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BASIC AND TRANSLATIONAL—LIVER

Loss of Junctional Adhesion Molecule A Promotes Severe Steatohepatitis in Mice on a Diet High in Saturated Fat, Fructose, and Cholesterol

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BACKGROUND & AIMS: There is evidence from clinical studies that compromised intestinal epithelial permeability contributes to the development of nonalcoholic steatohepatitis (NASH), but the exact mechanisms are not clear. Mice with disruption of the gene (*F11r*) encoding junctional adhesion molecule A (JAM-A) have defects in intestinal epithelial permeability. We used these mice to study how disruption of the intestinal epithelial barrier contributes to NASH. **METHODS:** Male C57BL/6 (control) or $F11r^{-/-}$ mice were fed a normal diet or a diet high in saturated fat, fructose, and cholesterol (HFCD) for 8 weeks. Liver and intestinal tissues were collected and analyzed by histology, quantitative reverse-transcription polymerase chain reaction, and flow cytometry. Intestinal epithelial permeability was assessed in mice by measuring permeability to fluorescently labeled dextran. The intestinal microbiota were analyzed using 16S ribosomal RNA sequencing. We also analyzed biopsy specimens from proximal colons of 30 patients with nonalcoholic fatty liver disease (NAFLD) and 19 subjects without NAFLD (controls) undergoing surveillance colonoscopy. **RESULTS:** $F11r^{-/-}$ mice fed a HFCD, but not a normal diet, developed histologic and pathologic features of severe NASH including steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis, whereas control mice fed a HFCD developed only modest steatosis. Interestingly, there were no differences in body weight, ratio of liver weight:body weight, or glucose homeostasis between control and $F11r^{-/-}$ mice fed a HFCD. In these mice, liver injury was associated with significant increases in mucosal inflammation, tight junction disruption, and intestinal epithelial permeability to bacterial endotoxins, compared with control mice or $F11r^{-/-}$ mice fed a normal diet. The HFCD led to a significant increase in inflammatory microbial taxa in $F11r^{-/-}$ mice, compared with control mice. Administration of oral antibiotics or sequestration of bacterial endotoxins with sevelamer hydrochloride reduced mucosal inflammation and restored normal liver histology in $F11r^{-/-}$ mice fed a HFCD. Protein and transcript levels of JAM-A were significantly lower in the intestinal mucosa of patients with NAFLD than without NAFLD; decreased expression of JAM-A correlated with increased mucosal inflammation. **CONCLUSIONS:** Mice with defects in intestinal epithelial permeability develop more severe steatohepatitis after a HFCD than control mice, and colon tissues from patients with NAFLD have lower levels of JAM-A and higher levels of inflammation than subjects without NAFLD. These findings indicate that intestinal epithelial barrier function and microbial dysbiosis contribute to the development of NASH. Restoration of intestinal barrier integrity and manipulation of gut microbiota might be developed as therapeutic strategies for patients with NASH.

Keywords: Occludin; Claudin-4; Bacterial Translocation.

Nonalcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver failure in the United States, and its incidence is expected to increase in the near future.[1] One third of the US population, and a majority of obese individuals, develop nonalcoholic fatty liver (NAFL) or bland steatosis; a benign condition characterized by triglyceride deposition in hepatocytes.[2] Although asymptomatic, nearly 20% of individuals with NAFL progress to nonalcoholic steatohepatitis (NASH), and 15% of NASH patients develop cirrhosis.[2] In addition, NASH patients are at a higher risk for developing hepatocellular carcinoma.[3] Despite the growing incidence of NAFLD-related chronic liver disease, the lack of clarity in the mechanisms of NASH pathogenesis has hindered our ability to develop effective biomarkers as well as therapies for more severe forms of NAFLD. One major barrier to achieving this goal is the lack of a suitable murine model that faithfully recapitulates human NASH.

Abbreviations used in this paper: **ALT, alanine aminotransferase; AST, aspartate aminotransferase; αSMA, α smooth muscle actin; CRN, Clinical Research Network; FITC, fluorescein isothiocyanate; HFCD, high-fat, high-fructose, and high-cholesterol diet; HSC, hepatic stellate cell; IL, interleukin; JAM-A, junctional adhesion molecule A; LPS, lipopolysaccharide; MetS, metabolic syndrome; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PCR, polymerase chain reaction; rRNA, ribosomal RNA; TJ, tight junction; TLR, Toll-like receptor.**

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Several recent clinical studies have highlighted the potential role for compromised intestinal epithelial permeability in NASH pathogenesis.[3–5] In both human and animal NASH models, increased intestinal epithelial permeability correlates with increased serum endotoxin,[6,7] a potent inducer of hepatic inflammation.[8] Furthermore, increased intestinal epithelial permeability and small intestinal bacterial overgrowth are 2 frequently observed features of human NASH and NASH-related cirrhosis.[3] However, it is not clear whether intestinal epithelial barrier dysfunction is a cause or consequence of chronic systemic inflammation observed in NASH patients.

Here, we interrogated the role of intestinal epithelial permeability in NAFLD progression using junctional adhesion molecule A (JAM-A) knockout mice ($F11r^{-/-}$) that have a basal defect in the intestinal epithelial barrier.[9] JAM-A is a component of the tight junction (TJ) complex that regulates intestinal epithelial paracellular permeability.[9–11] Previous studies have shown that $F11r^{-/-}$ mice have leaky TJs that allow for translocation of gut bacteria to peripheral organs.[9,12,13] Interestingly, these mice do not develop spontaneous colitis when fed a normal diet, rendering them useful for studying the effect of a Western diet in NAFLD progression. Here, we show a critical role for intestinal epithelial permeability in NASH pathogenesis and our findings underscore the complex interplay between diet, intestinal epithelial barrier, and gut microbial composition in driving NAFLD progression.

Materials and Methods

Mice

C57BL/6 (control) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and $F11r^{-/-}$ mice were originally a gift from Dr Thomas N. Sato (Cornell University, New York, NY). $F11r^{-/-}$ mice were generated as previously described[14] and were backcrossed to C57BL/6 mice for 7 generations. The intestine-specific JAM-A knockout mice, $Villin^{Cre}F11r^{FL/FL}$, were obtained by crossing $Villin^{Cre}$ mice with $F11r^{FL/FL}$ mice. C57BL/6, $F11r^{-/-}$, $F11r^{FL/FL}$, and $Villin^{Cre}F11r^{FL/FL}$ mice were bred and maintained at Emory University Division of Animal Resources. All animal studies were approved by the Institutional Animal Care and Use Committee.

NAFLD Diet

To induce NAFLD, 5- to 6-week-old male control and $F11r^{-/-}$ mice were fed a high-fat, high-fructose, and high-cholesterol diet (HFCD) ad libitum for 8 weeks. The diet consisted of 0.2% cholesterol, 20% protein, 43% carbohydrates, and 23% fat (6.6% trans-fat) (TD.120330; Envigo, Indianapolis, IN).[15,16] In addition, 2.31% fructose was provided in drinking water. The normal diet is the standard mouse chow that contains 16% protein, 61% carbohydrates, and 7.2% fat. The $F11r^{FL/FL}$ and $Villin^{Cre}F11r^{FL/FL}$ mice were fed HFCD containing 2.31% fructose (TD.130885; Envigo) to eliminate the need for supplementing drinking water with fructose.

Microbiota Depletion

For antibiotic treatment, mice were given a combination of ampicillin (1 g/L), vancomycin (500 mg/L), neomycin sulfate (1 g/L), and metronidazole (1 g/L) for 8 weeks in drinking water.

In Vivo Permeability Assay

Intestinal permeability was assessed by in vivo fluorescein isothiocyanate (FITC)-dextran (FD4; Sigma-Aldrich, St. Louis, MO) permeability assay as described previously.[17] Mice fasted for 4 hours were gavaged with 0.6 mg/g body weight FITC-dextran (4 kilodaltons) solution and blood was collected by submandibular bleeding after 3 hours. Fluorescence intensity in the serum was measured using a Fluorescence Spectrophotometer (Synergy 2; Biotek, Winooski, VT). FITC-dextran concentrations were determined from a standard curve generated by serial dilutions of FITC-dextran.

Luminal Microbiota Analysis

For high-throughput 16S ribosomal RNA (rRNA) gene sequencing, control and $F11r^{-/-}$ mice were initially co-housed for 5 weeks to ensure uniformity of intestinal microbiota[18] before dietary intervention. Subsequently, control and $F11r^{-/-}$ mice were housed separately during the 8-week feeding period. At necropsy, cecum content was collected from all mouse cohorts and total DNA was extracted using a PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). The 16S rRNA genes were polymerase chain reaction (PCR) amplified using Hot Master PCR mix (5 Prime, Gaithersburg, MD) and 515F/806R primer pairs targeting the V4 region of the 16S rRNA gene.[19] The sequence of the forward and the reverse primers consisted of the following: 515f: 5'-AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3' and 806r: 5'-CAAGCAGAAGACGGCATACGAGATXXXXXXXXXXXXXAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3' respectively. Each reverse primer has a unique multiplex identifier barcode consisting of 12 bp Golay error correcting barcode, designated as XXXXXXXXXXXX, used to identify each sample in the multiplex sequencing run. The reaction conditions were 95°C for 2 minutes followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 90 seconds. Three independent PCRs per sample were purified with Ampure magnetic purification beads (Agencourt, Beverly, MA), pooled, and were sequenced using an Illumina Miseq Sequencing platform at the Emory Integrated Genomics Core. Detailed protocols for bacterial 16S rRNA gene amplification and sequencing can be found on the Earth Microbiome Project website (http://www.earthmicrobiome.org/emp-standard-protocols).[20,21]

16S rRNA Sequence Analysis

Paired-end 16S rRNA–V4 region sequencing data were subjected to the Quantitative Insights Into Microbial Ecology analysis pipeline.[22] Default Quantitative Insights Into Microbial Ecology parameters and workflow scripts were used to process raw data, normalization, clustering, taxonomic classification, and visualization as described previously.[22] The unweighted UniFrac distances between samples were calculated using 27,000–30,000 sequences per sample.[23] The variation between experimental group (β diversity) was assessed by principal coordinates analysis plots, and jack-knifed β diversity was used to estimate the uncertainty in principal coordinates analysis plots. α Diversity curves were calculated based on the number of observed species, and the Shannon diversity index was used to characterize species diversity in a community.

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