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**Takeshi Sasahara · Hiroko Maruyama · Masahito Aoki
Ritsuko Kikuno · Tomoko Sekiguchi · Akira Takahashi
Yoshinori Satoh · Hidero Kitasato · Yoko Takayama
Matsuhisa Inoue**

Apoptosis of intestinal crypt epithelium after *Cryptosporidium parvum* infection

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Abstract Using a neonatal mouse model of *Cryptosporidium parvum* infection, we investigated whether apoptosis of epithelial cells was induced in the small intestine. At the time when the number of *C. parvum* oocysts in the ileum was maximal, columnar goblet cells and absorptive cells showed a decrease in the ileal epithelium that was accompanied by a significant reduction in the height of the villi. A few apoptotic epithelial cells were also observed in the vicinity of the basal crypts where *C. parvum* was proliferating. Morphological changes of the villous structure and apoptotic epithelial cells associated with proliferation of the parasite were scarcely detected in the duodenum, cecum, and colon of the infected mice. These findings suggest that the loss of absorptive cells and goblet cells, and the apoptosis of intestinal epithelial cells, are common events in the ileum after *C. parvum* infection, and that epithelial apoptosis may have a significant role in the pathogenesis of cryptosporidiosis.

Key words *Cryptosporidium parvum* · Apoptosis · Intestinal crypt epithelium

T. Sasahara (✉) · T. Sekiguchi · Y. Satoh · H. Kitasato · M. Inoue
Department of Microbiology and Parasitology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan
Tel. +81-42-778-9350; Fax +81-42-778-8441
e-mail: ts011501@kitasato-u.ac.jp

Y. Takayama
Department of Infectious Diseases, Kitasato University School of Medicine, Kanagawa, Japan

A. Takahashi
Laboratory Animal Center for Medical Science, Kitasato University School of Medicine, Kanagawa, Japan

H. Maruyama
Department of Pathology, Kitasato University School of Allied Health Sciences, Kanagawa, Japan

M. Aoki · R. Kikuno
Kitasato Research Centre for Environmental Sciences, Kanagawa, Japan

Cryptosporidium parvum is an enteric protozoan that causes diarrhea in both immunocompetent and immunocompromised hosts. Following the release of sporozoites from an oocyst on the apical surface of the intestinal epithelium, these sporozoites attach to the epithelial cells and produce superficial parasitophorous vacuoles, so that *Cryptosporiodium* is an intracellular infection, but is extracytoplasmic.[1–3] Infection causes villous atrophy and crypt hyperplasia in vivo.[4,5] Although apoptosis of intestinal and biliary epithelial cell lines has been observed in vitro,[1,2,6,7] apoptosis of the intestinal epithelium in *C. parvum*-infected hosts has still not been observed directly in vivo. In the present study, we used a neonatal mouse model of *C. parvum* infection to investigate whether or not this parasite induced apoptosis of the intestinal epithelium, and we assessed the significance of such apoptosis in the pathogenesis of cryptosporidiosis.

Oocysts of *C. parvum* strain HNJ-1, originally isolated from a Japanese patient in 1989 and genotypically of the cattle type,[8] were harvested from infected SCID mice (C.B-17/Icr-scidjcl strain, Clea Japan, Tokyo, Japan). The oocysts were purified from the feces by the sequential discontinuous sucrose gradient method, as described previously,[9] suspended in Dulbecco's phosphate-buffered saline (PBS; Sigma, St. Louis, MO, USA) containing 200 μg/ml of gentamicin (Sigma), and stored at 4°C.

Litters ($n = 10$) of 7-day-old ddy mice were maintained with their dams (purchased from Japan SLC, Shizuoka, Japan) in a specific pathogen-free environment (the biohazard area of level P2 of the Laboratory Animal Center for Medical Science) and were divided into two groups. Experiments were performed according to the ethical guidelines of the Animal Care Committee of Kitasato University School of Medicine. Each group of five neonatal mice was orally inoculated with 10^4 oocysts in 50 μl of PBS or received PBS alone as a control. On day 7 after inoculation, the mice were killed by exposure to CO_2.

Because the ileum is colonized by *C. parvum* and always harbors the greatest number of developing parasites,[9,10] approximately 1 cm of the terminal ileum was removed from each mouse, fixed in 10% neutral buffered formalin, and

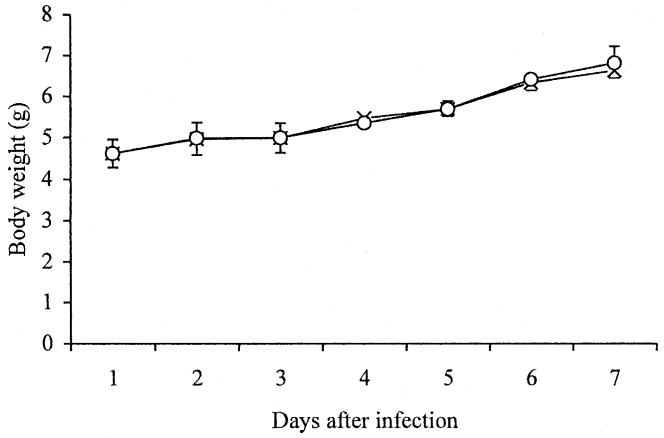

Fig. 1. Changes in body weight in infected and control mice. *Open circles*, infected mice; *crosses*, control mice

embedded in paraffin for sectioning. In some experiments, the duodenum, cecum, and colon were also removed from the infected mice and sectioned in the same manner. Sections were stained with hematoxylin and eosin (H&E; Sigma), as well as with a mouse monoclonal anti-*C. parvum* oocyst antibody (Wako Pure Chemical Industries, Osaka, Japan) and peroxidase (POD)-labelled goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA) for the detection of *C. parvum* oocysts. The sections were also used for the detection of apoptosis by the indirect TUNEL method with an in situ Cell Death Detection kit (Boehringer Mannheim, Mannheim, Germany). Sections prepared for the detection of *C. parvum* oocysts and apoptotic cells were counterstained with methyl green (Sigma). The stained sections were examined under a light microscope (BH-2; Olympus, Tokyo, Japan). The height of the villi in the villous epithelium of infected and control mice was determined by light microscopy with an (10×) ocular micrometer. As previously reported,[9] the number of *C. parvum* oocysts in the ileum was maximal (about 10^6) by 7 days after inoculation (data not shown). However, the weight gain of infected and control mice was similar up to 7 days after infection (Fig. 1), and symptoms such as severe diarrhea or piloerection were not observed during this period.

As shown in Fig. 2A,B, H&E-stained sections of the ileum revealed that the number of columnar absorptive cells (containing eosinophilic granules) and goblet cells (containing clear apical vacuoles) was decreased in all of the infected mice ($n = 5$), accompanied by a significant reduction in the height of the villi (Fig. 3), while these changes did not occur in the control mice ($n = 5$). Many small spherical protozoal particles (2–5 μm) showing basophilic staining were seen on the apical surface of epithelial cells from the ileal crypt epithelium of the infected mice (Fig. 2B), and immunohistochemical staining revealed *C. parvum* oocysts in the ileal sections (Fig. 2D). In contrast, there was little or no evidence of such severe changes of the villous structure or proliferation of oocysts in the epithelium of the duodenum, cecum, or colon obtained from infected mice (data not shown). These observations suggested that attachment of *C. parvum* to specific sites on the apical membrane of the ileal epithelium and its proliferation may lead to a unique influence on host ileal epithelial cells that results in structural changes, probably due to modification of cytoskeletal elements. These structural abnormalities might lead to dysfunction of the intestinal barrier, with an increase of its permeability, as well as malabsorption of vitamin B_{12} and fat, changes that were previously demonstrated in children and AIDS patients with symptomatic cryptosporidial infection.[5] However, the severe villous atrophy and crypt hyperplasia that occur in AIDS patients[4,5] were not observed in our infected mice. It has been reported that recombinant HIV-1 TAT protein, a protein released in a biologically active soluble form by HIV-1-infected T cells and macrophages, enhances *C. parvum*-associated apoptosis.[11] Thus, it is possible that the severe structural changes in the ileal epithelium may be caused by synergistic effects of pathogens after dual infection with human immunodeficiency virus type 1 (HIV-1) and *C. parvum*.

There have been reports that *C. parvum* induces the apoptosis of cultured human intestinal epithelial cell lines,[1,2,6] as well as biliary epithelial cells and gallbladder epithelium obtained by biopsy in an AIDS patient with biliary cryptosporidiosis.[7] To detect the apoptosis of epithelial cells in the ileal, where a large number of oocysts was found, ileal sections from the infected mice were stained by the indirect TUNEL method. As shown by the arrows in Fig. 2E,F, ileal epithelial cells showing signs of apoptosis were frequently detected near the basal crypts where *C. parvum* was proliferating in infected mice, but not in normal mice. There were four or five apoptotic cells in each *C. parvum*-infected crypt. These results provide the first evidence that epithelial cells lining the basal crypts of the ileum undergo apoptosis in *C. parvum*-infected neonatal mice, and suggest the need for further studies of the pathways leading to apoptosis and its role in the pathogenesis of cryptosporidiosis.

During the differentiation and maturation of intestinal epithelial cells, apoptosis may act as a regulator of cell migration toward the villous surface, but it is uncertain whether or not it is responsible for epithelial cell sloughing from the tips of the villi.[12] In the present study, there was no evidence of apoptotic cells in the ileal epithelium of the control mice, suggesting that apoptotic cells might be rapidly phagocytased and digested by local histiocytes. On the other hand, a few apoptotic cells were observed in the infected mice, suggesting that these cells might not have been removed from the site of infection by phagocytes due to excessive epithelial apoptosis. It has also been reported that apoptosis in *C. parvum*-infected cells is increased by the inhibition of nuclear factor-κB (NF-κB) and is attenuated at moderate levels by strongly proapoptotic agents such as staurosporine.[2] Thus, it is also possible that *C. parvum* may prevent the induction of a high level of epithelial apoptosis to promote the development and proliferation of the parasite, and it may also limit the host inflammatory response, which is detrimental to its survival in the intestine.

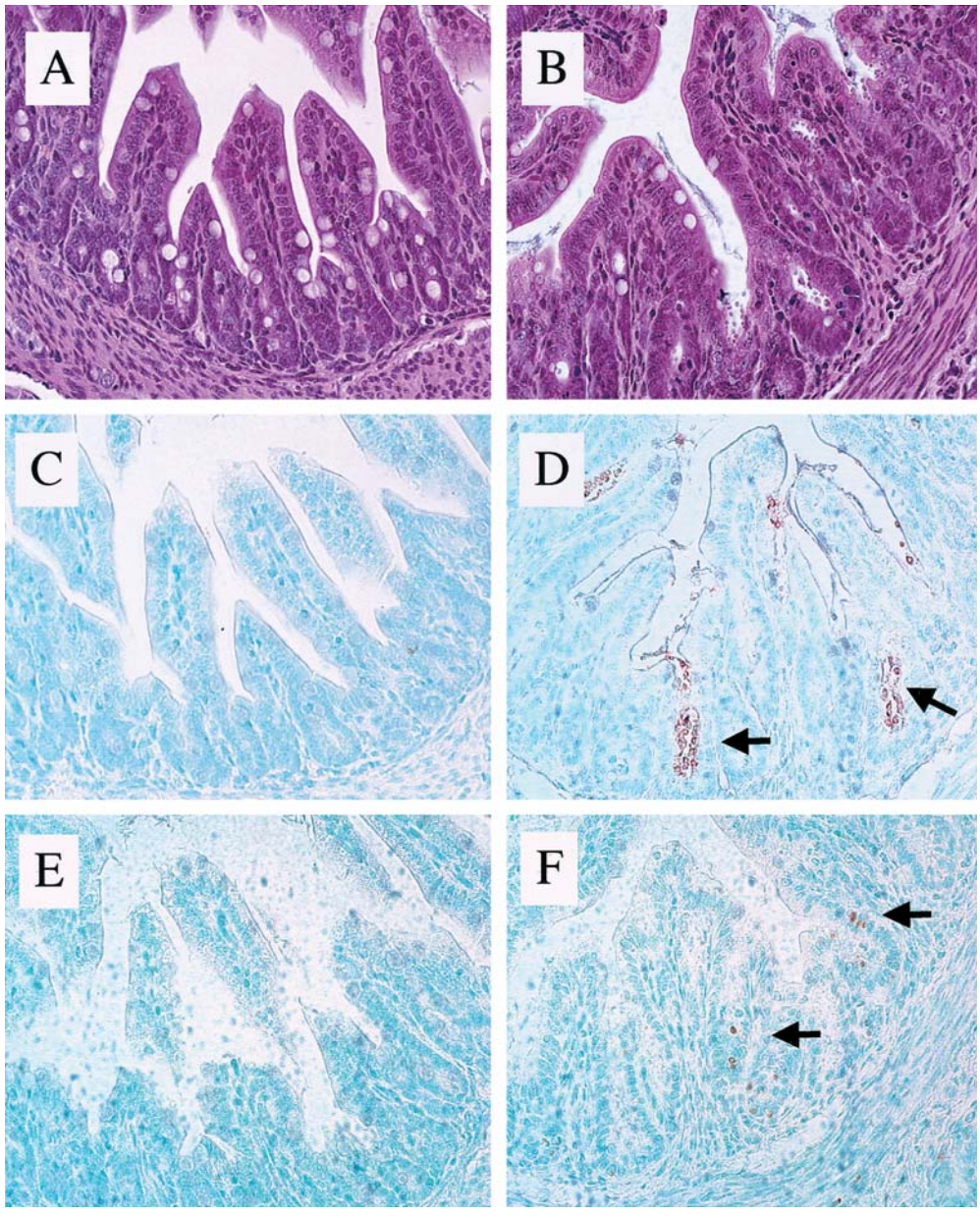

Fig. 2A–F. Detection of apoptotic epithelial cells in the ileum of mice infected with *Cryptosporidium parvum*. Ileal sections from normal mice (**A**,**C**,**E**) and infected mice (**B**,**D**,**F**) were subjected to the following stains. **A**,**B** H&E; **C**,**D** immunohistochemical staining with anti-*C. parvum* oocyst antibody. *Arrows* in **D** show *Cryptosporidium* (*brown*) adhering to the surface of the villi. **E**,**F** TUNEL staining for the detection of apoptotic cells. *Arrows* in **F** show apoptotic cells (*brown*) lining the basal crypts of the ileum. **A**–**F**, ×200

The subcellular mechanisms by which specific pathogens induce apoptosis of host epithelial cells remain obscure. Suggested candidates for microbe-related apoptosis pathways include an increase of protease activity (e.g., caspases),[1] up-/down-regulation of proapoptotic cytokines (e.g., tumor necrosis factor-α),[7] and down-regulation of antiapoptotic proteins (e.g., Bcl-2).[13] It has recently been reported that epithelial cells undergoing apoptosis are influenced by adjacent cells with *C. parvum* infection via the Fas receptor-Fas ligand death system[7] and via CD40-CD40 ligand interaction.[14] On the other hand, it was also reported that cells undergoing apoptosis are limited to those directly infected by the pathogen and that the process is dependent on the activation of caspases.[2] It was unclear whether or not *C. parvum* infection was directly cytopathic and induced the apoptosis of ileal epithelial cells in the present study. These reports, taken together with our findings, however, suggest that the subcellular mechanisms of apoptosis related to infection with *C. parvum* appear to be complicated and may involve unknown virulence factors (e.g., a *C. parvum* enterotoxin) or mediators released from infected cells.

It still remains unclear whether the occurrence of apoptosis benefits either the parasite or the host in cryptosporidiosis. Removal of infected epithelial cells by apoptosis may assist the host in maintaining the integrity of the epithelial barrier. On the other hand, there has been a recent increase in reports suggesting that apoptosis could be involved in the pathogenesis of *C. parvum* infection.[1,2,7] For example, infection of epithelial cell monolayers with *C. parvum* leads to an increase in permeability to large molecules.[7] The possibility thus exists that part of the dysfunction of the intestinal barrier may be due to the apoptosis of

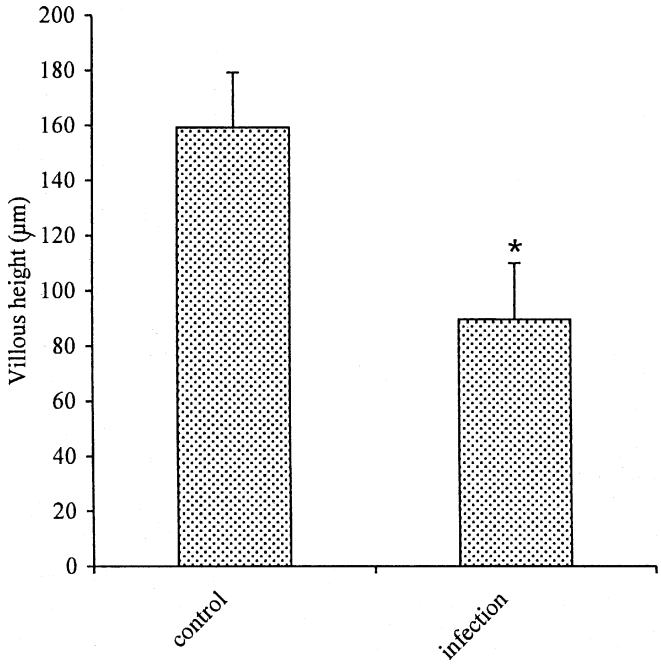

Fig. 3. Height of the ileal villi in infected and control mice. The mean height (μm), ± standard deviation, of 50 villi was calculated for each mouse. * Significant difference ($P < 0.05$)

infected cells, so it would be interesting to determine whether the inhibition of apoptosis in vivo by a caspase inhibitor such as z-VAD-fmk[1,15] can ameliorate the symptoms (diarrhea, etc.) of cryptosporidiosis. Future studies are planned at our laboratory to evaluate the effect of several caspase inhibitors on *C. parvum* infection in a neonatal mouse model.

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