Apoptosis of Enterocytes Induced by Inoculation of a Strain of Attaching and Effacing Escherichia coli and Verotoxin

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ABSTRACT. When verotoxin (VT)-producing attaching and effacing Escherichia coli (AEEC, serotype O5: H-1) were inoculated perorally into 10-day-old rabbits, attaching of the E. coli to enterocytes and effacing of their microvillous portion were observed extensively from the ileum to the colon. Subsequent apoptotic changes of the infected enterocytes were demonstrated by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) reaction and electron microscopy. Apoptosis was also induced in cultured Vero cells by inoculation of VT extracted from the AEEC. This study clarified that VT-producing AEEC induce apoptosis of enterocytes, causing mucosal damage. — key words: apoptosis, attaching and effacing Escherichia coli, verotoxin.

Attaching and effacing Escherichia coli (AEEC) is a representative diarrheagenic E. coli [7, 8]. AEEC colonizes in the lumen of the intestinal tract and adheres directly to enterocytes, resulting in the effacement of the apical surface microvilli. Some strains of AEEC produce verotoxin (VT), which possesses a distinctive cytotoxicity to Vero cells [6]. An E. coli strain of serotype O5, one of the VT-producing AEEC, has been frequently identified as a cause of dysentery in calves [9].

Apoptosis, which can be distinguished from necrosis, is known as a typical type of cell death. The apoptotic cell death is characterized by nuclear pyknosis, cytoplasmic condensation and karyorrhexis, and is easily induced by extracellular factors, such as toxins, ischemia, and irradiation [4, 12]. Similar morphological changes of enterocytes were observed so frequently in the intestine of AEEC-infected animals [8, 9, 12, 13]. Therefore, we now hypothesize that the morphological changes of AEEC-infected intestine may be caused by an enhanced apoptotic cell death of enterocytes. Although previous reports [5, 10] have suggested the presence of apoptosis in the intestine infected with VT-producing E. coli, they did not show any evidence for apoptosis at a molecular level. In this paper, we first demonstrate that the attaching of AEEC (serotype O5: H-) is closely related to apoptotic cell death of rabbit enterocytes in vivo, and that VT obtained from AEEC induces the apoptosis of Vero cells in vitro.

The E. coli (serotype O5: H-) strain used in the present inoculation study was isolated from the diarrheal feces of a calf with natural infection in Hokkaido, Japan. The strain has been shown to produce VT, but not to express heat-stable toxin (ST), heat-labile toxin (LT), or any fimbrial adhesion of K88, K99 and 987P. The E. coli attaching and effacing (eae) gene was detected by polymerase chain reaction [11]. The E. coli were grown on trypticase soy broth (BBL, Bectom Dickinson, U.S.A.) in a rotary shaker at 37°C for 18 hr. Suspensions of the trypticase soy broth were adjusted to a concentration of approximately 10⁶ CFU/ml in 10% NaHCO₃. Three 10-day-old Japanese white rabbits were perorally inoculated with 2 ml of the suspension via a tuberculin syringe. As a control, another three 10-day-old rabbits were inoculated with 2 ml of the tryptase soy broth. All the rabbits were sacrificed 3 days after inoculation. Various regions of the intestine from the duodenum to rectum were fixed in 20% neutral buffered formalin, and embedded in paraffin according to a conventional method. Paraffin sections 4 μm in thickness were stained with hematoxylin and eosin (HE). Using HE-stained sections, the presence and frequency of apoptotic cells in the epithelium were studied. Ten microscopic fields were randomly selected in each section at a magnification of x 480 under a light microscope. The total number of apoptotic cells in ten fields was counted, and the values were given as the mean ± standard deviation (n=3). To check the fragmentation of DNA in situ, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) reaction [1] was applied to paraffin sections using an apoptosis detection kit (Takara, Tokyo, Japan). After TUNEL reaction, the sections were counterstained with hematoxylin. For electron microscopy, the formalin-fixed colons were post-fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and 1% O₂O₄. The specimens were processed according to a routine method and observed under a Hitachi H-7000 transmission electron microscope.

Vero cells provided from National Institute of Animal Health, Japan, were grown for 48 hr at 37°C with 5% CO₂ in Eagle MEM supplemented with 10% fetal calf serum using a chamber slide (16 chambers, Lab-Tec, Nunc Inc., Naperville III.). E. coli were cultured with trypticase soy broth in a rotary shaker at 37°C for 18 hr and centrifuged at 10,000 rpm for 15 min. Supernatants were filtered through 0.45 μm membrane filters and 20 μl of the supernatant was added to the chamber. As a control, 20 μl of medium was added to the chamber. The chamber slide was incubated in 5% CO₂ for 3 days at 37°C and fixed with 4% paraformaldehyde. After incubation, TUNEL reaction was carried out on the chamber slide.

For detection of DNA fragmentation in agarose gel electrophoresis, Vero cells were grown in two 25 mm² tissue
culture flasks as described above, and 0.5 ml of the VT extraction from the E. coli and the medium for the control were added to each flask. After incubation for 3 days, Vero cells were scratched and pelleted. DNA extraction of the pellets was carried out as described previously [1]. The sample was subjected to electrophoresis on a 2% agarose gel. To detect DNA fragments, the gel was stained with ethidium bromide.

Clinically, diarrhea, indicated as so-called wet tail, was observed at 3 days in all the E. coli-inoculated rabbits, but not in the control. Histologically, the superficial epithelia in the colon of inoculated rabbits were irregular in outline due to the protrusion of enterocytes into the lumen. Numerous E. coli adhered to the luminal surface of enterocytes showing the polypous extrusion (Fig. 1), while no bacilli adhered to enterocytes in the control. Similar changes were also found in the ileum, but not in the duodenum and jejunum. The epithelial cells attached by E. coli were shrunken and showed increased cytoplasmic eosinophilia. Their nuclei were condensed and fragmented into smaller pieces. These changes were in agreement with figures in apoptotic cell death. The apoptotic cells, frequently termed apoptotic bodies, desquamated into the lumen or were engulfed by adjacent intact epithelial cells (Fig. 2). The frequency of occurrence of apoptotic cells in the epithelium was 37.0 ± 4.6 in inoculated rabbits, while in control it was 7.3 ± 1.5.

Applying the TUNEL method to sections of the colon from the inoculated animals, many apoptotic bodies were positive in the superficial epithelium (Fig. 3). Some apoptotic bodies detectable by the TUNEL method were observed in the superficial epithelium of the control rabbits, however, their frequency of occurrence was clearly elevated in AEEC-infected rabbits. In inoculated rabbits, only a few TUNEL-positive cells were found in the crypt epithelium without E. coli attaching, and their frequency was the same as control animals.

Ultrastructural observation of the inoculated-rabbit colon demonstrated attaching of numerous E. coli on the epithelial cell surface and absence of microvilli in these areas. Apoptotic changes of nuclei, characterized by the condensation of chromatin along the nuclear envelope, were found in some infected enterocytes which were extruded into the lumen and formed a domed or polypous protrusion (Fig. 4). Such epithelial cells possessed irregular-shaped vacuoles and lipid droplets of various sizes in the cytoplasm.

Vero cells inoculated with VT showed typical fragmentation of nuclei into smaller pieces under a light microscope. TUNEL method demonstrated that many positive stains were detected in Vero cells, while TUNEL-positive nuclei were hardly observed in the control (Fig. 5). Agarose gel electrophoresis of DNA extracted from VT-inoculated Vero cells showed the characteristic ladder pattern of DNA fragments (Fig. 6).

AEEC is known to adhere to intestinal epithelial cells and induce some morphological changes of the target cells, represented by the effacement of microvilli, and followed

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Fig. 1. Colonic mucosa obtained from an E. coli-inoculated rabbit. A semi-thin epon section is stained with toluidine blue. Numerous E. coli (arrows) adhere to the apical surface of enterocytes which protrude into the lumen. × 600.

Fig. 2. Apoptotic epithelial cells showing nuclear pyknosis (arrowheads) desquamate into the lumen of the colon. Some apoptotic bodies are found within intact epithelial cells (arrows). HE. × 500.

Fig. 3. Apoptotic bodies (arrows) in the superficial epithelium of the colon are positive to the TUNEL reaction. The section is slightly counterstained with hematoxylin. × 250.
E. coli induces apoptosis

of apoptosis has been suggested in rabbits infected with enterohemorrhagic E. coli O157; H7, DNA fragmentation, unique to apoptotic events, was not proved [10]. Irradiation and some agents such as colonic carcinogens are known to induce apoptosis in intestinal epithelial cells predominantly in the crypt region throughout the intestine [3]. In the AEEC-inoculated rabbits, no increase of apoptotic bodies was found in the crypt epithelium without attaching of E. coli. The present study further clarified by TUNEL method and agarose gel electrophoresis that the cytotoxicity of VT to Vero cells in vitro was associated with apoptosis, suggesting that VT is an inducer of apoptosis to target cells with AEEC infection, and plays an important role in pathogenesis of diarrhea.

Apoptotic bodies are rapidly incorporated and degraded by adjacent intact cells or professional phagocytic cells. The apoptotic bodies formed in the epithelial lining are usually extruded from the surface, rather than being phagocytosed within the tissue [4]. On the other hand, Han et al. [2] reported that there were species differences in the disposal of apoptotic enterocytes; in the rat and mouse, effete epithelial cells are exfoliated into the lumen, while those in the guinea pig and monkey are phagocytosed by lamina propria macrophages. The mechanism responsible for disposing of effete epithelial cells in the normal rabbit intestine remains unclear. From our observations, it appears that the removal of AEEC-induced apoptotic bodies in the rabbit intestine is accomplished by both exfoliation into the lumen and engulfment by adjacent epithelial cells.

REFERENCES