Involvement of Apoptosis in the Endotoxemic Lesions of the Liver and Kidneys of Piglets

Yasuyuki NAKAJIMA, Osamu MIKAMI, Miyako YOSHIOKA, Shozo ARAI, Katsuhiro MIURA, Yasushi KOIKE*, Masumi SATO*, Masaru KOBAYASHI and Emiko NAKAJIMA¹⁾

National Institute of Animal Health, 3 Kannondai, Tsukuba 305–0856, and ¹⁾STAFF Institute, Kamiyokoba, Tsukuba 305–0854, Japan

(Received 6 October 1999/Accepted 23 February 2000)

ABSTRACT. The involvement of apoptosis was evaluated in lesions of endotoxemic piglets. A single injection with *E. coli* O111:B4 lipopolysaccharide (LPS) induced foci of coagulative necrosis in the liver and kidneys. No significant change was observed in these organs at 1.5 hr after LPS injection, but at 6 hr, epithelial cells with chromatin condensation or fragmentation and apoptotic bodies were visible. Foci of coagulative necrosis were formed within 24 hr after LPS inoculation. In and adjacent to the necrotic foci, dead hepatocytes with nuclear condensation or fragmentation were scattered. These dead cells were positively stained by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) methods. Electronmicroscopy revealed apoptotic cells with condensed or fragmented homogeneous nuclear chromatin, and necrotic cells with irregularly destroyed nuclei and cytoplasmic membranes. Apoptotic cell death were also observed in parietal cells of the stomach and lymphocytes in the lymphatic system. DNA ladders with approximately 200-bp multimers were observed in hepatic, renal and thymic samples prepared after 6 and 24 hr of LPS injection by agarose gel electrophoresis. These results suggest that apoptosis is involved in the pathology of swine endotoxemia.—**KEY WORDS:** apoptosis, endotoxemia, hepatic necrosis, piglet.

J. Vet. Med. Sci. 62(6): 621–626, 2000

In the domestic swine industry, acute and chronic gram negative-bacterial infections cause severe damage. A common component of the cell wall of gram-negative bacteria, lipopolysaccharide (LPS), induces diarrhea, edema in the lung and/or gastrointestinal tract, mononuclear cell infiltration and fibrosis in the lung, and/or systemic lymphatic degeneration [6, 14, 16]. Focal necrosis in the visceral organs, such as the liver or kidneys, frequently occurs with multiple fibrin microthrombosis by LPS injection [13]. The pathophysiology is not due to direct toxicity of LPS but to cytokines induced through LPS stimulation [5, 11]. The kinetics of endogenous tumor necrosis factor (TNF) are related to the occurrence of disseminated intravascular coagulation and focal necrosis in the visceral organs during endotoxemia of piglets [13].

Both accidental cell death (necrosis) and suicidal cell death (apoptosis) are possible mechanisms of organ damage and failure [2], because these two types of cell death were observed in necrotic lesions in the case of infectious disease or intoxication [7, 17]. Apoptosis is characterized by DNA fragmentation by the action of endonucleases synthesized in the affected cell itself [10]. In mouse models, apoptosis in the liver is induced by LPS through the interaction of TNF, or through the Fas-Fas ligand system in association with hepatic focal necrosis [8, 9, 15]. The effects of LPS differ depending on the animal species. In swine, lymphocytic depression by LPS has been shown to be related to apoptosis [12, 14]. However, whether apoptosis

is involved in the major organ lesions during endotoxemia has been unclear in swine [5]. In this study, we evaluated the involvement of apoptosis in the visceral organ lesions of piglets with endotoxemia.

MATERIALS AND METHODS

Inoculation: *Escherichia coli* LPS (O111: B4, Difco, Detroit, MI, U.S.A.) at 100 µg/kg of body weight was dissolved in 5 ml of sterile 0.85% saline solution. The control used was 5 ml of sterile 0.85% saline solution. Seven 1-month-old Landrace piglets were used. To allow gentle sampling, a cannulation with a polyurethane catheter was inserted into the jugular vein 5 days before LPS injection, as previously described [13]. Five of the seven piglets were injected with LPS. One of the piglets was died of shock at 1.5 hr after injection and necropsied. One was necropsied at 6 hr by bleeding under anaesthesia, and the remaining three were necropsied at 24 hr. The other two served as control and were necropsied at 24 hr. The experiments were conducted according to the guidelines for animal experiments of the National Institute of Animal Health.

Histopathology and in situ labeling for DNA fragmentation: Specimens from various organs were fixed in 10% buffered formalin. Paraffin sections were stained with hematoxylin and eosin, phosphotungstic-acid hematoxylin, or a kit (Apop Tag, Oncor, Gaithersburg, MD, U.S.A) for TUNEL [1]. For electron microscopy, the liver and kidneys were double-fixed in 0.25% glutaraldehyde and 1% osmium tetroxide and embedded in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate, and observed with a JEM-100CX (JEOL,

* PRESENT ADDRESS: KOIKE, Y., Kumamoto Central Livestock Hygiene Center, 1666 Shidumenihata, Shimoituki, Kumamoto 961–4215. SATO, M., Kyusyu Branch, National Institute of Animal Health, Chuzan, Kagoshima 891–0105, Japan.

Tokyo, Japan).

Agarose gel electrophoresis: Specimens from the liver, kidneys and thymus were frozen at -30°C . The tissues were homogenized in 1 ml of a DNA isolation reagent (DNA ZOL, Life Technologies, Grand Island, NY, U.S.A.). The lysates were centrifuged at $10,000 \times g$ for 10 min at 4°C , and the DNA in the supernatant was precipitated in 100% ethanol and resuspended in distilled water. The DNA solution was treated with 5 mg/ml Rnase A for 2 hr at 50°C , and was then electrophoresed at 100 V for 30 min in 2% agarose gel (Life Technologies, Grand Island, NY, U.S.A.). The gel was stained in 0.5 $\mu\text{g/ml}$ ethidium bromide solution for 15 min. DNA bands were visualized by fluorescence under UV light.

RESULTS

Histopathological lesions: At 1.5 hr after the injection, mild cloudy swelling of the hepatocytes was observed with swelling of the sinusoidal cells, and white blood cells in the sinusoids increased in number. After 6 hr, hepatocytes with chromatin condensation near the nuclear lining or fragmented nuclear chromatin, and apoptotic bodies originating from hepatocytes were scattered mainly around the central veins, as well as were mildly formed microthrombi (Fig. 1). Similar apoptotic changes were present in the renal tubules and lymphocytes in the thymus and intestine. Fragmentation of nuclear DNA was detected in these cells by the TUNEL method, but the frequency was low.

Twenty-four hr after the LPS injection, foci of coagulative necrosis with microthrombosis had been formed in the liver and kidneys in 2 piglets, but the other had a minimal degenerative change. The foci of coagulative necrosis in the liver were centrilobular. In

Fig. 1. Foci composed of apoptotic bodies (arrows) and hepatocytes with nuclear condensation (arrow heads) are scattered in the liver 6 hr after LPS injection. Liver. H-E. $\times 630$.

some hepatocytes in and around the necrotic foci, segregation of nuclear chromatin attached to the nuclear membrane and apoptotic bodies were observed (Fig. 2A). Also, nuclei of some hepatocytes showed irregular chromatin fragmentation or had been disappeared. The cytoplasm of these hepatocytes were swollen. Fragmentation of nuclear DNA was detected in and around the necrotic foci by the TUNEL method (Fig. 2B).

In the kidneys, foci of coagulative necrosis were also formed mainly in the proximal tubules, and apoptotic or dead cells with compacted or fragmented nuclear remnants often sloughed off into the lumen (Fig. 3A). The lesions

Fig. 2. In and adjacent to the necrotic foci, many apoptotic hepatocytes with fragmented or condensed nuclei (arrows), as well as the necrotic hepatocytes (arrow heads), are seen 24 hr after LPS injection (A: Liver. H-E. $\times 400$). TUNEL-positive nuclei showing DNA fragmentation are scattered in the necrotic foci (B: Liver. TUNEL. $\times 400$).

Fig. 3. Apoptotic epithelial cells with condensed or fragmented nuclei can be seen in the renal proximal tubules 24 hr after LPS injection (A: Kidney. H-E. $\times 400$). Many epithelial cells in the tubules show DNA fragmentation (B: Kidney. TUNEL. $\times 400$).

was less severe than in the liver, but the nuclear morphology of affected cells was similar to that found in the liver. TUNEL-positive nuclei were scattered in these necrotic foci and in the glomeruli (Fig. 3B).

Similar apoptotic lesions were seen in the gastric mucosa, mainly parietal cells in the base were affected (Figs. 4A to D). The lymphatic organs such as the thymus spleen and lymphnodes also showed nuclear DNA fragmentation by the TUNEL method.

Ultrastructural morphology: After 24 hr, two types of cell death with different morphologies were observed in the necrotic foci. Necrotic hepatocytes with irregularly clotted chromatin and degenerated nuclear membranes, and destroyed organelles in the cytoplasm were observed dominantly. Clotted chromatin attached to the nuclear membrane was occasionally observed in the hepatocytes, and some of these nuclei showed a half-moon appearance composed of homogeneously condensed chromatin and flocculated chromatin (Fig. 5A). The cytoplasmic organelles and membrane of the apoptotic hepatocytes were frequently swollen or degenerated. The lesion of apoptotic and necrotic hepatocytes occasionally were infiltrated by neutrophils. Fibrin strands, platelets and thrombocytes were often observed in the sinusoids.

In the renal tubules, apoptotic epithelial cells with compacted or segregated nuclear chromatin, compacted and shrunken cytoplasmic organelles, and loss of microvilli were also observed. Such apoptotic cells could be engulfed by adjacent cells or sloughed off into the lumen (Fig. 5B). Necrotic cells with irregularly clotted nuclear chromatin and destroyed cytoplasmic organelles were also observed.

Detection of oligonucleosomal DNA fragmentation: The agarose gel electrophoresis patterns of hepatic, renal and thymic samples prepared after 6 and 24 hr of LPS injection showed DNA ladders with approximately 200-bp

multimers (Fig. 6). Though the patterns of renal sample were faint according to the size of the lesions, the ladder was able to be distinguishable.

DISCUSSION

In the present study, morphologically typical necrotic and apoptotic cells were present in the necrotic foci of the liver and kidneys at 24 hr after the LPS injection. Apoptotic cells were dominantly observed in the gastric mucosa. The detection of DNA fragmentation by TUNEL and the presence of characteristic nuclear morphology confirmed the occurrence of apoptosis in the liver and kidneys during endotoxemia of piglets. This suggests that apoptosis is associated with or precedes the occurrence of coagulative necrosis in the endotoxemic lesions of the piglets. Since duration of apoptosis is very short and the affected cells are immediately engulfed by adjacent cells or phagocytized by macrophages [2, 10], the presence of apoptotic cells suggests a delay or disturbance in the clearance of these damaged cells in the liver and kidneys. The ruptured or swollen microorganella in the cytoplasm of cells with apoptotic nuclei suggest a condition of secondary necrosis.

Since apoptosis is characterized by controlled autodigestion of the affected cells [17], the occurrence of the apoptosis in the liver and kidney may function to weaken the organ damage by deletion of the affected cells. On the other hand, sequential inflammatory responses could be induced against the damaged tissues by the release of cellular component from damaged hepatic or renal tissues. Such responses could accelerate to develop the necrotic foci, coupled with action of the infiltrated neutrophils and an ischemic condition as suggested by the microthrombi. The significance of the apoptosis in the gastric lesions

Fig. 4. Many gastric epithelial cells (mainly parietal cells at the base) show apoptotic nuclear morphology of condensed chromatin attaching to the nuclear membrane (arrows) 24 hr after LPS injection (A: Stomach. H-E. $\times 400$). The epithelial cells show DNA fragmentation (B: Stomach. TUNEL. $\times 400$). The control shows no apoptotic morphology (C: Normal stomach. H-E. $\times 400$) and negative for TUNEL (D: Normal stomach. TUNEL. $\times 400$).

appears to be that it prevents the release of proteolytic enzymes or stomach acids from the epithelial cells to minimize organ toxicity [17]. Apoptosis also occurs in the thymus and other lymphatic organs of piglets after intravenous injection of endotoxin [14]. Our results may explain the event that the apoptosis in the lymphatic organs could damage immune functions of piglets through deletion of T-lymphocytes during and after endotoxemia.

The mechanisms that cause apoptosis in the present piglet model are not clarified, but possible ones include the actions of TNF or Fas-Fas ligand system [3, 8, 9, 15], and local ischemia due to microthrombosis and hemorrhage [13]. Of these, the most likely factor is TNF. Excessive production of systemic TNF result in shock, hemorrhage, and tissue injury in various organs [4]. Endogenous TNF activity induced by LPS is related to the severity of hepatic necrosis in endotoxemic piglets [13]. TNF induced

apoptosis in cultured hepatocyte from piglets in combination with D-galactosamine (unpublished). In murine shock models [8, 9], apoptosis of the hepatocytes induced by LPS precedes massive hepatic coagulative necrosis, and TNF is involved in the induction of the lesion. Local ischemia associated with disseminated intravascular coagulation may cause coagulative necrosis in organs, but multiple microthrombosis does not always precede to focal necrosis in the liver or kidneys of piglets during endotoxemia [13]. Fas-Fas ligand system also induce hepatocyte apoptosis [15], but LPS causes hepatocyte apoptosis in Fas deficient *lpr* mice [8].

ACKNOWLEDGEMENTS. We thank Dr. Y. Ando and Mr. T. Fujisawa for their help in preparing the photographs. This work was partly supported by a grant from the Ministry of Agriculture Forestry and Fishery of the

Fig. 5. Twenty-four hr after LPS injection, a typical apoptotic nucleus of a hepatocyte with a half moon-appearance (Ap) is visible. The cytoplasm shows secondary necrosis in organelles. A neutrophil (N) is seen in lower right (A: Liver. electron microscopy. $\times 4,800$). An apoptotic cell with fragmented nuclear remnant (Ap) is visible in the cytoplasm of a renal proximal tubule epithelial cell (B: Kidney. electron microscopy. $\times 3,200$).

Fig. 6. DNA ladder formation. Samples from the liver, kidney and thymus from a piglet 6 hr after LPS injection. Agar gel electrophoresis.

Japanese Government.

REFERENCES

1. Ansari, B., Coates, P.J., Greenstein, B.D. and Hall, P.A. 1993. *In situ* end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. *J. Pathol.* 170: 1–8.
2. Alison, M.R. and Sarraf, C.E. 1994. Liver cell death: Patterns and mechanisms. *Gut* 35: 577–581.
3. Böhlinger, I., Leist, M., Gantner, F., Angermüller, S., Tiegs, G. and Wendel, A. 1996. DNA fragmentation in mouse organs during endotoxic shock. *Am. J. Pathol.* 149: 1381–1393.
4. Fong, Y. and Lowry, S.F. 1990. Tumor necrosis factor in the pathophysiology of infection and sepsis. *Clin. Immunol. Immunopathol.* 55: 157–170.
5. Ghosh, S., Latimer, R.D., Gray, B.M., Harwood, R.J. and Oduro, A. 1993. Endotoxin-induced organ injury. *Crit. Care Med.* 21: s19–24.
6. Kurtz, H.J. and Short, E.C. 1976. Pathogenesis of edema disease in swine: Pathologic effects of hemolysin, autolysate, and endotoxin of *Escherichia coli* (O141). *Am. J. Vet. Res.* 37: 15–24.
7. Ledda-Columbano, G.M., Coni, P., Curto, M., Giacomini, L., Faa, G., Oliverio, S., Piacentini, M. and Columbano, A. 1991. Induction of two different modes of cell death, apoptosis and necrosis, in rat liver after a single dose of thioacetamide. *Am. J. Pathol.* 139: 1099–1109.
8. Leist, M., Gantner, F., Böhlinger, I., Tiegs, G., Germann, P.G. and Wendel, A. 1995. Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. *Am. J. Pathol.* 146: 1220–1234.
9. Leist, M., Gantner, F., Jilg, S. and Wendel, A. 1995. Activation of the 55 kDa TNF receptor is necessary and sufficient for TNF-induced liver failure, hepatocytes apoptosis, and nitrite release. *J. Immunol.* 154: 1307–1316.
10. Majno, G. and Joris, I. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.* 146: 3–15.
11. Morrison, D.C. and Ryan, J.L. 1987. Endotoxins and disease mechanisms. *Ann. Rev. Med.* 38: 417–432.
12. Nakajima, Y., Ishikawa, Y., Momotani, E., Takahashi, K., Madarame, H., Ito, A., Ueda, H., Wada, M. and Takahashi, H. 1991. A comparison of central nervous lesions directly induced by *Escherichia coli* lipopolysaccharide in piglets, calves, rabbits and mice. *J. Comp. Pathol.* 104: 57–64.

13. Nakajima, Y., Momotani, E., Takahashi, H., Ishikawa, Y., Ito, T., Kanesaki, M. and Madarame, O. 1995. Endogenous tumor necrosis factor (TNF) production and modification of pathological lesions in experimental *Escherichia coli* endotoxemia of piglets. *Vet. Immunol. Immunopathol.* 45: 45–54.
14. Norimatsu, M., Ono, T., Aoki, A., Ohishi, K., Takahashi, T., Watanabe, G., Taya, K., Sasamoto, S. and Tamura, Y. 1995. Lipopolysaccharide-induced apoptosis in swine lymphocytes *in vivo*. *Infect. Immun.* 63: 1122–1126.
15. Ogasawara, J., Watanabe-Fukunaga, R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T. and Nagata, S. 1993. Lethal effect of the anti-Fas antibody in mice. *Nature (Lond.)* 364: 806–809.
16. Olson, N.C., Dodam, J.R. and Kruse-Elliott, K.T. 1992. Endotoxemia and gram-negative bacteremia in swine: Chemical mediators and therapeutic considerations. *J. Am. Vet. Med. Assoc.* 200: 1884–1893.
17. Thompson, C.B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science* 267: 1456–1462.