FULL PAPER *Anatomy*

Immunohistochemical Study on the Delayed Progression of Epithelial Apoptosis in Follicle-Associated Epithelium of Rat Peyer's Patch

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(Received 25 May 2007/Accepted 10 July 2007)

ABSTRACT. It is well known that some caspases in apoptosis is involved in determinant of terminal differentiation and maturation of various cells. Our previous study ultrastructurally clarified the differentiation into M cells from immature microvillous epithelial cells and the redifferentiation from M cells to microvillous epithelial cells in the follicle-associated epithelium (FAE) of rat Peyer's patch. In this study, the difference of epithelial apoptosis between the FAE of Peyer's patch and intestinal villi was immunohistochemically investigated in rat jejunoileum. As a result, cleaved caspase-3 was limited to several epithelial cells at the tip of FAE, whereas almost all of the epithelial cells were cleaved caspase-3 positive in intestinal villi. Cleaved caspase-9 was detected only in a few exfoliating or exfoliated epithelial cells of both FAE and intestinal villi. Nuclear DNA-fragmentation was detected only in several epithelial cells of the tip of FAE, while it was expressed from the middle regions in the intestinal villi. The DNase I expression of the epithelial cytoplasm was much weaker in FAE than in intestinal villi. Bcl-x expression was restricted in the apical cytoplasms of epithelial cells in the FAE, whereas it was restricted in whole cytoplasms in villous epithelial cells. These findings suggest that the progression of the apoptotic process in the epithelial cells of FAE is later than in the intestinal villi, so that the possibility of epithelial differentiation might be remained in the FAE, unlike in the intestinal villi.

KEY WORDS: differentiation, epithelial apoptosis, M cell, Peyer's patch, rat.

J. Vet. Med. Sci. 69(11): 1123–1129, 2007

Caspases are a group of cysteine proteases critical for apoptosis of eukaryotic cells and function in cell signaling events including apoptosis, cellular maturation and terminal differentiation [26]. That is, caspase-3 activity is necessary for maturation from the myoblast to the myotube in mouse skeletal myogenesis *in vitro* [12]. Caspase-3 activity is also involved in the human erythroblast maturation *in vitro* [30] and both caspase-3 and caspase-9 activity is involved in the differentiation of human monocytes to macrophages *in vitro* [23]. Caspase-14 strongly increases during the maturation of human keratinocytes *in vivo* and *in vitro* [11]. Thus, some caspases in apoptosis is involved in the maturation or the terminal differentiation of various somatic cells.

The epithelial cells proliferate from undifferentiated epithelial cells in intestinal crypts and differentiate to the special types of epithelial cells in intestinal crypts [4–7]. Almost all epithelial cells migrate through the villous axis and are finally exfoliated from the villous tips. On the way of the migration, apoptosis is induced in the villous epithelial cells. Namely, DNase I - specific mRNA expression was detected in the epithelial cells at the lower thirds of the intestinal villi [29], and the intracytoplasmic DNase I gradually increases toward the villous tips [20]. From these findings, it is speculated that almost all epithelial cells are in apoptotic process in the intestinal villi, where epithelial differentiation has already been determined.

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In the follicle-associated epithelia (FAEs) of mouse and rabbit Peyer's patches, epithelial cells migrate from the follicle-associated intestinal crypts (FAICs) to the apices of the FAEs [3, 24]. Apoptotic epithelial cells are exfoliated from the apices of the FAEs in porcine Peyer's patch and chicken cecal tonsil [18, 25]. In the migration process in the FAE, the differentiation to M cells from immature microvillous epithelial cells and the redifferentiation of M cells into mature microvillous epithelial cells continuously occurs [19]. From this finding, it is speculated that the determinant of epithelial differentiation is different in the FAE-FAIC axis from in the intestinal villi - intestinal crypt axis. However, the epithelial apoptosis has been hardly clarified in the FAE.

In this study, we immunohistochemically investigated the difference in the progression of the apoptotic process between in the FAE of Peyer's patch and in the intestinal villi to discuss the relationship between epithelial apoptotic progression and the differentiation of M cells in the FAE.

MATERIALS AND METHODS

Animals: Five male Wistar rats aged 7 weeks (Japan SLC Inc., Japan) were maintained under conventional laboratory housing conditions. They were permitted free access to water and food (Lab MR Stock; Nosan Corp., Japan). The animal facility was maintained under conditions of a 12 hr light/dark cycle at 21 ± 1°C and 50–60% humidity. Clinical and pathological examinations in all animals confirmed no

sign of disorder. This experiment was approved by the Institutional Animal Care and Use Committee (Permission number: 17–04–05) and carried out according to the Kobe University Animal Experimentation Regulations.

Tissue preparation: Immediately after euthanasia with an intraperitoneal injection of overdose pentobarbital sodium (Dainippon Sumitomo Pharma Co., Ltd., Japan), intestinal walls including Peyer's patches were rapidly removed, sliced, and immersion-fixed in cold periodate-lysine-paraformaldehyde fixative (PLP) for 24 hr at 4°C. The tissue blocks were snap frozen in liquid nitrogen with reference to an embedding method described by Barthel and Raymond [2]. Sections of 4 μm in thickness were cut using a Coldtome HM505E (Carl Zeiss, Germany) and were placed on slide glasses precoated with 0.2% 3-aminopropyltriethoxysilane (Shin-Etsu Chemical., Japan).

Antisera and normal sera: The following antisera and normal sera were used for immunohistochemistry: anti cleaved caspase-3 rabbit IgG, anti cleaved caspase-9 rabbit IgG, anti Bcl-x rabbit IgG, horseradish peroxidase (HRP)-conjugated anti rabbit IgG goat IgG, anti DNase I rabbit IgG which was conjugated with HRP by a peroxidase labeling kit (Dojindo Lab., Japan), bovine normal serum, chicken normal serum and rabbit normal serum (prepared in our laboratory).

Immunohistochemistry: The detection of antigens was conducted using the indirect method of enzyme immunohistochemistry, except for the detection of DNase I. Briefly, after rinsing with 0.05% Tween-added phosphate buffered saline (pH 7.4, PBS), the sections were incubated or not in proteinase K (Sigma-Aldrich, U.S.A.) solution. After rinsing with PBS, the sections were followed by immersion in absolute methanol and 0.5% H_2O_2 for 30 min, respectively. Following blocking with 1% normal serum for 1 hr at room temperature (r.t.), the sections were reacted with primary antibody for 18 hr at 4°C. Then the sections were incubated with secondary antibody for 1 hr at r.t. After rinsing with PBS, the sections were incubated with 3,3'-diaminobenzidine (DAB) containing 0.03% H_2O_2, and were counterstained with methyl green. Control sections were incubated with non-immunized rabbit serum instead of primary antiserum. The combination of enzyme and antisera is described in Table 1.

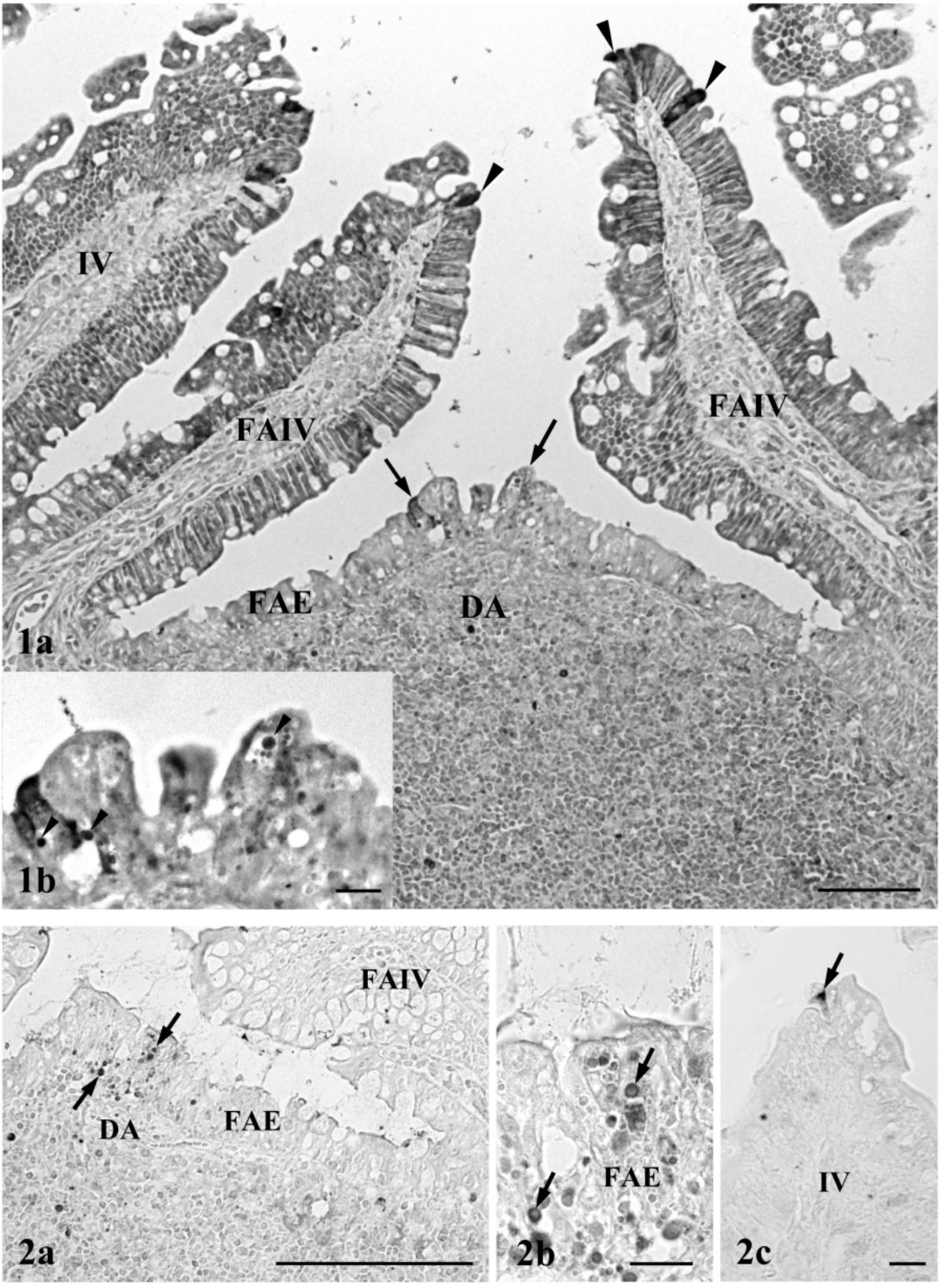
In situ nick-end labeling method: The bromodeoxyuridine triphosphate (BrdUTP) method was applied to detect the apoptotic epithelial cells in the Peyer's patches. Briefly, four sections were prepared (Sections 1, 2, 3, and 4). Sections 1 and 2 were used as positive controls for section 3. Section 4 was used as a negative control. After rinsing with 0.1% Tween-added PBS, the sections were pretreated with 1.5 μg/m*l* proteinase K solution (Sigma-Aldrich) for 10 min at 37°C. After treatment with both absolute methanol and 0.5% H_2O_2, sections 1 and 2 were incubated with 1.5 mg/m*l* DNase I solution (Boehringer Mannheim, Germany) for 10 min at 37°C. Sections 1 and 3 were incubated with terminal deoxynucleotidyl transferase (TdT) buffer (Roche Diagnostics, Switzerland) containing BrdUTP solution (0.1 nM BrdUTP in 4 m*l* distilled water; Sigma-Aldrich) for 2 hr at 37°C. All sections were then incubated with 1% normal bovine serum for 1 hr at r.t. and were treated with anti BrdU mouse monoclonal antibody (diluted at 1:100; Santa Cruz Biotechnol. Inc., U.S.A.) for 18 hr at 4°C. The sections were then reacted with anti mouse IgG rat IgG (diluted at 1:50; Jackson ImmunoRes. Lab., U.S.A.) for 1 hr at r.t. and treated with mouse peroxidase anti peroxidase complex (diluted at 1:50; Seikagaku Corp., Japan) for 1 hr at r.t. Finally, the sections were incubated with DAB and counterstained with methyl green.

RESULTS

Cleaved caspase-3: The cytoplasmic expression of cleaved caspase-3 in the microvillous columnar epithelial cells in the FAEs of the Peyer's patches was far weaker than that in the villous columnar epithelial cells in the

Table 1. Enzymes and antisera used in this immunohistochemistry

Antigen	Pretreatment	Normal serum	Primary antiserum	Secondary antiserum
Cleaved caspase-3	0.6 μg/m*l* proteinase K for 10 min at 37°C	Chicken (a gift from Tottori Univ., Japan) × 100	Anti cleaved caspase-3 rabbit IgG (Cell Signaling Technol., U.S.A.) × 200	HRP-conjugated anti rabbit IgG goat IgG (Chemicon Intern. Inc., U.S.A.) × 50
Cleaved caspase-9	0.6 μg/m*l* proteinase K for 5 or 10 min at 37°C	Chicken (a gift From Tottori Univ.) × 100	Anti cleaved caspase-9 rabbit IgG (Cell Signaling Technol.) × 50	HRP-conjugated anti rabbit IgG goat IgG (Chemicon Intern. Inc.) × 50
DNase I	–	Chicken (a gift from Tottori Univ.) × 100	HRP-conjugated anti DNase I rabbit IgG (Rockland Immunochem., U.S.A.) × 3200	–
Bcl-x	–	Bovine (Chemicon Intern. Inc.) × 100	Anti Bcl-x rabbit IgG (Transduction Lab., U.S.A.) × 400	HRP-conjugated anti rabbit IgG goat IgG (Chemicon Intern. Inc.) × 400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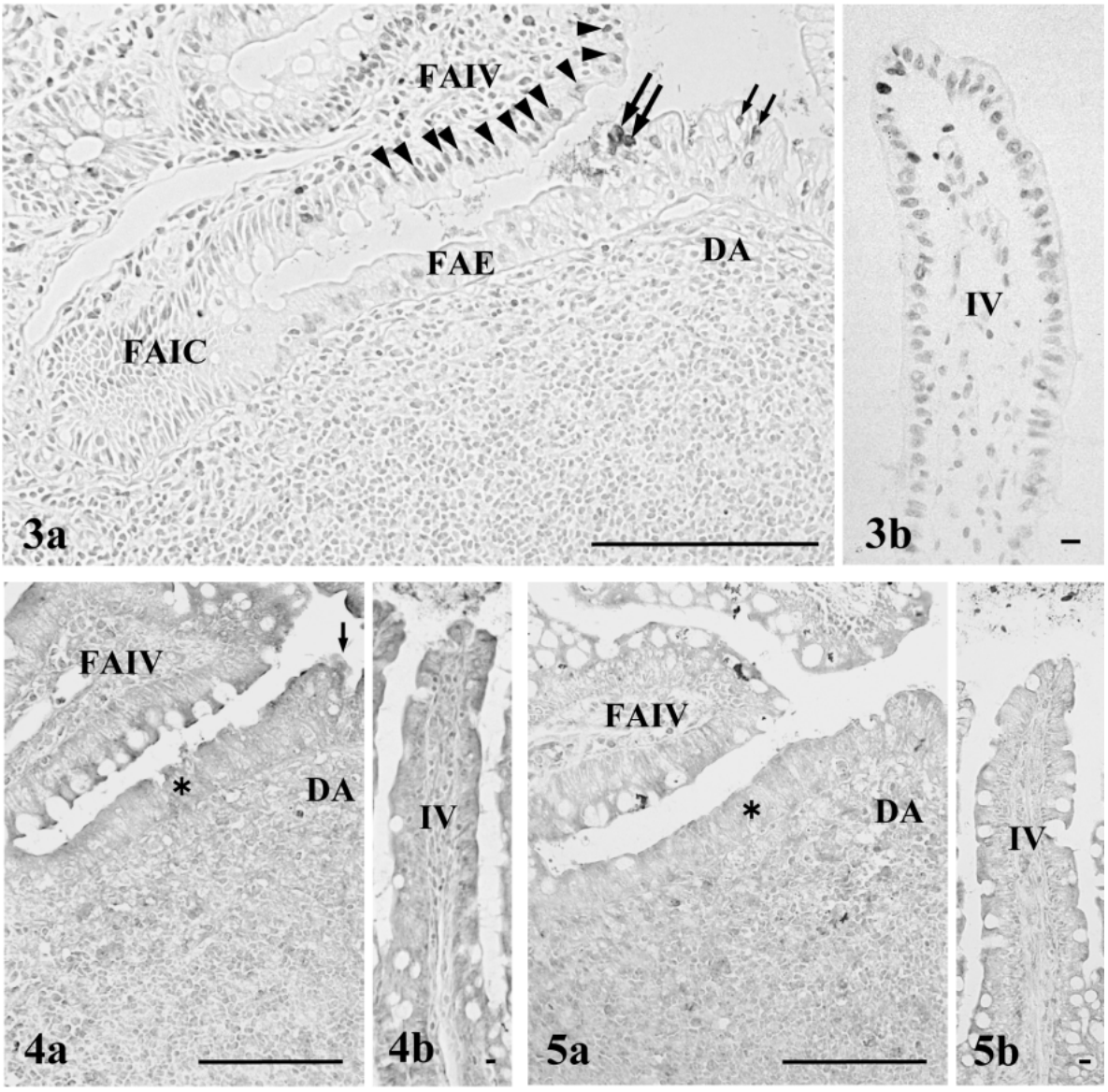

Fig. 1. Cleaved caspase-3 in a rat Peyer's patch. a) Cleaved caspase-3 expression is visible in the epithelial cells of the entire follicle-associated intestinal villus (FAIV) and the intestinal villus (IV), whereas cleaved caspase-3 expression is restricted in several epithelial cells in the apex of FAE (arrows). The strong expression is also visible at the tops of the intestinal villi (arrowheads). DA, dome area. Bar = 100 μm. b) Highly magnified micrograph of the top of FAE. Fragmented cytoplasmic droplets express strong cleaved caspase-3 positive (arrowheads). Bar = 10 μm.

Fig. 2. Cleaved caspase-9 in a rat Peyer's patch. a) Cleaved caspase-9 expression is almost negative in the follicle-associated intestinal villus (FAIV) and the FAE except for the fragmented cytoplasms (arrows). DA, dome area. Bar = 100 μm. b) Highly magnified micrograph of the top of FAE. Fragmented cytoplasmic droplets express strong cleaved caspase-9 positive (arrows). Bar = 10 μm. c) An exfoliating epithelial cell (arrow) exhibits cleaved caspase-9 positive in an intestinal villus (IV). Bar = 10 μm.

Fig. 3. Nuclear DNA fragmentation in a rat Peyer's patch. a) The nuclear DNA fragmentation is visible only in some epithelial cells (small arrows) and in exfoliating epithelial cells (large arrows) at the top of the FAE, but it is seen in the upper half of follicle-associated intestinal villus (FAIV) (arrowheads). DA, dome area; FAIC, follicle-associated intestinal crypt. Bar = 100 μm. b) The nuclear DNA fragmentation in the epithelial cell is gradually increased toward the apex of intestinal villus (IV). Bar = 10 μm.

Fig. 4. DNase I in a rat Peyer's patch. a) The intensity of the DNase I expression is weaker in the FAE (*) than in the FAIV and somewhat strong at the top of the FAE (arrow). DA, dome area; FAIV, follicle-associated intestinal villus. Bar = 100 μm. b) The intensity of the DNase I expression is somewhat stronger in the apical regions than in the base of the intestinal villi (IV). Bar = 10 μm.

Fig. 5. Bcl-x in a rat Peyer's patch. a) The intensity of the Bcl-x expression is very weak and is restricted in the apical cytoplasms of epithelial cells in the FAE (*). DA, dome area; FAIV, follicle-associated intestinal villus. Bar = 100 μm. b) In intestinal villus (IV), Bcl-x expression is somewhat stronger in the apical to middle cytoplasms than in the basal cytoplasms of the epithelial cells and decrease toward the apical regions of IV. Bar = 10 μm.

neighboring intestinal villi (FAIVs) (Fig. 1a). However, a few epithelial cells possessed highly concentrated cleaved caspase-3 in the apex of FAE, as well as exfoliating or exfoliated epithelial cells. In the immuno-positive epithelial cells, fragmented cytoplasms were strongly stained (Fig. 1b).

Almost all of the villous epithelial cells were strongly stained with anti cleaved caspase-3. The positive intensity of the cleaved caspase-3 in the apical regions was somewhat stronger than in the basal region of the intestinal villi, including FAIVs (Fig. 1a).

Cleaved caspase-9: Cleaved caspase-9 was never detected in the cytoplasms of epithelial cells in either the FAEs of the Peyer's patches or the intestinal villi, except in their apices (Fig. 2a). In the apices of the FAEs and the intestinal villi, cleaved caspase-9 was strongly concentrated in the fragmented cytoplasms of epithelial cells (Fig. 2b) and also in the exfoliating or exfoliated epithelial cells exclusively (Fig. 2c).

Nuclear DNA fragmentation: Epithelial nuclear DNA fragmentation was restricted in several epithelial cells at the apices of the FAEs in Peyer's patches (Fig. 3a), whereas the epithelial DNA-fragmentation was detected from the basal or middle to apical regions in the intestinal villi (Fig. 3b). The exfoliating and exfoliated epithelial cells also expressed the strong nuclear DNA fragmentation (Fig. 3a).

DNase I: DNase I was found at the apical cytoplasms of the epithelial cells in the FAE, whereas DNase I was expressed throughout the cytoplasms in the intestinal villi. The intensity of the DNase I expression was weaker in the FAEs than in the intestinal villi (Fig. 4a, b), and somewhat strong at the top of FAE (Fig. 4a). In the intestinal villi, the intensity of the DNase I expression was also somewhat stronger in the apical regions than in the base of the intestinal villi (Fig. 4b).

Bcl-x: Bcl-x expression was very weak and almost constant in the FAE, and restricted in the apical cytoplasms of the epithelial cells in the FAE (Fig. 5a). In the intestinal villi, Bcl-x was slightly expressed in the apical to middle cytoplasms of the epithelial cells and decreased toward the apical regions (Fig. 5b).

DISCUSSION

Differentiation of a cell is defined as a primitive cell that can produce two daughter cells with different lineages within one cell cycle transit [21]. Namely, differentiation of a cell is also defined as a change in the genetic expression patterns, a reprogramming of the genome or a heritable phenotypic change in daughter cells [17]. Cellular maturation is defined as a time course of cellular change after a differentiation event and is a quantitative change [17]. Recently, it has been shown that some caspases are involved in maturation or determination of the differentiation of various animal cells. In myoblast cultures which are derived from caspase-3 null mouse, a severe lack of myotube formation was evident relative to wild-type cells. Therefore, caspase-3 activity is necessary for maturation during the early stages of skeletal myogenesis [12]. Caspase-3 activation is also required for human erythroblast maturation after the basophilic stage *in vitro* [30]. An activation of both caspase-3 and caspase-9 is involved in the differentiation into macrophages after stimulation of macrophage-colony stimulating factor (M-CSF) in human peripheral blood monocytes [23]. Caspase-14 is involved in the maturation of keratinocyte, because caspase-14, which accumulates in the granular layer, is activated during the formation of stratum corneum *in vitro*, and cleaved caspase-14 is present in normal human epidermis *in situ* [11]. Thus, some caspases function in cell signaling events including apoptosis, cellular maturation and terminal differentiation [26]. In addition to this, Fas, Fas-L and Bax are involved in the induction of epithelial apoptosis in the rat small intestine [1]. In the rat small intestine, DNase I-specific mRNA is expressed in the epithelial cells at the lower third of the intestinal villi and DNase I protein was found in the epithelial cells at the middle and upper thirds of the intestinal villi [29]. In the present study, the cleaved caspase-3 rather than caspase-9 was expressed at almost all epithelial cells in the entire intestinal villi. However, the epithelial cells gradually store functional proteins, such as sucrase-isomaltase and maltase-glucoamylase, during epithelial migration along villous axes in rat intestines [10]. These findings suggest that epithelial apoptosis also progressed with the epithelial maturation in the rat small intestinal villi.

In rat Peyer's patches, apoptotic epithelial cells, such as chromatin-condensed and cytoplasm-shrunk cells, are frequently observed in the apical region but are scarcely observed in the basal to middle region of the FAE [8]. In rat, pig and mouse Peyer's patches and chicken cecal tonsils, nuclear DNA fragmentation of the epithelial cells was localized at the apices of the FAEs [9, 18, 22, 25]. In the present study, cleaved caspase-3 and -9 expression was restricted at the top, but never at the base and middle of FAE. These findings show that caspase expression and nuclear DNA fragmentation occur much later in the FAE than in the intestinal villi. This suggests that the epithelial maturation in the FAE is also late compared with that in the intestinal villi.

In human and rabbit Peyer's patches and chicken cecal tonsils, M cells were predominantly distributed at the lateral sides of the dome [13, 15, 16]. In rat Peyer's patches, M cells were also predominantly located in the lower half and decreased in number toward the apices of FAEs [8]. A detailed ultrastructural investigation suggests that typical M cells transit from immature microvillous epithelial cells and then into mature microvillous epithelial cells in the base to middle of the FAE of rat Peyer's patches [19]. In this study, the epithelial cells in the base to middle of the FAE did not express caspases and DNase I. This finding indicates that the differentiation of M cells from immature microvillous epithelial cells and the redifferentiation of M cells to mature microvillous epithelial cells are accompanied with no

apoptotic process in the FAEs as well as in the intestinal crypts.

Recently, intestinal villous M cells, which are special M cells, have been found in the intestinal villi of mice. Villous M cells accompanied by intraepithelial pockets were detected in the villous epithelium by lectin histochemistry by means of UEA-1, and take up some species of bacteria [14]. On the other hand, the epithelial cells in rat intestinal villi which take up both the luminal antigen and its specific antibody are epithelial cells with a late apoptotic stage themselves, unlike intestinal villous M cells [27, 28]. In the chicken cecal tonsil, no M cells exhibited any apoptosis signs [25]. In the present study, the epithelial cells expressed apoptosis in the entire intestinal villus, whereas the apoptosis of epithelial cells was restricted at the apex in the FAE. These findings might also suggest that the possibility of epithelial differentiation might be remained in the FAE, unlike in the intestinal villi.

ACKNOWLEDGMENTS. This work was financially supported in part by Grants-in-Aid for Scientific Research (nos. 16580239 and 19580339) from the Japan Society for the Promotion of Science.

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