The present case report is of an atypical elderly female patient. Generally, presence of endoscopic findings characteristic of eosinophilic esophagitis, such as liner farrows, multiple rings, white stipple-like exudates, and wrinkled pattern, are found frequently on diagnostic endoscopic study. However, endoscopic characteristics that facilitated diagnosis were only evident when NBI imaging, which can clarify the superficial structure of the esophageal mucosa, was employed in this case. In addition, her symptoms and eosinophilic infiltration of esophageal epithelium spontaneously disappeared without treatment. Although follow-up examinations of this patient are important, therapeutic intervention is considered not to be necessary, since the purpose of such treatment is to relieve unpleasant esophageal symptoms and normalize health-related quality of life.

The mechanism of spontaneous remission of eosinophilic esophagitis in the present case is not clear, though it is possible that an unintended change of lifestyle or dietary habit may have decreased antigen exposure. We were unable to identify a possible antigen, or an obvious change of lifestyle or dietary habit. The second possible mechanism is related to the chronic administration of hydrocortisone in this patient for postsurgical hypopituitarism. However, the dose of hydrocortisone administered is only 10 mg/day, which is not a pharmacological dose but rather a physiological replacement dose. In addition, administration of hydrocortisone had already been started 8 years before the appearance of symptoms and the diagnosis of the disease. Therefore, it is difficult to consider administration of hydrocortisone as a possible mechanism for spontaneous remission of eosinophilic esophagitis in this case. Nevertheless, reports of atypical cases are important to avoid unnecessary treatment and minimize therapy-related adverse effects.

Finally, in this case, thickened esophageal wall was not found even by CT/EUS examinations. According to the literature, in only part of the patients, thickened esophagus was found in patients with eosinophilic esophagitis. The absence of thickened esophageal wall may represent weak disease activity in this patient. In addition, as a second possible reason, disease activity may have at least partly regressed spontaneously at the time point when the CT/EUS examinations were done, since CT/EUS were done 1 month after the pathological diagnosis of this case. In future study, the relationship between possible spontaneous remission and absence of esophageal wall thickening should be investigated to predict clinical course of patients with eosinophilic esophagitis.

In summary, we diagnosed an atypical elderly female with eosinophilic esophagitis, in whom esophageal symptoms and esophageal eosinophilic infiltration spontaneously disappeared.

References

1. Furuta GT, Liacouras CA, Collins MH, Gupta SK, Justinich C, Putnam PE, et al. Eosinophilic esophagitis in children and adults: a systematic review and consensus recommendations for diagnosis and treatment. Gastroenterology. 2007;133:1342–63.
2. Dellon ES, Gibbs WB, Fritchie KJ, Rubinas TC, Wilson LA, Woosley JT, et al. Clinical, endoscopic, and histologic findings distinguish eosinophilic esophagitis from gastroesophageal reflux disease. Clin Gastroenterol Hepatol. 2009;7:1305–13.
3. Lamb CA, Kanakala V, Stirling RW. Attwood SEA. Clinical lesson: eosinophilic oesophagitis, a new diagnosis to swallow. Frontline Gastroenterol. 2010;1:25–9.
4. Dellon ES, Gibbs WB, Rubinas TC, Fritchie KJ, Madanick RD, Woosley JT, et al. Esophageal dilation in eosinophilic esophagitis: safety and predictors of clinical response and complications. Gastrointest Endosc. 2010;7:706–12.
5. Furuta K, Adachi K, Kowari K, Mishima Y, Imaoka H, Kadota C, et al. A Japanese case of eosinophilic esophagitis. J Gastroenterol. 2006;41:706–10.
6. Abe Y, Iijima K, Ohara S, Koike T, Ara N, Uno K, et al. A Japanese case series of 12 patients with esophageal eosinophilia. J Gastroenterol. 2010;46:25–30.
7. Prasad GA, Alexander JA, Schleck CD, Zinsmeister AR, Smyrk TC, Elias RM, et al. Epidemiology of eosinophilic esophagitis over three decades in Olmsted County, Minnesota. Clin Gastroenterol Hepatol. 2009;7:1055–61.
8. Straumann A, Spichtin HP, Grize L, Bucher KA, Beglinger C, Simon HU. Natural history of primary eosinophilic esophagitis: a follow-up of 30 adult patients for up to 11.5 years. Gastroenterology. 2003;125:1660–9.
9. Spergel JM, Brown-Whitehorn TF, Beausoleil JL, Franciosi J, Shuker M, Verma R, et al. 14 years of eosinophilic esophagitis: clinical features and prognosis. J Pediatr Gastroenterol Nutr. 2009;48:30–6.
10. Kelly KJ, Lazenby AJ, Rowe PC, Yardley JH, Perman JA, Sampson HA. Eosinophilic esophagitis attributed to gastroesophageal reflux: improvement with an amino acid-based formula. Gastroenterology. 1995;109:1503–12.
11. Markowitz JE, Spergel JM, Ruchelli E, Liacouras CA. Elemental diet is an effective treatment for eosinophilic esophagitis in children and adolescents. Am J Gastroenterol. 2003;98:777–82.
12. Kagalwalla AF, Sentongo TA, Ritz S, Hess T, Nelson SP, Emerick KM, et al. Effect of six-food elimination diet on clinical and histologic outcomes in eosinophilic esophagitis. Clin Gastroenterol Hepatol. 2006;4:1097–102.
13. Almansa C, Krishna M, Buchner AM, Ghabril MS, Talley N, DeVault KR, et al. Seasonal distribution in newly diagnosed cases of eosinophilic esophagitis in adults. Am J Gastroenterol. 2009;104:828–33.
14. Moawad FJ, Veerappan GR, Lake JM, Maydonovitch CL, Haymore BR, Kosisky SE, et al. Correlation between eosinophilic oesophagitis and aeroallergens. Aliment Pharmacol Ther. 2010;31:509–15.
15. Mishra A, Hogan SP, Brandt EB, Rothenberg ME. An etiological role for aeroallergens and eosinophils in experimental esophagitis. J Clin Invest. 2001;107:83–90.
16. Spergel JM, Andrews T, Brown-Whitehorn TF, Beausoleil JL, Liacouras CA. Treatment of eosinophilic esophagitis with specific food elimination diet directed by a combination of skin prick and patch tests. Ann Allergy Asthma Immunol. 2005;95:336–43.
17. Moawad FJ, Veerappan GR, Wong RK. Eosinophilic esophagitis. Dig Dis Sci. 2009;54:1818–28.
18. Rothenberg ME. Biology and treatment of eosinophilic esophagitis. Gastroenterology. 2009;137:1238–49.

19. Dohil R, Newbury R, Fox L, Bastian J, Aceves S. Oral viscous budesonide is effective in children with eosinophilic esophagitis in a randomized, placebo-controlled trial. Gastroenterology. 2010;139:418–29.

20. Straumann A, Conus S, Degen L, Felder S, Kummer M, Engel H, et al. Budesonide is effective in adolescent and adult patients with active eosinophilic esophagitis. Gastroenterology. 2010;139:1526–37.

Autophagy is required for toll-like receptor-mediated interleukin-8 production in intestinal epithelial cells

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Abstract. Autophagy is an evolutionarily conserved process that maintains cellular homeostasis via synthesis, degradation, and subsequent recycling of cellular products under various physiological conditions. However, the link between autophagy and the innate immune system remains unknown. In the present study, we evaluated Toll-like receptor (TLR)-mediated autophagy induction in intestinal epithelial cells (IECs) and its relationship to interleukin (IL)-8 production. IEC-6, HCT-15, RAW264.7, and THP-1 cells were cultured with or without various TLR ligands, followed by evaluation of the expressions of pro-inflammatory cytokines [IL-8, cytokine-induced neutrophil chemoattractants (CINC)-2β, macrophage inflammatory protein (MIP)-2] by real-time PCR and ELISA. To reveal the status of autophagy in IECs and macrophages, light chain 3 (LC3)-II expression was examined using Western blotting and immunofluorescence with confocal microscopy. Also, to evaluate the influence of TLR ligands on autophagy-mediated innate-immune responses, autophagy-related gene (Atg)7 specific siRNA was transfected into intestinal epithelial cells and IL-8 expression was determined following exposure to various TLR ligands. Cells treated with the TLR ligands produced considerable amounts of pro-inflammatory cytokines (IL-8, CINC-2β, MIP-2). Furthermore, the basal levels of LC3-II were markedly higher in IECs as compared to those in macrophages. Our findings indicated that autophagy induction following TLR ligand stimulation was not significantly evident in IECs as compared to macrophages. In addition, Atg7 gene expression silencingled to down-regulation of TLR-mediated IL-8 expression in IECs, which indicates a potential role of autophagy in generating innate-immune responses. In conclusion, autophagy may be an important intracellular machinery for inducing the innate immune system in IECs.

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Introduction

In mammalian systems, autophagy, a process that degrades cell components through the lysosomal machinery, thus helping to maintain a balance between synthesis, degradation, and subsequent recycling of cellular products is evolutionarily conserved (1). The process of autophagy is characterized by formation of double-membrane vesicles known as autophagosomes around a targeted region of the cell, mediated by the autophagy-related gene (Atg)12-Atg5-Atg16 complex and by microtubule-associated protein light chain 3 (LC3)-phospholipid conjugates (LC3-II) (2,3). The resultant vesicle then fuses with a lysosome and subsequently degrades the cellular contents (4,5). Although autophagy is recognized as a homeostatic process that enables eukaryotic cells to survive during starvation, recent studies have also revealed a variety of roles for autophagy in the regulation of cell death, differentiation, and anti-microbial responses (6-8).

Innate immunity is triggered by pattern recognition receptors (PRRs) that sense pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), flagellin, peptidoglycans, and bacterial DNA (9,10). The Toll-like receptor (TLR) family, an important class of PRRs, is well known to induce expression of various inflammatory genes in response to microbial components, which regulates the balance between activation and inhibition of the innate immune system (11). TLRs play essential roles in gut immunity, barrier function and healing during intestinal inflammation (12-16). In particular, the monolayer of intestinal epithelial cells (IECs) is endowed with a capacity for first-line defense against microbial pathogens, which contributes to the regulation of the gut innate immunity under physiological and pathological conditions (17-20).

The role of autophagy in the innate immunity has been emphasized in several recent publications. The link between autophagy and the innate immune system was shown by the discovery that intracellular pathogens can be eliminated from cells via a TLR-induced autophagy pathway, which may help to maintain normal homeostasis during pathogen infection (21-24). Also, genome-wide association studies recently indicated that autophagy is an essential factor in a variety of disease states including inflammatory bowel disease (IBD) (25,26). Suppression of autophagy can lead to inflammation and tissue damage resembling Crohn's disease (CD), consistent with the identification of allelic loss of Atg16L1 and immunity-related GTPase M (IRGM) as risk factors of CD development (27-29). Although TLR-dependent induction of autophagy has been noted in numerous studies (21-24), the role of autophagy in the gut innate immune system remains largely unknown. To understand the pathogenesis of innate immune-related gut disorders, it is considered important to clarify the crosstalk that occurs between the TLR-mediated pathway and autophagy in IECs, which respond directly to luminal microbial components.

In the present study, we evaluated TLR-mediated autophagy induction in IECs and compared it to that in macrophages, as well as its relationship to the production of interleukin (IL)-8. Our results indicate that IECs have a high basal level of autophagy even without stimulation by various TLR ligands, while we were interested to note that expression of the autophagy system was not altered when stimulated with those TLR ligands. In addition, we also revealed that a deficiency of the autophagy pathway caused by transfection with Atg7 siRNA significantly decreased TLR-mediated IL-8 production in IECs. These are the first known results to show that autophagy may be an essential system for regulation of TLR-mediated IL-8 production in IECs.

Materials and methods

Reagents. The following reagents and antibodies (Abs) were used in our experiments: purified flagellin from *S. typhimurium* (InvivoGen, CA, USA), purified LPS, *E. coli* LPS (InvivoGen), purified Pam2CSK4 (InvivoGen), rapamycin (Sigma, St. Louis, MO, USA), Lipofectamine™ RNAiMAX (Invitrogen, CA, USA), a human IL-8 enzyme immune assay (EIA) (Invitrogen), a non-radioactive cell proliferation assay (CellTiter 96® AQueous) (Promega, Madison, WI, USA), Atg7 siRNA and control siRNA (Santa Cruz, CA, USA), anti-LC3 Ab (MBL, Nagoya, Japan), anti-p62 Ab (MBL), anti-β-actin Ab (Sigma), anti-rabbit IgG (Santa Cruz), FITC-conjugated anti-rabbit IgG (DAKO, Glostrup, Denmark), propidium iodide (Sigma), E64D (Sigma), and pepstatin A (Sigma).

Cell cultures. The human colorectal cancer cell line, HCT-15, the human monocytic leukemia cell line, THP-1, the rat small intestine epithelial cell line, IEC-6, and the mouse macrophage cell line, RAW264.7, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and grown in RPMI-1640 (Invitrogen) or Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific, Logan, UT, USA) and penicillin-streptomycin-amphotericin B (Invitrogen). The cell lines were maintained at 37˚C in 5% CO_2 in a humidified incubator.

RNA extraction and real time-PCR. Total RNA was extracted from each sample using Isogen (Nippon Gene, Japan), equal amounts of RNA were then reverse transcribed into cDNA using a QPCR cDNA kit (Stratagene, CA, USA). All primers (Table I) utilized were flanked by intron-exon junctions using the NCBI BLAST tool and the Primer3 software. Quantitative real-time PCR was performed using a StepOne Real-Time PCR system with SYBR-Green PCR master mix (Applied Biosystems, CA, USA), according to the manufacturer's instructions. The levels of human IL-8, rat cytokine-induced neutrophil chemoattractants (CINC)-2β, and mouse macrophage inflammatory protein (MIP)-2 mRNA were normalized to that of β-actin using a sequence detector software (Applied Biosystems).

Protein extraction and Western blotting. Protein extraction and Western blotting assays were performed as previously described (30,31). Briefly, cells were harvested from cultured dishes and were lysed in RIPA lysis buffer. Protein concentration was determined using the BCA protein assy kit (Thermo Scientific). Total cell lysates (20 μg) were separated by Tris-glycine SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. After blocking with 10% skim milk (Difco, Detroit, MI, USA) in PBS (pH 7.4), the membrane was reacted with anti-LC3 (1:1000), anti-p62 (1:1000), or anti-β-actin Abs (1:5000) at room temperature for 1 h, then reacted with peroxidase-conjugated anti-rabbit (1:10000) or anti-mouse (1:5000) Abs at room temperature for 1 h. The resulting signals were imaged using an ECL (GE Healthcare, Buckinghamshire, UK). To inhibit degradation of LC3-II protein, E64D and pepstatin A were used for the cultured cells.

Confocal microscopy. After stimulation with TLR ligands for 16 h, cells were washed in PBS and then fixed with 4% paraformaldehyde (Sigma) for 10 min at room temperature. Fixed cells were washed with PBS, immersed in 100 mg/ml of Digitonin for 15 min at room temperature, washed in PBS, and blocked in blocking buffer (X909; DAKO, Carpinteria, CA, USA) for 1 h at room temperature. Cells were subsequently incubated with the anti-LC3 antibody (PM036, MBL) for 1 h at room temperature. After 3 washes in PBS, cells were incubated with FITC-anti-rabbit Ab (DAKO) for 30 min at room temperature and then washed 3 times in PBS and subsequently incubated with propidium iodide (Sigma). Cells were observed with a Laser Scanning Confocal Microscope (Olympus, FV300), photographed at a x120 magnification, and analyzed using the Olympus confocal microscope software.

Enzyme immune assay (EIA). Proteins extracted from HCT-15 cells and culture supernatants were used for the assays. IL-8 contents were measured using an IL-8 EIA kit, following the manufacturer's protocol. Briefly, appropriate sample amounts were transferred by pipette into appropriate wells of anti-human IL-8-coated microtiter strips, followed by addition of a second biotinylated monoclonal Ab, then incubation was performed at room temperature for 90 min. After removing the excess secondary Ab by washing, the samples

Table I. Primer sequences.

Gene (Accession no.)	Sequences (5'–3')
Human GAPDH (NM-002046)	
Forward	CCACATCGCTCAGACACCAT
Reverse	TGACCAGGCGCCCAATA
Human IL-8 (NM-000584)	
Forward	TGTGTGTAAACATGACTTCCAAGCT
Reverse	TTAGCACTCCTTGGCAAAACTG
Human Atg7 (NM-001136031)	
Forward	GATCCGGGGATTTCTTTCACG
Reverse	CAGCAATGTAAGACCAGTCAAGT
Rat GAPDH (NM-017008)	
Forward	AAGATGGTGAAGGTCGGTGT
Reverse	GATCTCGCTCCTGGAAGATG
Rat CINC-2β (NM-138522)	
Forward	GAGACGGGAATGCAATTTGTTT
Reverse	GGTCTGCTAGGAATGTTGTCGAT
Mouse β-actin (NM_007393)	
Forward	GATTACTGCTCTGGCTCCTAGC
Reverse	GACTCATCGTACTCCTGCTTGC
Mouse MIP-2 (NM_009140)	
Forward	TGTCAATGCCTGAAGACCCTGCC
Reverse	AACTTTTTGACCGCCCTTGAGAGTGG

were incubated with streptavidin-peroxidase, after which a substrate solution was added to produce color that was directly proportional to the concentration of human IL-8 present in the sample. Quantitative results were obtained from a standard curve produced from the experimental findings.

RNA interference. HCT-15 cells were grown in 24-well plates ($5x10^4$ cells/well), then custom siRNAs (Santa Cruz) targeting the human Atg7 gene or control siRNAs were transfected (50 nM/well), according to the manufacturer's protocol. The efficiency of target gene knock-down was assessed by real-time PCR and the results were compared to those of the negative control siRNA-transfected condition. In addition, the efficacy of the Atg7 gene knockdown on autophagy induction was assessed by Western blotting for the detection of LC3-II and p62.

Cell proliferation assay. A non-radioactive cell proliferation assay kit was used to assess cell viability after treatment with Atg7 siRNA. HCT-15 cells were treated with control or Atg7 siRNA, then incubated with TLR ligands for 16 h, after which a cell proliferation assay was performed according to the manufacturer's protocol. The formation of formazan was determined with an EIA plate reader (Bio-Rad, Hercules, CA, USA) at 490 nm at 4 h after adding the PMS/MTS solution.

Statistical analysis. All data are expressed as the mean ± standard error of the mean (SEM). Values were analyzed using the Student's t-test with the SPSS software version 10.1 (San Rafael, CA, USA). ANOVA was used for comparisons of multiple values. P-values <0.05 were considered to be significant.

Results

TLR ligands stimulate production of pro-inflammatory cytokines in cultured IECs and macrophages. Initially, we examined if IECs and macrophages can respond to TLR ligand stimulation (LPS for TLR4, flagellin for TLR5, Pam2CSK4 for TLR2). After 3 h of stimulation with the ligands, the gene expressions of IL-8, CINC-2β (rat counterpart of human IL-8), and MIP-2 (mouse counterpart of human IL-8) in cultured cells were examined by real-time RT-PCR. In both macrophage cell lines (RAW264.7, THP-1), all ligands tested in this study significantly induced the expressions of MIP-2 and IL-8 (Fig. 1A and B). On the other hand, though stimulation with flagellin and Pam2CSK4 markedly induced CINC-2β in IEC-6 cells and IL-8 in HCT-15 cells, stimulation with LPS in these cells resulted in relatively lower levels of the respective cytokines (Fig. 1C and D).

TLR-mediated autophagy induction in IECs and macrophages. The expression level of LC3-II, a mammalian Atg8 homologue widely used as an autophagosome marker and thought to be present on the membranes of autophagosomes, is generally correlated with the number of autophagosomes in a tested sample. In the present study, we assessed LC3-II protein levels in TLR-mediated IECs and macrophages using Western blotting and confocal microscopy. Rapamycin was used as a positive control for autophagy induction. As shown in Fig. 2A,

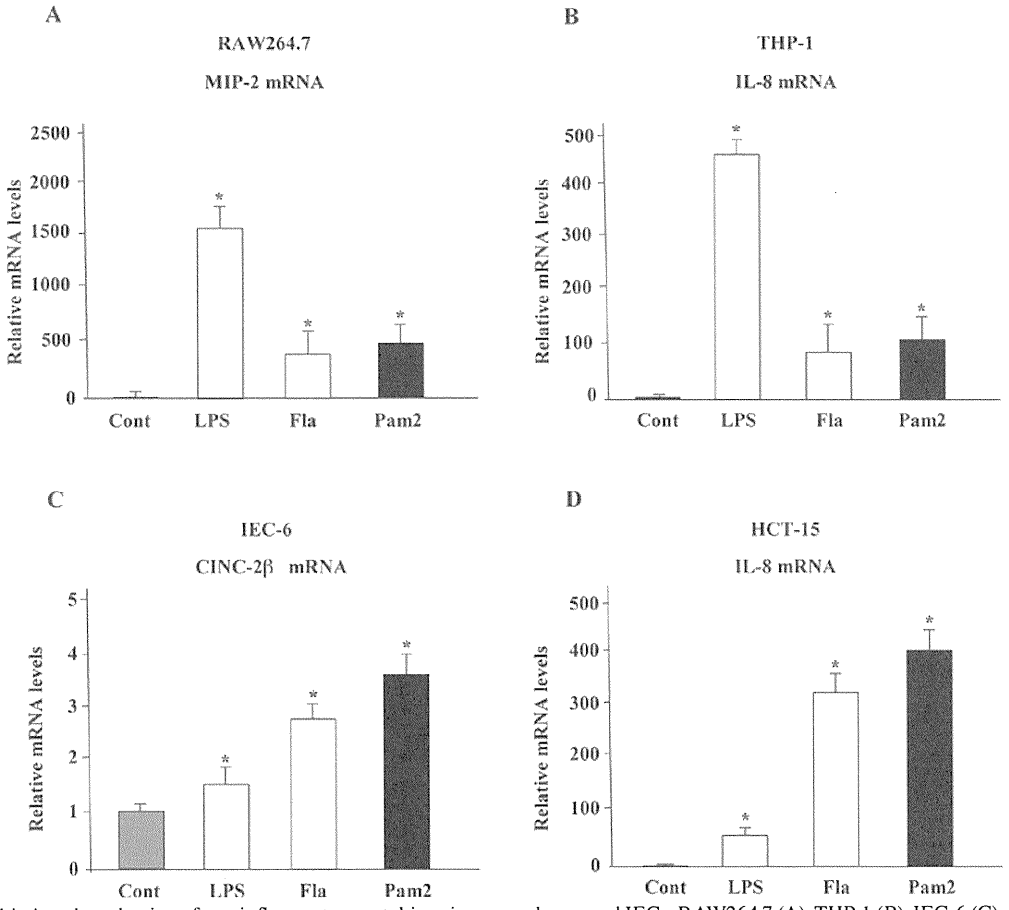

Figure 1. TLR ligand-induced production of pro-inflammatory cytokines in macrophages and IECs. RAW264.7 (A), THP-1 (B), IEC-6 (C), and HCT-15 (D) cells were incubated with various TLR ligands [LPS, 100 ng/ml; flagellin (Fla), 100 ng/ml; Pam2CSK4 (Pam2), 100 ng/ml] for 3 h. After ligand stimulation, total RNA was extracted from each sample and the expressions of IL-8, CINC-2β and MIP-2 were examined by real-time RT-PCR. All signals were standardized to the intensity of GAPDH. Error bars indicate the standard error of mean values obtained from 4 independent experiments. *P<0.01 vs. non-stimulated cells.

TLR4 (LPS) and TLR2 (Pam2CSK4) induced abundant levels of LC-3II in macrophages (RAW264.7, THP-1). In contrast, HCT-15 and IEC-6 cells have high basal levels of LC3-II protein in the absence of TLR ligand incubation, which were not changed even following stimulation with the TLR ligands (Fig. 2A). Furthermore, confocal microscopy revealed significant LC3-II immunoreactive signals indicating the presence of autophagosomes in the cytoplasm (green dots). The representative confocal microscopic images shown in Fig. 2B clearly show the results of Western blotting (Fig. 2B), indicating that IECs have a high basal level of LC3-II protein in the absence of TLR ligand incubation.

Effects of Atg7 siRNA on autophagy induction and p62 protein levels in HCT-15 cells. As shown in our experiments, IECs have a high basal level of LC3-II protein even after stimulation with TLR ligands, which was different from the basal autophagy levels observed in macrophages. Based on these findings, we focused on IECs (HCT-15 cells) and investigated the role of autophagy in TLR-stimulated IECs by using a gene knockdown method with an siRNA targeting Atg7. The siRNA used in this study showed a high gene knockdown efficacy, since basal Atg7 gene expression was decreased by 85-90% (Fig. 3A). The results of Western blotting clearly showed that knockdown of the Atg7 gene decreased LC3-II protein levels in both basal and rapamycin-treated conditions (Fig. 3B), confirming that RNA interference by Atg7 can inhibit autophagy induction in IECs. In addition, to clearly reveal the siRNA-induced deficiency of autophagy function, we examined p62 levels after treatment with Atg7 or control siRNA by Western blotting. The p62 protein is a selective substrate for autophagy that binds ubiquitin and LC3, which regulates the formation of protein aggregates in the cytoplasm. Once autophagy is induced in cells, p62 protein is immediately degraded. As shown in Fig. 3C, the cellular p62 protein level was increased after treatment with Atg7 siRNA, suggesting that Atg7 siRNA-mediated autophagy deficiency leads to an accumulation of p62 protein in the cytoplasm.

Knockdown of the Atg7 gene does not influence viability of HCT-15 cells in the presence of TLR ligands. After confirming the efficacy of Atg7 siRNA in autophagy induction, we next examined whether siRNA-induced autophagy deficiency influences cell viability before and after stimulation with TLR ligands in serum. The results of our cell proliferation assay clearly show that transfection of Atg7 siRNA does not influence cell viability in the serum-treated condition (Fig. 4A). In addition, treatment with the autophagy inducer rapamycin had no influence on cell viability (Fig. 4B).

Autophagy is required for TLR-mediated IL-8 secretion by HCT-15 cells. After confirming the efficacy of siRNA, the

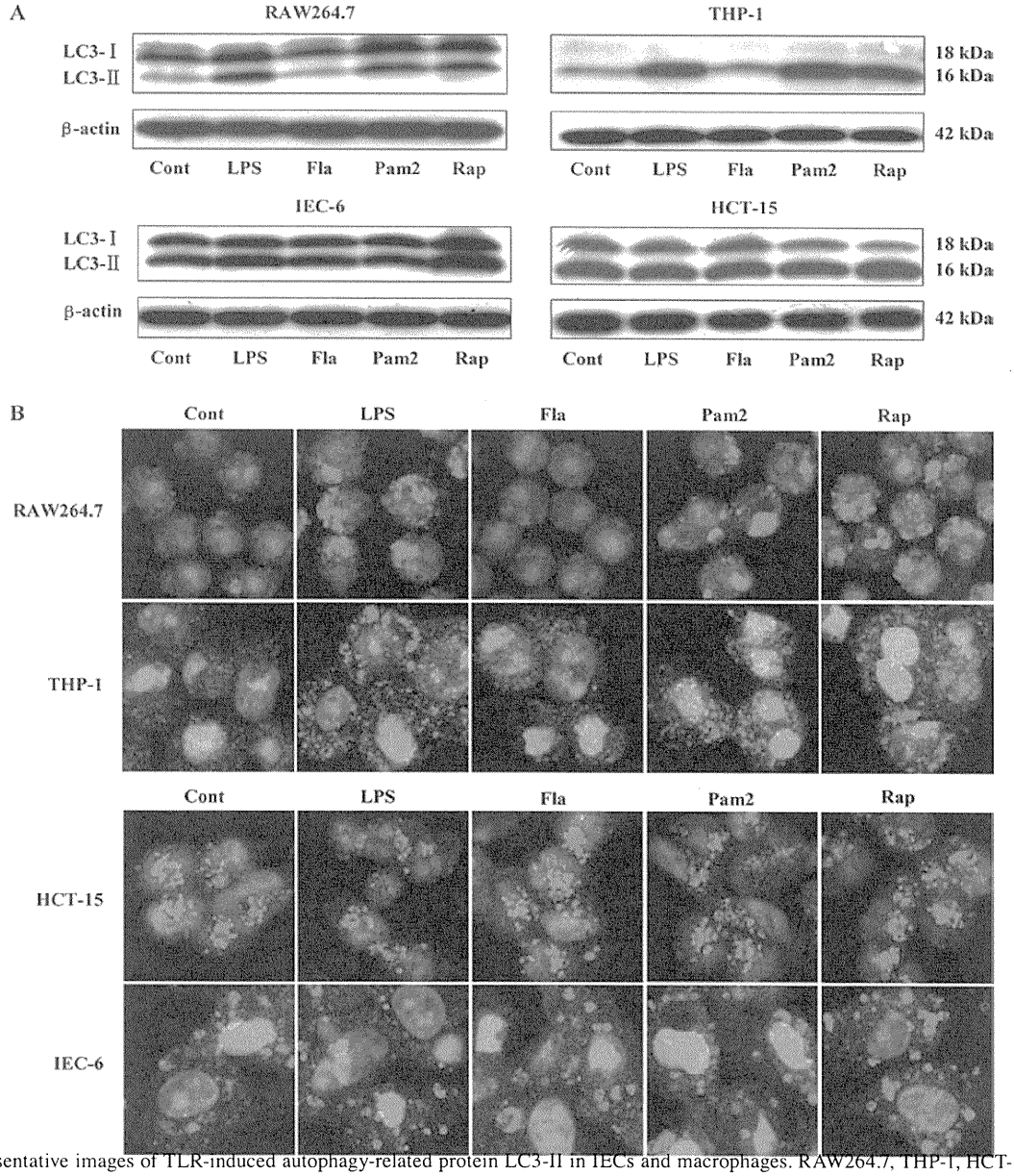

Figure 2. Representative images of TLR-induced autophagy-related protein LC3-II in IECs and macrophages. RAW264.7, THP-1, HCT-15, and IEC-6 cells were incubated with various TLR ligands [LPS, 100 ng/ml; flagellin (Fla), 100 ng/ml; Pam2CSK4 (Pam2), 100 ng/ml] for 16 h. Rapamycin was used as a positive control for autophagy induction. Total LC3-II protein levels and cellular localization were examined by Western blotting (A) and confocal microscopy (B), respectively. Immunoreactive signals (green dots) from LC3-II indicate the presence of autophagosomes in the cytoplasm (B).

role of autophagy in TLR-mediated inflammatory responses in IECs was examined. Control or Atg7 siRNAs were transfected into HCT-15 cells, after which the cells were stimulated with various TLR ligands for 16 h and the IL-8 contents in the culture supernatants were measured using EIA. As shown in Fig. 5A, TLR ligand-induced IL-8 mRNA expression in Atg7 siRNA-treated cells was significantly lower than that in control siRNA-treated cells. In addition, Atg7 siRNA treatment significantly inhibited TLR-mediated IL-8 protein production (Fig. 5A). To further confirm the role of autophagy, we induced autophagy in IECs by treatment with rapamycin and also examined the TLR-mediated production of IL-8. After stimulation with the TLR ligands, IL-8 mRNA expression and IL-8 contents in the culture supernatants of rapamycin-treated cells were significantly increased as compared to the control cells (Fig. 5B).

Discussion

In the present study, we found that IECs have a high basal level of autophagy, which did not increase following stimulation with various TLR ligands. In addition, our results clearly demonstrated that Atg7 siRNA-induced inhibition of basal autophagy significantly decreased TLR-mediated IL-8 production in IECs, suggesting that the presence of basal autophagy in IECs is essential for TLR-mediated inflammatory responses to maintain the innate immune system in the gut.

TLRs are sensors of microbial products that initiate host defense responses in various organs (9-12). There is abundant evidence indicating that TLRs play a vital role in maintaining intestinal epithelial homeostasis as well as regulating inflammation, while dysregulation of TLR-mediated signaling leads

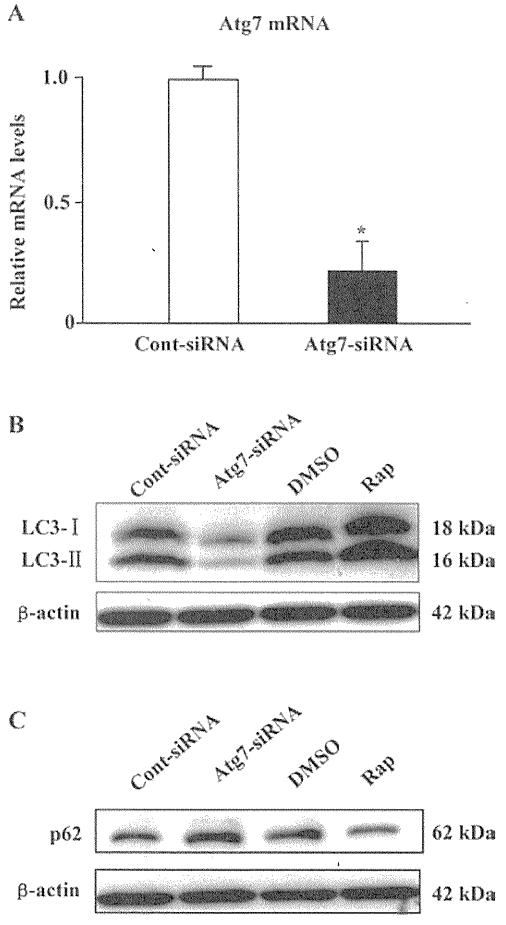

Figure 3. Efficacy of Atg7 siRNA on autophagy induction and p62 protein levels in HCT-15 cells. (A) The efficacy of Atg7 siRNA was evaluated by real-time PCR and compared to that of control siRNA. Error bars indicate the standard error of mean values obtained from 3 independent experiments. *P<0.01 vs. control siRNA. (B) and (C) Representative images of Western blotting showing LC3-II and p62 levels in HCT-15 cells treated with control siRNA, Atg7 siRNA, DMSO (vehicle), and rapamycin.

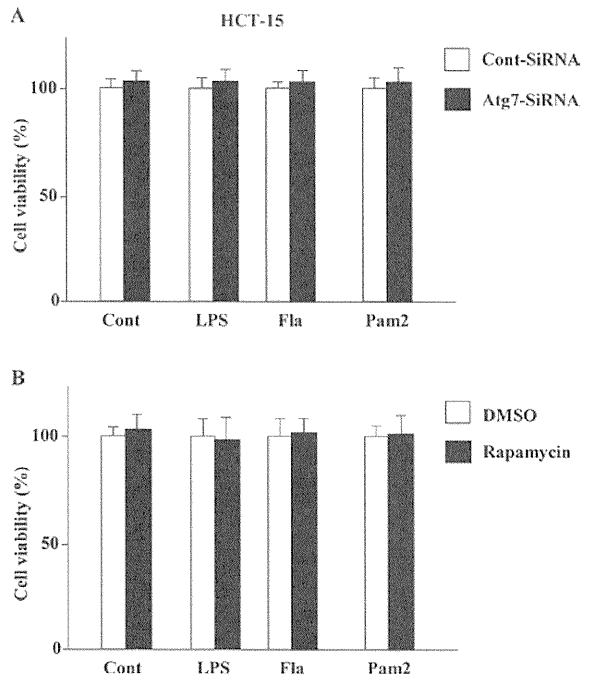

Figure 4. Results of cell proliferation assays. HCT-15 cells were treated with (A) siRNA and (B) rapamycin (Rap), then incubated with various TLR ligands [LPS, 100 ng/ml; flagellin (Fla), 100 ng/ml; Pam2CSK4 (Pam2), 100 ng/ml] for 16 h and cell viability was assessed. Transfection of Atg7 siRNA did not influence cell viability in the serum-treated condition. Error bars indicate the standard error of mean values obtained from 3 independent experiments.

to development of several gut immune disorders (13-19). Autophagy is also an important physiological process that controls a variety of cellular functions closely associated with the innate immune system (21-24). Although recent findings suggest crosstalk between autophagy and TLR signaling in the intestinal tract, that process remains largely unknown. To investigate this issue, we performed several different *in vitro* experiments in the present study.

To investigate the effects of stimulation with TLR ligands on autophagy induction, we initially used macrophage cell lines in our *in vitro* experiments. Stimulation with LPS and Pam2CSK, but not flagellin, significantly increased cellular LC3-II protein levels in RAW264.7 and THP-1 cells, showing that TLR4 and TLR2 are essential sensors for autophagy induction in macrophages. Recently, Xu *et al* reported that LPS induced autophagy in RAW264.7 cells, which was found to be regulated through a pathway that is dependent on the Toll-IL-1 receptor domain containing adaptor-inducing interferon-β (TRIF), while it was independent of the myeloid differentiation factor 88 (MyD88) (32). Delgado *et al* also demonstrated that activation of TLR4 and TLR7 was associated with autophagy induction in RAW264.7 cells. On the other hand, it has been reported that TLR5 is not a significant sensor for autophagy induction in RAW264.7 cells or bone marrow macrophages (23). These recent findings support our results obtained with the macrophage cell lines.

In contrast to those findings, we found that HCT-15 and IEC-6 cells have high basal levels of autophagy, as determined by the presence of converted LC3-II protein in the cells. Several studies have shown that basal levels of LC3-II protein were detected in cultured IEC lines, including IEC-6, IEC-18, Colon-26, and HT-29 (33-35). Others have also reported that immune-reactive signals of LC3-II were observed in epithelial cells from mice and human intestinal tissue sections. Although various stimuli including serum starvation, glutamine treatment, and γ-irradiation induce significant autophagy in IECs (33-36), little is known regarding the effect of TLR activation in that process. In the present study, we found that Pam2CSK and flagellin markedly stimulated pro-inflammatory cytokines in HCT-15 and IEC-6 cells. However, these TLR ligands did not have an influence on autophagy induction in these cells. Lee *et al* previously indicated an inability to detect induction of autophagy in TLR7-mediated plasmacytoid dendritic cells (pDCs) (37). Furthermore, Schmid *et al* noted that a high level of constitutive autophagosome formation was observed in DCs, which was not increased after incubation with various immunological agonists (38). Also, a recent study revealed that TLR ligands including LPS did not influence autophagy induction in mice primary cultured macrophages isolated from the peritoneal cavity and bone marrow (27). Together, these findings suggest the possibility that basal autophagy and TLR-mediated autophagy are cell type- and TLR ligand-dependent.

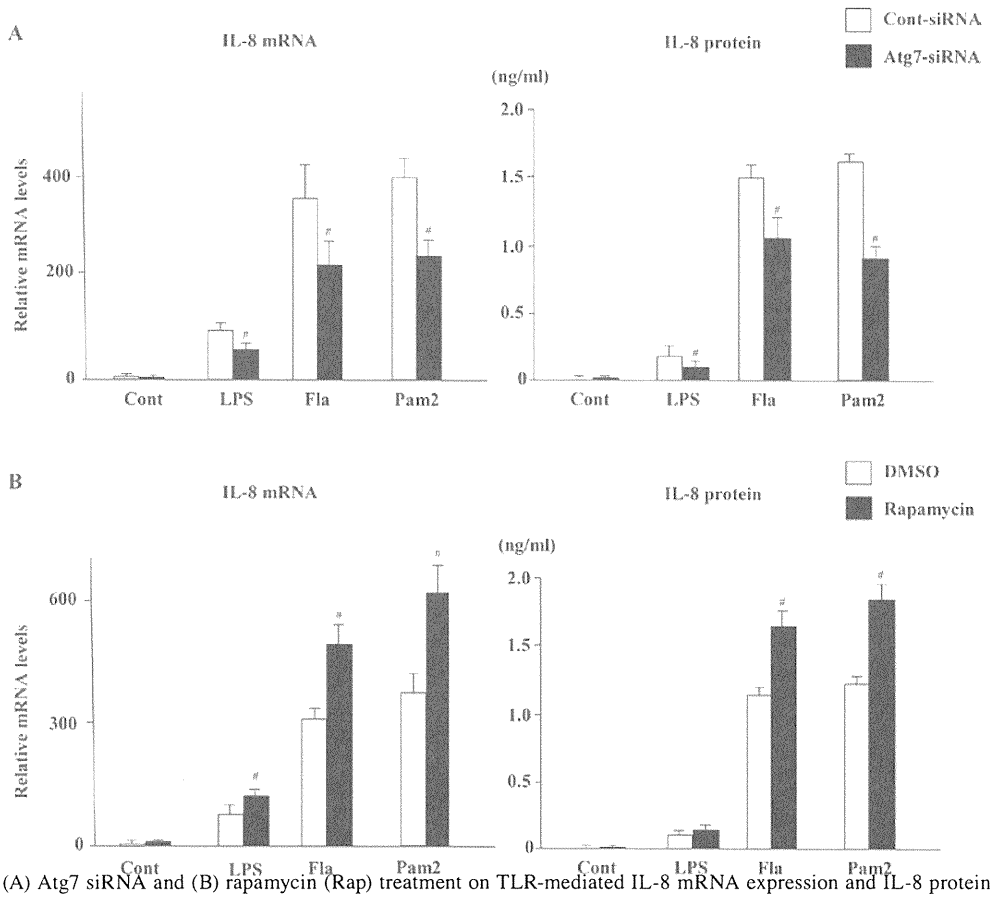

Figure 5. Effects of (A) Atg7 siRNA and (B) rapamycin (Rap) treatment on TLR-mediated IL-8 mRNA expression and IL-8 protein production in HCT-15 cells. HCT-15 cells were treated with siRNA or rapamycin, then incubated with TLR ligands [LPS, 100 ng/ml; flagellin (Fla), 100 ng/ml; Pam2CSK4 (Pam2), 100 ng/ml] for 3 h, after which IL-8 mRNA levels were evaluated by real-time PCR. All signals were standardized to the intensity of GAPDH. IL-8 protein contents in the culture supernatants at 16 h after treatment with TLR ligands were examined by EIA. Error bars indicate the standard error of mean values obtained from 3 independent experiments. #P<0.05 vs. control siRNA or DMSO.

Based on our results, we speculated that high basal levels of autophagy may have certain roles in TLR-mediated inflammatory responses in IECs. Thus, we employed an *in vitro* system using a gene knockdown method with an siRNA targeting Atg7, an essential gene for autophagy induction. Although gene knockdown of Atg7 did not influence cell viability, TLR ligand-induced IL-8 secretion by Atg7 siRNA-treated cells was significantly lower than that by control siRNA-treated HCT-15 cells. Sasaki *et al* demonstrated that the secretions of CCL2/monocyte chemotactic protein-1 (MCP-1) and CX3CL1/Fractalkine in cultured biliary epithelial cells induced by various stress factors, such as oxidative stress and serum starvation, were significantly decreased by treatment with the autophagy inhibitor 3-MA (39). Furthermore, a recent study using peripheral blood macrophages clearly revealed that 3-MA or Atg5 siRNA-mediated inhibition of autophagy suppressed influenza virus-induced CXCL10 and interferon-α (IFN-α) (40). These findings support our present results and suggest the possibility that autophagy is required for production of inflammatory cytokines in response to a variety of stimuli, including TLR ligands, in epithelial cells. The mucosal neutrophil response is induced when bacteria stimulate epithelial cells in various organs to secrete several chemokines such as IL-8. Hang *et al* also found that IL-8 receptor knockout mice have a dysfunctional migration of neutrophils leading to urinary tract infection, which results in tissue destruction (41). Taken together, autophagy may have an essential contribution to maintain innate immune response by regulating inflammatory cytokine production in IECs.

Several studies of crosstalk between autophagy-related genes and intestinal inflammation have been recently reported. Saitoh *et al* demonstrated that mice lacking Atg16L1 in hematopoietic cells are highly susceptible to dextran sulphate sodium (DSS)-induced experimental acute colitis (27). They also found enhanced levels of LPS-induced IL-1β production in macrophages isolated from this mice strain, indicating that Atg16L1 is an important autophagy-related gene for the inhibition of TLR4-mediated inflammatory response. Apart from the present evidence regarding autophagy functions in macrophages, Cadwell *et al* found that Atg16L1 or Atg5 mutation leads to disruption of the Paneth cell granule exocytosis pathway, suggesting that abnormal Paneth cell functions may be associated with the pathogenesis of CD (42). Thus, we consider that autophagy has various essential roles in gut immunity, which may be dependent on the cell type and the type of autophagy-regulatory genes.

In summary, we investigated TLR-mediated autophagy induction in IECs, as well as the role of autophagy in IL-8 production in TLR-activated IECs. We have demonstrated that IECs have a high basal level of autophagy, which essentially regulates TLR-mediated IL-8 production in these cells. Our findings provide new insight into crosstalk that occurs

between autophagy and TLR signaling in IECs. Additional investigations will be necessary to elucidate the precise roles of autophagy in the pathogenesis of intestinal immune disorders.

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References

1. Kundu M and Thompson CB: Autophagy: basic principles and relevance to disease. Annu Rev Pathol 3: 427-455, 2008.
2. Mizushima N and Klionsky DJ: Protein turnover via autophagy: implications for metabolism. Annu Rev Nutr 27: 19-40, 2007.
3. Virgin HW and Levine B: Autophagy genes in immunity. Nat Immunol 10: 461-470, 2009.
4. Jung CH, Ro SH, Cao J, *et al*: mTOR regulation of autophagy. FEBS Lett 584: 1287-1295, 2010.
5. Deretic V: Autophagosome and phagosome. Methods Mol Biol 445: 1-10, 2008.
6. Deretic V: Autophagy in infection. Curr Opin Cell Biol 22: 252-262, 2010.
7. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI and Deretic V: Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. Cell 119: 753-766, 2004.
8. White E, Karp C, Strohecker AM, Guo Y and Mathew R: Role of autophagy in suppression of inflammation and cancer. Curr Opin Cell Biol 22: 212-217, 2010.
9. O'Neill LA, Fitzgerald KA and Bowie AG: The Toll-IL-1 receptor adaptor family grows to five members. Trends Immunol 24: 286-290, 2003.
10. Li X and Qin J: Modulation of Toll-interleukin 1 receptor mediated signaling. J Mol Med 83: 258-266, 2005.
11. Kawai T and Akira S: The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11: 373-384, 2010.
12. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S and Medzhitov R: Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118: 229-241, 2004.
13. Abreu MT, Fukata M and Arditi M: TLR signaling in the gut in health and disease. J Immunol 174: 4453-4460, 2005.
14. Uematsu S and Akira S: Immune responses of TLR5(+) lamina propria dendritic cells in enterobacterial infection. J Gastroenterol 44: 803-811, 2009.
15. Mayer L: Evolving paradigms in the pathogenesis of IBD. J Gastroenterol: 45: 9-16, 2010.
16. Gribar SC, Anand RJ, Sodhi CP and Hackam DJ: The role of epithelial Toll-like receptor signaling in the pathogenesis of intestinal inflammation. J Leukoc Biol 83: 493-498,2008.
17. Ortega-Cava CF, Ishihara S, Rumi MA, *et al*: Strategic compartmentalization of Toll-like receptor 4 in the mouse gut. J Immunol 170: 3977-3985, 2003.
18. Ishihara S, Rumi MA, Kadowaki Y *et al:* Essential role of MD-2 in TLR4-dependent signaling during *Helicobacter pylori*-associated gastritis. J Immunol 173: 1406-1416, 2004.
19. Oshima N, Ishihara S, Rumi MA, *et al*: A20 is an early responding negative regulator of Toll-like receptor 5 signalling in intestinal epithelial cells during inflammation. Clin Exp Immunol 159: 185-198, 2010.
20. Hansen R, Thomson JM, El-Omar EM and Hold GL: The role of infection in the aetiology of inflammatory bowel disease. J Gastroenterol 45: 266-276, 2010.
21. Schmid D and Münz C: Innate and adaptive immunity through autophagy. Immunity 27: 11-21, 2007.
22. Delgado MA and Deretic V. Toll-like receptors in control of immunological autophagy. Cell Death Differ 16: 976-983, 2009.
23. Delgado MA, Elmaoued RA, Davis AS, Kyei G and Deretic V: Toll-like receptors control autophagy. EMBO J 27: 1110-1121, 2008.
24. Saitoh T and Akira S: Regulation of innate immune responses by autophagy-related proteins. J Cell Biol 189: 925-935, 2010.
25. Ishihara S, Aziz MM, Yuki T *et al*: Inflammatory bowel disease: review from the aspect of genetics. J Gastroenterol 44: 1097-1108, 2009.
26. Engel MA and Neurath MF: New pathophysiological insights and modern treatment of IBD. J Gastroenterol 45: 571-583, 2010.
27. Saitoh T, Fujita N, Jang MH, *et al*: Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 456: 264-268, 2008.
28. Kuballa P, Huett A, Rioux JD, Daly MJ and Xavier RJ: Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. PLoS One 3: e3391, 2008.
29. McCarroll SA, Huett A, Kuballa P, *et al*: Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. Nat Genet 40: 1107-1112, 2008.
30. Aziz MM, Ishihara S, Mishima Y, *et al*: MFG-E8 attenuates intestinal inflammation in murine experimental colitis by modulating osteopontin-dependent alphavbeta3 integrin signaling. J Immunol 182: 7222-7732, 2009.
31. Aziz MM, Ishihara S, Rumi MA, *et al*: Prolactin induces MFG-E8 production in macrophages via transcription factor C/EBPbeta-dependent pathway. Apoptosis 13: 609-620, 2008.
32. Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE and Eissa NT: Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 27: 135-144, 2007.
33. Maynard AA, Dvorak K, Khailova L, *et al*: Epidermal growth factor reduces autophagy in intestinal epithelium and in the rat model of necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 299: G614-G622, 2010.
34. Sakiyama T, Musch MW, Ropeleski MJ, *et al*: Glutamine increases autophagy under Basal and stressed conditions in intestinal epithelial cells. Gastroenterology 136: 924-932, 2009.
35. Li J, Hou N, Faried A, Tsutsumi S, Takeuchi T and Kuwano H: Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells. Ann Surg Oncol 16: 761-771, 2009.
36. Gorbunov NV and Kiang JG: Up-regulation of autophagy in small intestine Paneth cells in response to total-body gamma-irradiation. J Pathol 219: 242-252, 2009.
37. Lee HK, Lund JM, Ramanathan B, Mizushima N and Iwasaki A: Autophagy-dependent viral recognition by plasmacytoid dendritic cells. Science 315: 1398-1401, 2007.
38. Schmid D, Pypaert M and Münz C: Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. Immunity 26: 79-92, 2007.
39. Sasaki M, Miyakoshi M, Sato Y and Nakanuma Y: Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. Lab Invest 90: 835-843, 2010.
40. Law AH, Lee DC, Yuen KY, Peiris M and Lau AS: Cellular response to influenza virus infection: a potential role for autophagy in CXCL10 and interferon-alpha induction. Cell Mol Immunol 7: 263-270, 2010.
41. Hang L, Frendéus B, Godaly G and Svanborg C: Interleukin-8 receptor knockout mice have subepithelial neutrophil entrapment and renal scarring following acute pyelonephritis. J Infect Dis 182: 1738-1748, 2000.
42. Cadwell K, Liu JY, Brown SL, *et al*: A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 456: 259-63, 2008.

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RAPID COMMUNICATION

Eosinophilic esophagitis investigated by upper gastrointestinal endoscopy in Japanese patients

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Abstract
Background The prevalence of eosinophilic esophagitis (EE) is increasing rapidly in Western countries. Several case series of EE have also been reported in Japan. However, the prevalence of EE in Japanese patients as investigated by upper gastrointestinal endoscopy is unknown. Therefore, we carried out a prospective multicenter study to address this issue.
Methods From July to December 2010, 23,346 patients who had undergone routine upper gastrointestinal endoscopy in 17 institutions were enrolled. In patients with symptoms suggesting EE, such as dysphasia, food impaction, and heartburn, and/or in patients in whom endoscopic findings suggested pathology, esophageal biopsy samples were collected, and the numbers of eosinophils in the squamous epithelium were counted.
Results During the study period of 6 months, 4 patients were endoscopically and histologically diagnosed with EE. The prevalence of EE was calculated to be 17.1/100,000.
Conclusion The prevalence of EE in Japanese patients by upper gastrointestinal endoscopy has now been documented.

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Keywords Eosinophilic esophagitis · Prevalence · Japanese population

Introduction

Eosinophilic esophagitis (EE), first described in 1977 [1], was recognized as a new disease in which patients experience esophageal eosinophilia and gastroesophageal reflux disease (GERD)-like symptoms that do not respond to the usual GERD management methods, such as the administration of proton pump inhibitors (PPIs) [2]. The prevalence of EE has been reported to be increasing rapidly in Western countries. Straumann et al. [3] reported that the proportion of patients with EE increased from 2/100,000 in 1989 to 23/100,000 in 2004 in Switzerland. Similarly, in the US, Prasad et al. [4] found that the prevalence of EE was 55/100,000 in 2006 and that the incidence of clinically diagnosed EE had increased markedly over the last 3 decades.

In Japan, the first case of EE was reported in 2006 [5], and other cases of EE have been reported since then [6, 7]. The prevalence of EE has not yet been investigated in the Japanese population. The prevalence of EE in endoscopy-examined cases was recently reported in the USA as 6.5% [8]. Therefore, to determine the prevalence of EE in Japanese patients investigated by routine upper gastrointestinal endoscopy, we analyzed patients in a prospective multicenter study.

Methods

In 17 institutions that ranged from primary medical clinics to tertiary-care referral centers in the San-In district of

western Japan, patients who underwent upper gastrointestinal endoscopy, either due to symptoms or as part of an annual medical check-up, were enrolled from January to December 2010. In patients who suffered from symptoms suggesting EE, such as dysphasia, food impaction and heartburn, and/or who had endoscopic findings suggestive of EE, such as wrinkle patterns, linear fissures, and white stipple-like exudates [9, 10], biopsy samples were taken from the upper, middle and lower esophagus, and the numbers of eosinophils in the squamous epithelium were counted. Photographs of typical endoscopic EE findings were provided beforehand to all participating endoscopists. All upper gastrointestinal endoscopies were performed by expert endoscopists. EE was histologically confirmed by the presence of more than 20 eosinophils in every high-power field [2]. In patients with histologically confirmed eosinophilic infiltration, the absence of other diseases that may cause esophageal eosinophilic infiltration was clinically confirmed.

Results

During the study period of 6 months, upper gastrointestinal endoscopy was carried out in 23,346 patients at 17 institutions, and 4 cases of EE were found. The prevalence of EE in Japanese patients investigated by upper gastrointestinal endoscopy was calculated to be 17.1/100,000. The characteristics of the 4 EE patients are listed in Table 1. None of the 4 cases had gastrointestinal eosinophilia. In two cases, PPI administration was at least partially effective for symptom resolution, although the long-term observation period was somewhat limited. In one case, fluticasone administration was markedly effective, but PPI administration was not effective. The remaining case experienced spontaneous disappearance of the subjective symptoms. These 4 EE patients were not critically ill and had no serious complications, such as stricture of the esophagus. In the present study, no endoscopy-negative EE patients had esophageal symptoms suggesting EE.

Discussion

EE is a chronic allergic disorder of the esophageal mucosa, possibly caused by antigens in the air and in food, with marked eosinophilic infiltration in the esophageal squamous epithelium [11, 12]. The prevalence of EE in the Japanese population is unknown, although it may be increasing, as is the case in Western countries. In the present study, the prevalence of EE was found to be 17.1/100,000 in patients investigated by upper gastrointestinal endoscopy. This prevalence of EE was higher than expected, since the reported prevalence of EE in Western countries was 23–55/100,000 in recent etiological studies [3, 4]. Our study is not an epidemiological one, and strong inclusion bias was present in the enrollment. However, the presence of EE in approximately one in 5,000 endoscopy-investigated cases is high enough to encourage endoscopists to carefully consider the possible presence of minute endoscopic findings suggesting EE in patients they see in their daily clinical practice.

Abe et al. [7] reported that the endoscopic recognition of EE is not difficult, based on the identification of characteristic findings, such as linear furrows, transient and constant concentric rings, and white exudates. Molina-Infante et al. [13] also reported that the specificity of endoscopy-identified multiringed esophagus for the diagnosis of EE was only 37%, whereas that of furrows or exudates was 90% in Spanish adults. In the present study, all of the patients with EE had characteristic endoscopic findings. These endoscopic findings and symptoms may be good markers for the diagnosis of EE, although calculations of their sensitivity and specificity were difficult because of the small number of patients with EE in our study. Future studies of larger numbers of patients will be necessary to confirm the value of these markers. In two cases found in the present study, PPI administration partially improved symptoms, as previously reported [7, 9, 14, 15]. Although one diagnostic guideline for EE published in the USA required a lack of responsiveness to high-dose PPI or normal pH monitoring [2], many investigators did not follow this, and used variable diagnostic criteria for EE [16]. In the presence of acid reflux, eosinophilic infiltration

Table 1 Characteristics of 4 cases of eosinophilic esophagitis

Case	Age	Gender	Symptoms	Endoscopic findings	Treatment
1	83	F	Dysphasia, heartburn	Wrinkle pattern, white stipple-like exudates	None
2	58	M	Heartburn, epigastric pain	Linear fissures, mucosal edema	PPI partially effective
3	51	M	Food impaction	White stipple-like exudates, small ulcerations	PPI partially effective
4	61	F	Chest pain, epigastric pain	Linear fissures, small ulcerations, white stipple-like exudates	PPI not effective, fluticasone effective

in the esophageal mucosa is reported to be aggravated though several mechanisms, including augmented production of the cytokine eotaxin-3 [17–19]. Therefore, as a first-line treatment of EE, PPIs may be considered for their safer drug profile than standard treatment with glucocorticoids.

Considering the results of the present study, EE should be considered more readily in clinical practice when patients are suffering from dysphagia, food impaction, or reflux symptoms. EE may be common in Japanese patients, since its prevalence in endoscopy-investigated cases was 17.1/100,000 patients.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Dobbins JW, Sheahan DG, Behar J. Eosinophilic gastroenteritis with esophageal involvement. Gastroenterology. 1977;72:1312–6.
2. Furuta GT, Liacouras CA, Collins MH, Gupta SK, Justinich C, Putnam PE, et al. Eosinophilic esophagitis in children and adults: a systematic review and consensus recommendations for diagnosis and treatment. Gastroenterology. 2007;133:1342–63.
3. Straumann A, Simon HU. Eosinophilic esophagitis: escalating epidemiology? J Allergy Clin Immunol. 2005;115:418–9.
4. Prasad GA, Alexander JA, Schleck CD, Zinsmeister AR, Smyrk TC, Elias RM, et al. Epidemiology of eosinophilic esophagitis over three decades in Olmsted County, Minnesota. Clin Gastroenterol Hepatol. 2009;7:1055–61.
5. Furuta K, Adachi K, Kowari K, Mishima Y, Imaoka H, Kadota C, et al. A Japanese case of eosinophilic esophagitis. J Gastroenterol. 2006;41:706–10.
6. Sano H, Iwakiri K, Kawami N, Tnaka Y, Umezawa M, Iizumi T, et al. Eosinophilic esophagitis: a case report with a review of the literature. Clin J Gastroenterol. 2010;3:279–84.
7. Abe Y, Iijima K, Ohara S, Koike T, Ara N, Uno K, et al. A Japanese case series of 12 patients with esophageal eosinophilia. J Gastroenterol. 2011;46:25–30.
8. Veerappan GR, Perry JL, Duncan TJ, Baker TP, Maydonovitch C, Lake JM, et al. Prevalence of eosinophilic esophagitis in an adult population undergoing upper endoscopy: a prospective study. Clin Gastroenterol Hepatol. 2009;7:420–6.
9. Remedios M, Campbell C, Jones DM, Kerlin P. Eosinophilic esophagitis in adults: clinical, endoscopic, histologic findings, and response to treatment with fluticasone propionate. Gastrointest Endosc. 2006;63:3–12.
10. Müller S, Pühl S, Vieth M, Stolte M. Analysis of symptoms and endoscopic findings in 117 patients with histological diagnoses of eosinophilic esophagitis. Endoscopy. 2007;39:339–44.
11. Mishra A, Hogan SP, Brandt EB, Rothenberg ME. An etiological role for aeroallergens and eosinophils in experimental esophagitis. J Clin Invest. 2001;107:83–90.
12. Spergel JM, Beausoleil JL, Mascarenhas M, Liacouras CA. The use of skin prick tests and patch tests to identify causative foods in eosinophilic esophagitis. J Allergy Clin Immunol. 2002;109:363–8.
13. Molina-Infante J, Ferrando-Lamana L, Ripoll C, Hernandez-Alonso M, Mateos JM, Fernandez-Bermejo M, et al. Esophageal eosinophilic infiltration responds to proton pump inhibition in most adults. Clin Gastroenterol Hepatol. 2011;9:110–7.
14. Dranove JE, Horn DS, Davis MA, Kernek KM, Gupta SK. Predictors of response to proton pump inhibitor therapy among children with significant esophageal eosinophilia. J Pediatr. 2009;154:96–100.
15. Peterson KA, Thomas KL, Hilden K, Emerson LL, Wills JC, Fang JC. Comparison of esomeprazole to aerosolized, swallowed fluticasone for eosinophilic esophagitis. Dig Dis Sci. 2010;55:1313–9.
16. Dellon ES, Aderoju A, Woosley JT, Sandler RS, Shaheen NJ. Variability in diagnostic criteria for eosinophilic esophagitis: a systematic review. Am J Gastroenterol. 2007;102:2300–13.
17. Paterson WG. Role of mast cell-derived mediators in acid-induced shortening of the esophagus. Am J Physiol. 1998;274:G385–8.
18. Kottyan LC, Collier AR, Cao KH, Niese KA, Hedgebeth M, Radu CG, et al. Eosinophil viability is increased by acidic pH in a cAMP- and GPR65-dependent manner. Blood. 2009;114:2774–82.
19. Blanchard C, Stucke EM, Burwinkel K, Caldwell JM, Collins MH, Ahrens A, et al. Coordinate interaction between IL-13 and epithelial differentiation cluster genes in eosinophilic esophagitis. J Immunol. 2010;184:4033–41.

総 説

好酸球性食道炎の診断と治療

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要 旨

好酸球性食道炎は食物や空気中の物質が抗原となってアレルギー反応が食道の粘膜上皮を中心におこり, 食道上皮中に多数の好酸球の浸潤がみられる稀な疾患である. 好酸球の上皮内浸潤を中心とした慢性炎症のため食道粘膜下層の浮腫や線維化がおこる. このため食道の運動に異常が生じ, 嚥下障害や食べ物のつまりを主とする症状が出現し食道狭窄が生じることもある. 内視鏡検査では食道粘膜の縦走溝, 白斑, 多発輪状狭窄がみられることもあるが, 確定診断は食道粘膜の生検による上皮内好酸球の存在(15-30個/高倍率視野)をもっておこなう. 治療には局所作用型のグルココルチコイドの食道粘膜への投与を第一選択とすることが多い.

Key words eosinophil／eotaxin／endoscopy／allergy／interleukin-13

I はじめに

好酸球性食道炎は食道の上皮層中に多数の好酸球の浸潤が慢性的に持続する疾患で, 好酸球による慢性炎症が原因となって食道扁平上皮の透過性の亢進, 増殖促進, 粘膜下層の浮腫と線維化がおこる. これらの変化のために食道の運動や知覚に異常がおこり, 嚥下障害, 食べ物が食道内につかえる, 胸やけなどの不快な症状が出現するとともに食道の狭窄が生じる可能性がある[1].

本疾患は好酸球性胃腸炎とは異なって病変が食道にみられる疾患として報告がなされてから, すでに30年が経過している[2),3)]. しかし小児に加えて成人にも発症例が増加しつつあることが注目され始めたのはごく最近のことである. 好酸球性食道炎に関しては早くから研究が開始されていた欧米での基礎, 臨床研究がほとんどで, 日本での研究は始まったばかりである. 著者は, 厚生労働科学研究費補助金, 難治性疾患克服研究事業にもとづく研究の一部として2009年に好酸球性食道炎の日本での疫学実態調査をおこなった[4]. そこで本総説では, 多くの研究成果が集積されつつある好酸球性食道炎の病態, 診断, 治療に関する欧米の最新のデータを紹介するとともに, 実態調査にもつづいた日本人患者の特徴についても解説をこころみる.

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Diagnosis and Treatment of Eosinophilic Esophagitis.

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II 病 態

好酸球性食道炎の病態に関しては, 患者の半数に喘息, アトピー性皮膚炎等のアレルギー性疾患の合併がみられ, かつ血中のIgEが上昇している例が多いためTh2系の免疫反応が活性化していること, 特に好酸球の産生増加と関係のあるIL-5の関与が大きいことが推定されていた[5),6)]. また, IL-5は好酸球を食道に集積させる上で重要な役割りを演じていることも示された. 皮膚のpin prick testやpatch testをおこなうと食物抗原やアスペルギルスに対して陽性反応を示す例が多いことが報告されている. さらに食物アレルギーの原因となりやすい乳製品, 小麦, 卵, 大豆, ピー

想定される好酸球性食道炎の病態

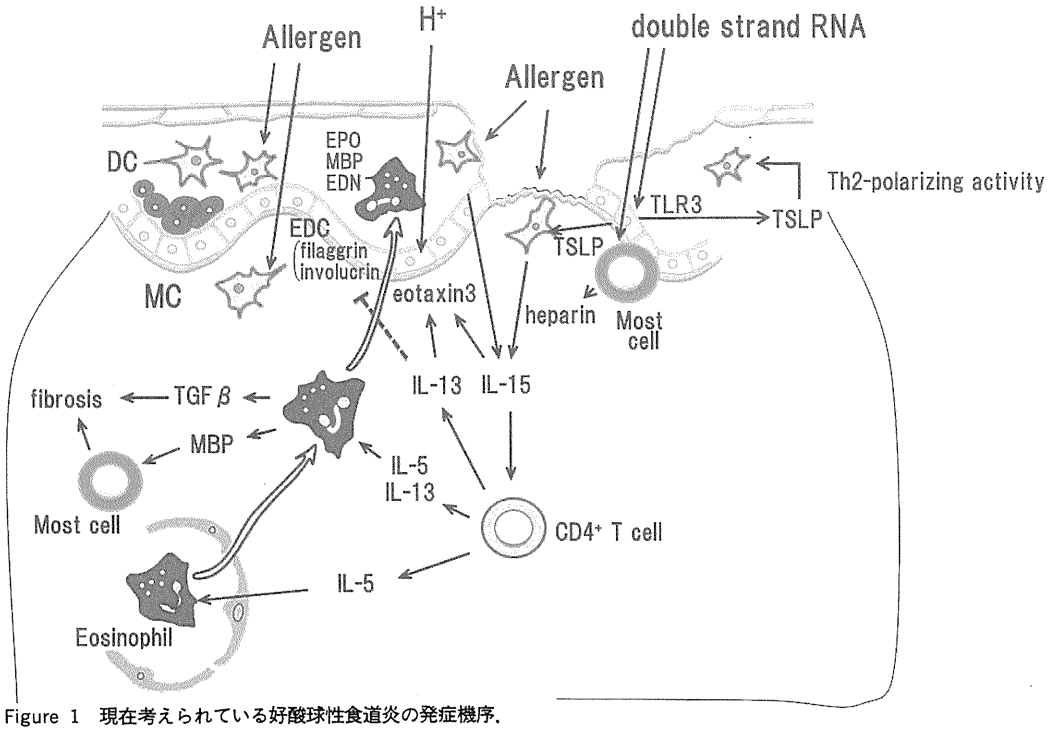


Figure 1 現在考えられている好酸球性食道炎の発症機序.

ナッツ, 魚/甲殻類などを食事から除く除去食またはアミノ酸成分栄養食に食事内容を変更すると, 小児例を中心に症状が軽快することも知られている[7),8)]. このため, これらの多種の食品や空気中の抗原が好酸球性食道炎の Th2 系の免疫反応の抗原となっていると考えられる.

系統的な病態の解明に向けた研究も多方面からおこなわれている. まず, 好酸球性食道炎例と健常者の食道粘膜から生検をおこない mRNA を抽出した後にマイクロアレイを用いて食道粘膜に好酸球性食道炎例で高発現する遺伝子と発現が低下する遺伝子を検索している. その結果, 好酸球性食道炎例では eotaxin-3 というケモカインの発現が正常例の 50 倍以上に増加していること, filaggrin という扁平上皮の分化とともに発現するたんぱくの遺伝子発現が 10 分の 1 に減少していることが明らかとされている[9)]. eotaxin-3 は, IL-5 とともに好酸球を食道上皮に浸潤させる強力なケモカインであること[10)], 食道粘膜上皮中に散在する樹状細胞や単球が産生する IL-15 が Th2 免疫反応を誘導するとともに食道扁平上皮に作用して eotaxin-3 の産生を亢進させること, IL-15 は健常者に比べて好酸球性食道炎患者の食道粘膜では産生量が 10 倍程度亢進していること[11)], Th2 細胞が産生する IL-5 は好酸球の食道への浸潤を誘導するが, 同様に Th2 細胞の産生する IL-13 は好酸球性食道炎患者では食道粘膜中の濃度も血中濃度も上昇していることが明らかとされた. また増加した IL-13 は扁平上皮細胞の eotaxin-3 産生を増加させるとともに分化した扁平上皮にのみ発現する filaggrin や involucrin などの epidermal differentiation complex に関係する遺伝子発現を低下させることが示された[12)~14)]. これらの成績を総合すると, 食物抗原や空気中の抗原で刺激された食道粘膜上皮中の樹状細胞が, 直接に IL-15 の産生を介して, また Th2 系の免疫反応を誘導してリンパ球に IL-5, 13 を大量に産生させて食道上皮の正常な分化を阻害するとともに, eotaxin-3 の発現を刺激して食道粘膜上皮中への好酸球浸潤を促進していることが示されたことになる (Figure 1).

好酸球性食道炎では家族性発症例と散発例にお

いて病像の違いはみとめられていないが[15]，家族内発症も報告されているため遺伝的な素因がありうるであろうと考えられる．遺伝的素因に関係する遺伝子を明らかとすることができれば本疾患の病態を明らかとする大きな手がかりとなるため，まず好酸球性食道炎例で食道での発現が50倍も増加しているeotaxin-3遺伝子の多型についての解析がなされた[9]．その結果，eotaxin-3遺伝子の3'untranslated regionのrs2302009のSNPにT→Gへの多型があれば好酸球性食道炎の有病率が上昇すること，すなわち好酸球性食道炎例は健常者の3.6%に比べて13.7%とこのSNP部位に有意に多くのGG多型を有することが明らかとなった．

ところが，好酸球性食道炎例でもeotaxin-3の遺伝子座にリスクを有する例はわずか13.7%であったるため，他のリスク遺伝子をもとめてgenome-wide association studyがおこなわれ，全遺伝子をカバーする550,000のSNPsの多型と好酸球性食道炎発症の関係が検討された[16]．その結果，5番染色体長腕上の5q22座に存在するSNPに有意な関与が指摘された．この部位にはWDR36とthymic stromal lymphopoietin (TSLP)という2つの遺伝子が存在しているが，好酸球性食道炎例で発現が亢進しているのはTSLPのみのためTSLPが好酸球性食道炎の発症に関与する遺伝子だろうと考えられている．TSLPは上皮細胞が産生するIL-7に似たサイトカインで，樹状細胞を活性化してTh2細胞を増加させることが知られている[17),18]．このため好酸球性食道炎の発症にはTSLPを介するTh2免疫系の活性化も重要であろうと考えられる．

さらに，興味のあることにTSLPの受容体の遺伝子多型や扁平上皮の分化蛋白であるfilaggrinの遺伝子多型も，好酸球性食道炎の発症と関係するとする報告がなされている[14),19]．したがって現在明らかになりつつあるTh2系の活性化とそれに続くeotaxin-3の産生や扁平上皮の分化障害が好酸球性食道炎の発症機序の重要なポイントである可能性は高いと考えられる．

このように，すべての発症機序が明らかとなったわけではないが，食物・空気中の抗原がeotaxin-3，TSLP，TSLP受容体，filaggrin等の遺伝子にリスク多型をもつ個人に作用すると，①上皮が未熟で透過性が高くなりやすい，②強いTh2免疫反応をおこしやすい，③好酸球を呼び寄せるサイトカインが産生されやすいため，食道の局所で強いTh2免疫反応がおこって好酸球の密な食道粘膜上皮内浸潤がおこり食道の慢性炎症に発展することになるのであろうと考えられる[20]．

III 診　断

好酸球性食道炎の診断は，食道に起因すると考えられる種々の症状がある例に上部消化管の内視鏡検査をおこない，食道粘膜の生検組織中に多数の上皮内好酸球をみとめ，好酸球が増加する他疾患を除外することができれば確定することができる．以下に診断の実際を順を追って述べる．

1）患者基本情報

好酸球性食道炎は男性に多く欧米の報告では76%が[1]，日本の厚生労働省研究班の調査では80%が男性であった[4]．年齢は，欧米の報告では14-89歳で平均38歳と若い年代層に多い．日本で成人を対象としておこなった調査では26-79歳で平均年齢は51歳であった[1),4]．本疾患の発症率は，米国での検討では1990年代前半は人口10万人あたり年間1人以下であったが，2000年代の前半では10人にまで増加しており急速に増加していると考えられている[21]．また好酸球性食道炎の有病率は人口の5%に達するという報告もある[22]．最近おこなわれたSystematic reviewでは人口10万人あたり30人前後（0.03%），好酸球性食道炎を疑うような症状のある例では2.8%程度が平均的な有病率であろうとされている[23]．日本でも2006年の第1例目の報告以来[24]その報告例が増加してきている．

2）家族歴，既往歴

アレルギー疾患を合併している例，あるいは過去に合併していた例が欧米での調査では半数以上にあたると報告されている[8),25]．日本での調査でも2009年に集積できた36例のうち44%にアレルギー疾患の合併があり，喘息は特に多く22%の例が喘息を合併していた[4]．米国では家族内集積に関する報告もあり，有名なOlmstead countyでの調査では成人例の23%，小児例の38%に家族歴があると報告されている[21]．日本国内での家族内集積の報告は今のところない．

3）主訴

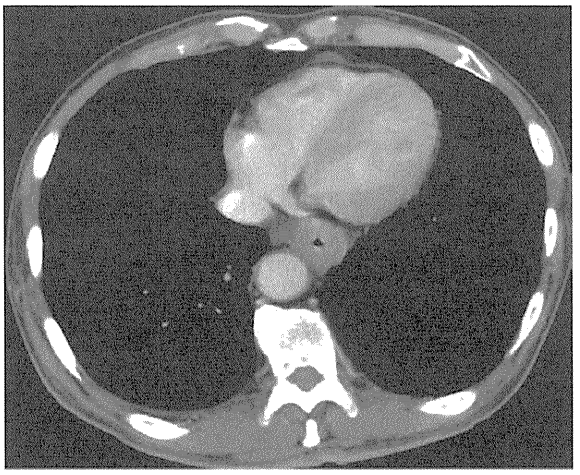


Figure 2 好酸球性食道炎例の胸部CT．食道壁の著明な肥厚をみとめる（文献24より引用）．

胸やけ，呑酸，嘔吐，嚥下困難，食物のつかえ，腹痛等の種々の症状が主訴となりうることが知られている．小児例では食べ物のつかえが主訴となることは少ないが，成人では患者の93%が嚥下障害，42%が食べ物のつかえ，38%が呑酸を訴えると欧米では報告されている[1),26),27)]．日本での調査でも成人では嚥下障害を訴える頻度が最も高く60%であった．

欧米で作成されている診断基準では，上記の症状が高用量のプロトンポンプ阻害薬（PPI）を用いた胃酸分泌抑制療法に反応しないことを診断に必要な項目の1つとしてあげているものが多い[1),28)]．これは，胃食道逆流症（GERD）例，特にmucosal breakを有しない非びらん性胃食道逆流症（NERD）例では，胸やけ，呑酸，嚥下困難等の症状を訴え内視鏡検査で食道に異常を発見することが困難であること，生検をおこなって検討するとGERD例でも食道粘膜に好酸球浸潤が存在することがあり，好酸球性食道炎とNERDの鑑別診断が時に困難であるため附加されたと考えられる診断の基準である[29)~32)]．好酸球性食道炎がPPI治療に反応しないことが確実であれば，この基準を用いることに問題はないが，実際にはPPIで症状が軽快する好酸球性食道炎の報告もみられる．好酸球性食道炎の発症において，食道上皮が産生するケモカインであるeotaxin-3の重要性についてはすでに解説したが，pH4程度の酸性条件下ではIL-13で刺激をした時に食道上皮細胞からのeotaxin-3の遊離が増加することが分かっている[14)]．健常者でさえ胃酸は食道内に短時間逆流している．そこで好酸球性食道炎例にPPIを投与すると食道内への生理的範囲の胃酸の逆流が減少し，eotaxin-3の遊離も減少して好酸球性食道炎の病理像および症状が軽快することが推定される．このため，PPIを投与することで症状が軽快しても，それだけで好酸球性食道炎を否定することは，必ずしも容易ではないと判断する方が正しいと考えられる．

4）血液検査

血液検査をおこなうと末梢血中での好酸球増加は，成人では10-50%，小児では20-100%に間でみとめると報告されている．日本の調査では，好酸球性食道炎例のうちに末梢血好酸球増加をみとめる例は約30%で，好酸球性胃腸炎例の90%と比較して低頻度であった[4)]．

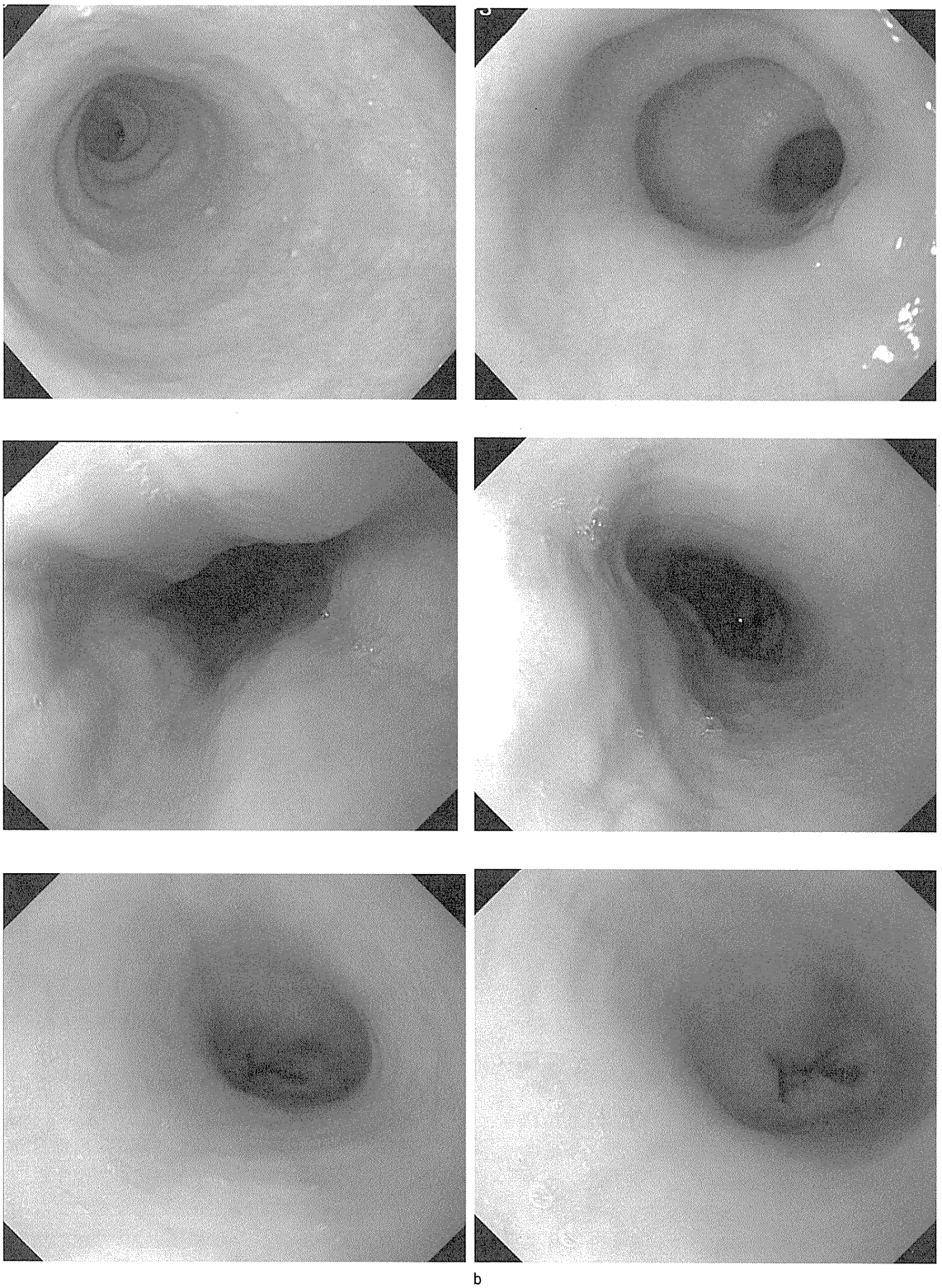
末梢血中のIgEも70%の例で高値を示すと報告されている．ところが，末梢血中の好酸球もIgEも血液データのみで好酸球性食道炎の診断をおこなったり，病勢を判定するには感度や特異度に問題があり参考データと考えられる．そこで，末梢血中のeotaxin-3や好酸球由来のeosinophil-derived neurotoxinの測定をおこなって好酸球性食道炎の診断マーカーとしようとするこころみもおこなわれている[30),33)]．

5）食道レントゲン検査，CT検査

胸やけや呑酸が主訴となっている場合にはまずGERDの存在が疑われる．GERDの診断には食道レントゲン検査の有用性は低いため，本検査がおこなわれることは少ない．ところが，嚥下障害を訴える例や小児例では食道レントゲン造影検査がおこなわれることもある．レントゲン検査では，食道の狭窄やその口側の拡張が描出されることがあり，狭窄の様子が1-2 mmの輪状の多発性狭窄となる場合がある[28),34)]．またCT検査をおこなえば食道壁の肥厚を同定することが可能であり[35)]，日本における研究班の集計でも好酸球性食道炎例の64%において食道壁の肥厚が同定されている（Figure 2）[4)]．

6）内視鏡検査

内視鏡検査では種々の食道粘膜の異常が報告されており，117例（9例が小児で，108例が成人）の好酸球性食道炎の内視鏡像を集計したMüller S



b

Figure 3　好酸球性食道炎例の食道内視鏡写真.
a：日本で第1例目の食道内視鏡像．白斑と多発の輪状狭窄をみとめる（文献24より引用）.
b：最近経験した別の好酸球性食道炎例の食道内視鏡像．縦走溝をみとめる.

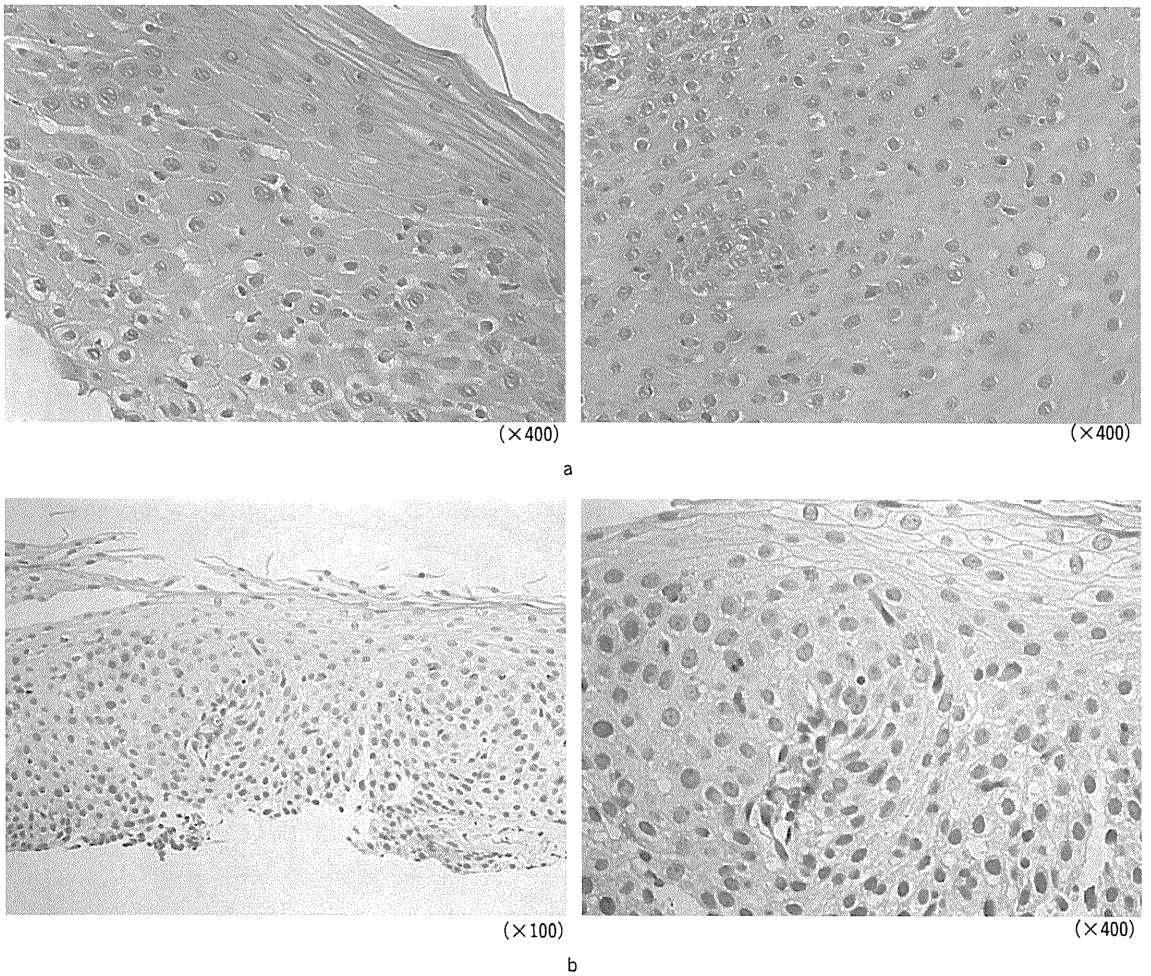


Figure 4 好酸球性食道炎例の食道生検組織像.
a：日本での第1例目の食道粘膜生検組織. 1視野（HPF）あたり20個以上の上皮内好酸球をみとめる（文献24より引用）.
b：最近経験した別の好酸球性食道炎例の食道粘膜生検組織.

らの報告によると，26%の例に白色の点状浸出物の附着，26%に縦走する溝状裂孔，26%に発赤，19%に輪状の狭窄，16%に局在性の狭窄，15%にしわ状パターンを示す食道粘膜，14%にcobble stone-likeのパターンを示す食道粘膜，14%に肉芽，10%になだらかな凹凸を有する粘膜，7%に血管透視の不良，4%に長い狭窄，2%にscarがみられたとされている[27]. ただし，彼らの報告によると25%の例では内視鏡検査で異常を発見することができなかったとされており，内視鏡検査で好酸球性食道炎を疑うことはできるが好酸球性食道炎の存在を否定をすることはできないことが分かる[36]. さらに，生検をおこなうと食道粘膜が薄く，クレープのように広い範囲にわたってはがれることが特徴の1つであるとする報告もみられる[37]. 日本での集計でも内視鏡像では，白斑が最も多く次いで縦走する溝状の陥凹が多く観察されている（Figure 3）.

内視鏡検査の感度は十分ではないため，内視鏡検査が正常であっても好酸球性食道炎を否定することはできないが，特異度に関しても十分ではなく好酸球性食道炎で観察される内視鏡像は他の疾患においても観察される像である. 実際，白斑を食道カンジダ症と考え，好酸球性食道炎の診断に至るまでに長期を要した例の報告や正診されるまでGERDと診断されていた例の報告がみられ

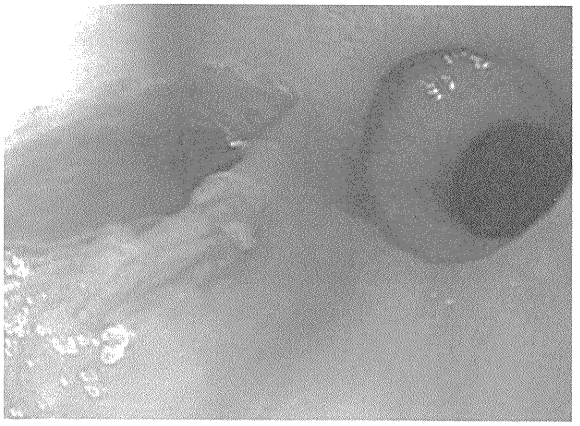


Figure 5　生検組織採取後の好酸球性食道炎例の食道内視鏡像．粘膜の大きな欠損をみとめる（文献 24 より引用）．

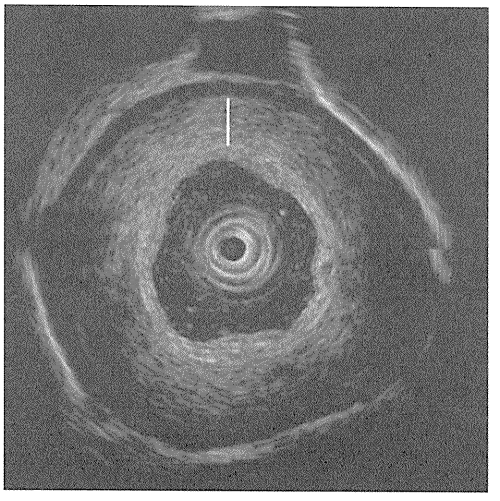


Figure 6　好酸球性食道炎例の超音波内視鏡検査．著明な食道壁の肥厚をみとめる（文献 24 より引用）．

る[38),39)]．

多数例を集積し，より詳細な内視鏡検査をおこなって内視鏡診断の感度と特異度を向上させていく努力が必要であろうと考えられる．

7）内視鏡下生検診断

内視鏡検査で異常がみつかる場合だけではなく内視鏡検査でまったく異常がなくても，好酸球性食道炎を疑わせる嚥下障害，胸やけ等の症状がある場合には生検をおこなうことが必要である．好酸球性食道炎例の 25-30% は内視鏡検査で異常を発見できず[27)]，診断のよりどころが生検診断となるため，生検をおこなっておくことが重要となる（Figure 4）．

好酸球性食道炎例の食道生検をおこなった報告では，上部食道では食道上皮内に 1-157（平均 63.3）個の好酸球/400 倍の高倍率視野（HPF）をみとめ，中部食道では 3-182（平均 62.1）個/HPF，下部食道では 18-244（平均 91.5）個/HPF の好酸球がみられるとされている[27)]．多くの報告では有意差には至らないが下部食道の方が上部食道より浸潤好酸球数は多いと報告されている[40)]．好酸球性食道炎の診断確定のための食道上皮内好酸球数の算定をどのようにおこなうかについてのコンセンサスはないように思われる．20/HPF 以上の好酸球が生検組織の 1 つ以上にみられれば診断基準を満たすとするものが多いが，15/HPF～30/HPF まで報告により基準にばらつきがみられる[1)]．

1 人の患者からおこなう生検個数に関しては，診断の基準を 15/HPF 以上の食道上皮内好酸球とした場合には 1 個の生検では，生検診断の感度はわずか 55% であり，感度を 100% とするには 5 個の生検組織を採取することが必要であると報告されている[40)]．このため，好酸球性食道炎を疑う症状を有する例では内視鏡検査時に異常が発明されれば，その部位から，発見されなくても複数個の生検組織を採取するべきだと考えられる．特に白斑の存在部位を生検すると好酸球が上皮内に集積した eosinophilic microabscess が得られる可能性が高いとされている[41)]．

好酸球性食道炎例の食道上皮には多数の好酸球の浸潤以外に，eosinophilic microabscess，重層扁平上皮の内腔側に近い部分への多数の好酸球の浸潤，基底層細胞の過形成，上皮乳頭の延長などが観察される．また上皮内に好酸球とともに mast cell の浸潤が多いことも最近注目されている[42)]．GERD においても，これらのいくつかの特徴はみられるが，GERD 例では好酸球数が 20/HPF 以上となることはなく，好酸球の浸潤は主に上皮の基底層側で，microabscess や mast cell は見当たらないと報告されている[29)]．

好酸球性食道炎を疑って生検をおこなう場合には，食道粘膜が弱く傷つきやすくなっており生検で上皮が大きくはがれやすく傷が大きくなりやす