Histol Histopathol (2011) 26: 811-820

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Immunohistochemical study of the apoptosis process in epidermal epithelial cells of rats under a physiological condition

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Summary. Epidermal homeostasis is maintained by both epithelial proliferation in the stratum basale (SB) and the apoptosis of epithelial cells under physiological conditions. In this study, the induction and regulation mechanisms of epidermal apoptosis were immunohistochemically investigated in the epidermis from Wistar rat's palm and foot pad by using several apoptotic related proteins under a physiological condition. The results showed that Fas and Fas-L were expressed in cellular membranes of the stratum spinosum (SS), whereas TNF-R1 did not show any membranous expression in any epidermal layers. TNF-α was not observed in the epidermis. Caspase-10, cleaved caspase-3 and DNase-1 were found in the epithelial cytoplasms from the SS to stratum granulosum (SG), whereas caspase-8 was not detected in the epidermis. XIAP and Bak were found in the cytoplasm from the SS to SG, and the intensity of Bak-positivity was stronger in the SG than the SS, whereas Bid, Apaf-1 and cleaved caspase-9 were restricted in the SG. Homogenous cytoplasmic immunoreactivity of Bcl-2 was found in the SB and the intensity was gradually decreased from the SB to the SG. The granular-cytoplasmic immuno-positivity of cytochrome C gradually altered into homogenous cytoplasmic expression in the upper half of the SG. Single-stranded DNA was rarely detected in the upper portion of the SG. These results suggest that epidermal apoptosis is induced by the interaction between Fas and Fas-L and the activation of caspase-10, and might initially proceed through a mitochondrial-independent pathway, and that a mitochondrial-dependent pathway finally accelerated under physiological conditions.

Key words: Apoptosis, Epidermis, Immunohisto-chemistry, Rat

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Introduction

The epidermis is the outermost layer of the body and is situated at the interface between the external environment and the tissues. Epidermal homeostasis is crucial for maintaining the basic barrier functions to protect the internal environment from the external one. The homeostasis of the epidermis is maintained by a delicate balance between the proliferation rate and exfoliation rate of epithelial cells. Imbalances in the physiological turn-over of keratinocytes are responsible for many skin disorders (Lippens et al., 2009). Epidermal cell death in development is accomplished by apoptosis (Raj et al., 2006).

The generation of the normal epidermis is executed by a so-called epidermal proliferative unit, which contains one stem cell, the transient amplifying cells and other cells (Potten, 1974; Mackenzie, 1997). The newly generated epidermal epithelial cells are moved outward with their several structural changes and finally become squamous keratinized cells (Lippens et al., 2005; Fuchs, 2008). In general, the differentiation of a cell is defined as a change in the genetic expression patterns, a reprogramming of the genome or a heritable phenotypic change in daughter cells. Cellular maturation is defined as a time course of cellular change after a differentiation event and is a quantitative change (Lagitha, 1979). Therefore, we refer to the structural change process of epidermal epithelial cells as keratinocyte maturation, though it has also been referred to as terminal

differentiation (Lippens et al., 2000; Rendl et al., 2002).

Normal epidermal epithelial cell maturation proceeds accompanied by apoptosis (Candi et al., 2005; Raj et al., 2006) and control of the maturation might also be involved in the process of apoptosis (Haake and Polakowska, 1993; Chaturvedi et al., 2006). In addition, the apoptosis process contributes to crucial functional and homeostatic processes in cells of various tissues. These processes include the denucleation process of lens fibers (Ishizaki et al., 1998), the maturation process of erythroblasts (De Maria et al., 1999; Testa, 2004), the formation process of platelets from megakaryocytes (De Botton et al., 2002), and holocrine secretion in sebaceous glands (WróBel et al., 2003), as well as esophageal (Bennett et al., 1999) and intestinal epithelial cell maturation (Onishi et al., 2007).

Epidermal apoptosis can be triggered by Fas (Aragane et al., 1998), TNF-R1 or TNF-related apoptosis inducing ligand (TRAIL)-receptor through ligand recognition under UV exposure (Qin et al., 2004), or by other harmful stimuli, such as chemical, physical, or biological factors (Raj et al., 2006). On the other hand, Fas and Fas-L are detected in the normal epidermis, UV-irradiated epidermis (Leverkus et al., 1997) or various other lesional epidermises (Sayama et al., 1994). After induction, the apoptotic process progresses up to DNA fragmentation through the activation of caspase-3 by a mitochondrial-independent or -dependent manner (Green and Kroemer, 1998; Allombert-Blaise et al., 2003; Milhas et al., 2005). Moreover, some cutaneous disorders cause changes in the progression of apoptosis (Raj et al., 2006). Therefore, the clarification of the apoptosis mechanism provides valuable fundamental knowledge in regulation and treatment in skin disorders. However, there has been no clear and detailed explanation of the histo-physiological mechanism of the induction and progression of the apoptotic process in normal epidermal epithelial cells. In this study, to provide a histo-physiological clarification of the epithelial process of apoptosis, we immunohistochemically investigated the expressions of apoptosis-related proteins in the normal rat epidermis under a physiological condition.

Materials and methods

Experimental animals

Fourteen male Wistar rats, aged 7 weeks (Japan SLC Inc., Hamamatsu, Japan), were used as experimental animals. They were maintained under conventional laboratory housing conditions of a 12-hour 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, Japan). They were examined clinically and pathologically to confirm that they had no signs of disorders. This experiment was approved by the Institutional Animal Care and Use Committee (permission number: 19-05-07) and was completed in accordance with the 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. Normal wild snake serum, prepared in our laboratory from the Japanese four-lined snake *Elaphe quadrivirgata*, was applied as a blocking agent for immunohistochemistry.

The following primary antiserums 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, LLC, 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.), HRP-conjugated anti DNase-1 rabbit IgG (Santa Cruz Biotechnology; HRP was conjugated in our laboratory), anti XIAP rabbit IgG (IMGENEX Corp., 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, LLC, Littleton, CO, U.S.A.), anti ssDNA rabbit IgG (Dako, Glostrup, Denmark), anti Bcl-2 goat IgG (Santa Cruz Biotechnology), anti Bak rabbit IgG (BD Biosciences, San Jose, CA, U.S.A.), and anti Bax mouse IgG (Santa Cruz Biotechnology). The primary sera for control sections were normal goat IgG (PeproTech, Rocky Hill, NJ, U.S.A.) and normal rabbit IgG (BioVision, Mountain View, California, U.S.A.). The following secondary antiserums were used: HRP-conjugated anti goat IgG mouse IgG (Chemicon International, Billerica, MA, U.S.A.), HRP-conjugated anti rabbit IgG goat IgG $F(ab')_2$ (Chemicon International), and HRP-conjugated anti mouse IgG2b (γ2b chain specific) rat $IgG1_k$ (Beckman Coulter, Inc., Brea, CA, U.S.A.).

Tissue preparation

Histological sampling was carried out from 9:00 a.m. All animals were deeply anesthetized by intraperitoneal pentobarbital sodium injection, and then perfusion fixation was performed with 0.1 M phosphate buffered 4% paraformaldehyde fixative. Soon after the perfusion, skin samples were obtained from the palm and footpad. All the samples were cut into small tissue blocks with about 2-3 mm in width and were immersed in the same fixative for another 6 hours at 4°C. After the fixation, the samples were snap-frozen in liquid nitrogen according to the embedding method described by Barthel and Raymond (1990). Then, 4 μm-thick sections were cut using a Coldtome HM505E (Carl Zeiss, Jena, Germany) and placed on glass slides precoated with 0.2% 3-aminopropyltriethoxysilane (Shin-Etsu

Chemical., Tokyo, Japan).

Immunohistochemistry

The antigens were detected in the epidermal sections by using a 1- or 2-step method of enzyme-immunohistochemistry. The combination and dilution of enzyme and antisera are described in Table 1. All the sections were stored at -30°C until use in the immunohistochemical process. First, all the sections were rinsed three times in 0.05% of Tween-added phosphate buffered saline (TPBS, pH 7.4), then incubated for 10 minutes at 37°C with proteinase k (0.6 μg/ml), if necessary. Then they were immersed in absolute methanol and 0.5% H_2O_2 for 30 minutes, respectively. After washing in TPBS, the sections were blocked with normal snake serum for 1 hour, followed by an incubation of primary antibody for 18 hours at 4°C and incubation with secondary antibody for 1 hour at room temperature. Finally they were incubated with 3, 3'-diaminobenzidine containing 0.03% H_2O_2 and counterstained with methyl green. The negative control sections were incubated with TPBS or IgGs instead of primary antiserums. The IgGs were from the non-immunized sera of animal species that produced the primary antibody.

Evaluation of immunoreactivity

In each immunostaining with different primary serum, the intensity of immunoreaction was evaluated in consideration with the results of negative control sections. Briefly, the intensity which is equal to that in negative control section was estimated at "negative" and the highest intensity is categorized as "strong". In addition, the intermediate intensity between negative and strong was categorized as "weak".

Results

Fas

In the stratum basale (SB), the basal portion of the stratum spinosum (SS) and the stratum corneum (SC),

Table 1. Chemical reagents used in the immunohistochemical analysis.

Antigen	Pre-treatment	Normal serum and dilution	Primary antiserum and dilution	Secondary antiserum and dilution
Fas	0.6 μg/ml Proteinase-K for 10 min at 37°C	Normal wild snake serum x 0.01	Anti Fas goat IgG x 0.01	HRP-conjugated anti goat IgG mouse IgG x 0.005
TNF-R1	0.6 μg/ml Proteinase-K for 10 min at 37°C	Normal wild snake serum x 0.01	Anti TNF-R1 rabbit IgG x 0.000125	HRP-conjugated anti rabbit IgG goat IgG $F(ab')_2$ x 0.005
Fas-L	0.6 μg/ml Proteinase-K for 10 min at 37°C	Normal wild snake serum x 0.01	Anti Fas-L goat IgG x 0.01	HRP-conjugated anti goat IgG mouse IgG x 0.005
TNF-α	0.6 μg/ml Proteinase-K for 10 min at 37°C	Normal wild snake serum x 0.01	Anti TNF-α rabbit IgG x 0.00025	HRP-conjugated anti rabbit IgG goat IgG $F(ab')_2$ x 0.005
Caspase-10	-	Normal wild snake serum x 0.01	Anti Caspase-10 goat IgG x 0.005	HRP-conjugated anti goat IgG mouse IgG x 0.005
Caspase-8	-	Normal wild snake serum x 0.01	Anti Caspase-8 goat IgG x 0.005	HRP-conjugated anti goat IgG mouse IgG x 0.005
Cleaved caspase-3	-	Normal wild snake serum x 0.01	Anti Cleaved caspase-3 rabbit IgG x 0.01	HRP-conjugated anti rabbit IgG goat IgG $F(ab')_2$ x 0.005
XIAP	-	Normal wild snake serum x 0.01	Anti XIAP rabbit IgG x 0.0005	HRP-conjugated anti rabbit IgG goat IgG $F(ab')_2$ x 0.005
DNase-1	-	Normal wild snake serum x 0.01	HRP-conjugated anti DNase-1 rabbit IgG x 0.000625	-
Bid	-	Normal wild snake serum x 0.01	Anti Bid goat IgG x 0.01	HRP-conjugated anti goat IgG mouse IgG x 0.005
Cytochrome C	-	Normal wild snake serum x 0.01	Anti Cytochrome C goat IgG x 0.01	HRP-conjugated anti goat IgG mouse IgG x 0.005
Apaf-1	-	Normal wild snake serum x 0.01	Anti Apaf-1 goat IgG x 0.01	HRP-conjugated anti goat IgG mouse IgG x 0.005
Cleaved caspase-9	-	Normal wild snake serum x 0.01	Anti Cleaved caspase-9 rabbit IgGx 0.01	HRP-conjugated anti rabbit IgG goat IgG $F(ab')_2$ x 0.005
Bcl-2	-	Normal wild snake serum x 0.01	Anti Bcl-2 goat IgG x 0.01	HRP-conjugated anti goat IgG mouse IgG x 0.005
Bak	-	Normal wild snake serum x 0.01	Anti Bak rabbit IgG x 0.00025	HRP-conjugated anti rabbit IgG goat IgG $F(ab')_2$ x 0.005
Bax	-	Normal wild snake serum x 0.01	Anti Bax mouse IgG x 0.01	HRP-conjugated anti mouse IgG_{2b} rat $IgG1_k$ x 0.005
ssDNA	-	Normal wild snake serum x 0.01	Anti ssDNA rabbit IgG x 0016	HRP-conjugated anti rabbit IgG goat IgG $F(ab')_2$ x 0.005

no epithelial cells expressed Fas. In the rest of the SS, Fas was immunopositive in the cell membranes with the highest intensity, but in the upper layers of SS, the cell membrane-immunopositivity was gradually changed into cytoplasmic-immunopositive expression. In the stratum granulosum (SG), Fas was detected in the epithelial cytoplasm (Fig.1A, a).

TNF-R1

From the SB to the middle portions of the SS, granular-shaped and strongly immunopositive expression of various sizes was found in the epithelial cytoplasm (Fig. 1B, b). From the middle portions of the SS to the SG, the TNF-R1 immunopositive reaction gradually changed into weak and homogeneous cytoplasmic immunoreaction and the SC expressed no TNF-R1.

Fas-L

In the SB, the basal portion of the SS and the SC, no epithelial cells expressed Fas-L. In the middle portion of the SS, cell membrane-immunopositive expression with high intensity was clearly seen. The membrane-immunopositivity was gradually changed into cytoplasmic immunopositivity toward the SG with rising intensity (Fig. 1C, c).

TNF-α

Positive immunoreaction was not detected in any portions of the epidermis (Fig. 1D, d).

Caspase-10

In the SB, the basal portion of the SS and the SC,

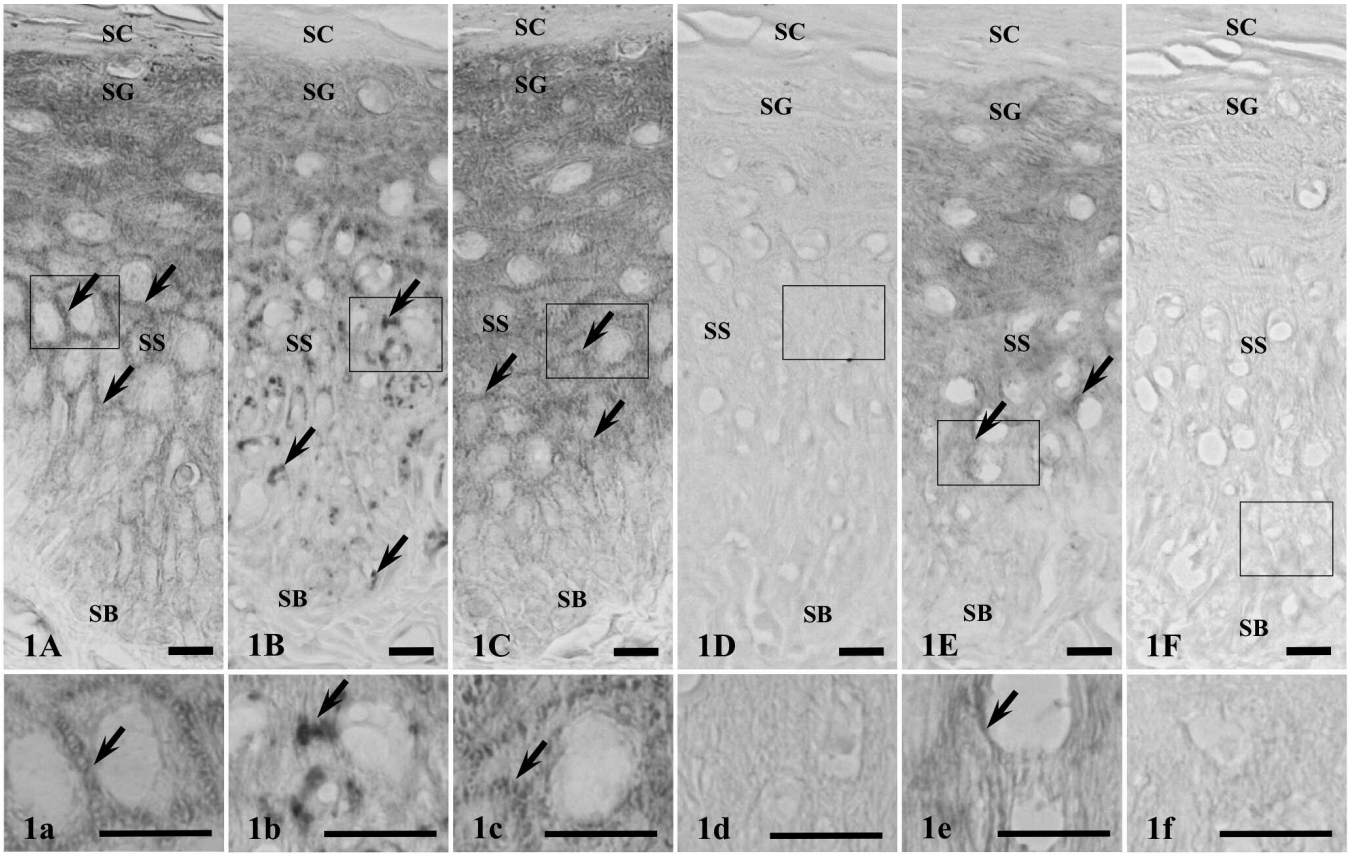

Fig. 1. The immunopositivities of apoptosis-related receptors, ligands and initiator caspases in normal rat epidermis. Fas is visible as strong cell membrane immunopositive expression (arrows) in the middle portion of the stratum spinosum (SS) (**A**). TNF-R1 is visible as strong, granular-shaped cytoplasmic immunopositive expression (arrows) from the stratum basale (SB) to the middle portion of the SS (**B**). Fas-L shows strong immunopositivity in cell-membrane (arrows) from the middle portion of the SS (**C**). These positivities gradually change into homogeneous epithelial cytoplasmic immunopositive expression toward the stratum granulosum (SG) (**A, B and C**). TNF-α is not noticeable in the normal epidermis (**D**). Caspase-10 is visible from the middle portion (arrows) of the SS with gradually increasing cytoplasmic immunopositivity toward the SG (**E**). Caspase-8 is not noticeable in the normal epidermis **(F)**. High-magnification photographs (**a-f)** of the areas outlined with squares in **Fig. 1A-F**. SC, stratum corneum. Bar: 10 μm.

immunopositive expression was not detected. From the middle portion of the SS, immunopositive-caspase-10 expression was found in the epithelial cytoplasm with low intensity and increased toward the SG (Fig. 1E, e).

Caspase-8

Positive immunopositive expression was not found in any layers of the epidermis (Fig. 1F, f).

Cleaved caspase-3

In the SB, the basal portion of the SS and the SC, the epithelial cells showed no immunopositive reaction. But from the middle portion of the SS, cleaved capase-3 was found as weakly immunopositive expression in the epithelial cytoplasm and the positive intensity was gradually increased toward the SG (Fig. 2A, a).

XIAP

In the SB, the basal portion of the SS and the SC, the epithelial cells were negative for XIAP. Homogeneous cytoplasmic immunopositive expression was detected in the rest of the epidermal epithelial cells. The intensity of positive expression was increased toward the SG (Fig. 2B, b).

DNase-1

No immunopositive reaction was found in the SB, the basal portion of the SS or the SC. From the middle portion of the SS, however, homogeneously and weakly positive immunoreaction was found in the epithelial cytoplasm and the positive intensity increased toward

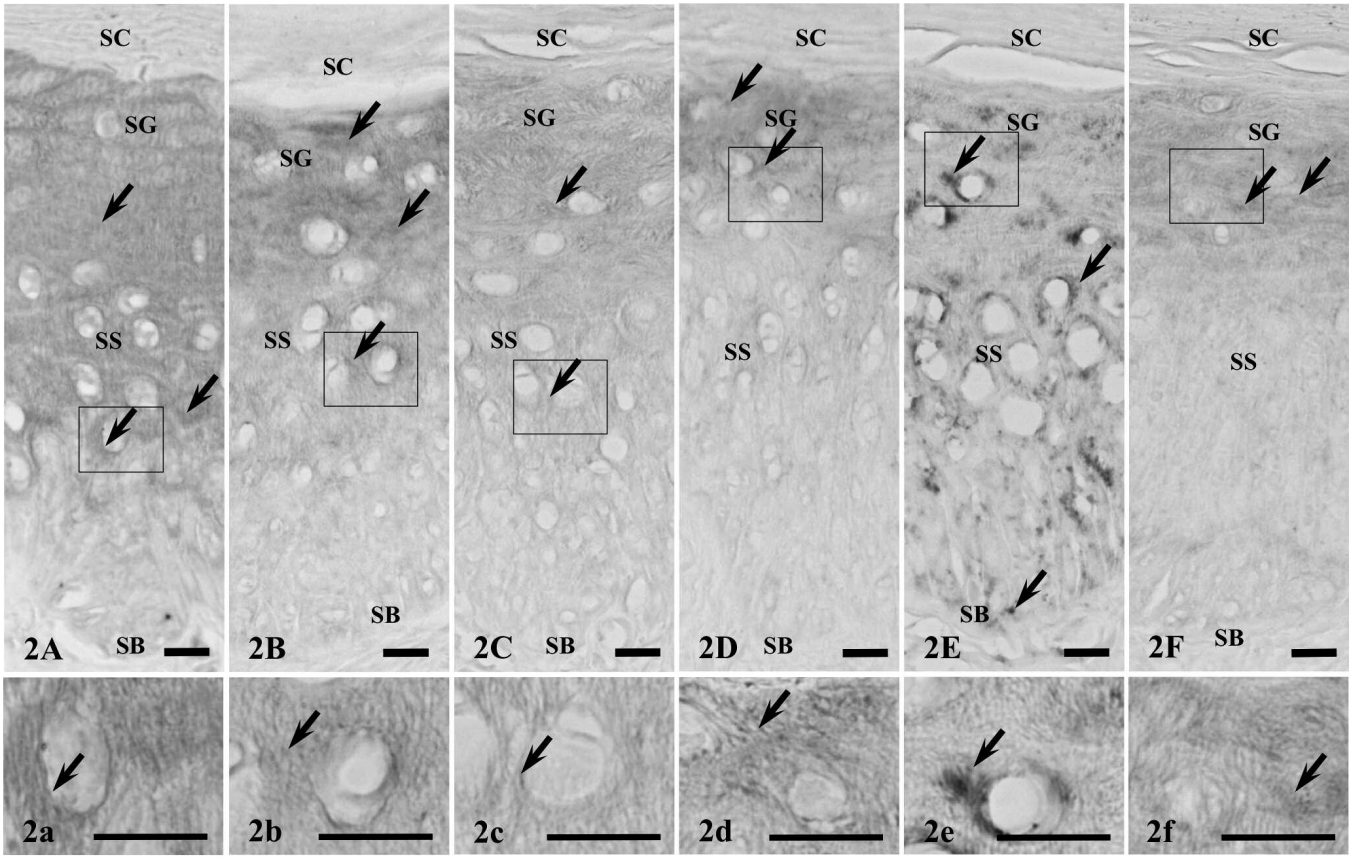

Fig. 2. The immunopositivities of mitochondrial-independent and -dependent apoptosis-related proteins in normal rat epidermis. Cleaved caspase-3 **(A)**, XIAP **(B)** and DNase-1 **(C)** are visible as homogeneous cytoplasmic immunopositivity (arrows) from the middle portion of the stratum spinosum (SS). These positivities increase toward the stratum granulosum (SG) (**A, B and C**). Bid can be seen as strongly positive cytoplasmic immunoreaction (arrows) in the upper portion of the SS and the SG **(D)**. Cytochrome C is immunopositively visible as small, granular-shaped cytoplasmic expression in the SB and the basal portion of the SS and large, strong, granular-shaped expression in the rest of the SS (arrows). The positivity fades out in the SG (**E**). Apaf-1 is immunopositively observable as homogenous cytoplasmic expression (arrows) in the upper portion of the SS and the SG (**F**). High-magnification photographs (**a-f)** of the areas outlined with squares in **Fig. 2A-F**. SC, stratum corneum. Bar: 10 μm.

the SG (Fig. 2C, c).

Bid

From the SB to the middle portion of the SS and the SC, the epithelial cells showed no immunopositive reaction. In the upper portion of the SS and the SG, Bid was strongly immunopositive in the epithelial cytoplasm (Fig. 2D, d).

Cytochrome C

In the SB and the basal portion of the SS, small granular-shaped, weak immunopositive expression was found in the epithelial cytoplasm. In the middle and the upper portion of the SS, large, granular-shaped, and strongly positive cytochrome C immunoreaction was detected in the epithelial cytoplasm and also in the perinuclear region of each epithelial cell. From the upper portion of the SS, however, the granular immunopositive expression gradually changed into homogeneous cytoplasmic immunoreaction and disappeared toward the SG. The SC was negative for cytochrome C (Fig. 2E, e).

Apaf-1

From the SB to the middle portion of the SS and the SC, no positive immunoreaction was detected. From the

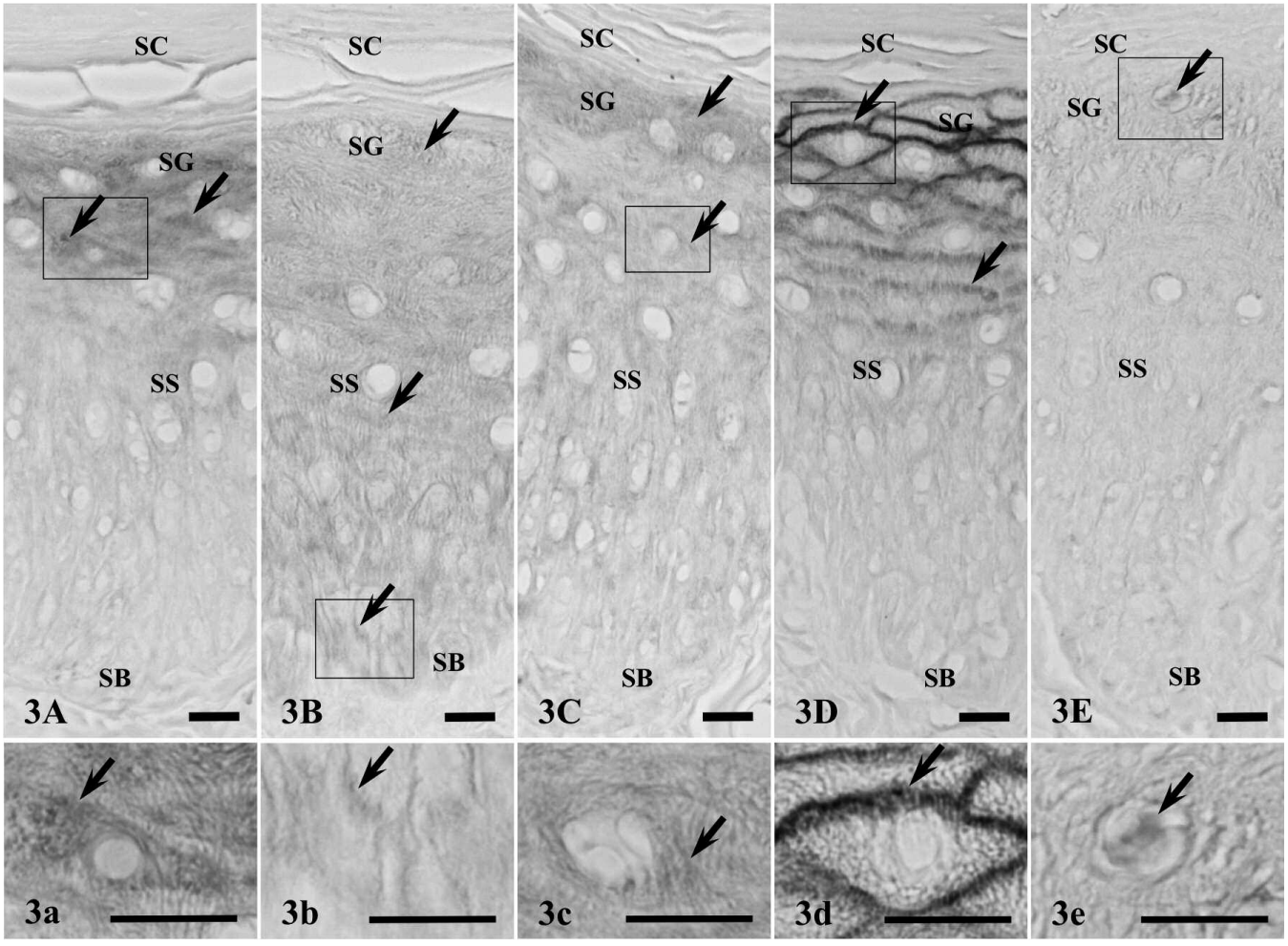

Fig. 3. The immunopositivities of cleaved caspase-9, regulator proteins of apoptosis and fragmented DNA in normal rat epidermis. Cleaved caspase-9 shows weak cytoplasmic immunopositive expression from the middle portion of the stratum spinosum (SS). The intensity and the frequency of the positivity are increased toward the stratum granulosum (SG) (arrows) **(A)**. Homogenous cytoplasmic immunopositivity of Bcl-2 is observed in the stratum basale (SB), SS and SG (arrows) **(B)**. A weak, cytoplasmic immunopositivity is visible for Bak from the middle portion of the SS, and the intensity is increased toward the SG (arrows) **(C)**. Bax is not visible from the SB to the middle portion of the SS. However, from the upper portion of the SS, weak cytoplasmic and strong cell membrane immunopositivity (arrows) is visible **(D)**. ssDNA is not observable in the SB or the SS. In the uppermost portion of the SG, nuclear positivity (arrow) can be seen **(E)**. High-magnification photographs (**a-e**) of the area outlined with a square in **Fig. 3A-F**. SC, stratum corneum. Bar: 10 μm.

upper portion of the SS, weakly positive expression was detected in the epithelial cytoplasm and the intensity of immunopositive expression gradually increased toward the SG (Fig. 2F, f).

Cleaved caspase-9

In the SB and the basal portion of the SS and the SC, no positive immunoreaction was detected. From the middle portion of the SS, cleaved caspase-9-positive expression was detected in the epithelial cytoplasm, but the frequency and intensity were low. However, in the upper portion of the SS and the SG, strongly positive immunoreaction was frequently found in the epithelial cytoplasm (Fig. 3A, a).

Bcl-2

From the SB to the SG, homogenous and immunopositive expression was found in the epithelial cytoplasm. The intensity of the cytoplasmic positive expression was gradually decreased toward the SG. In the SC, no immunopositive expression was detected (Fig. 3B, b).

Bak

In the SB, the basal portion of the SS and the SC, the epithelial cells expressed no immunopositive reaction. In the middle portion of the SS, weakly positive expression was detected in the epithelial cytoplasm. In contrast, in the upper portion of the SS and the SG, strongly Bak-positive immunoreaction was found in the epithelial cytoplasm (Fig. 3C, c).

Bax

From the SB to the middle portion of the SS and the SC, no positive immunoreaction was detected. In the upper portion of the SS and the SG, weakly positive expression in the epithelial cytoplasm and strongly positive Bax-immunoreaction around the cellular membranes were detected (Fig. 3D, d).

ssDNA

In the SB, the SS and the SC, no positive immunoreaction was detected. But in the uppermost portion of the SG, very sporadic immunopositive reaction was found in the nucleus (Fig. 3E, e).

Discussion

Nonlesional epidermis and unstimulated cultured keratinocytes might express minimal levels of Fas (Sayama et al., 1994). Fas and its ligand are expressed in cultured keratinocytes, but apoptosis is considered to be a rare event in the normal epidermis (Viard-Leveugle et al., 2003). No characteristic morphological and biochemical apoptotic markers, such as cytoplasmic/nuclear condensation, formation of apoptotic bodies and caspase activation, appear in normal keratinocytes with senescence (Gosselin et al., 2009). Apoptosis might be blocked during keratinocyte maturation to prevent premature apoptotic cell death and to allow correct cornification (Lippens et al., 2009). On the other hand,

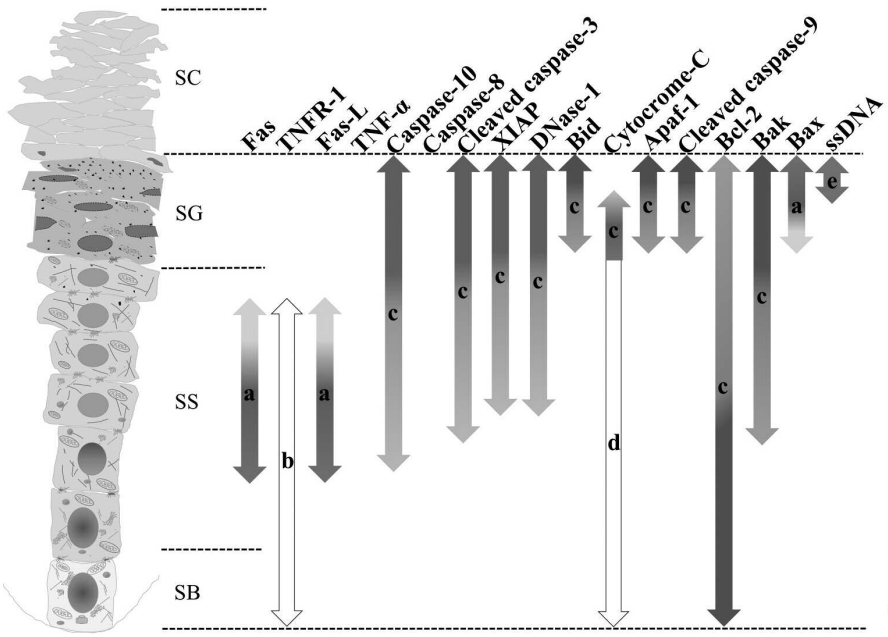

Fig. 4. Schematic summary of the immunohistochemical expression of apoptosis-related proteins in normal epidermis. a, cell membrane immunopositivity; b, no cell membrane immunoreaction; c, homogeneous cytoplasmic immunopositivity; d, cytoplasmic granular immunoreaction; e, nuclear immunopositivity; SB, stratum basale; SS, stratum spinosum; SG, stratum granulosum; SC, stratum corneum.

UV irradiation and other harmful stimuli trigger apoptosis (Olson and Everett, 1975) through a mitochondrial-dependent apoptotic pathway by the interaction of Fas or TNF-R1 with their ligands in the epidermis (Raj et al., 2006). Thus, the induction and progression of the apoptotic process in the normal epidermis has been unclear, and its *in vivo* mechanisms have never been clarified in detail. In this study, the proteins related to both the mitochondrial-independent and -dependent pathways of apoptosis were immunohistochemically clarified in the rat normal epidermis in chronological order. Our findings have tentatively elucidated the normal progression of apoptotic pathways in the normal epidermis.

The TNF-R superfamily comprises the so-called death receptors, namely TNF-R1, Fas (Gruss and Dower, 1995), the TRAIL-receptors and so on (Ashkenazi and Dixit, 1998). TNF-R1, a type-1 membrane protein, is a receptor for TNF-α and responsible for induction of apoptosis (Gruss and Dower, 1995). The TNF superfamily receptor and ligand are present in normal (Lotz et al., 1996; Pan et al., 1997; Emery et al., 1998) and psoriasis lesional tissues (Kristensen et al., 1993). In the present study, however, TNF-R1 showed granular deposits in the epithelial cytoplasm, and its ligand, TNF-α, was never detected in the epidermis (Fig. 4). Conversely, Fas, which is another apoptosis-inducing, type 1 membrane receptor (Yonehara et al., 1989), showed cell membranous immunopositive expression in the middle portion of the SS, and its ligand, Fas-L, also exhibited cell membrane and cytoplasmic immunopositive expressions in a manner similar to Fas (Fig. 4). Thus, under physiological conditions, TNF-R1 might be an inactive receptor and the Fas-Fas-L system might induce apoptosis from the middle portion of the epidermis.

In general, the FADD which contains the death domain and is responsible for a signal from death receptors (Muzio et al., 1996), conducts the activation of caspase-8 or caspase-10 (Wang et al., 2001; Kischkel et al., 2001; Sprick et al., 2002; Boatright et al., 2004), which leads to further progression of apoptosis through a mitochondrial-dependent apoptotic pathway or through a mitochondrial-independent apoptotic pathway (Milhas et al., 2005). These pathways cause activation of caspase-3 which may finally cause cytoplasmic events and nuclear events (Hengartner, 2000) via activating DNase (Zheng et al., 1998) in Fas-mediated apoptosis. The XIAP inhibits apoptosis by binding with caspase-3, caspase-7 and caspase-9 (Deveraux and Reed, 1999). In our results, although caspase-8 showed no immunopositive expression, caspase-10, cleaved caspase-3, DNase-1 and XIAP presented clear cytoplasmic immunopositive expression from the middle portion of the epidermis (Fig. 4). These findings suggest that caspase-10 initiation plays a role in the apoptotic process and that further progression of the apoptotic process might be inhibited by XIAP, even though caspase-3 is finally activated through the mitochondrial-independent pathway from the middle portion in the rat epidermis.

Bid is a pro-apoptotic member of the Bcl-2 protein family which is oligomerized in the outer membranes of mitochondria and induces the release of cytochrome C from mitochondria (Waterhouse and Green, 1999; Grinberg et al., 2002). Activated Bid may directly induce conformational changes of Bax and Bak, and further, its oligomerization can lead to the formation of pores on the outer mitochondrial membrane that allow the release of cytochrome C (Martinou and Green, 2001). Cytochrome C is gradually released during *in vitro* keratinocyte maturation (Allombert-Blaise et al., 2003). In the presence of dATP/ATP, cytochrome C triggers the assembly of a protein complex called "apoptosome"-containing Apaf-1 and cleaved caspase-9; together these assemblies cause the activation of caspase-3 (Zou et al., 1997; Czerski and Nuñez, 2004). In the present study, activated Bid, cleaved caspase-9 and Apaf-1 were restricted to the upper portion of the SS and in the SG, while the immunopositive intensity of Bak and Bax was stronger in the same area (Fig. 4). Interestingly, the granular immunopositive expression of cytochrome C changed into homogenous expression and finally disappeared at the upper layers of the SS and the SG of the epidermis (Fig. 4). This result strongly suggested that cytochrome C is released from the mitochondria in the upper part of the epidermis and that the mitochondrial-dependent apoptotic pathway is acutely progressed in the upper part of the epidermis. This progression was considered to have potently enhanced the activation of caspase-3. Eventually, both the mitochondrial-independent and mitochondrial-dependent apoptotic pathways may collectively cause DNA fragmentation of epidermal epithelial cells around the uppermost layers of the SG in the rat epidermis under physiological conditions.

Acknowledgements. This work was financially supported in part by a Grant-in-Aid for Scientific Research (no. 23580403) from Japan Society for the Promotion of Science.

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Accepted January 3, 2011