FULL PAPER *Anatomy*

Alteration in the Apoptosis Process of Rat Esophageal Epithelium with Hyperproliferation of Indigenous Bacteria under a Physiological Condition

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ABSTRACT. The apoptosis process in rat esophageal epithelium was investigated using enzyme-immunohistochemistry and transmission electron microscopy. As a result, Fas and Fas-L were expressed in the epithelial cell membrane and cytoplasm from the stratum spinosum (SS) to the stratum granulosum (SG). No TNF-R1 show immunopositivity in the cell membranes. TNF-α and caspase-8 were not observed in any layer. Caspase-10, cleaved caspase-3, XIAP and DNase-1 were found in the epithelial cytoplasm from the SS to the SG, whereas Bid, Apaf-1 and cleaved caspase-9 were detected only in the SG. Cytochrome c was observed as cytoplasmic granular positivity from the stratum basale (SB) and altered into homogeneous immunopositivity in the SG. Bcl-2 and Bcl-X immunopositivity was detected in cytoplasm from the SB to the SG. Immunoreactions of Bak in the cytoplasm and Bax beneath the cell membrane were observed from the upper portion of the SS with increasing intensity toward the SG. In the sites with the hyperproliferation of indigenous bacteria, TNF-R1, TNF-α and caspase-8 were detected in the SG and the immunopositive intensities of Bid, Apaf-1 and cleaved caspase-9 were altered to be strong. Prominently swollen cells and decreased mitochondria were ultrastructurally confirmed in the uppermost layers of stratum corneum. These findings suggest that the Fas–Fas-L-interaction initially induces apoptosis through a mitochondria-independent pathway and secondarily through a mitochondria-dependent pathway, leading to eventual epithelial cell death in the rat esophageal epithelium. The bacterial stimuli probably enhance the mitochondria-dependent pathway through the TNF-R1–TNF-α interaction.

KEY WORDS: apoptosis, esophageal epithelium, immunohistochemistry, indigenous bacteria, rat.

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The esophageal epithelium of humans corresponds to a non-keratinized stratified squamous epithelium [25, 50], but in rodents, to a keratinized squamous epithelium [23]. In the normal esophageal epithelium, dynamic homeostasis is maintained by both epithelial generation and epithelial apoptosis. The generation of epithelial cells in the esophageal epithelium is executed by a proliferative unit, which is composed of both stem cells and transient amplifying cells [27, 35]. The proliferative unit exists in the stratum spinosum (SS) as well as in the stratum basale (SB) [5, 40, 47]. The newly generated epithelial cells migrate upward, meanwhile gradually losing their cytoplasmic organelles and desmosomes, elongating and becoming more folded in shape; puncturing and desquamation finally take place at the surface [23, 50]. These morphological changes, which are generally called "maturation" [23] or "terminal differentiation" [2, 19], are also deeply associated with apoptosis [19]. In the normal human esophagus, epithelial apoptosis is initiated by Fas and Fas-L interactions [2]. Further, under a physiological condition, the up-regulation of apoptosis in epithelial cells is considered to be conducted in the upper layers of the esophageal epithelium [22, 39], whereas the down-regulation of apoptosis by Bcl-2 and Bcl-X occurs throughout the esophageal epithelium except in the stratum corneum (SC) [19, 42]. DNA fragmentation, which designates apoptotic cell death, is found in the uppermost epithelial layers of the normal human esophagus [2, 19]. The above knowledge suggests that the apoptotic process occurs in the esophageal epithelium under physiological conditions. But the detailed histophysiological mechanism of the induction and progression of apoptosis has never been clarified.

The esophageal epithelium is occasionally affected by biliopancreatic or gastroesophageal reflux, that causes the hyperproliferation of epithelial cells, and that in a chronic situation may break the balance of epithelial homeostasis and stimulate esophageal carcinogenesis [16, 20, 31, 34].

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Additionally, various species of indigenous bacteria residing in the entire alimentary tract influence metabolic activity and play vital physiological roles [4, 26]. Over 200 species of indigenous bacteria reside in the oral cavity. The saliva contains 10^6/m*l* of transient bacteria shed from oral surfaces such as the tongue and cheek [13]. *Lactobacilli* prefer to reside on the stratified squamous epithelium in the upper digestive tract of piglets [32], horses [49], mice [38] and rats [46]. The human distal esophagus also possesses various bacterial species on its surface [33]. Thus, the esophageal stratified squamous epithelium is probably stimulated by the flowing or residing bacterial populations. However, the influences of bacterial stimuli on the apoptotic process of esophageal epithelial cells have never been clarified. This study makes a detailed histophysiological clarification of the fundamental induction and progression of apoptosis and the influence of the hyperproliferation of indigenous bacteria on the apoptotic process of rat esophageal epithelial cells under a physiological condition.

MATERIALS AND METHODS

Experimental animals: Eleven 7-week-old male Wistar rats that were confirmed as having no signs of clinical and pathological disorders (Japan SLC, Hamamatsu, Shizuoka, Japan), were used. They were maintained under conventional laboratory housing conditions of a 12-hr light/dark cycle at 23 ± 1°C and 50–60% humidity. All animals were permitted free access to water and commercial foods (Lab MR Stock; Nosan Corporation, Yokohama, Kanagawa, Japan). This experiment was approved by the Institutional Animal Care and Use Committee of Kobe University (Permission number: 19-05-07) and was completed in accordance with Kobe University Animal Experimentation Regulations.

Chemical reagents: Pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan) was used as an anesthetic agent. Proteinase-K (Sigma-Aldrich, St. Louis, MO, U.S.A.) was applied as a pre-treatment agent, and normal wild snake (Japanese four-lined snake; *Elaphe quadrivirgata*) serum prepared by our laboratory was applied as a blocking agent in the immunohistochemistry.

The following primary antisera were used: Anti Fas goat IgG (R&D systems, Minneapolis, MN, U.S.A.), anti TNF-R1 rabbit IgG (EMD Chemicals, Gibbstown, NJ, U.S.A.), anti Fas-L goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti TNF-α rabbit IgG (Abbiotec, San Diego, CA, U.S.A.), anti caspase-10 goat IgG (Santa Cruz Biotechnology), anti caspase-8 goat IgG (Santa Cruz Biotechnology), anti cleaved caspase-3 rabbit IgG (Cell Signaling Technology, Danvers, MA, U.S.A.), Horseradish peroxidase (HRP)-conjugated anti DNase-1 rabbit IgG (Santa Cruz Biotechnology; HRP was conjugated in our laboratory), anti XIAP rabbit IgG (Imgenex, San Diego, CA, U.S.A.), anti Bid goat IgG (Santa Cruz Biotechnology), anti cytochrome c goat IgG (Santa Cruz Biotechnology), anti Apaf-1 goat IgG (Santa Cruz Biotechnology), anti cleaved caspase-9 rabbit IgG (Novus Biologicals, Littleton, CO, U.S.A.), anti ssDNA rabbit IgG (Immuno-Biological Laboratories, Fujioka, Gunma, Japan), anti Bcl-2 goat IgG (Santa Cruz Biotechnology), anti Bcl-X mouse IgG_{2a} (Chemicon International, Billerica, MA, U.S.A.), anti Bak rabbit IgG (BD Biosciences, San Jose, CA, U.S.A.), and anti Bax mouse IgG (Santa Cruz Biotechnology). The following secondary anti serums were used: HRP-conjugated mouse anti goat IgG (Chemicon International), HRP-conjugated goat anti rabbit IgG F $(ab')_2$ (Chemicon International) and HRP-conjugated rat anti mouse IgG2b (γ2b chain specific) (Beckman Coulter, Fullerton, CA, U.S.A.). Control sections were incubated with normal goat IgG (PeproTech, Rocky Hill, NJ, U.S.A.) or normal rabbit IgG (BioVision, Mountain View, CA, U.S.A.) as the primary antiserum.

Tissue preparation for light microscopic immunohistochemistry: Histological sampling, tissue preparation and immunohistochemistry were performed in 8 animals as described in our previous immunohistochemical study [43]. Briefly, after deep anesthesia by intraperitoneal pentobarbital sodium injection and perfusion fixation with 0.1 M phosphate buffered 4% paraformaldehyde fixative, esophageal tissue blocks were obtained and embedded in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek Japan, Koto, Tokyo, Japan). Subsequently, the embedded tissues were cut into 4 μm-thick sections.

Immunohistochemistry was performed as follows. After the elimination of intrinsic peroxidase activity with immersion in absolute methanol and 0.5% H_2O_2 each for 30 min, the sections were incubated with wild snake serum for 1 hr at room temperature (r.t.). Then the sections were reacted with each primary antibody for 18 hr at 4°C, followed by incubation with HRP-conjugated secondary antibodies for 1 hr at r.t. except for in DNase-1 detection. Finally, the sections were colorized with 3, 3′-diaminobenzidine containing 0.03% H_2O_2 and weakly counterstained with methyl green. The negative control sections were incubated with 0.05% of Tween-added phosphate buffered saline (pH 7.4), or non-immunized IgGs instead of primary antisera.

Transmission electron microscopy: The esophagus was extracted from 3 rats after anesthesia and immediately sliced and immersed in 2.5% glutaraldehyde 2.0% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 24 hr at 4°C. Post fixation was performed with 1.0% OsO_4 in PB for 2 hr at r.t., and small specimens were dehydrated and embedded in a Quetol 812-mixture. Afterward, ultrathin sections were cut using an ultramicrotome (Sorvall MT-1, DuPont, Newtown, CT, U.S.A.). The sections were stained with both uranyl acetate and lead citrate and observed under transmission electron microscope (Hitachi H-7000, Kokubunji, Tokyo, Japan) at an accelerating voltage of 75 kV.

Observation: After direct or indirect enzyme immunohistochemistry performed for each apoptosis-related protein, the intensity and the areas of positive expression in all sections were compared between the epithelium without hyperproliferation of indigenous bacteria (No-IB-Ep) and the epithelium with hyperproliferation of indigenous bacteria, which resided on and invaded into the SC (Hyper-IB-Ep).

In each immunostaining with a different primary serum, the intensity of immunoreaction was evaluated with regard to the results of negative control sections. Briefly, an intensity that was equal to that in the negative control section was judged as "negative" and the highest intensity was categorized as "strong". The intensities between negative and strong were defined as "weak" and "moderate".

The immunohistochemical results were presented in figures. In cases of the similar results in No-IB-Ep and Hyper-IB-EP, however, the figures in the No-IB-Ep were presented as representative figures.

RESULTS

Fas: In the SB, the basal portion of the SS and the SC, no epithelial cells expressed immunopositivity for Fas. In the rest of the SS and the stratum granulosum (SG), Fas was immunopositive in the cell membrane and cytoplasm of epithelial cells (Fig. 1a). There was no difference in the positive reaction and the intensity of the No-IB-Ep and the Hyper-IB-Ep.

Fas-L: In both the No-IB-Ep and the Hyper-IB-Ep, epithelial cells from the SB to the middle portion of the SS and in the SC showed no immunopositive reactions. In the apical portion of the SS and the SG, a strongly immunopositive reaction was found in the cell membrane and cytoplasm of epithelial cells of both the No-IB-Ep and the Hyper-IB-Ep (Fig. 1b).

TNF-R1: In the No-IB-Ep and the Hyper-IB-Ep, various sizes of granular-shaped and strongly immunopositive expressions were scattered in the epithelial cytoplasms of the SB and the basal portion of the SS. From the middle portion of the SS to the SG in the No-IB-Ep, the immunopositivity gradually changed into a weak and homogeneous cytoplasmic reaction. No immunopositivity was detected in the SC (Fig. 1c). In the Hyper-IB-Ep, strongly immunopositive expression in the cell membrane and cytoplasm was detected in the SG and in the basal portion of the SC. No immunopositivity was detected in the rest of the SC (Fig. 2a).

TNF-α: In the No-IB-Ep, no immunopositive expression was detected in any strata of the esophageal epithelium (Fig. 1d). In the Hyper-IB-Ep, however, moderate cytoplasmic immunopositivity was found in the SG (Fig. 2b).

Caspase-10: Immunopositive expressions in both the No-IB-Ep and the Hyper-IB-Ep, were similar. No immunopositive reaction was detected from the SB to the middle portion of the SS or in the SC. From the apical portion of the SS to the SG, moderately immunopositive expression of caspase-10 was found in the epithelial cytoplasm, with the intensity gradually increasing toward the SG (Fig. 1e).

Caspase-8: No immunopositive expression was detected in any strata of the No-IB-Ep (Fig. 1f). In Hyper-IB-Ep, however, a weakly immunopositive reaction was found in the epithelial cytoplasm of the SG (Fig. 2c).

Cleaved caspase-3: No positive expression was observed in the SB, the basal portion of the SS or the SC of the esophageal epithelium. From the apical portion of the SS to the SG, moderately immunopositivity for cleaved capase-3 was frequently found in the cytoplasm, with the intensity gradually increasing toward the SG (Fig. 3a). No difference in intensity between the No-IB-Ep and the Hyper-IB-Ep was observed.

XIAP: In the No-IB-Ep and Hyper-IB-Ep, the SB, the basal portion of the SS and the SC were negative for XIAP. Likewise in both, homogeneous positive cytoplasmic expression in the middle portion of the SS began as a weak immunopositivity and increased toward the SG (Fig. 3b).

DNase-1: Similar immunopositive reactions were found in the No-IB-Ep and the Hyper-IB-Ep. Namely, no expression was detected from the SB up to the middle portion of the SS and in the SC. From the apical portion of the SS to the SG, homogeneous and moderate immunopositivity was found in the epithelial cytoplasm. Its intensity increased toward the SG (Fig. 3c).

Bid: In both the No-IB-Ep and the Hyper-IB-Ep, no immunopositive reaction was detected in the strata except for the SG. In the SG, Bid was detected as homogeneous and weak immunopositivity in the epithelial cytoplasms of the No-IB-Ep (Fig. 3d). In Hyper-IB-Ep, the positive reaction was higher in the SG than that in the No-IB-Ep (Fig. 2d).

Cytochrome c: From the SB to the apical portion of the SS, large, granular-shaped and strongly-positive cytochrome c immunopositivity was detected in the epithelial cytoplasm. The granular expression in the apical portion of the SS gradually altered into homogeneous immunopositivity toward the SG. The SC was negative for cytochrome c (Fig. 3e). The immunopositivity of the SG was higher in the Hyper-IB-Ep than in the No-IB-Ep (Figs. 2e).

Apaf-1: In the Hyper-IB-Ep and the No-IB-Ep, no immunopositive reaction was detected in the strata except for the SG. Weak and homogeneous immunopositivity of the cytoplasm was detected in the SG (Fig. 3f), where the intensity was higher in the Hyper-IB-Ep than in the No-IB-Ep (Fig. 2f).

Cleaved caspase-9: In the strata except for the SG, no immunopositive reaction was detected in either area of epithelium. In the SG, moderate cleaved caspase-9 immunopositivity was detected in the epithelial cytoplasm of both No-IB-Ep (Fig. 4a) and the Hyper-IB-Ep. The intensity was higher in the Hyper-IB-Ep than in the No-IB-Ep (Fig. 2g).

Bcl-2: Homogenous and moderate immunopositivity was found in the epithelial cytoplasm of all strata except for the SC. The intensities of the No-IB-Ep and Hyper-IB-Ep were similar. No immunopositivity was detected in the SC (Fig. 4b).

Bcl-X: Homogeneous and moderately positive expression was found in the epithelial cytoplasm of all the strata except the SC. No differences were detected between the No-IB-Ep and the Hyper-IB-Ep (Fig. 4c).

Bak: From the SB to the middle portion of the SS and in the SC, no positive reaction was observed. Weakly immunopositive expression was detected in the cytoplasm of the apical portion of the SS and gradually changed into

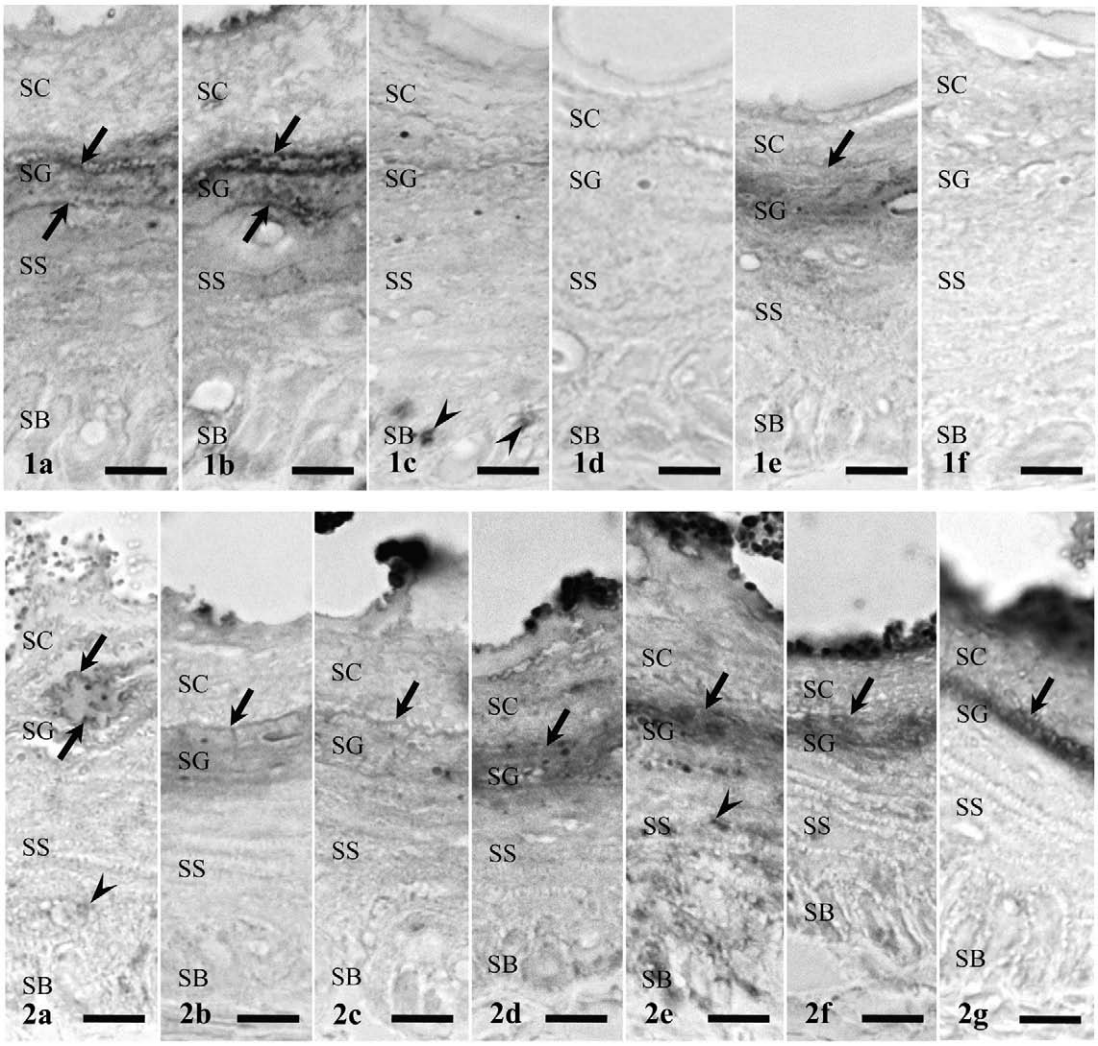

Fig. 1. Immunopositivities of receptors, ligands and initiator caspases in No-IB-Ep. a) A clear membranous immunopositivity for Fas (arrows) is visible in the upper layers of the SS and the SG. b) Fas-L immunopositivity is also noticeable in cell membranes (arrows) in the same places where Fas is detected. c) TNF-R1 is visible as granular immunopositivity (arrowheads) in the SB and the basal portion of the SS. d) TNF-α is not seen in any layer of the epithelium. e) Homogeneous cytoplasmic immunopositivity for caspase-10 is noticeable in the apical portion of the SS and SG (arrow). f) Caspase-8 is not seen in any layer of the epithelium. Bar=5 μm.

Fig. 2. Immunopositivities of apoptosis-related proteins in Hyper-IB-Ep. Numerous bacteria are seen on the epithelial surfaces. a) TNF-R1 is visible as a granular-shaped cytoplasmic immunopositive expression (arrowhead) in the SB and the basal portion of the SS, whereas clear cell membranous and cytoplasmic immunoreactivity (arrows) is also visible in the SG and the basal portion of the SC. b) TNF-α is located as a diffuse cytoplasmic immunopositivity (arrow) in the SG. c) Caspase-8 is seen as a weak and diffuse cytoplasmic immunopositivity (arrow) in the SG. d) Bid shows stronger immunoreactivity in the apical portion of the SS and the SG (arrow) than in the No-IB-Ep (Fig. 3d). e) Cytochrome c is seen as a granular-shaped immunopositivity (arrowhead) in the SB and the SS, whereas a homogeneously stronger cytoplasmic immunopositivity (arrow) is visible compared with that of No-IB-Ep (Fig. 3e). f) Apaf-1 is visible as a stronger cytoplasmic immunopositivity in the SG than in the SG (arrow) of No-IB-Ep (Fig. 3f). g) Stronger cytoplasmic immunopositive expression is noticeable for cleaved caspse-9 in the SG and the SC (arrow) compared with those of No-IB-Ep (fig. 4a). Bar=5 μm.

moderate immunoreaction in the SG. No differences were detected between the No-IB-Ep and the Hyper-IB-Ep (Fig. 4d).

Bax: Similar patterns of immunoreaction were found in the No-IB-Ep and the Hyper-IB-Ep. Namely, no positive expression was detected from the SB to the middle portion of the SS and in the SC. Homogenous, weakly positive cytoplasmic expression was detected in the apical portion of the SS, and strongly positive expression was detected around the epithelial cell membranes in the SG (Fig. 4e).

ssDNA: In the SB, the SS and the upper layers of SC, no expression was detected. In the outermost layers of the SG

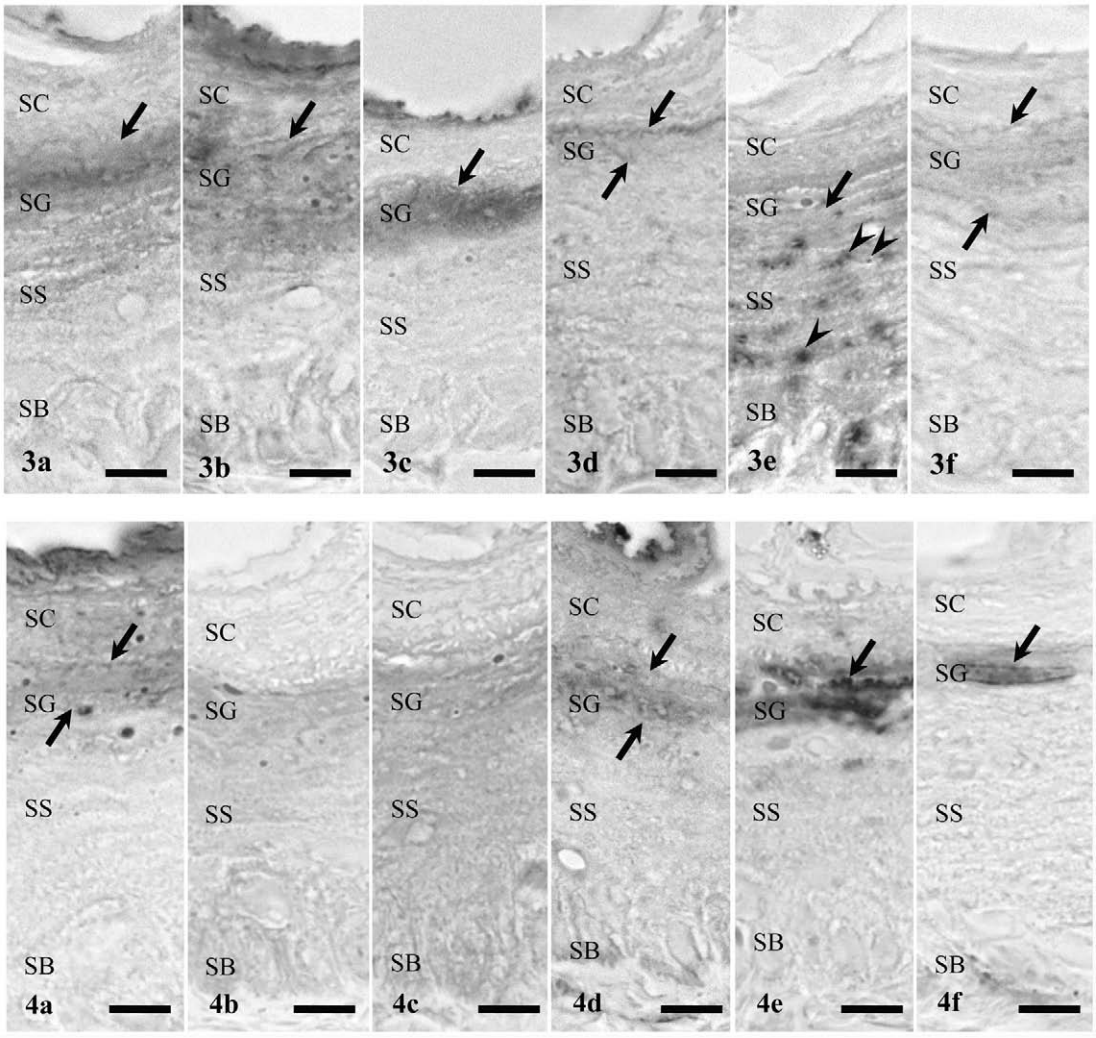

Fig. 3. Immunopositivities of mitochondrial-independent and -dependent apoptosis-related proteins in the No-IB-Ep. a) Homogeneous cytoplasmic immunoreactivity for cleaved caspase-3 is visible from the apical portion of the SS to the SG (arrow). b) XIAP is seen as a homogeneous and weak cytoplasmic immunopositivity from the basal portion of the SS to the SC (arrow). The strongest immunoreaction is visible in the apical portion of the SS and the SG. c) A moderate and diffuse immunopositivity of DNase-1 is seen in the epithelial cytoplasm of the apical portion of the SS and the SG (arrow). d) Bid is visible as a weak cytoplasmic immunopositivity in the SG (arrows). e) Cytochrome c is visible as a granular-shaped strong cytoplasmic immunoreaction (arrowheads) from the SB to the apical portion of the SS. In the SG, the immunoreaction is partially changed into weak and homogeneous cytoplasmic expression (arrow). f) Apaf-1 is visible as a weak immunopositive cytoplasmic immunoreaction (arrows) in the SG. Bar=5 μm.

Fig. 4. Immunopositivities of mitochondrial-dependent apoptosis-related proteins, regulator proteins and fragmented DNA in No-IB-Ep. a) Cleaved caspase-9 shows a moderate cytoplasmic immunopositivity in the SG (arrows) and a weak immunoreaction in the SC. b) Bcl-2 is visible as a moderate and homogeneous cytoplasmic immunopositivity from the SB to the SG. c) Bcl-X is visible as a moderately and homogeneous cytoplasmic immunopositivity from the SB to the SG. d) Bak is located as a moderate cytoplasmic immunopositivity in the SG (arrows). e) Bax is visible as a strong cytoplasmic immunoreaction in the apical portion of the SS and SG (arrow). f) ssDNA, indicating DNA fragmentation (arrow), is visible in the apical portion of the SG. Bar=5 μm.

and the basal layers of the SC, nuclear immunopositivity was rarely found in either the No-IB-Ep or Hyper-IB-Ep (Fig. 4f).

The immunohistochemical results of all apoptosis-related proteins were the same in all 8 rats. The immunohistochemical results are summarized in Fig. 5.

Ultrastructural differences in the No-IB-Ep and the Hyper-IB-Ep: In the No-IB-Ep, a few mitochondria and residuals of the organelles were found in the epithelial cells of the basal portion of the SC, but no organelles were observed in the other portions in the SC. Bacteria were seldom found in the intercellular spaces in the SC (Fig. 6a).

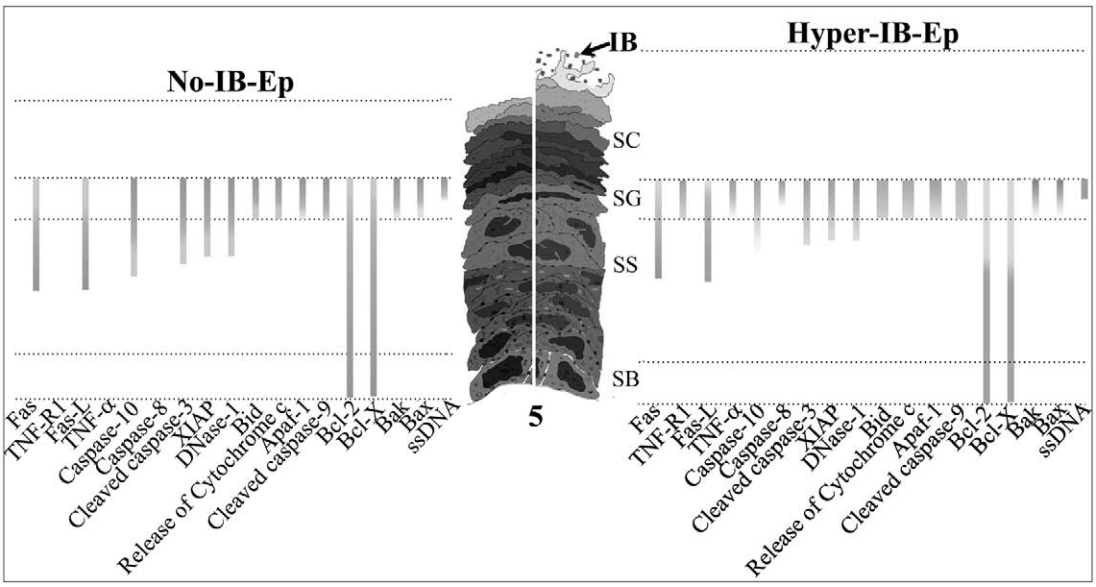

Fig. 5. Schematic summary of the expression of apoptosis-related proteins in No-IB-Ep and Hyper-IB-Ep. Each vertical column indicates the immunopositive intensity of the apoptosis-related protein. IB, indigenous bacteria.

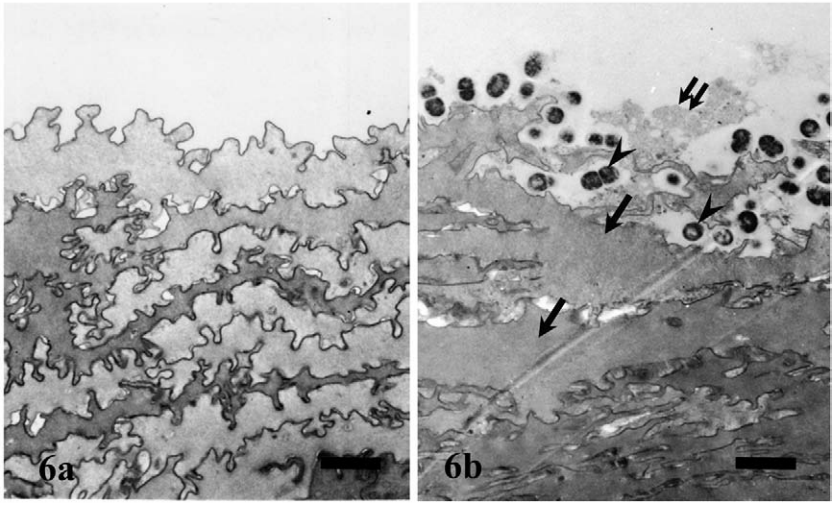

Fig. 6. Ultrastructures of epithelial cells in the apical portion of the SC of No-IB-Ep and Hyper-IB-Ep. a) The uppermost epithelial cells are slightly swollen and possess no cell organelles in No-IB-Ep. b) Numerous indigenous bacteria (arrowheads) have invaded into the enlarged intercellular spaces in the SC of Hyper-IB-Ep. The outermost epithelial cells are prominently swollen (arrows) and punctured (double arrow). Bar=1 μm.

In the Hyper-IB-Ep, however, the mitochondria in the SG was reduced in number comparing with those in the No-IB-Ep. The bacteria invaded into the superficial intercellular spaces which prominently enlarged. In addition, the outermost epithelial cells were also swollen comparing with those in the No-IB-Ep (Fig. 6a, b). No residues of organelles were visible in the entire SC.

DISCUSSION

The TNF-R superfamily comprises the so-called death receptors, such as Fas and TNF-R1 [1, 12, 24, 48], whose cytoplasmic regions are essential for fundamental apoptosis-induction processes [18]. In the present study, Fas and Fas-L were detected in the esophageal epithelium in both the No-IB-Ep and the Hyper-IB-Ep. In addition, a granular positivity for TNF-R1, which is probably an inactive form,

and no TNF-α was detected in the esophageal epithelium of No-IB-Ep. These findings confirm the hypothesis of Bennett *et al*. [2] that the Fas–Fas-L interaction plays a key role in the induction of apoptosis in esophageal epithelial cells under physiological conditions.

After the interaction of Fas–Fas-L, the FADD, which is responsible for receiving signals from death receptors [17, 30], generally activates caspase-8 or caspase-10 as the initiatory caspase [21, 44]. In the present study, caspase-10 was detected in the upper epithelial cells from the apical portion of the SS in both the No-IB-Ep and the Hyper-IB-Ep while caspase-8 was absent from the No-IB-Ep. These findings suggest that caspase-10 acts as the dominant initiatory caspase of the epithelial apoptotic process in the rat esophagus under a physiological condition.

Activated caspase-10 leads to further progression of the apoptotic process through a mitochondrial-independent or -dependent apoptotic pathway [29]. These two pathways result in the activation of caspase-3 which may lead to further cytoplasmic and nuclear events in Fas-mediated apoptosis [14, 51]. The cleaved caspase-3 further activates DNase (CAD), which initiates the degradation of DNA after entering the nucleus [9]. However, XIAP can inhibit the apoptosis process by binding with caspase-3, caspase-7 or caspase-9 [8]. In the present study, cleaved caspase-3, XIAP and DNase-1 were detected in the epithelial cells from the apical portion of the SS to the SG in both the No-IB-Ep and Hyper-IB-Ep. These findings suggest that the further progression of apoptosis might be suppressed by XIAP, in spite of the activation of caspase-3 in the apical portion of the SS through the mitochondria-independent pathway, in the rat esophageal epithelium of both No-IB-Ep and Hyper-IB-Ep.

Bax and Bak are the principal up-regulatory proteins of apoptosis, which oligomerize together to cause rupture of the mitochondrial membranes and release of cytochrome c [7, 10, 28, 41]. Conversely, Bcl-2 and Bcl-X act as the dominant down-regulatory proteins of the mitochondrial-dependent pathway of apoptosis [3, 15]. In the present study, both Bcl-2 and Bcl-X were found in all strata except for the SC, and both Bax and Bak showed their highest positive intensity in the SG. Therefore, Bcl-2 and Bcl-X might block the completion of the apoptotic process together with XIAP in the apical portion of the SS until the appearance of Bax and Bak in the rat esophageal epithelium.

Bid is a pro-apoptotic member of the Bcl-2 protein family which undergoes a truncation in structure and directly leads to conformational changes of Bax and Bak after translocating to the outer membranes of mitochondria; these changes may lead to release of cytochrome c from mitochondria [10, 45]. In the presence of dATP/ATP, cytochrome c triggers the assembly of a protein complex called "apoptosome", which causes the activation of caspase-3 [6, 52]. Activated caspase-3 may eventually cause DNA fragmentation with DNase-1 [14], which could be efficiently confirmed by the detection of ssDNA [11]. In the present study, Bid, Apaf-1 and cleaved caspase-9 were detected in the SG of both the No-IB-Ep and Hyper-IB-Ep.

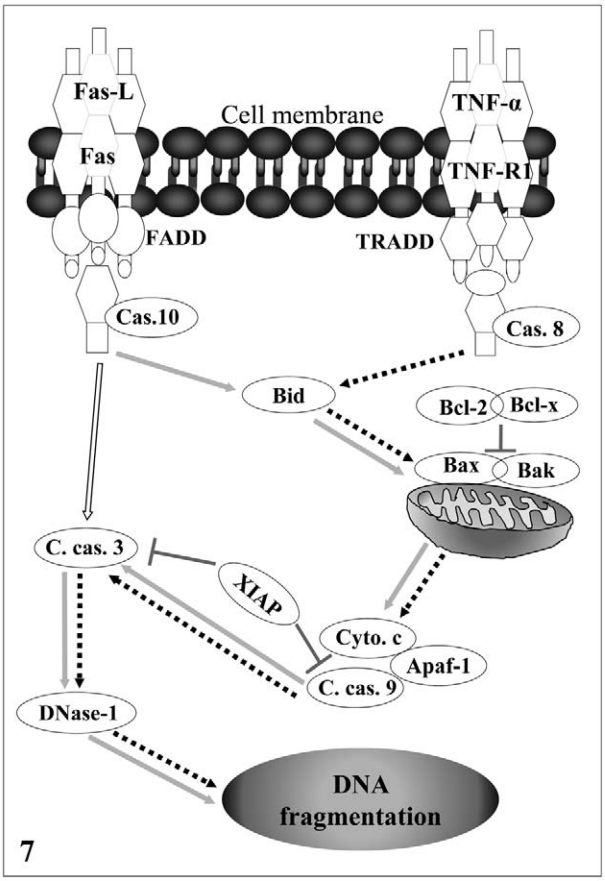

Fig. 7. Schematic cascade for the fundamental apoptosis progression in No-IB-Ep and Hyper-IB-Ep in rat esophageal epithelium. A white arrow and grey arrows indicate the mitochondrial-independent pathway and the mitochondrial-dependant pathway from Fas, respectively. Black dotted arrows illustrate the additional pathway from TNF-R1 in the Hyper-IB-Ep. T-shaped arrows indicate the inhibitory action of protein. C. cas. 3, cleaved caspase-3; Cas. 8, caspase-8; C. cas. 9, cleaved caspase-9; Cas. 10, caspase-10; Cyto. c, cytochrome c.; FADD, Fas-associated protein with death domain; TRADD, Tumor necrosis factor receptor type 1-associated death domain.

And the alteration from granular cytoplasmic positivity of cytochrome c to homogeneous cytoplasmic positivity suggests the release of cytochrome c from mitochondria in the SG of both the No-IB-Ep and Hyper-IB-Ep. Moreover, our electron microscopic findings confirmed the lower number of mitochondria in the SG of Hyper-IB-Ep than in the No-IB-Ep, coincident with the increase in swollen and punctured outermost epithelial cells. Additionally, ssDNA was positively found only in the uppermost portion of the SG. These results clarify that the activation and the progression of mitochondrial-dependent apoptosis probably occur in the SG of both No-IB-Ep and Hyper-IB-Ep and that the eventual DNA fragmentation might be fulfilled due to the collective potency of both mitochondria-independent and -dependent pathways in the rat esophageal epithelium

(Fig. 7).

The lifespan of villous columnar epithelial cells and the length of small intestinal villi are reduced under the hyperproliferation of indigenous bacteria in the rat small intestine [36, 37]. The migration speed of columnar epithelial cells is probably a crucial factor in the regulation of indigenous bacteria settlement in the rat small and large intestine [36, 37]. In the stratified squamous epithelium of the rat esophagus, several cocci of indigenous bacteria adhere and invade into the superficial layer of the SC [46]. In the present study, TNF-R1 and TNF-α were positively detected in the apical portion of SG in the Hyper-IB-Ep. Furthermore, in the Hyper-IB-Ep, caspse-8 immunopositivity was detected and the positive intensities of Bid, cytochrome c, Apaf-1 and cleaved caspase-9 were higher than those in the No-IB-Ep. Interestingly, the swelling of superficial epithelial cells were more prominent in the Hyper-IB-Ep than in the No-IB-Ep. These findings suggest that the TNF-R1–TNF-α interaction secondarily promotes the mitochondria-dependent pathway and that caspase-8 might act as the initiatory caspase, providing a potential enhancement to primarily induced apoptosis (Fig. 7).

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