Microbial Pathogenesis 123 (2018) 250–258

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Diet-induced obesity precipitates kidney dysfunction and alters inflammatory mediators in mice treated with Shiga Toxin 2

Lisa M. Harrison[a,*], Dennis W. Gaines[a], Uma S. Babu[a], Kannan V. Balan[a], Renate Reimschuessel[b], Andrew B. Do[a], Marion R. Pereira[a], Elmer C. Bigley III[a], Martine Ferguson[a], Akshita Mehta[a], Kristina M. Williams[a]

[a] Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, Food and Drug Administration, 8301 Muirkirk Road, Laurel, MD, 20708, USA

[b] Office of Research, Center for Veterinary Medicine, Food and Drug Administration, 8401 Muirkirk Road, Laurel, MD, 20708, USA

ARTICLE INFO

Keywords:
Shiga toxin
Obesity
Inflammation
Kidney damage
Foodborne pathogen

ABSTRACT

Shiga Toxin (Stx)-producing *E. coli* (STEC) continue to be a prominent cause of foodborne outbreaks of hemorrhagic colitis worldwide, and can result in life-threatening diseases, including hemolytic uremic syndrome (HUS), in susceptible individuals. Obesity-associated immune dysfunction has been shown to be a risk factor for infectious diseases, although few studies have addressed the role of obesity in foodborne diseases. We hypothesized that obesity may affect the development of HUS through an alteration of immune responses and kidney function. We combined diet-induced obese (DIO) and HUS mouse models to look for differences in disease outcome between DIO and wild-type (WT) male and female C57 B l/6 mice. Following multiple intraperitoneal injections with endotoxin-free saline or sublethal doses of purified Stx2, we examined DIO and WT mice for signs of HUS development. DIO mice receiving Stx2 injections lost more body weight, and had significantly higher ($p < 0.001$) BUN, serum creatinine, and neutrophil counts compared to WT mice or DIO mice receiving saline injections. Lymphocyte counts were significantly ($p < 0.05$) lower in Stx2-treated obese mice compared to WT mice or saline-treated DIO mice. In addition to increased Stx2-induced kidney dysfunction, DIO mouse kidneys also had significantly increased expression of IL-1α, IL-1β, IL-6, TNF-α, MCP-1, and KC RNA compared to saline controls ($p < 0.05$). Serum cytokine levels of IL-6 and KC were also significantly higher in Stx2-treated mice compared to saline controls, but there were no significant differences between the WT and DIO mice. WT and DIO mice treated with Stx2 exhibited significantly higher degrees of kidney tubular dilation and necrosis as well as some signs of tissue repair/regeneration, but did not appear to progress to the full pathology typically associated with human HUS. Although the combined obesity/HUS mouse model did not manifest into HUS symptoms and pathogenesis, these data demonstrate that obesity alters kidney function, inflammatory cells and cytokine production in response to Stx2, and may play a role in HUS severity in a susceptible model of infection.

1. Introduction

Illnesses caused by foodborne pathogens are a major public health problem in the United States [1,2]. In particular, Shiga toxin-producing *E. coli* (STEC) continue to cause foodborne illnesses that can lead to Hemorrhagic Colitis (HC) and Hemolytic Uremic Syndrome (HUS), a leading cause of acute renal failure in children in the developed world [3]. STEC colonize the intestinal mucosa through various adhesion factors, including intimin, which is an adhesion factor necessary for the attaching and effacing (A/E) histopathology associated with enteropathogenic and enterohemorrhagic *E. coli* infections [4–7]. Following colonization, STEC secrete Shiga toxins (Stxs), which are multifunctional exotoxins capable of inhibiting protein synthesis as well as activating signaling pathways that lead to inflammatory and apoptotic responses in numerous cell types [8]. Uncontrolled pro-inflammatory and apoptotic pathways in intestinal, kidney, and brain cells can result in tissue damage in humans and primates [9,10], further leading to the development of HC, HUS and/or central nervous system complications [11–13].

HUS is a human disease that usually occurs in immunocompromised

* Corresponding author. Office of Applied Research and Safety Assessment, CFSAN, US FDA, 8301 Muirkirk Road, MOD-1, Laurel, MD, 20708, USA.
E-mail address: lisa.plemons@fda.hhs.gov (L.M. Harrison).

https://doi.org/10.1016/j.micpath.2018.07.015
Received 23 March 2018; Received in revised form 12 July 2018; Accepted 13 July 2018
0882-4010/ Published by Elsevier Ltd.

populations such as young children or the elderly, but a recent outbreak in Germany in 2011 involved a Shiga toxin-producing enteroaggregative *E. coli* (Stx-EAEC) strain that was able to cause HUS in adults [14–17]. Equally alarming was the higher incidence of HUS (22%) in the 2011 outbreak since HUS usually occurs at a rate of 15% in children under 10 years of age [18]. As new pathogenic STEC strains emerge and existing pathogenic *E. coli* strains acquire new virulence factors, the populations that are susceptible as well as the severity of disease can change. *In vivo* models of STEC infection can help assess the public health risk of foodborne disease in susceptible populations. As of 2014, obesity is prevalent in over one third of the US adult population [19]. Several studies have demonstrated that obesity is associated with immune dysfunction [20–22], making it a risk factor for numerous diseases, including infectious diseases [23–27]. Diet is a primary factor in the development of obesity and thus there is particular interest in studying the diet-induced obese (DIO) population [28]. Despite the interest, only a few studies have examined the effects of obesity on susceptibility to infections with foodborne pathogens. For instance, leptin and leptin-receptor deficient obese rats have been found to be susceptible to intravenous infection with the foodborne pathogen *Listeria monocytogenes* [24], but obesity caused by congenital leptin deficiency in humans is rare [29]. Also, only two other studies have demonstrated that mice fed lipidic diets are more susceptible to subcutaneous or intraperitoneal *Salmonella* Typhimurium infections [25,30].

In order to determine whether diet-induced obesity affects immune responses and severity of HUS caused by the foodborne pathogen STEC, we combined a HUS mouse model [31] with an obese mouse model [23], and examined kidney function and pathology as well as cellular and inflammatory effects. By understanding the immune responses and pathology in the combined models of disease, we can determine whether obesity plays a role in the severity of disease caused by Stxs, and develop more effective public health strategies to reduce occurrences of foodborne illnesses in susceptible individuals and populations.

2. Materials and methods

2.1. Mice

Three-week old male & female C57 Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). For each of the three trials, we purchased 24 mice including 12 females and 12 males. Following a 1-week acclimation period, the 12 male and 12 female mice in each trial were further split into two dietary groups, which were designated the wild-type (WT) and diet-induced obese (DIO) groups (6 males and 6 females in each group). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. The protocol was approved by the Food and Drug Administration-Center for Food Safety and Applied Nutrition-Institutional Animal Care and Use Committee (FDA-CFSAN-IACUC). All mice were maintained at the FDA Module-1 animal facility using procedures approved by the FDA-CFSAN-IACUC.

2.2. Diets

Starting at 4 weeks of age, 6 male and 6 female mice from each trial were fed a standard grain-based diet (SD; 2018 Teklad Global 18% Protein Rodent Diet from Envigo, Madison, WI), with 18% of kcal from fat. The other 6 male and 6 female mice from each trial were fed a high-fat purified diet (HFD; D12492 Research Diets, Inc., New Brunswick, NJ) with 60% of kcal from fat [23]. In the HFD, fat was substituted for the carbohydrates in the SD. Body weights were monitored weekly during the 16-week diet regimen.

2.3. Blood glucose

Prior to starting the treatments (day −1), and following an 8 h fasting period, blood glucose levels were determined using blood from submandibular puncture and the AlphaTrak Blood Glucose Monitor set to code 7 (Originally manufactured by Abbott Laboratories, Abbott Park, IL; Current AlphaTrak 2 Blood Glucose Monitor is manufactured by Zoetis, Parsippany, NJ). Mice were considered to be diabetic if their blood glucose levels were 300 mg/dL or greater [32].

2.4. Treatment

Mice (~20 weeks old) were injected with either 50 µL 0.9% endotoxin-free saline solution (Technova, Hollister, CA) or 50 µL 1 ng purified Stx2 (BEI Resources, Manassas, VA) per 20 g body weight on days 0, 3, and 6 as previously reported by Sauter et al. [31]. On day 5, mice were transferred to diuresis metabolic cages (Tecniplast, Buguggiate, Italy) for urine collection on days 6, 7 and 8. Following urine and blood (submandibular puncture) collection, mice were euthanized on Day 8 and spleens and kidneys were harvested. Body weights were recorded daily following the initial treatment to monitor weight loss.

2.5. Cell and biochemical analyses of blood

Blood was collected by submandibular puncture from each mouse for cell count analyses and biochemical determinations of blood urea nitrogen (BUN) and serum creatinine in microtainers containing Lithium Heparin (Becton Dickinson, Franklin Lakes, NJ). All cell and biochemical analyses of blood were performed by Comparative Clinical Pathology Services, LLC (Columbia, MO).

2.6. Serum cytokine analyses

Blood was also collected (day 8) by submandibular puncture for serum cytokine analyses using microtainers containing a clot activator gel for serum separation (Becton Dickinson), and allowed to coagulate for at least 30 min prior to centrifugation for 1.5 min at ~ 15,000 × g at 4 °C. Separated serum samples were stored at −80 °C until further analyses. Serum samples were analyzed, in duplicate, using the Bio-Plex Mouse Cytokine Group I 7-plex Assay (#Y60-00DE9C; Bio-Rad) and the Bio-Plex 200 Multiplex Reader (Bio-Rad), according to the manufacturer's instructions. Observed concentration values were used for analyses to identify differences in serum cytokine expression between Stx2- and saline-treated mice.

2.7. Histopathology

Half of one kidney was fixed in Carnoy solution for at least 2 h, and washed twice with 70% ethanol before being stored in 70% ethanol. Fixed kidney tissues were embedded in paraffin blocks, sectioned into 4 µm sections, and processed for hematoxylin and eosin (H&E) staining by Histoserv, Inc. (Germantown, MD). Stained sections for each animal were scored (1 = minimal, 2 = mild, 3 = moderate, 4 = marked, 5 = severe) for tubular dilation, tubular necrosis, and glomerular changes by a pathologist who was blinded to the treatment groups. A representative tissue section (20× magnification) for each treatment group was selected to demonstrate the resulting pathology.

2.8. Quantitative real-time RT-PCR

Small sections of kidneys frozen in liquid N_2 were cut and used for RNA isolation. Briefly, a small portion of frozen tissue wrapped in aluminum foil was pulverized into powder with a hammer and immediately placed in 2 mL RLT buffer (QIAgen, Gaithersburg, MD) containing β-mercaptoethanol. Samples were vortexed briefly and homogenized using the Polytron PT-MR 2100 (Kinematica, Littau-

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