NF-κB Activation, Rather Than TNF, Mediates Hepatic Inflammation in a Murine Dietary Model of Steatohepatitis

AILEEN DELA PEÑA,* ISABELLE LECLERCQ,† JACQUELINE FIELD,* JACOB GEORGE,* BRETT JONES,‖ and GEOFFREY FARRELL*

*Storr Liver Unit, Westmead Millennium Institute, University of Sydney at Westmead Hospital, Sydney, NSW, Australia; †Laboratoire de Gastroentèrologie, Universitè Catholique de Louvain, Brussels, Belgium; and ‖Hepatology Unit, Royal North Shore Hospital, St. Leonards, NSW, Australia

Background & Aims: **We explored the roles of nuclear factor-κB (NF-κB) and tumor necrosis factor (TNF) α (TNF-α) as mediators of inflammation in a nutritional model of steatohepatitis.** ***Methods:*** **Wild-type (wt), TNF null (−/−), and TNF receptor (R)-1$^{-/-}$ mice were fed a methionine- and choline-deficient (MCD) diet for up to 5 weeks. Liver injury (serum alanine aminotransferase [ALT]), hepatic inflammation, triglycerides, and lipid peroxide levels were determined. Hepatic NF-κB activation and expression of TNF and intercellular adhesion molecule-1 (ICAM-1) were assayed.** ***Results:*** **Irrespective of genotype, MCD diet-fed mice developed hepatic lipid peroxidation and serum ALT elevation; at day 10, livers from wt, TNF$^{-/-}$, and TNFR-1$^{-/-}$ mice showed equivalent steatohepatitis. NF-κB/DNA binding was enhanced in hepatic nuclear fractions from MCD diet-fed wt mice compared with dietary controls; there were corresponding increases of ICAM-1 and TNF messenger RNA (mRNA). Likewise, NF-κB activation and ICAM-1 expression were enhanced by MCD dietary feeding in TNF$^{-/-}$ and TNFR-1$^{-/-}$ mice compared with respective controls. To establish whether NF-κB is a primary mediator of inflammation in experimental steatohepatitis, we overexpressed a mutant, nondegradable IκB (mIκB), 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 of NF-κB transcriptional activation substantially protected against development of steatohepatitis, with significant reductions in liver injury and hepatic inflammation.** ***Conclusions:*** **In the MCD dietary model of steatohepatitis, NF-κB is activated early and is an important proinflammatory mediator of lesion development, but steatohepatitis occurs independently of TNF synthesis and TNFR-1 activation.**

Nonalcoholic steatohepatitis (NASH), part of the spectrum of metabolic (nonalcoholic) fatty liver disorders (NAFLD), is characterized by steatosis, hepatic inflammation, and liver cell injury.[1] Recruitment and perpetuation of liver inflammation and liver cell injury appear to be the key events that discriminate NASH from steatosis and other benign forms of NAFLD. Possible mechanisms for NASH involve uncontrolled accumulation of fat in the liver, which could directly cause lipotoxicity, and induction or amplification of one or more proinflammatory and injury-inducing processes. Among the latter, oxidative stress and dysregulated cytokine production have been proposed as components of the pathogenic mechanisms of steatohepatitis.[2–5]

In alcoholic steatohepatitis (ASH), which shares pathologic similarities to NASH, the proinflammatory cytokine tumor necrosis factor (TNF) α appears to play a critical role in hepatic inflammatory recruitment.[2] Most of the key biologic effects of TNF (cell death, cell cycle induction, inflammatory recruitment) are exerted by its type 1 receptor (TNFR-1); TNFR-1 activation triggers several intracellular signals, including activation (transfer to the nucleus and DNA binding) of nuclear factor-κB (NF-κB).[6] Increased serum TNF and hepatic TNF messenger RNA (mRNA) levels have been reported in patients with ASH.[7,8] Likewise, studies using TNFR-1 gene-deleted (−/−) mice[9] and mice treated with anti-TNF antibodies[10] have indicated that TNF may be required to induce hepatic cellular injury and inflammation in response to alcohol. Increased serum TNF and hepatic expression levels of both TNF and TNFR-1 mRNA have also been reported in humans with NASH.[3,4,11] The potential sources of TNF in this syn-

Abbreviations used in this paper: Ad5mIκB, adenovirus-expressing mIκB superrepressor; Ad5LacZ, adenovirus-expressing LacZ; ASH, alcoholic steatohepatitis; CTL, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; ICAM-1, intercellular adhesion molecule-1; IκB, inhibitor of NF-κB; MCD, methionine and choline deficient; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NF-κB, nuclear factor-κB; NPC, nonparenchymal cell; TBARS, thiobarbituric acid-reactive substance; TNF, tumor necrosis factor; TNFR-1, tumor necrosis factor receptor-1; wt, wild-type.

0016-5085/05/$30.00
doi:10.1053/j.gastro.2005.09.004

drome include adipocytes, which exhibit up-regulation of TNF levels in obese persons[12] and hepatocytes in response to gut-derived bacterial products.[11] In animal models of NAFLD, such as the *ob/ob* mouse, a single injection of lipopolysaccharide causes acute focal hepatic inflammatory lesions associated with release of TNF.[5,13,14] However, the hypothesis that TNF could play a pathogenic role in the chronic lobular inflammatory reaction that characterizes metabolic or nutritional forms of steatohepatitis has not yet been tested directly.

In experimental ASH, the cause for TNF release and subsequent hepatic inflammatory accumulation has been related to NF-κB activation in Kupffer cells.[15–17] To date, there are no studies of NF-κB activation in animal models of NAFLD that exhibit pathology resembling NASH.[18,19] In the present study, we induced steatohepatitis by feeding mice a diet deficient in methionine and choline (MCD diet)[20,21] and used this model (1) to analyze hepatic TNF expression and release; (2) to determine the relationship between TNF expression and NF-κB transcriptional activity; and (3) to establish, by use of gene-deleted mice, whether TNF and TNFR-1 activation is essential for steatohepatitis development. Because steatohepatitis appeared to be similar in wt, $TNF^{-/-}$, and $TNFR\text{-}1^{-/-}$ mice, we studied activation of NF-κB and related genes during intake of the MCD diet. Finally, we tested whether NF-κB itself could be a key proinflammatory mediator in this form of steatohepatitis by causing adenoviral-mediated expression of a mutant (nondegradable) form of IκBα (mIκBα).[22] This protein binds cytosolic NF-κB to prevent its migration to the nucleus, thus blocking transcriptional activation of NF-κB-regulated genes.[22] The results provide evidence for a direct, mechanistic role for NF-κB in mediating inflammatory recruitment in this model of murine nutritional steatohepatitis, whereas the role, if any, of TNF is dispensable.

Materials and Methods

Animal Procedures and Dietary Induction of Steatohepatitis

All animal studies were approved by the Western Sydney Area Health Service Animal Care and Ethics Committee and conformed to the highest international standards of humane care of animals in biomedical research. Animals had unrestricted access to food and water, were housed in temperature and humidity controlled rooms, and were kept on a 12-hour light/dark cycle. Female C57BL6/J mice (wt) (Animal Resource Centre, Canning Vale, Western Australia), C57BL6/J $TNF^{-/-}$ mice (Centenary Institute of Cancer Medicine and Cell Biology, Sydney, Australia), and C57BL6/J $TNFR\text{-}1^{-/-}$ mice (James Cook University, Townsville, Australia) were randomly divided into experimental groups and fed either the methionine- and choline-deficient diet (MCD group) or the same diet supplemented with choline bitartate (2 g/kg) and DL-methionine (3 g/kg) (control {CTL} group) (catalog No. 960439 and 960441, respectively; ICN Biomedicals, Costa Mesa, CA). Experimental periods were for 10 days to 5 weeks. $TNF^{-/-}$ mice have been backcrossed 10 times against the wt strain; $TNFR\text{-}1^{-/-}$ mice have been backcrossed 5 times against the wt strain. In separate experiments, hepatic nonparenchymal cells (NPC; composed of Kupffer, endothelial, stellate, and inflammatory cells) were separated from hepatocytes by differential centrifugation of a cell suspension prepared by in situ collagenase type 2 (Worthington, Lakewood, NJ) perfusion of the liver. Contamination of the NPC fraction by hepatocytes was less than 5%, as determined by phase contrast microscopy and cell counting. Adenoviral vectors[22] were a kind gift from Dr David A. Brenner, departments of medicine and of biochemistry and biophysics and Center for Gastrointestinal Biology and Disease, University of North Carolina, Chapel Hill, NC. Adenovirus containing either the mIκB "superrepressor" (Ad5mIκB) or β-galactosidase as transfection control (Ad5LacZ) were resuspended as 1.5×10^{10} plaque-forming units in 100 μL sterile saline and delivered via lateral tail vein injection to lightly anesthetized female wt animals 2 days prior to exposure to the MCD diet.

Preparation of Tissue and Cellular Fractions

At the end of the experimental period, mice were anesthetized (ketamine 100 mg/kg, xylazine 20 mg/kg, intraperitoneally [IP]), blood was collected by cardiac puncture, and serum was prepared for determination of alanine aminotransferase (ALT) levels. Livers were rapidly excised and perfused with ice-cold phosphate-buffered saline (PBS). A portion of fresh tissue was fixed in 10% neutral-buffered formalin for histologic analyses, and another was used immediately to prepare nuclear proteins. The remaining liver was snap frozen in liquid nitrogen and stored at −80°C prior to molecular studies.

Morphologic Studies

Sections (4 μm thick) cut from paraffin-embedded tissue were stained with H&E, were scored blindly for hepatic steatosis and necroinflammation, and were graded (none, mild, moderate, severe) as previously described.[18] Polymorphs were stained with nitrosylated pararosanilin (Sigma, St Louis, MO) containing 0.028% (wt/vol) naphthol AS-D chloroacetate in 1 mol/L Sorenson's buffer.[23] Activated macrophages were stained by periodic acid-Schiff with diastase.[24] Cell counts were expressed as the average number of positive-stained cells per section (3 sections counted) at ×20 magnification. To determine expression of proteins of interest, hemagglutinin (HA) tagged to adenoviral-expressed proteins was immunostained in liver sections with 1:500 dilution of anti-HA antibody (Roche Molecular Biochemicals, Indianapolis, IN) in 2% bovine serum albumin (BSA).