Rapid Apoptotic Changes in the Gastric Glandular Epithelium of Rats Administered Intraperitoneally with Fusarenon-X

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ABSTRACT. Fusarenon-X (FX) 1.5 mg/kg was administered intraperitoneally (ip) to 6-week-old male Wistar rats for examination of pathologic effects on the glandular stomach. Rats ip-treated with sterilized physiological saline were used as control. FX-administered rats showed dilatation of the stomach with increased fluid contents after 1–4 hr. Light microscopically, a few apoptotic karyopyknosis were seen in chief cells in the basal region at 1 hr postadministration (PA) and mitotic inhibition was evident after 2 hr PA. Marked apoptosis of nuclear pyknosis and cytoplasmic inclusions in both zymogenic and oxyntic cells developed from basal to middle regions of the gastric mucous membrane at 2–4 hr PA with a peak at 3 hr. Apoptotic changes of differentiating neck cells and surface epithelia were less evident. Electron microscopy revealed that the chief cells were the main target of FX-induced apoptosis. The parietal cells were secondarily involved because they phagocytosed chief cell-derived apoptotic bodies. In situ detection of DNA breaks by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction revealed the positive nuclei after 1 hr PA, which increased with time and reached a peak at 3 hr PA, in accordance with apoptotic changes in histological study. Agarose gel electrophoresis of DNA isolated from the gastric mucosa of FX-administered rats showed ladder pattern of DNA fragments after 1.5 hr PA with the maximum distinctness at 3 hr PA. — KEY WORDS: apoptosis, DNA fragmentation, fusarenon-X, rat stomach, TUNEL.

Trichothecenes [2] are mycotoxins produced by various species of Fusarium molds which have been studied as the main causative agents of alimentary toxic aleukia in human [4, 8] and moldy corn toxicosis of farm animals [17]. Fusarenon-X (FX) [20], a potent trichothecene mycotoxin elaborated by Fusarium nivale [15], has strong cytoxicity to rapidly growing cells especially of lymphoid organs and intestines [14, 19] by inhibiting protein and DNA syntheses [12, 20]. In studying toxicity of intraperitoneally (ip) administered FX in mice, Shimizu et al. found rapidly developing hypoglycemia and cell death in the intestinal crypt cells after 2 hr postadministration (PA) [16]. In preliminary study of FX-intoxication, we have ascertained that the same kind of cell death is observed in rat stomach and that vulnerability of the stomach is higher in rats than in mice as reported formerly [14, 19].

Recent studies on apoptosis elucidated sensitivity of intestinal epithelium to many toxic substances [1, 7, 13]. However, FX-induced gastric lesions in the rat have not been elucidated yet as of apoptotic nature. In this paper, we describe pathological changes of the glandular stomach in rats ip-treated with FX in combination with detection of apoptotic endonucleolysis by TUNEL technique and DNA gel electrophoresis.

MATERIALS AND METHODS

Animals: Seventy-two male 6-week-old Wistar rats weighing 170 ± 10.0 g purchased from the Kumamoto Agency of Japan SLC Co., Ltd., Shizuoka, Japan, were devided into the respective experimental and control groups for examination of pathomorphologic changes and DNA fragmentation of the glandular stomach in FX-treated rats. They were kept in cages each containing 4–5 animals. A commercial breeder pellets for rats and mice (Japan Agricultural Products Co., Ltd., Yokohama) and tap water were given ad libitum. The room temperature and humidity during the experiment were conditioned 24 ± 1.0 °C and 50–60%.

Toxin administration: The experimental group was intraperitoneally (ip) administered with 1.5 mg/kg of FX (97.4% purity, Wako Pure Chemical Industries Ltd., Osaka) dissolved in sterile physiological saline at 0.5 mg/ml. The control group received ip 0.5 ml of saline.

Pathomorphological examination: Forty-eight rats, consisting of equal number of FX-treated and control groups, were sacrificed at 0, 0.5, 1, 2, 3, and 4 hr PA of the toxin by exsanguinating from the jugular vein under light ether anesthesia. The stomach with contents was removed and weighed using Mettler P1200 (Mettler Inc., Darmstadt, Germany). Data were analysed using Student’s t test. Tissues of glandular stomach were fixed in Bouin’s fluid or 10% neutral buffered formalin and processed for paraffin sectioning followed by hematoxylin eisin (HE) and periodic acid-Schiff hematoxylin staining for photomicroscopy.

For electron microscopy, small pieces of the glandular stomach were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffered solution pH 7.4 for 20 hr and postfixed with 1% osmium tetroxide in the same buffer for 2 hr. After dehydration through an upgrading series of ethanol and clarification by propylene oxide, the samples were embedded in the Epon 812 resin. Ultrathin sections stained with uranyl acetate and lead citrate were examined using a transmission electron microscope H-7000 (Hitachi Co., Ltd., Tokyo).

DNA fragmentation study: Twenty-four rats of the same
breed, age and sex were used. Each 3 FX-treated rats were sacrificed at 0, 0.5, 1, 1.5, 2, 3, and 4 hr PA by the same way as noted above and 3 non-treatment control rats at 0 hr. Small pieces of the glandular stomach were fixed with 4% paraformaldehyde (PFA) or 10% neutral buffered formalin solution and embedded in paraffin. For detection of apoptotic DNA strand breaks, paraffin sections were stained using a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method (In Situ Cell Death Detection Kit, POD, Boehringer Mannheim GmbH, Mannheim, Germany).

The remaining tissues of the glandular stomach at each experimental period were frozen on dry ice after rinsing with cold sterilized physiological saline for DNA extraction.

TUNEL histochemistry: Deparaffinized and rehydrated sections were incubated with 20 µg/ml proteinase K (Sigma Chemical Co., St, Louis, MO, U.S.A.) for 20 min at 37 °C. After being washed with distilled water for 5 min, the sections were rinsed with PBS for 3 min 3 times. They were immersed in blocking solution (0.3% H2O2 in methanol) for 30 min at room temperature, followed by rinsing with PBS for 5 min 3 times. Blocked sections were incubated with the reaction mixture of TUNEL in a humidified chamber for 30 min at 37 °C. The sections were washed with redistilled water for 4 min and with PBS for 5 min 3 times. Then the slides were incubated with DAB substrate solution at room temperature until desirable color intensity developed (5–10 min). After being washed with PBS for 3 min, the sections were counterstained with methyl green, dehydrated, clarified and mounted for light microscopy. Non-treated control tissue pretreated with DNase I (Sigma Chem. Co., St, Louis, MO, U.S.A.) and that eliminated TdT reaction were processed in the same staining procedure for positive and negative control, respectively.

Extraction of DNA and agarose gel electrophoresis: DNA isolation was performed according to a modified method of Gavrieli et al. [5]. After partially thawing the frozen tissue weighing 1–1.5 g, the gastric mucous membrane was scraped off from the smooth muscle with a stainless steel spatula and homogenized in 20 ml of DNA extraction buffer (100 mM Tris-HCl, pH 7.5, 20 mM EDTA, 100 mM NaCl), which was incubated overnight at 37 °C after mixing with 0.5% (w/v) sodium dodecyl sulfate (SDS) and 250 µg/ml proteinase K (Merck, Darmstadt, Germany). Then the DNA was purified with phenol and chloroform thrice in each purification and ethanol precipitation. The vacuum dried sediments were redissolved in TE buffer, and the DNA concentration was determined by the absorbance at 260 nm. DNA (about 50 µg) was subjected to electrophoresis for approximately 1.5 hr at 50 V through 1.6% agarose gel containing 10 µl of 10 µg/ml of ethidium bromide. BioMarker™ EXT (BioVentures, Inc., Murfreesboro, TN, U.S.A.) was used as the DNA molecular weight marker. DNA band (fragmentation) was visualized by a ultraviolet (312 nm) transilluinator and photographed.

RESULTS

FX-treated rats showed a remarkable dilatation of the stomach and intestines with increased fluid contents at 1–4 hr PA, during which the stomach weight with contents was significantly different from that of the control (Fig. 1).

Mitotic figures in the glandular stomach were normally seen in the neck cell region followed by more basal region. There were almost no changes at 0.5 hr PA. At 1 hr PA, mitotic figures in the basal region decreased and a few pyknotic nuclei were recognized in the chief cells (Fig. 2a).

After 2 hr PA, mitotic figures vanished from all regions of the gastric mucous membrane and a number of cells in the basal region showed karyopyknosis and various-sized spherical cytoplasmic inclusions with basophilic and/or eosinophilic components, often surrounded by a halo (Fig. 2b). Both chief and parietal cells were involved in this type of changes. At 3 hr PA, the above noted changes extended to the middle region and most of the chief cells and many parietal cells looked affected (Fig. 2c). At 4 hr PA, the characteristic cellular changes ceased to extend and the cytoplasmic inclusions showed decomposing appearance within the enclosing vacuoles and the chief cells looked

![Fig. 1. Changes of stomach weight with contents of FX-treated and control rats. Mean of the 4 stomachs was symbolized by a black square (FX) or black circle (control). Vertical bars indicate standard deviations. Significant difference: * P<0.05, ** P<0.01.](image-url)
Fig. 2a, 2b, 2c and 2d. Photomicrographs of the rat stomach tissue sections after ip administration of FX. HE. × 264. 2a. Scattered karyopyknotic cells in the basal region at 1 hr PA. 2b. Markedly increased karyopyknosis and various-sized spherical cytoplasmic inclusions with basophilic and/or eosinophilic components, often surrounded by a halo in the chief and parietal cells at 2 hr PA. 2c. At 3 hr PA, the cytoplasmic spherical shrinkage and nuclear fragmentation reach a peak, extending from the basal to the middle region, and most of the chief cells and many parietal cells look affected. 2d. At 4 hr PA, the characteristic cellular changes ceased to extend and the cytoplasmic inclusions show decomposing appearance within the enclosing vacuoles. The chief cells looked slightly decreased in number.

Fig. 6a, 6b, 6c and 6d. Photomicrographs of a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method (TUNEL) of the rat stomach tissue sections. TUNEL counterstained with methyl green. × 132. 6a. TUNEL positive nuclei indicating FX-induced endogenous nuclear fragmentation are sparsely scattered in the basal region at 1 hr PA. 6b. TUNEL-positive nuclei become numerous at 2 hr PA, extending from basal to middle regions of the glandular stomach. 6c. At 3 hr PA, the number of the positive nuclei reached a peak and they are heavily distributed in the middle region with extension to the basal and neck regions. 6d. At 4 hr PA, the positive nuclei slightly decreased in number, distributing mainly from middle to basal regions.
slightly decreased in number by this kind of cell death (Fig. 2d). During the course of these morphological changes, no infiltration of inflammatory cells was observed except for eosinophil infiltration seen at the base of the gland in all experimental and control animals. Pyloric glands were rather resistant to ip-treated FX intoxication. These changes were constantly seen in every experimental rat.

In electron microscopy of the stomach in FX-administered rats, there were only non-specific changes in the chief cells at 0.5 hr PA such as focal dilatation of rough endoplasmic reticulum and partial vacuolar breakdown of mitochondria. By scrutiny were observed early apoptotic changes with nuclear and cytoplasmic fragmentation in the basal chief cells at 1 hr PA (Fig. 3). There were compacted nuclear chromatin separated from homogeneous nucleoplasm and membrane-bounded spherical condensations of the cytoplasmic organelles. No morphological abnormalities were recognized in the capillary wall distributed in the gastric mucous membrane. At 2 hr PA, apoptotic bodies derived from chief cells were phagocytosed by chief cells and parietal cells. Fresh apoptotic shrinkage, condensation and fragmentation of the nucleus was also seen in chief cells, but not in the parietal cells (Fig. 4). These apoptotic cells became more numerous after 3 hr PA. Fresh nuclear changes peaked at 3 hr PA and decomposition of the apoptotic bodies in the vacuoles progressed at 4 hr PA (Fig. 5). Eosinophils occasionally had apoptotic nuclei. There were no changes in the gastric endocrine cells.

TUNEL histochemistry disclosed a distinct pattern of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeled nuclear staining in the uppermost surface epithelium throughout the observation period (Figs. 6a and 6c). Cells showing FX-induced endogenous nuclear fragmentation were sparsely scattered in the basal region at 1 hr PA (Fig. 6a), which became numerous at 2 hr PA extending from basal to middle regions of the glandular stomach (Fig. 6b). At 3 hr PA, the number of the positive nuclei reached a peak, the distribution of which centered in the middle region with extension to the basal and neck regions (Fig. 6c). At 4 hr PA, the positive nuclei slightly decreased in number, distributing mainly from middle to basal regions (Fig. 6d). Some animals showed almost the same number and distribution as seen in those at 3 hr PA. The rim of the intracytoplasmic vacuole containing remnants of apoptotic bodies showed positive reaction.

Electrophoresis of low molecular weight DNA extracted from the gastric mucosa of FX-treated rats disclosed that DNA fragmentation, shown as the ladder pattern, became faintly recognizable at 1.5 hr PA, distinct at 2 hr PA and very distinct at 3 hr PA, and at 4 hr PA less clear than that of 2 hr PA. Control and FX 0–1 hr PA showed no apoptosis-specific DNA cleavage (Fig. 7).

DISCUSSION

In the present paper, we demonstrated that FX-induced apoptotic changes in gastric mucosae ascertained by light and electron microscopy were consistent with nuclear DNA fragmentation proved by in situ TUNEL reaction and agarose gel electrophoresis.

The morphological studies showed that ip-administered FX could develop cell shrinkage and chromatin condensation in the mucosa of rat stomach, which began to appear at 1 hr PA, became numerous after 2 hr PA, and reached a peak at 3 hr PA. TUNEL reaction substantiated that the changes of distribution of positive nuclei coincided with those of the histological apoptotic cells, extending from basal to middle regions. Ladder pattern of the extracted DNA in agarose gel electrophoresis almost corresponded with changes of the number of the TUNEL-positive nuclei, although the first recognition was at 1.5 hr PA instead of 1 hr PA in TUNEL reaction. This may be due to a technical difficulty to gain enough quantity to form visible pattern at 1 hr PA. The decline of the ladder pattern at 4 hr PA may indicate that DNA with typical molecular weights decreased in amount because of its degradation. A slight decrease in TUNEL positive nuclei at 4 hr PA, especially at the basal region, might be attributable to decomposition of nick ends coincident with breakdown of apoptotic bodies in dilated phagosomal vacuoles.

Rapid development of FX-induced apoptosis from basal to middle regions of the gastric mucous membrane may be related to ip route of administration. Both in rats and mice, the intestinal crypt cell damage caused by sublethal toxic doses of FX was rapid and severe when administered ip, whereas it was slight to moderate when administered perorally (unpublished data). The apoptotic process might be triggered immediately after FX-administration, since FX is a direct cytotoxin showing contagious injury to the skin [18]. Another route of injury might be via blood, for the concentration in the blood was appreciably high in the first 2 hr, during which about 50% of intravenously injected [14C]-labeled trichothecene was rapidly absorbed and excreted in the urine and feces [11]. Thus, the neck cells, the most actively dividing cells in the stomach, might have shown a slower and lower susceptibility to ip-treated FX, protected by the distance from basal diffusion and hematogenous supply of the toxin.

Such a rapid extension of apoptotic areas is correspondent with the report that the apoptotic process takes about 3 hr to complete [3]. FX-induced gastric apoptosis seems to be too rapid to be ascribed to programed cell death, most of which begins 12 to 24 hr after administration of an initiating trigger [6].

Electron microscopic study revealed that the main target of the toxin was the chief cells, while the parietal cells, not involved in apoptosis by themselves, participated in phagocytosis of the chief cell-derived apoptotic bodies. The chief cell with higher synthetic function might have shown higher susceptibility to FX. The acid secreting parietal cell was spared, although pH at the surface of the fundic gland elevated after 2 hr PA (data to be published). The difference of susceptibility found in the chief and parietal cells in FX-induced apoptosis remains to be clarified.
Fig. 3. Electron micrograph of a rat stomach at 1 hr PA. Early apoptotic changes with nuclear fragmentation and cytoplasmic shrinkage in the basal chief cells. Note compacted nuclear chromatin and membrane-bounded spherical condensations of the cytoplasmic organella. Uranyl acetate and lead citrate stain. Bar = 2 \( \mu m \). B = basement membrane and the connective tissue at the base of the gland.

Fig. 4. Electron micrograph of a rat stomach at 2 hr PA. Apoptotic bodies derived from chief cells are phagocytosed by chief cells and parietal cells. Fresh apoptotic nuclear fragmentation and cytoplasmic shrinkage are seen in chief cells. Uranyl acetate and lead citrate stain. Bar = 2 \( \mu m \).

Fig. 5. Electron micrograph of a rat stomach at 4 hr PA. Note remarkable decomposition of apoptotic bodies phagocytosed by a parietal cell. Uranyl acetate and lead citrate stain. Bar = 2 \( \mu m \).

Fig. 7. 1.6% agarose gel electrophoresis of low molecular weight DNA extracted from gastric mucosae of rats treated with FX or saline. Lane M: DNA fragment size marker indicating 100 to 2,000 base pairs (bp); CON, FX0, FX0.5, FX1, FX1.5, FX2, FX3, FX4 represent no-treatment control, 0, 0.5, 1, 1.5, 2, 3, and 4 hr PA, respectively. From 1.5 to 4 hr PA, DNA fragmentation typical of apoptosis is observed, showing ladder pattern distinct at 2 hr, the most distinct at 3 hr. At 4 hr PA, the pattern becomes less obvious than that at 2 hr PA.
The TUNEL-positive nuclei in the uppermost surface epithelium of the rat stomach may be analogous to those found in the villus tip of rodent small intestine, which are morphologically intact cells going through the process of programmed cell death [5]. The distribution and number of these cells were not affected by FX-treatment.

Apoptosis seems to be the essential nature of FX-induced cytotoxicity in the gastrointestinal tract of rats, though at lethal doses areas of hemorrhage and necrosis appear in a longer observation period than the present one (own data). The cytotoxic mechanism of FX seems to have more similarity to cycloheximide or puromycin [12], both showing rapid crypt cell injury and mitotic inhibition through inhibition of protein and DNA syntheses, than cytosine arabinoside, adriamycin and other antitumor agents [1, 13].

Inhibition of protein and DNA syntheses, than cytosine arabinoside, adriamycin and other antitumor agents [1, 13].

Further study will be required on peroral administration which may reflect more natural intoxication of FX.

REFERENCES