-1 and TNF synthesis. Such blockade substantially protected against development of steatohepatitis, with reductions in liver injury and hepatic inflammation. Others have produced conflicting findings. The emerging concepts of metabolic stress provide some evidence that pro-inflammatory pathways in NASH could originate from stressed hepatocytes via activation of NF-κB. Alternatively, TNF-α, IL-1β and other cytokines released from NF-κB-activated Kupffer cells could activate NF-κB in neighboring hepatocytes [39].

JNK

The c-Jun N-terminal kinases (JNKs) are members of a larger group of serine/threonine (Ser/Thr) protein kinases known as the mitogen-activated protein kinase (MAPK) superfamily, which also includes the extracellular signal-regulated kinases (ERKs), or classical MAPKs and the p38 MAPK. JNKs bind and phosphorylate c-Jun on Ser63 and Ser73 within its transcriptional activation domain [40]. MAPK kinase (MKK) is responsive to stress stimuli, mainly inflammatory signals, but also to a lesser extent, to ultraviolet irradiation, heat and non osmotic shock [41]. The mammalian JNKs are encoded by three distinct genes (*jnk1, jnk2, jnk3*). Complexity is generated by splicing, which results in up to 10 isoforms varying in size from 46 kDa to 56 kDa. JNK1 and JNK are found in all cells of every tissue. JNK3 is found mainly in the brain, but is also found in the heart and the testis. JNK1 is involved in apoptosis, neurodegeneration, cell differentiation and proliferation, inflammatory conditions and cytokine production mediated by activation protein-1 (AP-1) such as regulated upon activation, normal T-cell expressed, and secreted cytokine, interleukin-8 and granulocyte-macrophage colony-stimulating factor. JNK1 has been found to regulate Jun protein turnover by phosphorylation and activation of the ubiquitin ligase Itch (polyubiquitination marks proteins for degradation by the proteasome) [42]. The JNK proteins lead to varied and seemingly contradictory cellular responses; particularly, JNKs have been reported to have a role in the induction of apoptosis, but have also been implicated in enhancing cell survival and proliferation. These opposing roles of JNKs have been attributed to the observation that JNKs activate different substrates based on a specific stimulus, cell type or temporal aspects [43]. The enzymatic activity of JNK is induced in response to diverse stimuli, such as cytokines TNF-α, IL-1, transforming growth factor (TGF-β), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), intracellular and extracellular pathogens, lipopolysaccharide (LPS), peptidoglycan and bacterial unmethylated CpG DNA that activates Toll-like receptors (TLRs), reactive oxygen species (ROS), pathologic and environmental stress (ischemia, hypoxia and ionizing radiation), toxins, drugs, ER stress and metabolic changes, including obesity and hyperlipidemia. JNK appears always to be activated in lipotoxicity and in both experimental and human forms of NASH. In a classical study Schattenberg et al. demonstrated that activation of JNK1 was essential for inflammatory recruitment in MCD-induced steatohepatitis model. Saturated fatty acids activate JNK in primary hepatocytes and tumor cells of hepatocyte lineage, and this was a crucial pathway to cell death by the mitochondrial apoptosis [44]. Larter et al. using the foz/foz diabetes/metabolic syndrome mice model described that both JNK1 and JNK2 are activated with NASH, but not in the controls. Additionally, nutritional or pharmacological actions that lowered hepatic free cholesterol virtually abrogated JNK activation in association with improvement of liver injury, hepatocyte apoptosis and macrophage accumulation; These observations are consistent with the suggestion that JNK signaling activation is a key injury and inflammatory pathway in metabolic syndrome-related NASH [45,46].

Innate Immunity in NAFLD

In the context of immunity, pattern-recognition is the art of discriminating friend or foe and innocuous from noxious. Innate immune system can respond to key molecules released by damage cells, thus eliminating them. The mechanism by which stressed or dead cells trigger inflammation and adaptive immune responses involves damage-associated molecular patterns (DAMPS), also named alarmins. Intracellular pro-inflammatory DAMPS include high-mobility group *gel* box 1 (HMGB1), heat shock proteins, fibrinogen and fibrinonectin, and mitochondrial products such as formyl peptides and mitochondrial DNA [47,48]. Although they differ from pathogen-associated molecular patterns (PAMPs), some DAMPS can be recognized by similar receptors, particularly TLRs (TLR4 responds to both HMGB1 and lipopolysaccharide). Eight Toll-like receptors (TLRs) are expressed in mammalian liver with varying levels of expression on Kupffer cells (KCs), hepatocytes, hepatic stellate cells (HSCs), sinusoidal endothelial cells (SECs). Most are expressed on the cell surface, but TLRs 3, 7, 8, 9 and 13 are intracellular (endosomal) proteins. Individual TLRs interact with different combinations of adapter proteins (e.g. MD2, Myeloid differentiation factor 2) and activate transcription factors such as NF-κB, AP-1 (via JNK) and interferon responsive factors (IRF) [49,50]. In the Figure 1, it is shown that MyD88 is shared by almost all TLRs and recruits members of the IL-1 receptor-associated kinase family. When released from necrotic cells, HMGB1 stimulates KCs and monocytes to produce pro-inflammatory mediators by acting as an endogenous ligand for TLR4, although it might do that by forming highly

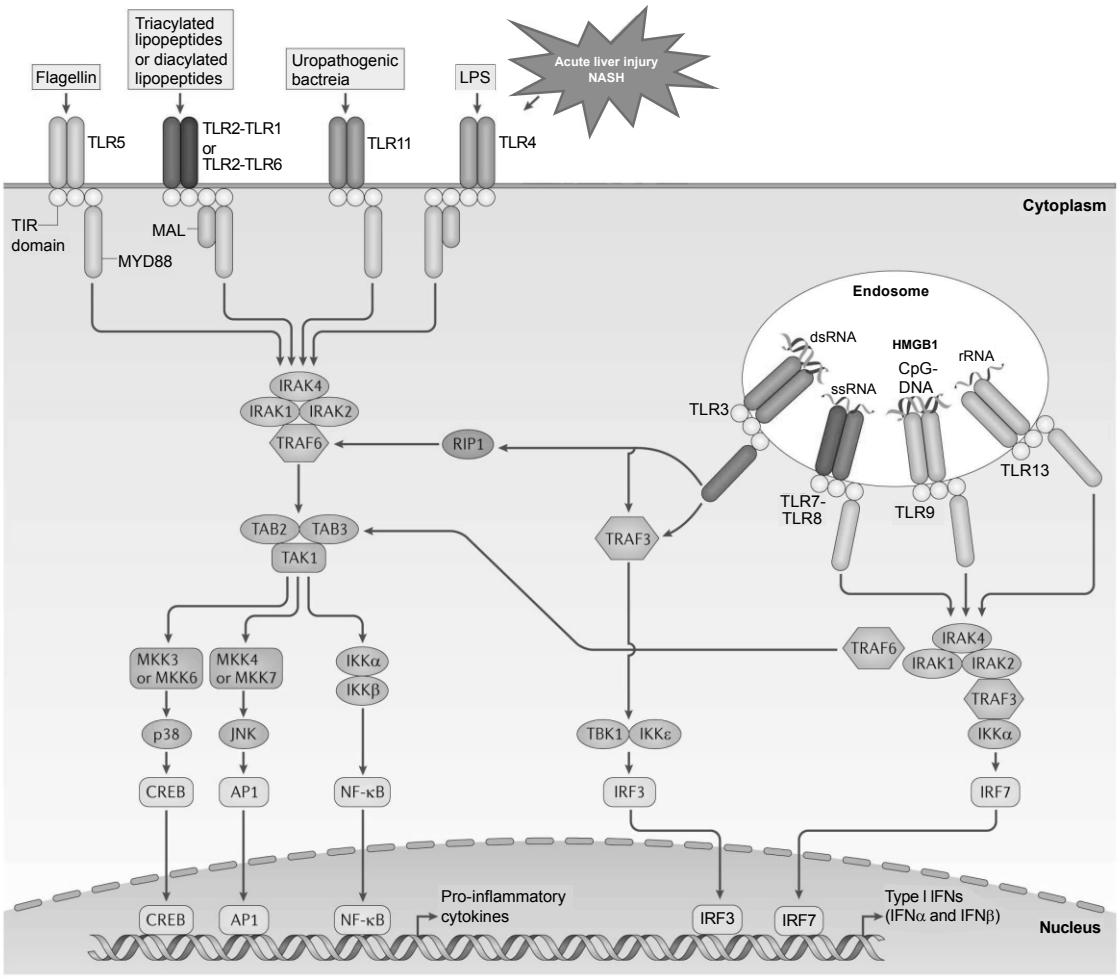

Figure 1: TLR signaling involves JNK and NF-κB-p65 activation. TLR constitute a family of receptors involved in pro-inflammatory signaling in the innate immune system, responsible for the recognition of PAMPs and DAMPs. A detailed knowledge of how mammalian Toll-like receptors (TLRs) signal has developed over the past 15 years. TLR5, TLR11, TLR4, and the heterodimers of TLR2–TLR1 or TLR2–TLR6 bind to their respective ligands at the cell surface, whereas TLR3, TLR7–TLR8, TLR9 and TLR13 localize to the endosomes, where they sense microbial and host-derived nucleic acids. TLR4 localizes at both the plasma membrane and the endosomes. TLR signalling is initiated by ligand-induced dimerization of receptors. Following this, the Toll–IL 1 resistence (TIR) domains of TLRs engage TIR domain-containing adaptor proteins (either myeloid differentiation primary-response protein 88 (MYD88) and MYD88 adaptor-like protein (MAL), or TIR domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM)). TLR4 moves from the plasma membrane to the endosomes in order to switch signalling from MYD88 to TRIF. Engagement of the signalling adap¬tor molecules stimulates downstream signalling pathways that involve interactions between IL 1R associated kinases (IRAKs) and the adaptor molecules TNF receptor-associated factors (TRAFs), and that lead to the activation of the mitogen-activated protein kinases (MAPKs) JUN N-terminal kinase (JNK) and p38, and to the activation of transcription fac¬tors. Two important families of transcription factors that are activated downstream of TLR signalling are nuclear factor κB (NF κB) and the interferon-regulatory factors (IRFs), but other transcription factors, such as cyclic AMP-responsive element-binding protein (CREB) and activator pro¬tein 1 (AP1), are also important. A major consequence of TLR signalling is the induction of pro-inflammatory cytokines, and in the case of the endosomal TLRs, the induction of type I interferon (IFN).

dsRNA: Double-stranded RNA; IKK: Inhibitor of NF-κB kinase; LPS: Lipopolysaccharide; MKK: MAP kinase kinase; RIP1: Receptor-interacting protein 1; rRNA: Ribosomal RNA; ssRNA: Single-stranded RNA; TAB: TAK1 binding protein; TAK: TGFβ-activated kinase; TBK1: TANK-binding kinase 1.

Modified from O´Neill et al. [48].

inflammatory complexes with other molecules (ssDNA, endotoxin, IL-1β, nucleosomes). TLR4 is involved in alcoholic liver injury and is also up-regulated in MCD steatohepatitis and fructose-induced hepatic steatosis. Interestingly, pathological effect of TLR4 in Kupffer cells is achieved by inducing reactive oxygen species (ROS)-dependent activation of X-box binding protein-1 (XBP-1) [51]. Saturated FFA can also bind to TLR4. TLR9 is located within the cell and is most response to unmethylated CpG containing DNA, but it also binds HMGB1. TLR9-deficient mice are protected from steatohepatitis in de CDAA (choline-deficient amino acid-defined) diet model.TLR2 expression by hepatocytes can be induced by lipopolysaccharide, TNF-α, and IL-1β via NF-κB activation, while signaling cross-talk between TLR4 and TLR9 amplifies the inflammatory response to macrophages [50].

Kupffer Cells

Scavenger receptors comprise a large family of structurally diverse

proteins that are involved in many homeostatic functions. They recognize a wide range of ligands, from pathogen-associated molecular patterns (PAMPs) to endogenous, as well as modified host-derived molecules (DAMPs). The liver deals with blood micro-organisms and DAMPs released from injured organs, thus performing vital metabolic and clearance functions that require the uptake of nutrients and toxins. Many liver cell types, including hepatocytes and Kupffer cells, express scavenger receptors that play key roles in hepatitis C virus entry, lipid uptake, and macrophage activation, among others [52].

KCs are specialized tissue macrophages in the liver. They not only contribute to insulin resistance in fatty liver disease but connect the inflammatory responses in many liver diseases. KCs are particularly sensitive to gut-derived endotoxin, acting trough CD14, TLR2 and TLR4 and adapter proteins such as MD2 to activate NF-κB via MyD88. In chimeric mice with KCs derived from MyD88$^{-/-}$ bone marrow donors, there was amelioration of the inflammation and fibrosis induced in the CDAA model of steatoheaptitis compared with Wild type mice, demonstrating a key role for KC activation. Ablation of KCs reduces severity of liver injury and inflammation in alcohol-related liver injury in rodents. Besides, in the HF- fed mouse model, ablation of KC reduce severity of steatosis by the releasing hepatocytes from IL-1β and NF-κB-dependent suppression of peroxisome proliferator-activated receptor-α activity [52,53].

Inflammasome

The inflammasome are a group of multimeric protein complexes that consist of an inflammasome sensor molecule, the adaptor protein ASC and caspase 1. Inflammasome formation is triggered by a range of substances that emerge during infections, tissue damage or metabolic imbalances. Once the protein complexes have formed, the inflammasomes activate caspase 1, which proteolytically activates the pro-inflammatory cytokines interleukin 1β (IL 1β) and IL 18. In addition, inflammasome activation causes a rapid, pro-inflammatory form of cell death called pyroptosis. The nucleotide-binding domain, leucine rich repeat containing (NLRP3) inflammasome (also named cryopyrin or NALP3) is express by myeloid cells and is up regulated by PAMPs. It requires a caspase recruitment domain, and can recruit pro-caspase 1 in the presence of the adapter protein ASC (apoptosis-associated speck-like CRD-domain containing protein). Once all components of the NALP3 inflammasome are assembled in the cytosol, caspase 1 is released and can promote the cleavage and production of pro-inflammatory cytokines to encourage and maintain inflammation. NALP3 inflammasome can be activated by several endogenous and exogenous agonists. Relevant to NASH, palmitic acid induces activation of the NALRP3-ASC inflammasome to activate caspase 1 and cause production of IL-1β and IL-18. Other important agonists include uric acid crystals, which can precipitate in the extracellular space of dying cells, and extracellular DNA [54,55]. Mice genetically deficient in any of three inflammasome components *Casp1, Nlrp3 (NOD-like receptor family, pyrin domain con-taining 3*) or *Asc* (*apoptosis-associated speck-like protein containing a carboxy-terminal CARD; also known as Pycard*), developed more hepatic inflammation and increased serum levels of alanine aminotransferase and aspartate aminotransferase than control, wild-type mice when fed a methionine–choline diet, which induces fatty liver [56,57].

Lipotoxicity

The lipotoxicity is a metabolic term coined by Unger 15 years ago to describe the toxic effects of the excessive free fatty acids over a pancreatic beta cell [20]. However, it now seems likely that the steatotic hepatocytes in NASH contain excess of lipid molecules other than triacylglycerides (TG), and there is mounting evidence that such non-TG lipid molecules are implicated in the pathogenesis of NASH by the processes of lipotoxicity [55]. Lipotoxicity is the mechanism proposed for triggering fibrogenesis in NASH. Hepatocellular damage results in the induction of pro-inflammatory and pro-fibrogenic cytokines, activation of adjacent HSCs and subsequent deposition of type I collagen. In NASH, this typically occurs within the lobules at the site of hepatocellular injury resulting in a pericellular sub-sinusoidal fibrosis maximal in centrilobular areas [58].

Lipidomic analyses of human fatty livers have identified free cholesterol (FC), but not free fatty acids (FFA), diacylglycerides (DAG) or ceramide among the potential lipotoxic molecules that accumulate selectively in NASH. Evidence for the toxic effects of excess lipid in the liver is found in animal models and in human disease. In a mouse model of impaired β-oxidation due to lack of mitochondrial trifunctional protein, moderate to severe lipid accumulation in the liver may lead to cell dysfunction, manifest as failure to appropriately carry out gluconeogenesis [59]. In these mice, neonatal hypoglycemia contributes to excess early mortality. In humans, triglyceride and FFA accumulation in the liver is associated with NASH, characterized by an inflammatory response with evidence of hepatocyte damage and fibrosis that can progress to cirrhosis [60]. Some potential lipotoxic lipid species implicated in NASH have been explored, particularly saturated FFA and FC, but also very long chain polyunsaturated fatty acids (PUFA), sucrose and fructose. These studies demonstrate the unmistakable potential of such lipid molecules to kill cells of hepatocyte lineage, by directly or indirectly activating JNK and the mitochondrial/lysosomal dell death pathway, and also stimulate pro-inflammatory signaling via NF-κB and AP-1 (JNK/activator protein-1) [61]. In general, saturated long chain fatty acids, such as palmitic and stearic acids, are more toxic than mono-unsaturated FFA. The most compelling evidence that hepatocyte may be the source of liver inflammation in NASH comes from studies in obese rodents with insulin resitance that leads to hyperinsulinemia and diabetes. Foz/foz mice exhibit hyperphagia with early onset obesity and insulin resistance. Feeding them a high carbohydrate, HF (High-fat) diet with 0.2% of cholesterol accelerates onset of diabetes with 70% reduction in serum adiponectin. The resultant liver pathology shows NASH with fibrosis. By 24 weeks, HF-fed *foz/foz* mice developed severe steatohepatitis (marked steatosis, alanine aminotransferase elevation, ballooning, inflammation, fibrosis), whereas dietary and genetic controls showed only simple steatosis [62]. While steatosis was associated with hepatic lipogenesis, indicated by increased fatty acid synthase activity, steatohepatitis was associated with significantly higher levels of CD36, indicating active fatty acid uptake, possibly under the influence of peroxisome proliferator-activated receptor-γ. A high fat diet rich in Trans fats combined with high-fructose corn syrup equivalent and physical inactivity also caused obesity-related steatosis with moderate necro-inflammation changes [20,63]. Fructose has a selective hepatic metabolism, and provokes a hepatic stress response involving activation of c-Jun N-terminal kinases and subsequent reduced hepatic insulin signaling. As high fat diet alone produces obesity, insulin resistance, and some degree of fatty liver with minimal inflammation and no fibrosis, the fast food diet which includes fructose and fats produces a gene expression signature of increased hepatic fibrosis, inflammation, endoplasmic reticulum stress and lipoapoptosis [64].

Differences in the development of NASH have recently been linked to genetic susceptibility. The single nucleotide polymorphism (rs738409) in the human patatin-like phospholipase domain containing

3 gene (PNPLA3 or adiponutrin) results in a I148M variant and is a strong predictor of steatosis, inflammation, and fibrosis across different populations, being independent of body mass, insulin resistance, or serum lipid levels. The expression of PNPLA3 is regulated by nutrition: fasting inhibits, and high-carbohydrate diet feeding increases, PNPLA3 expression. In humans, PNPLA3 is predominantly expressed in liver, while in mice the strongest expression is observed in adipose tissue. PNPLA3 possesses triglyceride hydrolase and DG transacylase activity, and converts lysophosphatidic to phosphatidic acid form. By modulating lipid intermediates, dysfunctional PNPLA3 promotes the accumulation of lipotoxic substrates, which lead to lipoapoptosis and inflammation [65,66].

Oxidative Stress

Oxidative stress is considered one of the most important pathogenic factors in the development of NASH. Reactive oxygen species (ROS) are produced from oxygen binding with electrons released from the mitochondrial respiratory chain. Structural mitochondrial abnormalities, including giant mitochondria, loss of mitochondrial cristae, and paracrystalline inclusions, are commonly observed in NASH livers. These mitochondrial abnormalities are associated with the impairment of electron transport, resulting in further ROS formation. Mitochondrial fatty acid oxidation also generates ROS, which may damage the mitochondria themselves and lead to mitochondrial DNA damage. Decreased activities of mitochondrial respiratory chain complexes also increase TNF-α expression and decrease mitochondrial ATP synthesis, leading to further hepatotoxicity and cell death. FFAs are another main source of oxidative stress. Hepatic FFA oxidation promotes progression of NASH. NASH patients were demonstrated to have increased beta-oxidation of FFAs in mitochondria with increased production of ROS. In addition, NASH patients have increased levels of oxidative stress compared with simple steatosis patients. Oxidation of FFAs in hepatic microsomes appears particularly likely to generate excessive ROS. It has been reported that CYP2E1 is persistently overexpressed in the liver of NASH patients compared with simple steatosis patients. It has also been reported that insulin resistance directly induces an increase in CYP2E1, which inhibits tyrosine phosphorylation of both IRS-1 and IRS-2. These mechanisms may lead to the development of more insulin resistance and increased ROS [20,67]. The excess of ROS generation produces lipid peroxides, which further harm the respiratory chain component and the membrane transport capacity, triggering a vicious circle which involves antioxidant depletion and the incapacity of mitochondria to inactivate ROS. Overall, these events contribute to the enhancement of lipid peroxidation, which leads to liver cells injury and inflammation and conspicuous hepatic fibrosis and, consequently, to the development of steatohepatitis progression. In addition to TNF-α production, lipid peroxidation induces the production of other cytokines, such as transforming growth factor-β (TGF-β) and interleukin-8 (IL-8) [68].

Clinical Lmpact

Patients with NAFLD, both adults and adolescents, have multiple risk factors for cardiovascular disease (CVD). In the majority of patients, NAFLD is closely associated with insulin resistance, hypertension, atherogenic dyslipidemia, dysglycemia and being overweight or obese, which are all established risk factors for CVD. Patients with NAFLD also have an increased prevalence of chronic kidney disease (CKD), which is another known risk factor for CVD. NAFLD is strongly associated with type 2 diabetes mellitus and abdominal obesity. As lifestyles have become increasingly sedentary and dietary patterns have changed, the worldwide prevalence of NAFLD has increased dramatically and is projected to be the principal etiology for liver transplantation within the next decade. The majority of patients with NAFLD only have simple steatosis; however, a notable minority of patients with NAFLD progress to more advanced disease that is characterized by NASH and subsequent fibrosis and cirrhosis or, in some cases, hepatocellular carcinoma. The presence of NAFLD *per se* is associated with an increased risk of all-cause mortality (OR 1.40, 95% CI 1.23–1.60, $P<0.00001$), histological subgroup analysis indicates that simple steatosis seems to be a fairly benign condition and that NASH is more strongly associated with excess liver-related morbidity [69,70].

Considerations

Over the past two decades, obesity has become a major public health challenge worldwide. It is clear that as-yet-unrecognized factors governing energy homeostasis must be uncovered in order to protect against the onslaught of metabolic diseases associated with excess adiposity. In fact, the benefits gained from current therapies targeting obesity-related diseases (e.g. hypertension, coronary heart disease, hyperlipidemia and type 2 diabetes) are in danger of being outweighed by the negative effects of increased adiposity. Thus, it is urgently necessary to develop systematic and comprehensive approaches to facilitate the identification of factors that play crucial roles in regulating energy homeostasis. Moreover, it is time to consider the utilization of novel models of steatohepatitis using fish models such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). Substantial advances in unraveling the molecular pathogenesis of NAFLD have recently been achieved through unbiased forward genetic screens using small fish models [70,71]. It is of relevance to better understand the underlying mechanisms involved in NASH in order to apply new knowledge to potential novel therapeutic approaches. The transition from simple steatosis towards NASH represents a key step in pathogenesis, as it will set the stage for further severe liver damage.

The diagnosis of NASH is challenging, as most affected patients are symptom free and the role of routine screening is not clearly established. A complete medical history is important to rule out other causes of fatty liver disease (alcohol abuse, medications, other). Plasma aminotransferase levels and liver ultrasound are helpful in the diagnosis of NAFLD/NASH, but a liver biopsy is often required for a definitive diagnosis.

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