Induction of apoptosis in tilapia, *Oreochromis aureus* Steindachner, and in TO-2 cells by *Staphylococcus epidermidis*

S-L Huang^{1,2}, I-C Liao³ and S-N Chen²

¹ Lu-Kang Branch, Taiwan Fisheries Research Institute, Changhua, Taiwan

² Department of Zoology, National Taiwan University, Taipei, Taiwan

³ Taiwan Fisheries Research Institute, Keelung, Taiwan

Abstract

This study describes apoptosis induced *in vivo* and *in vitro* in tilapia, *Oreochromis aureus* Steindachner, by *Staphylococcus epidermidis*. In an *in vivo* experiment, tilapia were challenged using viable *S. epidermidis* and its cultured supernatant, respectively. Apoptosis was predominantly detected in lymphocytes and macrophages in spleen and kidney. Apoptotic figures were observed occasionally in the brain, liver, gonad, mesentery, stomach, intestine and skeletal muscle of infected fish. In an *in vitro* experiment, TO-2 cells treated with brain heart infusion broth supernatant of the bacteria after 48 h revealed that the supernatant was able to induce the cell apoptosis. Fragmented DNA was also detected 48 h after treatment using 1.5% agarose gel electrophoresis. The smallest multimeric DNA fragment was approximately 180-bp in length. Results of both the *in vitro* and *in vivo* experiments showed that products of the bacteria could induce tilapia cell apoptosis.

Introduction

Pathogenicity initiated by *Staphylococcus epidermidis* in cultured tilapia in Taiwan was confirmed in a previous study (Huang, Chen, Shei, Liao & Chen 1999). In the pathological study performed on the tissues of infected fish, numerous electron-dense particles were observed in the brain, spleen, kidney, pancreas, liver, intestine, stomach, mesen-

tery, gonad and skeletal muscle of diseased tilapia. The particles were suspected to be apoptotic bodies caused by infection with *S. epidermidis* (Huang *et al.* 1999).

Since there was no report on apoptosis induced by *S. epidermidis*, it was unknown whether the bacteria caused the fish cell apoptosis. To demonstrate bacterial induction of apoptosis, tilapia and TO-2 cells were treated by *S. epidermidis* viable cells and/or the supernatant. The results showed that the bacteria's supernatant can induce apoptotic bodies forming either in tilapia or TO-2 cells, thus suggesting the electron-dense particles observed previously were indeed apoptotic bodies.

Materials and methods

Bacteria

Staphylococcus epidermidis strain LK0728, previously isolated from the spleen of diseased tilapia in July 1993 in Lukang, Changhua, Taiwan, was used in this study.

Bacterium and its supernatant

Collection of the bacteria was performed by using 6-cm discs of dialysis membrane (molecular weight cut-off of 6000–8000, Spectra/Por 1) overlaid on brain heart infusion agar (BHIA) and by spreading 0.2–0.3 mL of overnight cultured broth with a sterilized glass rod (Inamura, Muroga & Nakai 1984). The bacteria were collected by washing the

Correspondence Professor SN Chen, Department of Zoology, National Taiwan University, Taipei, Taiwan 107

dialysis membrane in 5 mL of saline (0.85% NaCl) after 24 h of incubation at 30 °C.

Bacterial suspension at a density of 3.24×10^9 cfu mL⁻¹ was centrifuged at $5000 \times g$ for 20 min, at 4 °C (Hitachi 05PR22; Hitachi, Tokyo, Japan). The supernatant was filtered through a sterilized 0.22-µm filter. The filtrate was designated as saline supernatant.

Conversely, *S. epidermidis* cultured on brain heart infusion broth (BHIB) at 30 °C for 24–36 h was diluted to a density of 3.24×10^9 cfu mL⁻¹. The bacteria suspension was centrifuged at $5000 \times g$ at 4 °C for 20 min and the supernatant was collected and filtered through a sterilized 0.22-µm filter. The filtrate was designated as BHIB supernatant.

***In vivo* induction of apoptosis**

Experimental animals

Tilapia, *Oreochromis aureus* Steindachner, fry were reared in a $2 \times 3 \times 1$ m tank with aeration until the body weight was 60–70 g. To judge the health of experimental fish, 10 fish randomly selected were evaluated for several parameters using routine methods before experimentation. These parameters included fish behaviour, external appearance, internal appearance, bacterial isolation and histopathological examination. Healthy tilapia were transferred to and kept in 1000-l tanks with aeration. The fish were allowed to acclimatize in the tank for at least 2 weeks prior to experimentation. During the course of the experiment, fish were fed with commercial tilapia food and reared at a room temperature of approximately 28 °C.

Challenge

Tilapia used for the challenge experiment were divided into eight groups, each of 10 fish. Six groups of the experimental fish were injected intraperitoneally (i.p.) with 0.5 mL of a bacterial suspension at a concentration of 1.34×10^3 , 1.34×10^4 , 1.34×10^5 , 1.34×10^6 , 1.34×10^7 and 1.34×10^9 cfu mL⁻¹, respectively. In addition, one group was injected with 0.5 mL of saline supernatant and another was administered saline as a control.

Transmission electron microscopy (TEM)

Kidney, liver and spleen were dissected from the experimentally infected tilapia, fixed in 2.5% glutaraldehyde for 2 h, post-fixed with 1% osmium tetroxide at 4 °C for 1 h, dehydrated in a graded series of ethanol and absolute acetone, and embedded in Spurr's resin (Spurr 1969). Ultrathin sections were then cut with a Sorvall MT 5000 (CN, USA) ultramicrotome and stained with uranyl acetate and lead citrate, according to procedures described by Frasca & Parks (1965). The sections were then examined using a Hitachi H-600 electron microscope at 75 kV.

***In vitro* induction of apoptosis**

Cell line cultures

The TO-2 (tilapia ovary) cell line was used in this experiment (Chen, Chi, Ueno & Kou 1983). The cells were routinely cultured in Leibovitz's 15 (L-15) medium (Gibco, New Zealand) containing 10% foetal bovine serum (FBS, Gibco) (L-15 growth medium) at 28 °C.

Phase contrast microscopy

TO-2 cells incubated with diluted BHIB supernatant (BHIB supernatant mixed with an equal volume of L-15 growth medium) for each consecutive incubation period were observed under an Olympus IM phase contrast microscope. The control used BHIB instead of BHIB supernatant.

Scanning electron microscopy (SEM)

For SEM observations, TO-2 cells grown on round coverslips were used. To investigate the effect of extracellular products of *S. epidermidis*, the cells were exposed to diluted BHIB supernatant. Selected incubation periods, including 4, 7, 16, 20, 24, 30, 48, 60, 72, 84, 96 and 108 h were used. After each incubation period, the coverslips were fixed in 2.5% glutaraldehyde at 4 °C for 2 h and post-fixed in 1% osmium tetroxide for 1 h, dehydrated in a graded series of ethanol and absolute acetone, and then passed through critical-point-drying using liquid CO₂. After drying, the coverslips were mounted on stubs, coated with carbon, and examined in a scanning electron microscope (S-520 Hitachi, Japan).

Fluorescent stain

Since apoptosis is based on DNA fragmentation, direct morphological evaluation of cells stained with fluorescent dye under light microscopy is required (Eastman 1995). TO-2 cells cultured on a chamber slide (glass slide, polystyrene chambers, Nunc) were exposed to diluted BHIB supernatant for 48 h at 28 °C. The treated cells were then fixed in pre-cooled 70% ethanol and stained with 5 $\mu\text{g mL}^{-1}$ of propidium iodide (PI) or 0.1% acridine orange (AO) (Fraker, King, Lill-Elghanian & Telford 1995).

DNA extraction

TO-2 cells (5.8×10^7 cells per 150-cm² flask) subcultured post-24 h were washed twice with Ca^{++} , Mg^{++} -free phosphate-buffered saline. Subsequently, 40 mL of the diluted BHIB supernatant was added to the flask. The cultures were incubated at 28 °C for 24, 36, 48, 60, 72 or 84 h, respectively, before harvesting. After each incubation period, the cells, including suspended cells, were collected and lysed in 1 mL of lysis buffer solution (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) at 4 °C for 1 h. The cell lysates were then centrifuged at $27\,000 \times g$ for 20 min at 4 °C and the supernatants collected. Extracted genomic DNA was prepared by adding two volumes of phenol-chloroform-isopropyl alcohol (25:24:1) into the supernatant. The DNA was precipitated in absolute ethanol at –20 °C overnight. Subsequently, the DNA was collected by centrifugation ($16\,000 \times g$, 10 min) and washed with cold 70% ethanol, and then air-dried. The crude DNA was resuspended in 0.5 mL of RNase buffer containing 20 $\mu\text{L mL}^{-1}$ of ribonuclease A. Digestion of RNA was progressed at 37 °C for 30 min. Genomic DNA obtained using the above methods was dissolved in Tris-EDTA buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0) and electrophoresed in a 1.5% agarose gel.

Results

In vivo experiment

TEM examination

TEM observations showed that *S. epidermidis* was phagocytosed by macrophages in the spleen 48 h after i.p. injection (Fig. 1).

In vivo experimental fish, challenged using the viable bacteria after 48 h, demonstrated cytoplasmic-condensed or cytoplasmic-vacuolized cells scattered throughout the spleen. Morphological characteristics of apoptosis, such as extensive chromatin clumping, nuclear fragmentation, cytoplasmic condensation and membrane blebbing were observed in splenic lymphocytes. During early stages of apoptosis, condensed chromatin with sharply delineated edges abutted the inner surface of the nuclear envelope (Fig. 2). Convolution of the cell and nuclear outlines and small masses of condensed chromatin were also observed. Multiple nuclear fragments, enclosed by double membranes and with varying chromatin content, were also noted in the lymphocytes. Apoptotic bodies observed in the lymphocytes could be distinguished from bacteria within macrophages since the latter possessed a significant cell wall. Apoptotic bodies were also detected in the kidney and liver. Apoptosis, however, was most severe in the spleen and kidney. More extensive apoptosis in these tissues was observed after challenge with a higher dose of the viable bacterium or saline supernatant of the bacterium.

Figure 1 TEM micrograph showing *Staphylococcus epidermidis* phagocytosed by a lymphocyte in the spleen. Arrow indicates the bacterial mass. Dividing bacteria with distinctive cell walls were observed (bar = 2 μm).

Figure 2 TEM micrograph of a lymphocyte in the spleen of tilapia 48 h after challenge by *S. epidermidis*. Condensed chromatin with sharply delineated edges abuts the inner surface of the nuclear envelope (bar = 800 nm).

In vitro experiment

Phase microscopy examination

In the *in vitro* experiment, TO-2 cells treated with the BHIB supernatant showed gradual changes, including rounding, shrinking, detachment and floating into the medium, and numerous small particles budding from the cell. With increasing incubation time, more and more small oval-shaped particles appeared at the cell surface (Fig. 3) and were released into the medium. The percentage of detached cells increased and reached 50–70% after 96 h of incubation. In contrast, no such particles were found in the control groups.

TEM examination

At 4–7 h after BHIB supernatant exposure, an abnormally convoluted nuclear outline was observed in a few cells. Other observable abnormal features after 24 h of incubation included chromatin aggregation, abnormal convolution and indentation of the nuclear outline, vacuolized and condensed cytoplasm, and lobulation or fragmentation of the nuclei – these being similar to the features of apoptosis. Numbers of the abnormal

cells increased with time. Furthermore, oval-shaped electron-dense particles in the cells were readily observed after 48 h of incubation.

SEM examination

TO-2 cells treated with the diluted BHIB supernatant after 4 h were distinguishable from normal TO-2 cells by their altered morphology. Normal TO-2 cells had numerous microvilli on the surface (Fig. 4A). TO-2 cells showed surface protuberances on the nuclear region after 4 h of incubation (Fig. 4B). Numerous protuberances were observed on the surface after treatment for 7 h (Fig. 4C). Round to oval-shaped bodies were observed budding from the cell surface after 16 h of treatment (Fig. 4D). The extent of such morphological alterations of TO-2 cells increased with incubation time. Sixty hours after incubation, microvilli were hard to observe and many oval-shaped bodies budding from the cell surface were observed (Fig. 4E). The bodies covered the whole cell surface after 72 h of incubation (Fig. 4F).

Fluorescent stain

Morphology of apoptotic cells, as characterized by AO and PI staining, showed that apoptotic cells with ‘dots’ of condensed chromatin appeared green, and the ‘dots’ of condensed chromatin showed a red colour by PI staining.

Figure 3 Phase contrast micrograph of TO-2 cells after 48 h of treatment with the supernatant of *S. epidermidis*. Numerous small particles are seen budding from the cells (arrow) (bar = 50 µm).

Figure 4 Scanning electron micrographs of TO-2 cells. (A) Normal TO-2 cells. Note numerous microvilli on the cell surface. (B) TO-2 cells after 4 h of treatment with the supernatant of *S. epidermidis*. Note the surface protuberance (arrow). (C) TO-2 cells after 7 h of treatment with the supernatant. Some cells show obvious surface protuberances. (D) TO-2 cells after 16 h of treatment, round to oval-shaped bodies are seen budding from the surface. Extensive apoptosis morphologies were observed after 60- and 72-h treatments as shown in E and F, respectively (bar = 7.5 μ m).

1.5% agarose gel electrophoresis

Results of genomic DNA electrophoresis of TO-2 cells using a 1.5% agarose gel are shown in Fig. 5. Fragmented DNA exhibited the typical 'ladder' pattern when TO-2 cells were incubated with the diluted BHIB supernatant. Fragmented DNA was detected extensively after 48 h of incubation, whereas no significant fragmented DNA was detected after 24 or 36 h of incubation. The concentration of DNA fragments increased with incubation time. The smallest multimeric DNA fragment was approximately 180 bp.

Discussion

Apoptosis is a physiological and pathological process of cell deletion that functions as an essential mechanism of normal tissue homeostasis and it may play a critical role in relation to disease status (Kerr, Wyllie & Currie 1972; Columbano 1995; Wertz & Hanley 1996). According to the description by Kerr *et al.* (1972), the morphological characteristics of apoptosis occurring in infected cells consist of extensive chromatin clumping, nuclear fragmentation, cytoplasmic condensation and membrane blebbing. Under TEM, apoptotic bodies appear as membrane-bound electron-dense particles resulting from nuclear fragmentation. At the early stages, apoptosis is characterized by convolution of nuclear outline and segregation of condensed, sharply circumscribed chromatin. This condensation and fragmentation is caused by DNA digestion (Kerr *et al.* 1972). In the next phase, the nucleus fragments and further condensation of cytoplasm is associated with extensive cell surface protrusion. Subsequently, apoptotic bodies of vary-

Figure 5 Gel electrophoresis of genomic DNA of TO-2 cell using 1.5% agarose showing fragmented DNA after 48 h of treatment with the *S. epidermidis* supernatant. The amount of the DNA fragment increased with time. Lanes 1–6, treated with supernatant after 24, 36, 48, 60, 72 and 84 h, respectively. Lane 7 is a control and lane 8 is a 100-bp DNA ladder marker.

ing size and composition separate from the surface protuberances.

In this study, the processes of apoptosis were observed either in tissues or in TO-2 cells after 48 h of treatment using *S. epidermidis* or its culture supernatant. The affected cells observed under TEM revealed typical apoptosis characteristics, such as chromatin clumping, nuclear fragmentation and cytoplasmic condensation. Membrane blebbing, which is also a characteristic of apoptosis, was observed in supernatant-treated TO-2 cells under SEM examination.

In this study, nuclear fragmentation was also observed in cells stained with PI or AO. In TO-2 cells treated with the supernatant for 48 h, fragmented DNA was also detected using agarose gel electrophoresis. The smallest multimeric DNA fragment observed, approximately 180 bp, has also been considered a characteristic of apoptosis (Kerr *et al.* 1972). Thus, it suggested that the bacterial supernatant could cause apoptosis both in tilapia tissue *in vivo* and in the TO-2 cell.

Although it is not known which component caused the cell to undergo apoptosis in this experiment, lipopolysaccharide-induced apoptosis in thymic lymphocytes *in vivo* was observed by Yokoshi, Inoue, Yokoo, Kimura & Kato (1989). Further experiments are necessary to determine the components in this study that induced the cell apoptosis.

In a previous study (Huang *et al.* 1999), electron-dense particles were revealed by TEM in numerous tissues, including the brain, spleen, kidney, pancreas, liver, intestine, stomach, mesentery, gonad and skeletal muscle of *S. epidermidis*-infected fish. These particles were identical in morphology to the apoptotic bodies observed in this study. Apoptotic bodies were easily distinguished from melanosomes by both their morphology and their location in the cell. Melanosomes, observed under TEM, are oval to long, rod-shaped structures with a single-layer membrane and they are usually scattered throughout the cytoplasm. Apoptotic bodies are double membrane-bound, round to oval in shape, and of varying size, and they are usually concentrated together in the centre of the cell. Moreover, the apoptotic cell usually has a denser cytoplasm than the melanocyte. Apoptotic bodies can also be distinguished from *S. epidermidis*

since the cell wall of the bacterium is very distinctive. Those electron-dense particles found in various tissues in naturally infected tilapia with *S. epidermidis* were considered to be apoptotic bodies.

The supernatant of *S. epidermidis* was shown to induce tilapia cell apoptosis and cause TO-2 cell death. This suggests that the pathogenicity of the bacterium was due to the toxicity of the bacterial product that induced the apoptosis. The functional components of this entity are still unknown and need to be investigated further.

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