International Immunopharmacology 39 (2016) 92–96

Contents lists available at ScienceDirect

International Immunopharmacology

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

S100G expression and function in fibroblasts on colitis induction

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ARTICLE INFO

Article history:
Received 19 May 2016
Received in revised form 4 July 2016
Accepted 18 July 2016
Available online 25 July 2016

Keywords:
Interleukin-10
Inflammatory bowel diseases
S100G
Fibroblasts
Monocyte chemotactic protein-1

ABSTRACT

Supplementation with interleukin (IL)-10, an important anti-inflammatory cytokine, has shown disappointing efficacy for inflammatory bowel diseases (IBD). IL-10 may down-regulate the expression of other anti-inflammatory mediators following colitis induction. We used a colitis model characterized by hapten-protein visualization, which indicates the site of hapten-protein formation after colitis induction for histological and gene expression analyses. Under IL-10 deficiency, following colitis induction inflammatory changes were reduced, and S100G expression was elevated. S100G was expressed in fibroblasts, and S100G expression was down-regulated by IL-10. S100G suppressed the production of monocyte chemotactic protein-1 (MCP-1) through the inhibition of NF-κB activation. Therefore, S100G, also known as Calbindin-D9k, may be an important anti-inflammatory mediator in fibroblasts following colitis induction, and down-regulation of S100G expression might be one reason for the insufficient performance of IL-10 supplementation.

1. Introduction

Inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, are chronic disorders of the gastrointestinal tract [1]. Although the precise pathogenesis of IBD still remains unclear, cytokines and chemokines are mediators of inflammation [2,3]. Tumor necrosis factor α (TNFα) is a critical pro-inflammatory cytokine, and anti-TNFα therapy has shown overwhelming efficacy for IBD [2]. Interleukin (IL)-10 is considered to be the most important anti-inflammatory cytokine [4–6]. IBD are associated with polymorphisms in IL-10 and IL-10 receptor genes [5], and chronic changes in inflammation are observed in IL-10-deficient mice [6]. However, IL-10 supplementation has shown disappointing results in clinical trials for IBD [4]. Marlow et al. suggested the following five potential explanations: 1) an insufficient dosage of IL-10, 2) the presence of different pathogeneses among IBD patients, 3) its prevention of only the establishment of IBD, 4) its failure to suppress all the pro-inflammatory mediators, and 5) IL-10′s immunostimulatory effects, such as interferon γ (IFNγ) production [4]. We would like to propose another possibility, namely that IL-10 may down-regulate the expression of other anti-inflammatory mediators. IL-10-deficient mice gradually develop inflammatory changes from the proximal region of the colon, and the chronic inflammation reaches the distal region when the mice become 12–16 weeks old under specific pathogen-free conditions [6,7]. To investigate gene expression under IL-10 deficiency following colitis induction, we selected 8-week-old IL-10-deficient mice that did not show any signs of colitis, such as diarrhea or rectal prolapse, for colitis induction with 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl).

NBD-Cl-induced colitis is characterized by hapten-protein visualization [8] (Fig. 1). NBD-Cl binds to proteins through the substitution reaction of 4-Cl with amino or thiol groups of amino acids residues [9]. NBD-Cl alone shows no fluorescence, but NBD-proteins are fluorescent (excitation 460 nm, emission 535 nm), similar to fluorescein and GFP [9]. NBD-proteins also work as hapten-proteins; NBD-proteins are endocytosed by macrophages and are presented to T cells for T cell activation, leading to colitis induction [8]. Therefore, a NBD-Cl-enema treatment induces inflammation in the distal region of the colon, and the site of hapten-protein formation is detected via fluorescence after colitis induction for histological and gene expression analyses. Using this unique model, we investigated inflammatory changes and gene expression under IL-10 deficiency at the site of hapten-protein formation.

2. Materials and methods

2.1. Mice

We obtained BALB/c mice from Japan SLC (Shizuoka, Japan) and IL-10-deficient mice on the BALB/c background from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were kept in a 12-h light/dark cycle with controlled humidity (60–80%) and temperature (22 ± 1 °C) under specific pathogen-free conditions. Food and water were freely

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; hpf, high-power field; IBD, inflammatory bowel diseases; IDO1, indoleamine 2,3-dioxygenase 1; IL, interleukin; MCP-1, monocyte chemotactic protein-1; NBD-Cl, 4-chloro-7-nitro-2,1,3-benzoxadiazole.

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http://dx.doi.org/10.1016/j.intimp.2016.07.017
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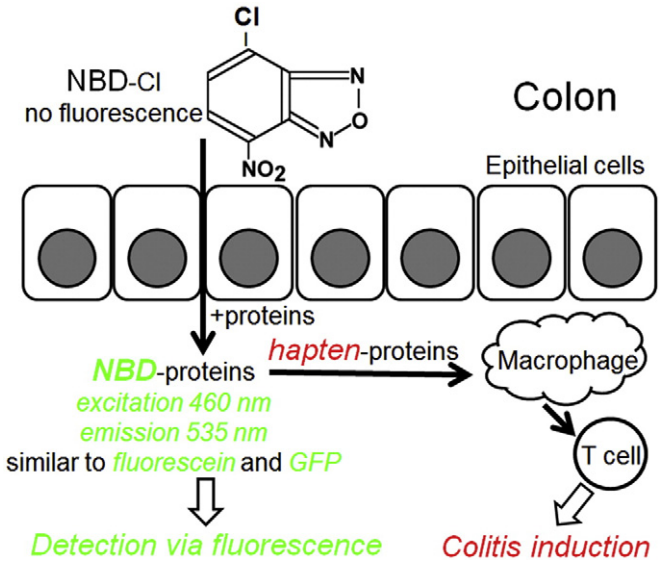

Fig. 1. Illustration of NBD-Cl-induced colitis. Unlike NBD-Cl, NBD-proteins fluoresce, so that NBD-protein formation can be detected in the colonic mucosa under fluorescence observation. NBD-proteins also work as hapten-proteins; NBD-proteins are endocytosed by macrophages and are presented to T cells for T cell activation, leading to colitis induction.

available. Under the specific pathogen-free conditions, IL-10-deficient mice showed signs of colitis, such as diarrhea and rectal prolapse, at 12–16 weeks old as reported previously [6,7]. All animal experiments were performed according to the guidelines of the Institute for Laboratory Animal Research and with the approval of the ethics committee of Nagoya University.

2.2. Colitis induction

NBD-Cl (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in dimethyl sulfoxide to a concentration of 200 mg/ml for the stock solution, which was stored at −80 °C. The NBD-Cl stock solution was diluted with ethanol and then with distilled water at a ratio of 1:100:100 to prepare a 50% ethanol solution containing 1 mg/ml NBD-Cl as the NBD-Cl enema.

For the experimental group, we selected 8-week-old male IL-10-deficient mice that did not show any signs of colitis, such as diarrhea or anal prolapse. Age-matched male wild-type mice were used as the controls. Colitis was induced with the NBD-Cl enema as described previously [8]. Briefly, we lightly anesthetized mice with isoflurane (Abbott Laboratories, Abbott Park, IL, USA) and inserted a rubber catheter (2 mm outer diameter) fitted onto a 1 ml syringe via the anus. The tip was positioned 2 cm proximal to the anus. Then, 100 μl of the NBD-Cl enema was slowly administered to the mice through the catheter. The mice were kept in a head-down position for 30 s and then returned to their cages. Body weight was measured on days 0–2. The colon was dissected from the mice on day 2 for histological analysis and on day 1 for gene expression analysis, immunohistochemical staining and organ culture. We opened the colon longitudinally to remove stools by washing with phosphate-buffered saline, and obtained colon samples positive for NBD-proteins under fluorescence observation using the SZX-RFL2 fluorescence stereomicroscopy system (Olympus, Tokyo, Japan). Because NBD-proteins were detected in the portion of the colon 2–4 cm from the anus following the NBD-Cl-enema treatment as described previously [8], control samples were obtained at a similar portion of the colon dissected from mice that had not received the NBD-Cl-enema treatment.

2.3. Histological analysis

The colon samples were fixed overnight in 4% paraformaldehyde solution and embedded in paraffin to prepare sections (6 μm). After deparaffinization, sections were observed with the BZ-8000 fluorescence microscopy system (Keyence, Osaka, Japan) to confirm NBD-protein formation in the colon samples. Adjacent sections were stained with hematoxylin and eosin for histological evaluation of colitis. Colitis scores were determined by the following histological criteria [8]: 1) a low level of mononuclear cell infiltration with infiltration observed in <50% of the high-power field (hpf, ×200) and no structural changes observed; 2) a low level of mononuclear cell infiltration, crypt distortion and no destruction of the epithelia; 3) a high level of mononuclear cell infiltration with infiltration observed in ≥50% of the hpf and no destruction of the epithelia; 4) the focal destruction of the epithelia covering <1 hpf; 5) the destruction of the epithelia covering ≥1 hpf, and 6) the extensive destruction of the epithelia covering ≥2 hpf. All sections were blinded for scoring.

2.4. Gene expression analysis

RNA was isolated from the colon samples with an RNeasy Mini kit (QIAGEN, Hilden, Germany). Two-color microarray analysis was performed with a SurePrint G3 Mouse GE 8 × 60 K microarray (Agilent Technologies, Santa Clara, CA, USA) by Hokkaido System Science (Hokkaido, Japan).

For Western blot analysis, the colon samples were lysed in lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA pH 8.0, 1% Triton X-100, and 6.2 KIU/ml aprotinin). After centrifugation, protein concentrations in the supernatants were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA) to ensure equal amounts of total proteins in each lane for Western blot analysis using rabbit anti-S100G antibody (catalog number, sc-28,532; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-indoleamine 2,3-dioxygenase 1 (IDO1) antibody (ABGENT, San Diego, CA, USA), or anti-tubulin α antibody (Thermo Scientific, Fremont, CA, USA).

2.5. Immunohistochemical staining

Sections were prepared as described in the section entitled *Histological analysis*. After deparaffinization, sections were incubated with anti-S100G antibody and then with biotinylated anti-rabbit IgG antibody. After reaction with avidin-biotin-peroxidase, staining was visualized with diaminobenzidine tetrahydrochloride-Ni^{3+}, Co^{2+} (Vector Laboratories, Burlingame, CA, USA). L.A.B. solution was used to liberate antigens according to the manufacturer's instructions (Polysciences, Warrington, PA, USA).

2.6. Primary fibroblasts

Primary fibroblasts were obtained from BALB/c mouse embryos on day 13.5 of gestation as described previously [10]. After three passages, the cells were stocked in liquid nitrogen and used within five passages from the stocks. Unless otherwise specified, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 50 U/ml penicillin G, 50 μg/ml streptomycin, and 125 ng/ml amphotericin B.

2.7. Organ culture

Organ culture was performed as described previously [8]. Briefly, the colon samples (1 cm in length) were placed on culture insert filters (0.4 μm pore size) in 6-well plates (BD Falcon, Franklin Lakes, NJ, USA) containing RPMI1640 with 5% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, 10 μg/ml gentamicin, and 10 mM HEPES (600 μl in the upper chamber and 1400 μl in the lower