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Leptin antagonist ameliorates chronic colitis in IL-10$^{-/-}$ mice

Udai P. Singh[a,*], Narendra P. Singh[a], Hongbing Guan[a], Brandon Busbee[a], Robert L. Price[b], Dennis D. Taub[c], Manoj K. Mishra[d], Raja Fayad[e], Mitzi Nagarkatti[a], Prakash S. Nagarkatti[a]

[a] Pathology, Microbiology and Immunology, School of Medicine, University of South Carolina, Columbia, SC 29208, USA
[b] Department of Cell and Developmental Biology, University of South Carolina, Columbia, SC 29208, USA
[c] Laboratory of Molecular Biology and Immunology, NIA-IRP, NIH, Baltimore, MD 21224, USA
[d] Department of Math and Sciences, Alabama State University, 1627 Hall Street, Montgomery, AL 36104, USA
[e] Department of Exercise Science, Arnold School of Public Health, University of South Carolina, Columbia, SC 29208, USA

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ABSTRACT

Background: Although the etiology of two major forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are unknown and evidence suggests that chronic intestinal inflammation is caused by an excessive immune response to mucosal antigens. Previous studies support the role for TGF-β1 through 3 in the initiation and maintenance of tolerance via the induction of regulatory T cells (Tregs) to control intestinal inflammation. Leptin, a satiety hormone produced primarily by adipose tissue, has been shown to increase during colitis progression and is believed to contribute to disease genesis and/or progression.
Aim: We investigated the ability of a pegylated leptin antagonist (PG-MLA) to ameliorate the development of chronic experimental colitis.
Results: Compared to vehicle control animals, PG-MLA treatment of mice resulted in an (1) attenuated clinical score; (2) reversed colitis-associated pathogenesis including a decrease in body weight; (3) reduced systemic and mucosal inflammatory cytokine expression; (4) increased insulin levels and (5) enhanced systemic and mucosal Tregs and CD39$^+$ Tregs in mice with chronic colitis. The percentage of systemic and mucosal TGF-β1, -β2 and -β3 expressing CD4$^+$ T cells were augmented after PG-MLA treatment. The activation of STAT1 and STAT3 and the expression of Smad7 were also reduced after PG-MLA treatment in the colitic mice. These findings clearly suggest that PG-MLA treatment reduces intestinal Smad7 expression, restores TGF-β1-3 signaling and reduces STAT1/STAT3 activation that may increase the number of Tregs to ameliorate chronic colitis.
Conclusion: This study clearly links inflammation with the metabolic hormone leptin suggesting that nutritional status influences immune tolerance through the induction of functional Tregs. Inhibiting leptin activity through PG-MLA might provide a new and novel therapeutic strategy for the treatment of IBD.

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Introduction

It is generally agreed that inflammatory bowel disease (IBD) is a multifactorial disease state with immunological, environmental and genetic influences. IBD, especially Crohn's disease (CD), is commonly characterized by weight loss, anorexia and increased energy expenditure during the acute stages of intestinal inflammation (Silk and Payne-James 1989; Rigaud et al. 1994; De Gaetano et al. 1996; Ehrhardt et al. 1997). While the causes of IBD remain unknown, the disease has been associated with food intake, increased energy expenditure and a reduced absorption of nutrients (Greenberg et al. 1989). Recently, it was suggested that the anorexigenic hormone leptin, might play a role in the anorexia associated with chronic inflammatory diseases and cancer. Anorexia, malnutrition and altered body composition are well-established features associated with IBD.

Leptin is a hormone produced primarily by adipose tissue (Auwerx and Staels 1998) with a primary function of controlling appetite and adiposity. Leptin has recently been shown to play a role in the control of inflammation and autoimmunity (De Rosa et al. 2006) and emerged as a potential inducer of inflammatory cytokine expression, more specifically IL-1, TNF-α, and IL-6 (Grunfeld et al. 1996; Dixit et al. 2004). In humans, increases in leptin levels are associated with ulcerative colitis (Tuzun et al. 2004;

* Corresponding author at: Department of Pathology, Microbiology and Immunology, University of South Carolina, Columbia, SC 29208, USA. Tel.: +1 803 216 3423; fax: +1 803 216 3413.
E-mail address: Udai.singh@uscmed.sc.edu (U.P. Singh).

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Karmiris et al. 2006) and over-expression of leptin mRNA in mesenteric adipose tissue in IBD patients has been reported (Barbier et al. 2003). Colonic leptin induces epithelial wall damage and neutrophil infiltration, a typical characteristic of histological findings in acute intestinal inflammation (Sitaraman et al. 2004). Similarly, in rats, elevated plasma leptin concentrations correlates with an increase in intestinal inflammation (Barbier et al. 1998).

Despite strong evidence supporting a role for leptin in inflammation and autoimmunity, the precise mechanism of its action has been controversial and both direct and indirect mechanisms have been described (Lord et al. 1998; Palmer et al. 2006). Leptin can directly affect numerous immune cell types of both the innate and adaptive immune systems and can induce pro-inflammatory cytokine expression (Fernandez-Riejos et al. 2010). In a recent study, a direct link of leptin with Tregs anergy and hyporesponsiveness was described (De Rosa et al. 2007). Interestingly, an important source of leptin is Tregs themselves, which appear to express both leptin and its receptors (De Rosa et al. 2007). Mice with a genetic deficiency in leptin have a higher percentage and absolute number of circulating Tregs (Hasenkrug 2007) and the treatment of wild type mice with leptin neutralizing antibody produces an expansion of Tregs. In a recent study by Matarese et al. (2005b), an increase in leptin in multiple sclerosis (MS) patients correlated with a reduced number of regulatory T cells (Matarese et al. 2005a). These reductions in Tregs levels are likely a direct consequence of leptin binding to receptors of regulatory cells.

TGF-β signaling in Tregs is essential to efficiently control inflammation in the colon (Huber et al. 2004). Oral administration of haptenized colonic proteins protects mice from the induction of trinitrobenzene sulfonic acid (TNBS) colitis by the generation of mucosal T cells that produce TGF-β (Neurath et al. 1996; Fuss et al. 2002). TGF-β either directly down-regulates the differentiation and function of effector T cells or induces the expression of forkhead transcription factor (FoxP3) expression associated with Tregs (Chen et al. 2003; Fantini et al. 2004; Rao et al. 2005). TGF-β1 signaling occurs from receptors to the nucleus via a set of proteins termed Smads. The up-regulation of inhibitory Smad7 is associated with inhibition of TGF-β1-induced Smad signaling (Nakao et al. 1997). It has been shown that activators of either NF-κB or signal transducer and activator of transcription (STAT)-1 pathways can enhance Smad7 expression (Ulloa et al. 1999). The activation of STAT-1/STAT-3 pathways is well documented in playing an important role in both human IBD and experimental colitis (Suzuki et al. 2001; Schreiber et al. 2002; Lovato et al. 2003). Furthermore, oral administration of Smad7 antisense oligonucleotide reduced systemic Smad7 expression and restored TGF-β1 associated P-Smad3 expression in colons of mice in TNBS and oxazolone-induced models of colitis (Monteleone et al. 2012).

In the present study, we investigated whether the blockade of leptin by PG-MLA mediates the development and progression of chronic colitis in IL10$^{-/-}$ mice. We have studied the cellular and molecular mechanism of PG-MLA treatment mediated abrogation of colitis. The results from this study clearly suggest that PG-MLA increases systemic and mucosal Tregs through restoration of TGF-β1-3 signaling, supported in part by a decrease in Smad7 expression, STAT1/3 activation that leads to reduced inflammatory cytokine levels, histological scores, and severity of disease to abrogate chronic colitis.

Materials and methods

Human sera, and tissue collection

Blood from a total of 120 age-matched untreated IBD patients and 30 healthy donors (30 each from African American, European, Ashkenazi Jewish, Asian American), and colon tissue from 8 patients, two from each ethnic group and two from normal healthy donors were collected (Clinomics Biosciences, Inc. Pittsfield, MA). After providing information on the future use of serum in biomarker analysis, formal consent was obtained from all patients approved by the Clinomics Biosciences Research Ethics Committee. These patients did not receive any steroid treatments before blood was taken. Age, gender, and race/ethnic/geographic origin were considered. Average mean age was 47 and ranged from 40 to 71 years. Studies included enough serum/plasma samples from these groups to ascertain statistically significant differences. Each sample was analyzed in triplicate and compared with a standard curve using ELISA techniques to measure leptin concentrations.

Animals

Female IL-10$^{-/-}$ mice on the C57BL/6 background aged 8 to 12 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). Under standard laboratory conditions, IL-10$^{-/-}$ mice develop spontaneous colitis at 12 weeks of age. Animals were housed and maintained in isolator cages under normal light and dark cycles in conventional housing conditions to minimize animal pain and distress in the University of South Carolina School of Medicine animal facility Columbia, USA. Experimental groups consisted of 6 mice and studies were repeated 3 times. The body weights of mice were monitored twice on each Monday and Thursday in a week. Even though it has been reported that IL-10$^{-/-}$ mice develop colitis at age of 12 weeks under conventional housing conditions, we did not notice a major weight change until the onset of severe chronic colitis.

PG-MLA and leptin antagonist treatment

Mouse leptin antagonist (mutant L39A/D40A/F41A) and PG-MLA was purchased from Protein Laboratories Rehovot LTD (Tel Aviv, Israel). The mutant leptin was purified by proprietary chromatographic techniques and was greater than 98% pure determined by gel filtration analysis. The endotoxin levels of this mutant are found to be less than 0.05 ng/μg (0.5 EU/μg). The biopotency of leptin antagonist is increased by attaching it to polyethylene glycol (PEG) molecules resulting in reduced renal clearance and consequent prolongation of its half life cycle. Based on previous studies from our laboratory (Singh et al. 2003), serum amyloid A (SAA) and -IL-6 levels generally peaked during the 18th week, corresponding with the onset of chronic colitis. At this stage, mice received 200 μl by intraperitoneal injection of vehicle, MLA and PG-MLA (6.25 mg/kg body weight) twice a week on each Monday and Thursday till week 27 at the end-point of the experiment. At the experimental end-point, blood was collected by tail-vein bleedings and serum was obtained following centrifugation. For comparison, a similar treatment was also given to normal BL/6 mice.

Cytokine quantitation by Luminex™ analysis

At the experimental end point, level of IL-6, TNF-α, monocyte chemotactic protein1 (CCL2), lipopolysaccharide-induced CXC chemokine (CXCL5), insulin and resistin in the serum were determined by a Luminex Elisa assay kit as described by manufacturer protocol (Millipore Corporation, MA, USA). Briefly, IL-6, TNF-α, CCL2, CXCL5, insulin and resistin analyte beads in assay buffer were added into pre-wet vacuum wells followed by 25 μl of serum or standard solution, 25 μl of assay buffer, and 25 μl of assay beads, and incubated overnight at 4 °C with continuous shaking (at setting #3) using a Lab-Line™ Instrument Titer Plate Shaker (Melrose, IL). The filter bottom plates were washed and vortexed at 300 × *g* for 30 s. Subsequently, 25 μl of anti-mouse detection Abs were added to each well and incubated for 1 h at room temperature. Next, 25 μl