Apoptosis of Mouse Pancreatic Acinar Cells after Duct Ligation

Kazuhiro ABE and Satoshi WATANABE

Department of Anatomy and Department of Surgery, Hokkaido University School of Medicine, Sapporo, Japan

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Summary. It has been established that pancreatic exocrine acinar cells disappear after pancreatic duct obstruction. This study aimed to examine the relationship between the disappearance of the acinar cells and apoptosis after pancreatic duct ligation of the splenic lobe in dd-mice, six weeks of age. In some mice, the ligature was removed after two or three days. In addition to general light and electron microscopic examinations on the pancreatic tissues, paraffin sections stained with the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method were observed to detect nuclear DNA fragmentation.

Pancreatic acinar cells underwent apoptosis initiated with nuclear DNA damages three days after duct ligation and were completely deleted by seven days. Due to the elevation of the intraluminal pressure, the acinar cells showed interrupted secretion of their zymogen granules and disorganization of their rough endoplasmic reticulum, causing the cessation of granule formation before apoptosis started. These cytoplasmic changes prior to apoptosis are reversible, as observed after removal of the ligation. Apoptosis of the acinar cells was identified by TUNEL-labeling of the nuclei, the condensation and margination of nuclear chromatin, and round fragmentation of cell bodies, all irreversible changes.

Apoptosis of acinar cells seemed to stimulate the proliferation of duct cells, which comprised the main cell components in the exocrine pancreas after the disappearance of acinar cells.

The acinar cells in the mouse pancreas have been shown to undergo marked degeneration after ligation of the pancreatic duct, completely disappearing from the pancreas distal to the ligated site within one week (WATANABE et al., 1995). This disappearance of the acinar cells resembles the process of apoptosis (KERR et al., 1972; WYLLIE et al., 1980). Studies on apoptosis as programmed cell death have mainly focused on nuclear changes following nuclear DNA fragmentation and the falling off of the cells (MCCONKEY et al., 1990; ZAKERI et al., 1993), but cellular changes before the initiation of DNA fragmentation in the nucleus have received scant attention (WALKER, 1987). The induction of apoptosis and the relationship between the cells undergoing apoptosis and the surrounding cells are also topics of interest. This study thus is designed to examine: 1) whether the disappearance of the acinar cells after duct ligation is due to apoptosis or necrosis of the cells, and if it is the former case; 2) how the cells might change before they die by apoptosis; 3) how this proceeds; and 4) how it influences the surrounding tissue.

The pancreatic exocrine cell represent an interesting subject of study as it possesses highly differentiated structures being filled with various organelles, and we already have some quantitative data of its morphological changes after duct ligation.

In order to prove the nuclear DNA fragmentation we used the method of 3'-OH nick end labeling of biotinylated dUTP (TUNEL) (GAVRIELLI et al., 1992).

MATERIALS AND METHODS

A total of 112 female dd-mice were used in this study. All animals were maintained on commercial pellet food and tap water ad libitum. Most of the animals were subjected to pancreatic duct ligation at six weeks of age. The animals were anesthetized with pentobarbital sodium (Nembutal®) injected intraperitoneally. The pancreas was exposed through a median abdominal incision, and the pancreatic duct from the splenic lobe, the largest lobe of the pancreas, was ligated under the dissecting microscope avoiding any damage to the vasculatures, as described elsewhere (WATANABE et al., 1995). In 25 animals, small silicon clips were used for obstruction of the duct; these clips were removed either two or three days after the first operation. The animals were killed at certain days during the postoperative two weeks. Seven normal animals were...
also used as controls. The pancreatic tissues from the splenic lobe distal to the duct obstruction, from the gastric or duodenal lobe in the operated animal as the operated control, and the pancreas from the normal control all served for light (LM) and electron microscopic observations (EM).

For light microscopy, the pancreas was fixed with a Zenker-formalin-acetic acid solution (a mixture of 18 volumes of Zenker’s fluid, 2 volumes of formalin and 1 volume of acetic acid), embedded in paraffin, serially sectioned, and stained with periodic acid Schiff (PAS) and hematoxylin. Paraffin sections of the tissue fixed with 2% paraformaldehyde-15% picric acid in 0.1 M phosphate buffer (pH 7.4) (Zamboni’s solution) were stained with the TUNEL method to detect nuclear DNA fragmentation and methyl green for nuclei. Some pancreases were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and embedded in the epoxy resin mixture. Semithin sections 1 µm thick were stained with toluidine blue for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate for transmission electron microscopy.

RESULTS

Morphological changes after duct ligation

Normal acinar cells
The normal acinar cells were pyramidal in shape, and their apical half was occupied by zymogen granules (Fig. 1a, e). In paraffin sections stained with PAS and hematoxylin, the apical cytoplasm appeared foamy because of the negative stainability of zymogen granules for PAS (Fig. 1a). The basal half of the cytoplasm was basophilic by LM. This ergastoplasm was occupied with well developed and parallel arranged cisterns of the rough-surfaced endoplasmic reticulum (rER), as seen by EM (Fig. 2a). The lumen of the acini was very small.

Pancreas with ligated duct
First day after ligation: In the exocrine pancreas distal to the ligature, all the acini showed a decrease in the height of the acinar cells and a slight enlargement of the lumen one day after ligation. The acinar cells were filled with smaller, PAS-positive zymogen granules except for the narrow basal cytoplasm. Zymogen granules of decreased size and elevated density were abundant in the apical cytoplasm as it was evident in both semithin sections stained with toluidine blue and ultrathin sections under the electron microscope (Fig. 2b). A few basophilic globules, which were round lamellations of rER cisterns, appeared in the basal cytoplasm of all acinar cells. These varied in size; the largest ones were as large as the nuclei. Other profiles of rER were often irregularly arranged in the basal cytoplasm (Fig. 2b). The basal ergastoplasm in the presence of rER was narrower than the control. The nuclei of the acinar cells appeared unchanged. The interstitial tissue surrounding the acini and lobules was edematous and infiltrated with many neutrophiles and a few monocytes.

On the other hand, the pancreatic tissue in the lobes with open ducts in the operated animals showed rather inconspicuous changes; the height of the acinar cells was only slightly decreased and the basal ergastoplasm with rER became slightly narrower.

Second day: The acini became smaller in size and the lumen, larger (Fig. 1b, f). The acinar cells showed a slightly narrower apical cytoplasm with zymogen granules. The granules were PAS-positive and localized in the cytoplasm under the luminal surface (Fig. 1b, f). The ergastoplasm was reduced to the narrower basal portion. The rER showed distinctive round lamellations (Fig. 2c). The infiltrating cells in the interstitium were mainly monocytes or macrophages.

The acini in the lobes with open ducts appeared almost intact.

Third and fourth days: The lobules of the pancreatic tissue were significantly decreased in size because of atrophic changes in the exocrine acinar portion. The size of the acini became smaller and the lumen was irregularly dilated (Fig. 1c, g). The acinar cells were smaller in height and appeared irregularly cuboidal. The zymogen granules decreased in number (Figs. 1g, 3a, b); the cytoplasm immediately beneath the luminal surface was PAS-positive, but some other cells showed no PAS-positive lining irrespective of the presence by EM of small granules (Fig. 3). The basophilic stainability of the basal cytoplasm became faint, but small numbers of basophilic globules of rER could still be seen.

The nuclei of the acinar cells showed the condensation and margination of chromatin against the nuclear membrane to various degrees (Figs. 2d, 3a–c). The euchromatin was paler as compared with the control. This pale euchromatin was clearly identified in semithin epon sections stained with toluidine blue (Fig. 1g). Pyknotic and/or fragmented nuclei appeared in some acinar cells on Day 3 (Figs. 2d, 3a–c). Fragmented round cell bodies were packed with degraded cell organelles, and often pyknotic nuclei or nuclear debris were seen in the adjacent acinar cells or macrophages (Figs. 2d, 3a–c). The fragmented cell bodies, apoptotic bodies, showed different degrees of degradation. Such degenerating acinar cells increased
in number on Day 4. Macrophages distended by the engulfment of degenerating cells also appeared in the epithelium (Figs. 2d, 3a-c). In the interstitium, many round macrophages were observed, some of which phagocyted degenerated epithelial cells (Fig. 3d).

As the acini deteriorated, the pancreatic duct cells became predominant in the lobules; they morphologically resembled the centroacinar or intercalated duct cells. Different from the normal control, the accumulated duct cells had irregularly shaped ducts were continuous with the remaining acini.

Fifth and seventh days: The exocrine acinar portion of the pancreatic tissue was further reduced while the lobules became shrunken; the acini almost disappeared, while the epithelial elements in the terminal portions of the exocrine pancreas were replaced with irregularly arranged ductal structures consisting of accumulated ductal cells, resembling

Fig. 1. Exocrine pancreatic tissue in the normal control mice and mice subjected to pancreatic duct ligation. a-d. Paraffin sections stained with PAS-hematoxylin. ×800. e-h. Semithin-Epon sections stained with toluidine blue. ×800. a and e. Control. Acinar cells in the control pancreas show a foamy apical cytoplasm (a) with zymogen granules (e) and form a narrow lumen (arrowheads). b and f. Second day after duct ligation. The apical cytoplasm of acinar cells appears PAS-positive, being filled with small granules (f). Basophilic round bodies are seen in the basal cytoplasm. The lumen is larger than the control (arrowheads). c and g. Third day after duct ligation. Acini appear irregular, having irregularly dilated lumen (arrowheads). Acinar cells are small and the zymogen granules are significantly decreased (g). Note the paler euchromation in the nuclei of the acinar cells than that in the free cells (arrows). m Mitotic figures. d and h. Seventh day after duct ligation. Acinar cells have almost disappeared, remaining only a few cells with small numbers of granules (arrowhead). The large lumen is formed by ductal cells. m Mitotic figures.
Fig. 2. Electron micrographs of exocrine pancreas. a. Control acinar cells. Note the small numbers of zymogen granules accumulated around the small lumen (arrow). b. Acinar cells one day after duct ligation. The number of granules has increased. Note irregularities in the size and density of the zymogen granules increased and irregular arrangement of rER cisterns. c. Acinar cells and a macrophage (lower right) two days after duct ligation. The height of acinar cells is lowered. The acinar cells contain variable sizes and densities of zymogen granules. A round lamellation of rER is seen (arrow). d. Acinar cells and a macrophage (lower right) three days after duct ligation. Paler nuclei, as compared with the nucleus of a macrophage, are surrounded by disarranged rER cisterns in the rounded acinar cell. Asterisks indicate fragmented nuclei. a-d: $\times 4,000$
the centroacinar and/or intercalated duct cells. They also possessed irregularly shaped lumens (Fig. 1d, h). The duct cells had poorly developed cell organelles. There existed some small numbers of the acinar cell-like cells on Day 5, but few on Day 7 (Fig. 1h). On Day 7, mitotic figures were often identified in the epithelium of the duct-like structures (Fig. 1d, h). The interstitium around the duct-like structures had many macrophages, sometimes containing nuclear debris, and fibroblasts with larger nuclei.

After 7 days: The pancreatic tissue became more involuted. The exocrine tissue consisted only of duct-like structures (Fig. 1d, h) and the amount of the exocrine tissue was considerably decreased. In the duct-like structures, degenerating duct cells were found.

Fig. 3. A variety of apoptotic acinar cells three days after duct ligation. fn Fragmented nuclei. Small numbers of zymogen granules (z) are seen in the cytoplasm just under the luminal surface (a, b). Profiles of rER are irregularly arranged. Intraepithelial macrophages (im) containing apoptotic cell debris of acinar cells are found in the epithelial lining (a, b). Cell debris is also found in the epithelial cells (arrows) (a, c). Extracellular macrophages with no notable ingested cell debris (em) (a, b) and distended with ingested cell debris of apoptotic cells (*em) can be noticed (d). a, c, d: ×4,000, b: ×5,000
Fig. 4. Pancreatic lobules stained with the TUNEL method and methyl green. The labeled cells are seen as dark dots. a. First day after duct ligation. A few labeled cells are seen. b. Third day after ligation. Many labeled cells are found. c. Fifth day after ligation. Small numbers of labeled cells are seen in the lobules consisting of duct cells. ×200

Fig. 5. Labeling index for TUNEL. Numbers of labeled cells in the epithelium (black) and extracellular cells (shaded) per 1000 epithelial cells have been obtained.
The islet of Langerhans, endocrine pancreatic tissue, histologically showed no remarkable changes.

**Result of the nick end labeling (TUNEL) (Figs. 4, 5)**

In the control pancreas, TUNEL-positive nuclei were not demonstrated. The labeled nuclei first appeared in the cells located in the acini of the splenic lobe distal to the duct ligation one day after the operation, although rare (Figs. 4a, 5). The labeled nuclei slightly increased in number on Day 2, while they were more remarkable on Day 2.5. They rapidly increased in number and were numerous on Day 3.5 (Fig. 4b). At this time, TUNEL-positive nuclei or nuclear fragments appeared in the acinar cells or macrophages infiltrated in the acini (Fig. 5). Macrophages with the labeled inclusions were frequently seen in interstitial tissue. On Day 3.5, when the incidence of labeled nuclei and cellular inclusions peaked, approximately half of the cells were found in the interstitium (Figs. 4b, 5).

The acinar cells with labeled nuclei decreased rapidly after the peak occurred; they particularly became scarce after 5 days. However, interstitial macrophages having labeled inclusions remained frequent even at these stages. The TUNEL-positive nuclei were also noticed in the duct cells of the duct-like structures after 5 days (Fig. 4c); they were still seen in the duct cells and interstitial macrophages even on Day 14.

**Reopening of the duct**

In the pancreas subjected to duct reopening two days after duct obstruction, the dilated lumen of the acini was again narrowed and positive staining of PAS disappeared in zymogen granules of acinar cells one day after reopening, whereas pyknotic nuclei were occasionally identified in the acinar cells at this stage. The acinar cells returned to normal two days after the second operation, but small numbers of pyknotic nuclei were still notable in acini and interstitial macrophages (Fig. 6a).

In the pancreas subjected to duct reopening three days after the first operation, degenerative processes of the exocrine pancreas proceeded to show many pyknotic nuclei of the acinar cells one day after duct reopening. Simultaneously, however, regenerative processes were also seen in acini one day after reopening. Particularly, small, round, newly regenerated acini were distributed throughout the exocrine pancreas two days after reopening (Fig. 6b).

**DISCUSSION**

In a previous study, we have stereologically quantified the changes in the numbers of acinar cells and duct cells following the pancreatic duct ligation and demonstrated that the numbers of acinar cells do not change until three days after the operation, there after rapidly decreasing in number until completely disappearing by seven days (WATANABE et al., 1995). The present histological and cytological studies indicate that the deletion of the acinar cells from the duct ligated pancreas is definitely due to apoptosis. TUNEL staining represented that nuclear DNA damages initiating apoptosis occurred between two and three
days. With the increase in the labeled nuclei of acinar cells, macrophages containing labeled inclusions increased in number in the epithelium and interstitium, suggesting that apoptotic bodies derived from dying acinar cells were ingested to be digested, resulting in the disappearance of the acinar cells. The acinar cells sometimes contained apoptotic bodies with TUNEL-positive nuclei, indicating that they also phagocytosed the degenerating cells. The nuclear and cellular changes in the mouse pancreas with a ligated duct were similar to the apoptosis occurring in the rat pancreas after duct ligation (Walker, 1987). The process of apoptosis in the mouse pancreatic acinar cells progressed according to the general changes in apoptosis (Kerr et al., 1972; Wyllie et al., 1984).

Apoptosis of the pancreatic acinar cells was induced by ligation of the duct. Various apoptosis-inducing circumstances have been established under embryological, physiological, immunological, and pathological conditions (Wyllie et al., 1980; Zakeri et al., 1993; Suzuki et al., 1994; Iwanaga et al., 1994). The dilation of the acinar and ductal lumens in the present experiment suggests that the intraluminal pressure is elevated by the obstruction of the flow of the secretion and may injure the acinar cell function to induce apoptosis. Zymogen granules were also accumulated in acinar cells two days after ligation, indicating that secretion of the granules was prevented. The granules, however, decreased in number on Day 2.5 and nuclear apoptotic changes occurred, leading to cell death on Day 3.

The present study clarified that there were reversible cellular changes in the cytoplasm of the acinar cells prior to irreversible nuclear changes and subsequent cellular apoptotic changes. Profiles of rER in the acinar cells showed an irregular arrangement soon after duct ligation and became rounded to form lamellar bodies in almost all acinar cells on Day 1. This change was more distinctive on Day 2. While the changes in rER proceeded, the zymogen granules in the acinar cells were first accumulated and then reduced in number. As stated above, the increase in the numbers of zymogen granules in acinar cells may be due to the elevation of intraluminal pressure to interrupt the secretion, whereas the following decrease in their numbers may be associated with disarrangement of rER to disturb its protein synthesis. These cytoplasmic changes in acinar cells occurring until two days after duct ligation are considered to be reversible because reopening of the clipped duct at this stage led to the recovery of the acinar cells.

However, reopening of the obstructed duct on Day 3, when TUNEL-positive nuclei abundantly appeared in the acinar cells, was not effective for recovery of the acinar cells. Subsequent histologic events have shown that the regeneration of acini is derived from duct cells, because small acini seen throughout the tissue were similar to the acini that appeared in the pancreas with duct reopening on Day 7, when the exocrine portion of the pancreatic tissue consisted only of duct cells (Anbo, 1993). Thus, the findings suggest that reversible changes in rER are followed with irreversible nuclear changes.

Corresponding to the decrease in the number of acinar cells, the remaining duct cells proliferate; the duct cell number doubles that of the control (Watanabe et al., 1995). Thus the decrease in the cell number by apoptosis may be associated with cell proliferation. This tendency is compatible with the rat pancreas after duct ligation (Walker and Pound, 1983) and other tissues (Abe and Takan0, 1989; Grassilli et al., 1992; Troiano et al., 1994). It may be reasonable to assume that the cell death stimulates cell proliferation for tissue regeneration. At present, the precise stimulating factors for acinar cell growth remain unknown. However, it is interesting that duct cells cannot differentiate to acinar cells under continuous obstruction of the draining duct. This may be associated with the fact that the proliferated duct cells also undergo apoptosis.

The present study allows the conclusion that cellular alterations in exocrine pancreas due to the duct ligation consist of reversible and irreversible events. As reversible events, the pancreas duct ligation interrupts the intraductal flow of the secrete, which is closely associated with the elevation of the intraluminal pressure in the acini and duct prevention of acinar cell secretion, and accumulation of zymogen granules in the acinar cells. Profiles of rER then greatly alter, forming round lamellar bodies which may correspond with the cessation of the granule formation, resulting in a decrease in the number of granules by probable continuous slow discharge of the granules. These changes proceeding within two days are reversible, since they recovered to normal states by removing the obstruction of the duct. On the other hand, TUNEL-positive nuclei appeared in acinar cells, which are considered to be linked with apoptotic cell death. It is interesting that the apoptosis of acinar cells stimulates the proliferation of the duct cells that also undergo apoptosis when no differentiation of duct cells into acinar cells occurs. The later changes in acinar cells occurring after three days of duct obstruction are irreversible.
REFERENCES


Prof. Kazuhiro ABE
Department of Anatomy
Hokkaido University School of Medicine
Nishi-7, Kita-15, Kita-ku
Sapporo 060, Japan

阿 部 和 厚
060 札幌市北区北 15 条西 7 丁目
北海道大学医学部
解剖学第三講座