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Autophagy in the intestinal epithelium regulates *Citrobacter rodentium* infection

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ABSTRACT

Autophagy, a ubiquitous degradation pathway, is important for the survival and homeostasis of cells. Previous studies have demonstrated the role of autophagy in host defense against bacterial infection, but the importance of autophagy in the intestinal epithelium for the regulation of bacterial infection has not been fully elucidated. In this study, we showed that the essential autophagy protein Atg7 is required for resistance to *Citrobacter rodentium* infection in the intestinal epithelium. Infected mice in which Atg7 had been conditionally deleted from the intestinal epithelium exhibited greater clinical evidence of disease and higher expression levels of pro-inflammatory cytokine mRNA in the large intestine. Moreover, *C. rodentium* clearance was reduced in the Atg7 conditional knockout mice. These results demonstrate that autophagy in intestinal epithelial cells plays an important role in host defense against *C. rodentium* infection and the regulation of *C. rodentium* infectious colitis.

Introduction

Autophagy is a bulk degradation system that is used to recycle long-lived proteins and organelles and is important for the maintenance of the amino acid pool, which is essential for survival [1]. This evolutionarily conserved process is initiated by the formation of double-membrane cytosolic vesicles, so-called autophagosomes, which envelop cytoplasmic contents and deliver them to lysosomes [2]. Previously, it was reported that mice lacking the genes for Atg5 and Atg7, which code for essential autophagic machinery components, died within 24 h of birth [3,4]. In addition, autophagy is known to be involved in many physiological processes including the regulation of cell growth, anti-aging effects [1], surface antigen presentation [5], protection against genome instability, and the prevention of necrosis [6]. Autophagy dysfunction has been proposed to be associated with various diseases, such as cancer [7–9], neurodegeneration [10,11], cardiomyopathy [12,13], liver disease [9], autoimmune disease [14], and diabetes [15]. Moreover, autophagy also plays a crucial role in the control of inflammatory diseases. Recently, genome-wide association studies identified three Crohn's disease susceptibility genes, IRGM1, which encodes for an autophagy-stimulatory immunity-related GTPase, NOD2, an intracytoplasmic member of the family of proteins now known as the nucleotide-binding oligomerization domain (NOD)[1]-like receptor proteins, and ATG16L1, which is an autophagy execution gene, these are involved in autophagy [16]. Autophagy regulates several inflammatory transcriptional responses. For example, increased levels of the adaptor protein p62, which activates the pro-inflammatory transcription factor nuclear factor-κB (NF-κB), were observed in autophagy-deficient cells [17]. We also reported that lipopolysaccharide (LPS)-induced production of tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) mRNA was enhanced in the intestines of autophagy conditional knockout mice, and the LPS-induced nuclear translocation of NF-κB was also promoted in their intestinal epithelia [18], suggesting that a loss of autophagy in intestinal epithelial cells enhances the immune response to bacteria.

Citrobacter rodentium, a pathogen used in murine models of enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) infection, is classified as an attaching and effacing (A/E) pathogen. The colonization of colon epithelial cells by *C. rodentium* results in epithelial hyperplasia and mucosal inflammation [19]. Similar to EHEC and EPEC, *C. rodentium* is

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[1] *Abbreviations used:* NOD: nucleotide-binding oligomerization domain; NF-B: nuclear factor-B; LPS: lipopolysaccharide; TNF-α: tumor necrosis factor-α; IL-1, interleukin-1; EHEC: enterohemorrhagic *Escherichia coli*; EPEC: enteropathogenic Escherichia coli; GFP: green fluorescent protein; IFN-γ: interferon-γ; NIK: NF-κB inducing kinase; IKK: inhibitor of NF-κB kinase; TLR: Toll-like receptor; TRIF: Toll-interleukin-1 receptor domain containing adaptor-inducing interferon-β; ROS: reactive oxygen species; MAPK: mitogen-activated protein kinase; DSS: dextran sodium sulfate; SCV: *Salmonella*-containing vacuole; TTSS: type III secretion systems.

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generally localized on the surface of the mucosa and the superficial portion of the glands [20–22]. Its colonization of the colon reaches its peak at 7–10 days after its oral administration [23], and it induces the marked infiltration of inflammatory cells after infecting immunocompetent mice [24]. Such infected mice exhibit weight loss and diarrhea in association with crypt hyperplasia; goblet cell loss; and the mucosal infiltration of the epithelium by lymphocytes, macrophages, neutrophils, and mast cells [25,26].

In this study, we investigated whether autophagy in the intestinal epithelium affects *C. rodentium* infection. To determine the necessity of autophagy in host defense, conditional knockout mice in which Atg7 had been knocked out from the intestine [18] were orally infected with *C. rodentium*, and then the involvement of autophagy in bowel inflammation was examined.

Materials and methods

Reagents and mice

For fluorescent immunostaining, phalloidin conjugated with Alexa Fluor 647 was purchased from Invitrogen (Carlsbad, CA). Atg7flox/flox (Atg7F/F) mice were kindly provided by Dr. M. Komatsu and Dr. K. Tanaka (The Tokyo Metropolitan Institute Medical Science, Japan) [4].

Generation of mice in which the Atg7 gene had been conditionally deleted from the intestinal epithelium

F/F:vil-cre mice, in which Atg7 had been conditionally deleted from the intestinal epithelium, were generated according to the method described in our previous report [18]. All animal experiments were conducted in accordance with the guidelines of the Institute for Experimental Animals, Kobe University Graduate School of Medicine.

Induction of C. rodentium colitis

C. rodentium strain DBS100 (catalog number 51459; ATCC) was kindly provided by Dr. Gad Frankel (Division of Cell and Molecular Biology, Imperial College London, United Kingdom). Green fluorescent protein (GFP)-expressing *C. rodentium* (GFP-*C. rodentium*) was kindly provided by Dr. C. Sasakawa (Tokyo University, Japan). *C. rodentium* was cultured in LB broth medium for 6 h at 37 °C with shaking. After 6 h, the bacterial density was assessed using absorbance at an optical density of 600 nm and confirmed via the plating of serial dilutions. Four-week-old mice were orally inoculated with 5×10^8 CFU of *C. rodentium* using a gavage needle. Their body weights, the bacterial concentrations in their feces, and the histological findings of their colon were assessed for 3 weeks after inoculation. Their colonic tissues were stained with hematoxylin and eosin and then evaluated as described in the "Histological analysis of colon tissue" section below. Intestinal epithelial cells were also obtained from the entire mucosal layer of the large intestine and used for real-time PCR.

Bacterial counts

Fresh fecal pellets were collected from the mice at 7, 14, and 21 days after infection and then dissolved in phosphate-buffered saline (PBS) at a concentration of 100 mg/ml. The mixture was vortexed, and serial dilutions were plated on MacConkey agar plates. The bacterial colonies were counted at 24 h after the start of the culture.

Histological analysis of colon tissue

For histological analysis, terminal colon tissue was removed from the mice and soaked in 10% formalin in PBS. Next, paraffin-embedded sections were prepared and stained with hematoxylin and eosin. The histological analysis was performed in a blinded manner according to the method of a previous report [24]. For the histopathological grading of colitis, five criteria; i.e., hypervascularization, the presence of mononuclear cells, epithelial hyperplasia, epithelial injury, and the presence of granulocytes, were scored from 0 to 3, yielding an total score of between 0 (no colitis) and 15 (maximal colitis activity).

Immunofluorescent staining

The large intestine was frozen in O.C.T. compound (Sakura Finetek, Tokyo, Japan) and then sectioned into 10-μm thick slices. The sections were fixed with acetone and then blocked with 10% goat serum (Vector Labs, Burlingame, CA) in PBS for 30 min at room temperature. The sections were incubated with Alexa Fluor 647-labeled phalloidin (1:200) for 1 h at room temperature. After being mounted with Fluorescent Mounting Medium (Dako, Denmark), the sections were observed using a confocal microscope (LSM 5 PASCAL; Carl Zeiss, Jena, Germany) in a blinded manner.

Determination of anti-C. rodentium antibody in serum samples

Whole blood was collected from the mice that had been infected with *C. rodentium*. Serum samples were obtained by centrifugation at 16,000*g* for 10 min and stored at −20 °C until they were used in the subsequent experiments. To analyze the antigen-specific antibody response, 96-well plates were coated overnight at 4 °C with 100 μl of bicarbonate solution (pH 9.6) containing 10 μg/ml *C. rodentium* lysate. After being washed with PBS containing 0.05% Tween 20 (PBST), the plates were blocked by the addition of 1.5% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. The plates were then washed three times with PBST before the sera from individual mice were added and serially diluted in PBST containing 0.2% BSA, and then the plates were incubated for 2 h at 37 °C. To determine specific IgG titers, the plates were washed with PBST and then treated with 100 μl of an anti-mouse IgG-specific horseradish peroxidase (HRP) conjugate (Dako-Cytomation; Denmark A/S) diluted 1:5000 in PBST containing 0.2% BSA. After incubation for 1 h at 37 °C, the plates were washed with PBST, the bound antibody was detected by the addition of *o*-phenylenediamine substrate, and the absorbance of the samples was measured at 450 nm.

Quantitative real-time PCR

Total RNA from intestinal epithelial cells was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription (RT) was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. The reaction conditions involved 40 cycles of two-stage PCR consisting of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min after an initial denaturation step of 95 °C for 10 min. The primer sequences were as follows: mouse TNF-α, 5′-CATCTTCTCAAAATTCGAGTGACAA-3′ and 5′-TGGGAGTAGACAAGGTACAACCC-3′; mouse IL-1β, 5′-ACAGAATATCAACCAAC AAGTGATATTCTC-3′ and 5′-GATTCTTTCCTTTGAGGCCCA-3′; mouse gamma interferon (IFN-γ), 5′-TCAAGTGGCATAGATGTGGAAGAA-3′ and 5′-TGGCTCTGCAGGATTTTCATG-3′; and mouse β-actin, 5′-AG