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Histochemical and chronological analysis of mouse submandibular gland parenchyma subjected to abrupt reperfusion

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Summary. We examined the effects of abrupt reperfusion on the mouse submandibular gland parenchyma and determined the degree of recovery from tissue damage. A main trophic artery supplying the gland was ligated with silk thread, and the ligature was then released after a variable period. The gland was removed at various times after reperfusion and then examined immunohistochemically and ultrastructurally.

With reperfusion after 15 or 30 min of ligation, the tissue damage to the glands was slight or inapparent. With reperfusion after 1 or 3 h of ligation, collapse of the acini and the ducts was observed in parts of the lobules, but restoration of the parenchymal structures occurred, with the appearance of PCNA-positive cells, although there were differences in the level of restoration. After 6 h of ligation, most of the normal parenchymal cells had disappeared by the 5th and 7th days after reperfusion, and apoptosis and necrosis were present. These findings suggest that if interruption of the blood supply to the submandibular gland parenchyma is limited to within a few hours, then tissue repair after reperfusion is possible, although this will differ according to the level of damage, because the acini and the ducts reappear, probably with proliferation of parenchymal cells.

Key words: Abrupt reperfusion, Submandibular gland, Histological observation, Cell death, Tissue restoration

Introduction

Restoration of circulation in tissues and organs that have been subjected to interruption of blood flow is an obvious goal. However, studies have shown that overly-abrupt restoration of blood flow can worsen tissue damage (Egashira et al., 1992; Hoffmann et al., 1995, 1997; Hachiya, 1996; Matsumoto, 1997; Noda et al., 1998; Ono et al., 1998). It has also been reported that ischemic damage is caused more by a reduction in tissue oxygen level than by a decrease in blood flow rate. Moreover, the abrupt supply of oxygen to hypoxic tissues induces an excess of free radicals and the activation of neutrophils; as a result, the tissue damage progresses further (Hoffmann et al., 1995, 1997; Matsumoto, 1997; Ono et al., 1998; Nakajima et al., 2000; Shoyama et al., 2001). It has been shown in some organs that apoptosis is induced primarily in tissues subjected to reperfusion and is exacerbated by the generation of active oxygen and free radicals caused by the abrupt restoration of blood flow (Gottlieb et al., 1994; Hachiya, 1996; Nogae, 1996; Noda et al., 1998; Miura, 2001). However, there have been no detailed reports of the histological changes in salivary glands subjected to reperfusion. Therefore, we aimed to examine histochemically and chronologically the parenchymal structure of the mouse submandibular gland subjected to sudden reperfusion after interruption of flow in a main trophic artery.

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Materials and methods

Animals

All animal experiments followed the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. Male ICR mice aged 8 to 10 weeks were used. All of the mice were kept in plastic cages maintained at an ambient temperature of 23 °C with a 12-h light–dark cycle, and allowed free access to food and water.

Ligation and release of blood vessel

Following anesthesia of the mouse with an intraperitoneal injection of pentobarbital sodium, the main trophic artery supplying the right submandibular gland was isolated 2 to 3 mm away from the gland's hilus and ligated with silk thread (No. 6-0) under a

microsurgical microscope. The ligature was released at the end of the designated ligation period. Restoration of blood circulation was confirmed by a flowmeter. Ligation times were 15 and 30 min, and 1, 3, 6, and 12 h. The glands were removed when the animals were sacrificed 1, 3, 5, 7, 14, or 21 days after reperfusion.

Tissue preparation

The excised glands were immediately cut into small pieces. Some of the small pieces were fixed in 10% neutral formalin and then processed for paraffin embedding according to standard procedures. Other pieces were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M phosphate buffer, then post-fixed in 1% osmium tetroxide for 1 h and finally embedded in Epon. For comparison, samples from the glands of untreated mice were also prepared by the procedure described above.

Light microscopy

Samples that had been fixed in 10% neutral formalin were cut into 5-μm-thick sections.

Some of these sections were stained with alcian blue, pH 3.5, for examination of the histological architecture of the lobules in addition to the cell structure and the distribution of acinar cells. The sections were counterstained with Kernechrot.

Other 5-μm-thick sections were subjected to TUNEL staining using an apoptosis in situ detection kit (Wako, Japan). The sections were incubated with TdT and dUTP-digoxigenin without proteinase K pretreatment. Further incubation with anti-digoxigenin peroxidase sheep polyclonal antibody was carried out, and the reaction was developed using the DAB method. The sections were counterstained with Mayer's hematoxylin. Negative control sections were incubated with distilled water in the absence of TdT.

TUNEL-positive cells were detected in the acini, the granular duct, the intercalated duct and the striated duct in the unit area (0.28 mm^2) with a magnification of x 400. We calculated the total number of cells, because after a ligation time of 6 h, any clear difference in structure between the acini and the ducts was lost because of the damage. Three animals were used. The total number of TUNEL-positive cells in 6 areas in samples from one animal was counted, and then the average number of cells per area was calculated. The average value from all 3 animals was then calculated.

The remaining 5-μm-thick sections were immunostained with PCNA mouse monoclonal antibody PC-10 (DAKO JAPAN, Kyoto, Japan) for examination of proliferating cells. Biotinylated anti-mouse IgG+IgA+IgM polyclonal antibody (NICHIREI, Japan) was applied as the secondary antibody. The sections were incubated with streptavidin biotin peroxidase complex (NICHIREI, Japan), visualized by the 3,3'-diaminobenzidine (DAB) method and counterstained with Mayer's hematoxylin. The number of positive cells among 1500 each of acinar cells, granular duct cells, and duct-like structural cells in samples from three animals (500 per animal) were counted, and the rate of an appearance was calculated. The striated and intercalated ducts were excepted from measurement, because the distinction between them became difficult with the onset of tissue damage.

Electron microscopy

Thin sections post-fixed in osmium tetroxide were double-stained with uranyl acetate and lead citrate for examination under a JEOL 2000EX II transmission electron microscope. The main focus of observation was the changes in the structure of the parenchymal cells and, in particular, evidence of cell death.

Statistical treatment

Values acquired by TUNEL staining were assessed for statistical significance by Student's t test, with a P value of 1%.

Results

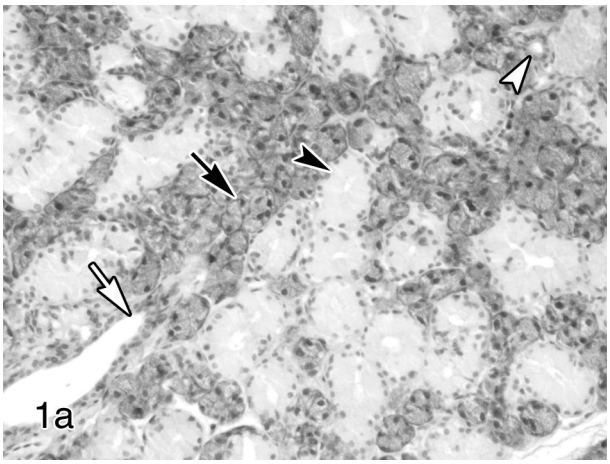
Light microscopy

Alcian blue staining

Ligation time 15 min: All acini and ducts from the 1st to the 21st day after release showed almost the same histological features as those in the glands of control animals (Fig. 1a). Accordingly, the influence of reperfusion following ligation was negligible.

Ligation time 30 min: The parenchyma, including the acini and ducts, looked almost normal in structure (Fig. 1b) at all observation times after release, although after the 3rd and subsequent days a small number of duct-like structures appeared in some lobules.

Fig. 1. Light micrographs of submandibular gland parenchyma with alcian blue staining. x 170. **a.** Control animal. Acinus: black arrow; granular duct: black arrowhead; striated duct: white arrow; intercalated duct: white arrowhead. **b**. Reperfusion time 3 days after 30-min ligation time. Glandular parenchyma looks almost normal. **c**. Reperfusion time 1 day after 1-h ligation time. Collapse of parenchymal cells and infiltration of migratory cells can be seen. **d.** Reperfusion time 7 days after 1-h ligation time. Duct-like structures (arrows) are seen to coexist with atrophied acini (arrowheads). **e.** Reperfusion time 14 days after 1-h ligation time. Normal acini and granular ducts have reappeared. **f.** Reperfusion time 21 days after 1-h ligation time. Duct-like structures (arrows) still exist. **g.** Reperfusion time 21 days after 6-h ligation time. Many connective tissue fibers have filled the lobule. **h.** Reperfusion time 1 day after 12-h ligation time. Severe collapse of glandular parenchyma is observed.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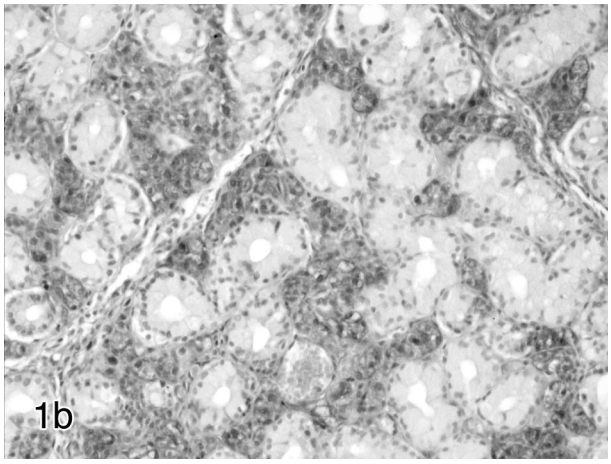
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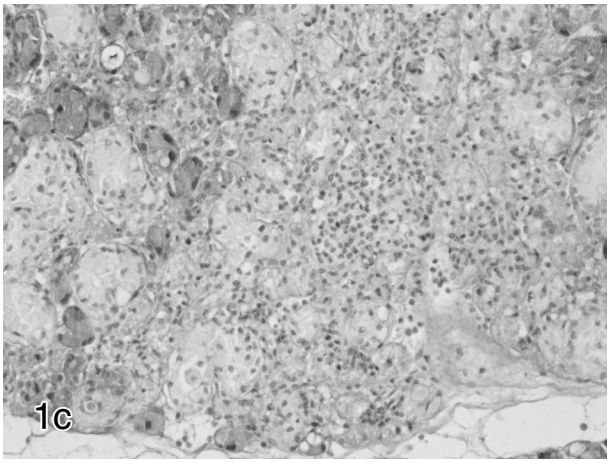
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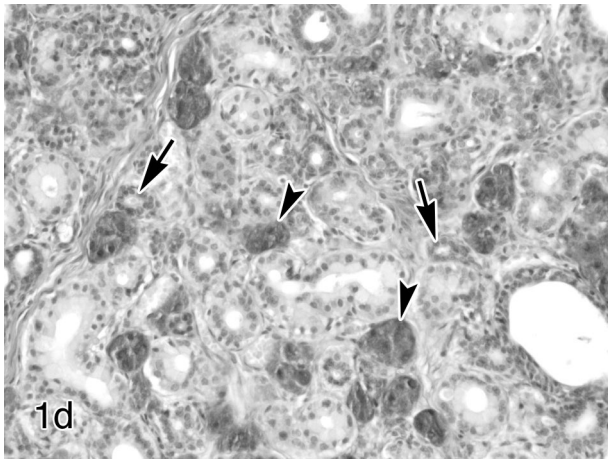
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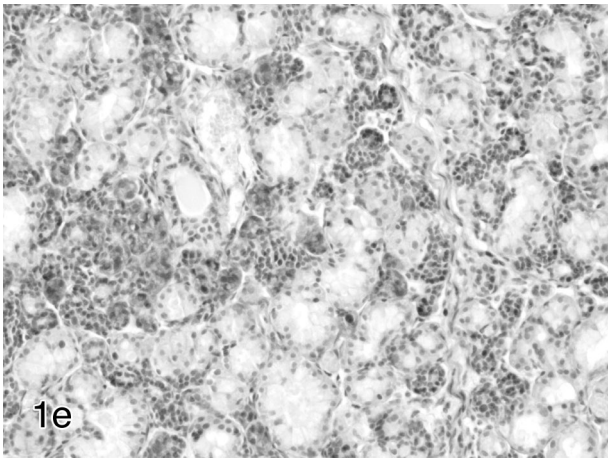
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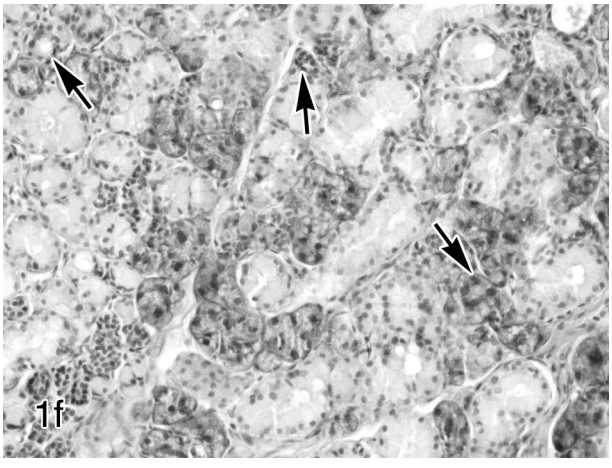
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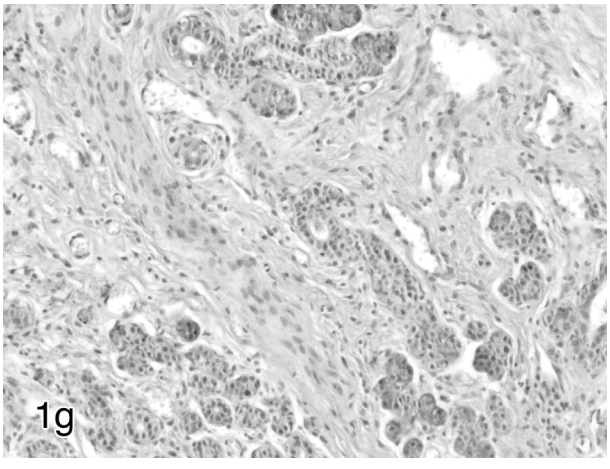
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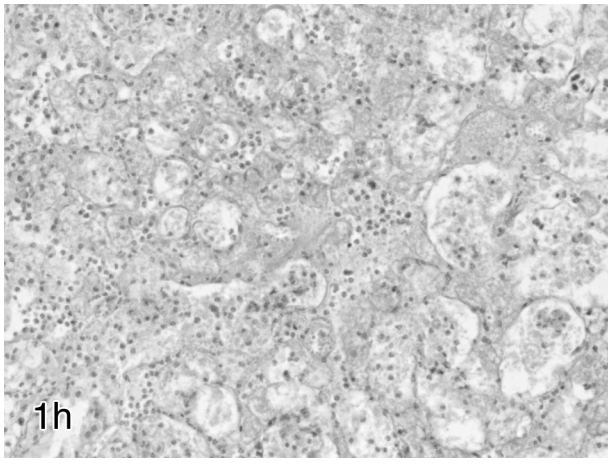
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Ligation time 1 h: On the 1st day after release (Fig. 1c), collapse of some parenchymal cells and infiltration of migratory cells, mostly neutrophils, were observed. On the 3rd day, a further increase in the numbers of migratory cells, atrophy of the acini, and extension of the lumina of the ducts were seen. On the 7th day (Fig. 1d), the appearance of duct-like structures and a further increase in atrophy of the acini were noticed. In addition, an increase in the quantity of connective tissue fibers in and among the lobules was seen. On the 14th day (Fig. 1e), normal acini and ducts began to appear. However, even on the 21st day (Fig. 1f), lobules with normal structure were not apparent because the duct-like structures were still present.

Ligation time 3 h: On the 1st day after ligature release, collapse of the acini and the duct and infiltration of migratory cells were observed extensively in the lobules in the center of the gland proper. On the successive 3rd, 5th, 7th, and 14th days, histological changes very similar to those seen at each respective interval after 1 h ligation occurred, but more extensive areas of the lobules were affected than after 1 h ligation.

Ligation time 6 h: On the 1st day after ligature release, collapse of the parenchymal cells and infiltration of migratory cells had already begun, as was seen after 3 h of ligation. On the 3rd day, formation of connective tissue fibers had progressed, and by the 5th and 7th days, the acini and the ducts had disappeared entirely, and the duct-like structures lay scattered in the peripheral regions of the gland proper. By the 14th and 21st days (Fig. 1g) almost all of the area of the gland was occupied by connective tissue fibers, and the duct-like structures were scattered in a few lobules, which had lost their original contour.

Ligation time 12 h: On the 1st day after release (Fig. 1h), severe damage had already extended to all of the area of the gland. It was difficult to define the various compositional elements of the parenchyma, such as the acini and ducts. Because we were unable to discern the existence of the gland proper, no further chronological observations of this sample were made.

TUNEL staining

Collapse of parenchymal cells was apparent from the 1st day after release in samples that had been subjected to at least 1 h of ligation, and we examined whether apoptosis participated in the death of these parenchymal cells. TUNEL staining showed a few positive cells after 1 h ligation (Fig. 2a) and more than twice as many cells after 3 h and 6 h (Figs. 2b, 3). When the ligation time reached 12 h, collapse of the parenchymal cells progressed much further, and checking for positive cells became difficult. Samples from untreated mice showed no TUNEL-positive cells.

PCNA staining

After ligation times of 30 min, 1 h, and 3 h, as the acini and ducts reappeared after release of ligation, we examined whether the proliferation of parenchymal cells participated in restoration of the glandular tissue.

Ligation time 30 min: We found several acinar cells and several granular duct cells positive for PCNA on the 5th day and on the 1st to the 5th day after release, respectively (Fig. 4a). However, by the 7th day the number of PCNA-positive acinar and granular duct cells had declined (Fig. 5).

Ligation time 1 h: PCNA-positive acinar and granular duct cells appeared from the 1st day after release, but thereafter the positive cells gradually decreased in

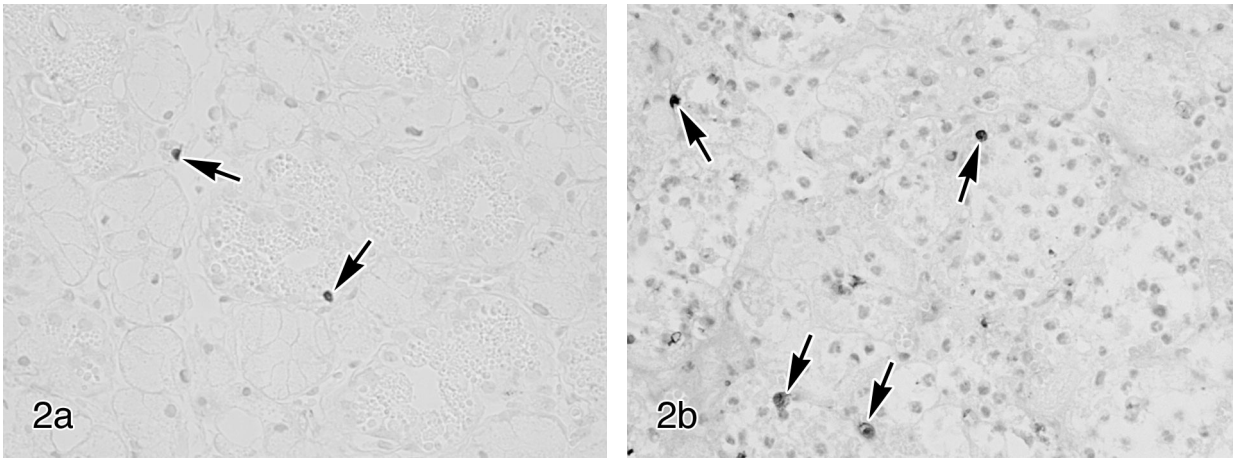

Fig. 2. Light micrographs of submandibular gland parenchyma with TUNEL staining. **a.** Reperfusion time 1 day after 1-h ligation time. There are a few TUNEL-positive cells (arrows). **b.** Reperfusion time 1 day after 6-h ligation time. There are more TUNEL-positive cells than in a (arrows). x 350

number each day. PCNA-positive cells were also detected in the duct-like structures and reached a peak on the 5th day after release (Fig. 4b). Afterward, PCNA-positive cell numbers in the duct-like structures gradually began to decline, but were still greater than the numbers of positive cells in the acini and the granular ducts (Fig. 5).

Ligation time 3 h: The number of PCNA-positive acinar and granular duct cells was greatest on the 1st day after release and decreased abruptly on the 5th day. PCNA-positive cells in the duct-like structures were as abundant on the 5th day after release as after a ligation time of 1 h, reached a peak on the 14th day (Fig. 4c), and then started to decrease in number by the 21st day (Fig. 5).

Ligation time 6 h: After 6 h of ligation, chronological observation was difficult because of severe damage to the glandular parenchyma, although a few PCNA-positive cells were found in the remaining duct-like structures. The acini and granular ducts from the untreated animals rarely contained PCNA-positive cells.

Transmission electron microscopy

Transmission electron microscopy was performed to clarify details of the apoptosis shown by TUNEL staining. Electron microscopy showed nuclei with the morphological features of apoptosis in the acinar and the duct epithelial cells, i.e., nuclei with highly condensed nuclear chromatin (Fig. 6a, b). In addition, electron microscopy demonstrated parenchymal cells with a necrotic appearance (Fig. 6c) because of swelling of the mitochondria, integration of rER, and rupture of the plasma membrane, although this could not be clarified by light microscopy.

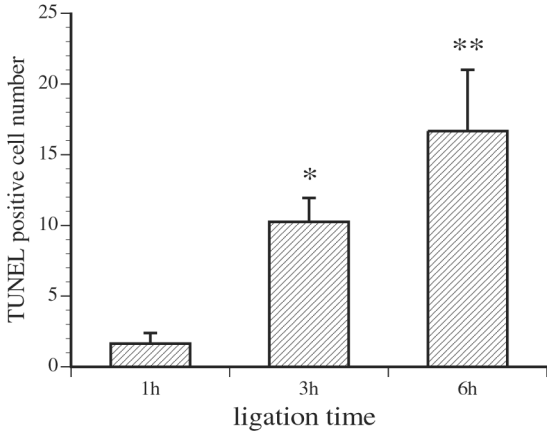

Fig. 3. Relationships between TUNEL-positive cell number and ligation time. Chronological changes in total number of TUNEL-positive cells in the acini, granular duct, striated duct, and intercalated duct 1 day after reperfusion of blood flow. 1h: vs 3h, p* <0.01; 1h vs 6h, p**<0.01

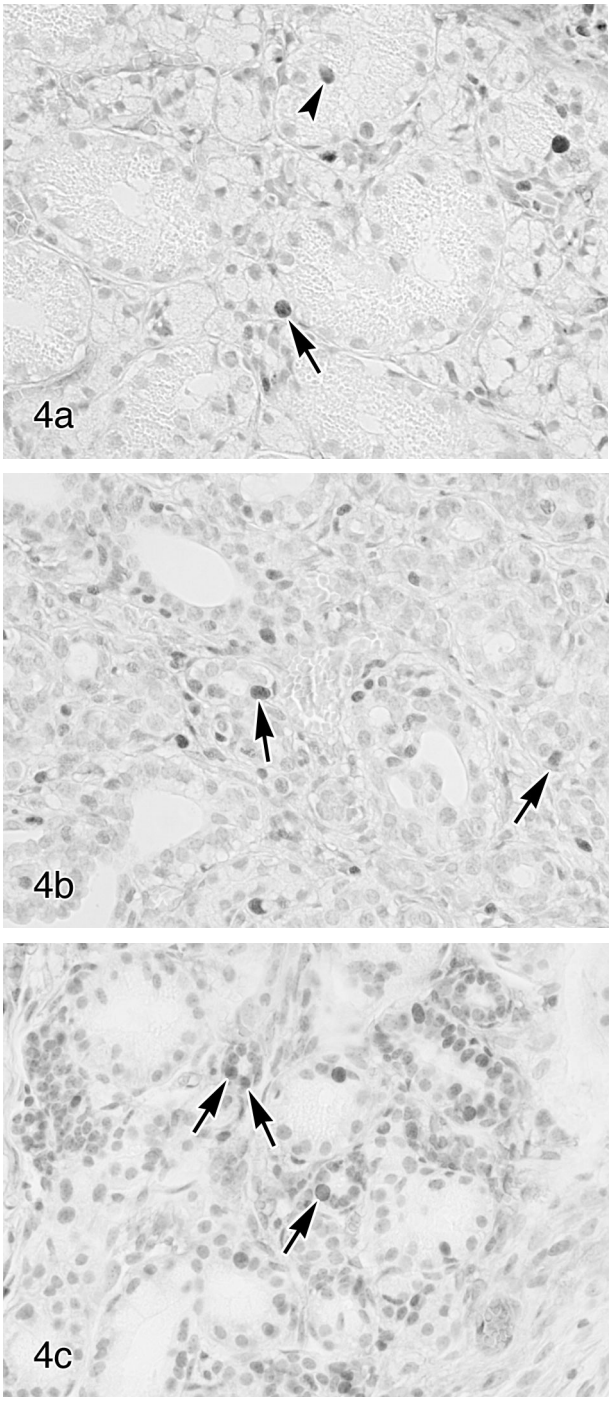

Fig. 4. Light micrographs of submandibular gland parenchyma with PCNA staining. **a.** Reperfusion time 5 days after 30-min ligation time. PCNA-positive cells can be seen in the acinus (arrow) and the granular duct (arrowhead). **b.** Reperfusion time 5 days after 1-h ligation time. PCNA-positive cells are visible in the duct-like structures (arrows). **c.** Reperfusion time 14 days after 3-h ligation time. PCNA-positive cells in the duct-like structures (arrows) are abundant. x 350

Discussion

Histological changes in the salivary gland parenchyma subjected to various periods of ligation of the main trophic artery have been reported (Standish and Shafer, 1957; Magoshi, 1998). Similarly, several reports have examined changes in the tissue of the salivary gland at various intervals after ligation of the main excretory duct (Burford-Mason et al., 1993; Burgess and Dardick, 1998; Magoshi, 1998; Takahashi et al., 1998;

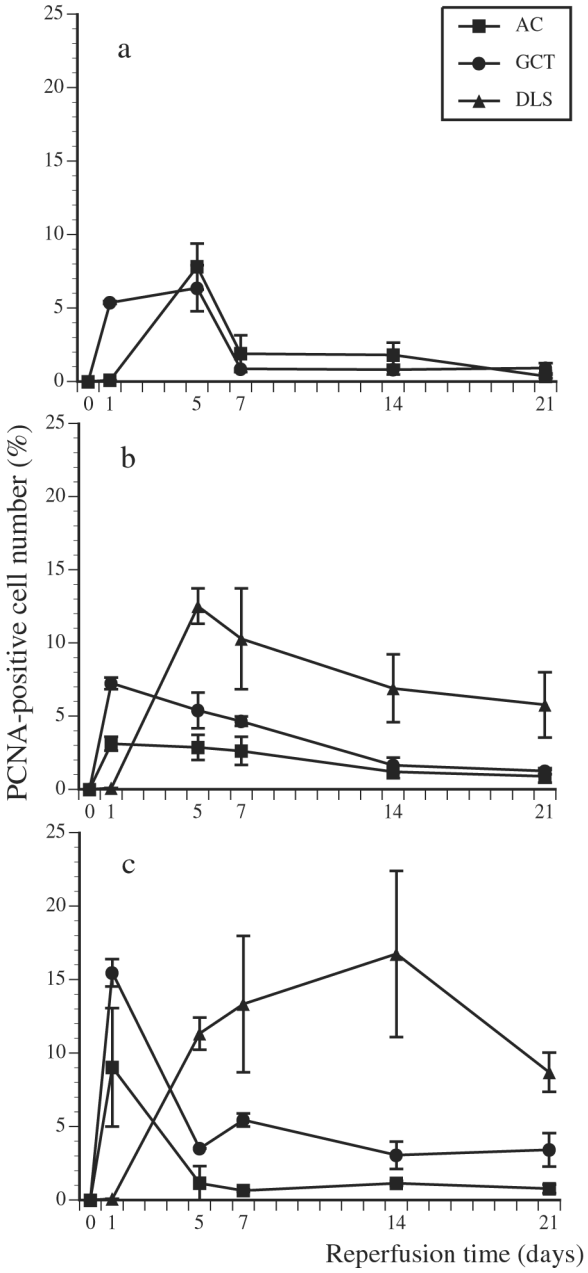

Fig. 5. Relationships between PCNA-positive cell number (%) and reperfusion time. 30-min ligation time **(a)**, 1-hour ligation time **(b)**, and 3-hour ligation time **(c)**. Chronological changes in the rate of appearance of PCNA-positive cells in the acinus (AC), granular duct (GCT), and duct-like structure (DLS) after reperfusion.

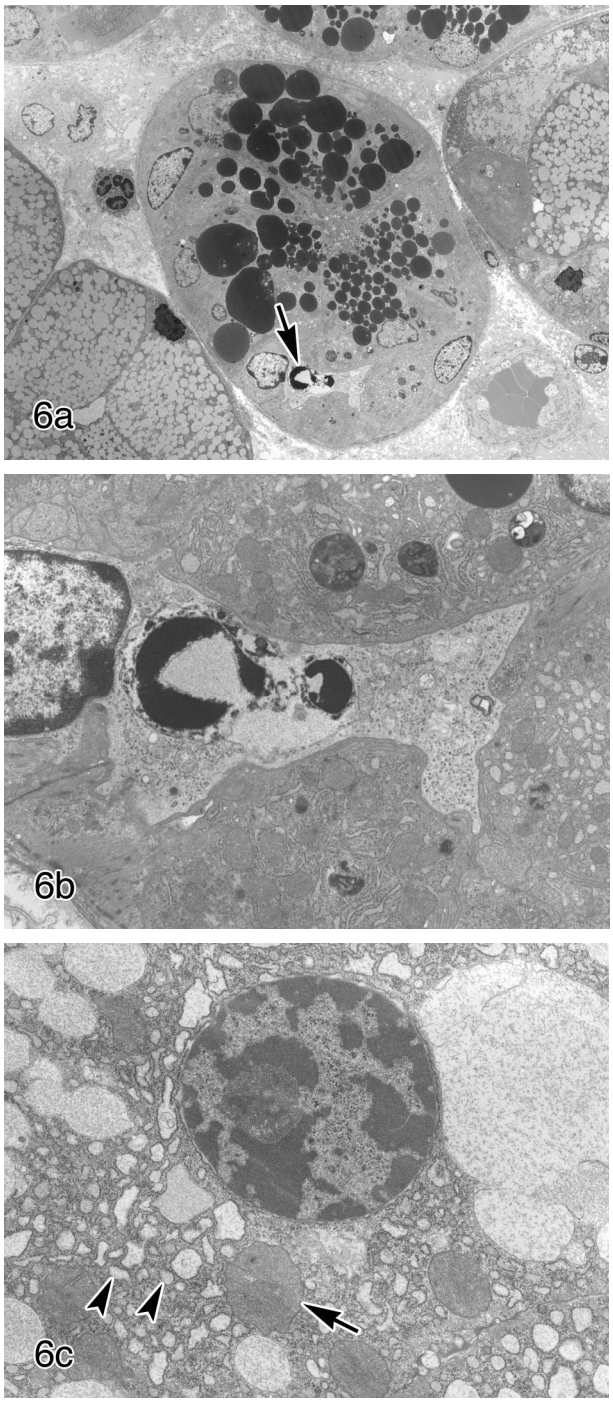

Fig. 6. Electron micrographs of submandibular gland parenchyma. **a.** Reperfusion time 1 day after 3-h ligation time. Granular duct with a cell (arrow) showing apoptotic features. x 900. **b**. Higher magnification of arrowed portion in a. x 5000. **c.** Reperfusion time 1 day after 6-h ligation time. Acinar cell showing necrotic features. Swelling of the mitochondria (arrow) and integration of rER (arrowhead) can be seen. x 10,000

Yagishita et al., 2001). Standish and Shafer (1957) demonstrated that the rat submandibular gland parenchyma after sustained ligation of a main trophic artery showed degenerative features as early as the first day after ligation, although a narrow rim of normal acini and ducts remained about the periphery of the gland proper on the 3rd day. In addition, the gland on the treated side became difficult to differentiate from the control on the 28th day, although there was a predominance of dilated ducts surrounded by a small island of connective tissue in the central areas of the gland or the smaller diameter of the gland. Magoshi (1998) have also described the survival of duct-like structures around the periphery of the rat submandibular gland proper on the 7th day after ligation of a main trophic artery and the subsequent appearance of acinar-like cells in addition to duct-like structures on the 21st day. In the present study, the rat submandibular gland parenchyma on the treated side also showed tissue damage and recovery to various extents according to the timing of ligation and reperfusion. However, the glands subjected to reperfusion after ligation for 6 h developed severe tissue damage, as a result of which the acinar cells did not reappear even by the 21st day. Furthermore, following ligation for 12 h, normal parenchymal cells were no longer evident even on the 1st day after release. Standish and Shafer (1957), who found normal acini and ducts in the peripheral region of the rat submandibular gland subjected to arterial ligation for 36 h, presumed that collateral vessels from the capsule continued to support the parenchymal cells arranged around the gland periphery. The degenerative change that was seen in the present study to extend throughout the gland after 1 day of reperfusion following 12 h of ligation might have been due to the peripheral parenchymal cells also being affected significantly by reperfusion. As a result, it was considered that collateral circulation from the capsule was not able to help the peripheral parenchymal cells.

In rat submandibular glands after ligation of the main excretory duct, Magoshi (1998) demonstrated that the acinar cells continued to survive in the peripheral region of the gland on the 3rd day after ligation, although with slight degeneration, and that normal acinar cells then reappeared on the 21st day. Yagishita et al. (2001) also reported the existence of acinar cells at the periphery of the rat submandibular gland on the 3rd day after ligation, and the appearance of duct-like structures on the 7th day. On the other hand, in the parotid gland, survival of amylase-positive cells and duct epithelial cells has been reported on the 7th day after ligation of the main excretory duct (Burford-Mason et al., 1993; Burgess and Dardick, 1998; Takahashi et al., 1998). In addition, striated ducts and a small number of acinar cells were also observed on the 28th day and 30th day after duct ligation, respectively. These previous studies of duct ligation seem to show survival of the parenchymal cells for 3 days or more after ligation, unlike the present study, in which all of the normal parenchymal cells disappeared after 1 day of reperfusion following 12 h of ligation. In addition, a new finding revealed by our study was that samples from tissues subjected to ligation times of 15 and 30 min showed slight or no damage, despite the abrupt reperfusion. Accordingly, we suggest that when the ligation time is short, there is little damage from sudden reperfusion.

TUNEL staining showed that apoptosis had already occurred after 1 h of ligation, and that the number of apoptotic cells increased with increasing ligation time. In addition, apoptotic cells were detected in samples from the 1st day after release, but were no longer apparent on the 3rd day after release (data not shown). Therefore, we consider that apoptosis following reperfusion arises more readily with extension of the ligation time and appears at an early stage after release of ligation. Hachiya (1996) found many TUNEL-positive cells in the skeletal muscles of the mouse hindlimb for a short time after reperfusion, and considered that this finding and an increase in the number of apoptotic cells was caused by the presence of an excess of free radicals generated by the abrupt restoration of oxygen supply. Remarkable development of apoptosis has also been demonstrated in the rat small intestine after reperfusion (Noda et al., 1998). The apoptosis seen in the mouse submandibular gland might also have been induced by the formation of free radicals. Transmission electron microscopy showed cell death with necrotic features in addition to apoptosis. Our results seem to indicate that death of the parenchymal cells in the mouse submandibular gland subjected to interruption and reperfusion of blood flow is brought out by apoptosis and necrosis.

One day after ligation of the trophic artery for 30 min, 1 h, or 3 h, PCNA-positive acinar and granular duct cells appeared in samples exposed to reperfusion. Therefore, we suggest that recovery of the glandular parenchyma with cell proliferation starts at an early stage after reperfusion. In addition, with a ligation time of more than 1 h, the epithelial cells of the duct-like structures exhibited a PCNA-positive reaction and increased in number, in contrast with the reduction in the number of acinar and granular duct cells. It has been reported that in the normal salivary gland, renewal of the acinar cells is achieved by proliferation and differentiation of the intercalated duct epithelial cells in addition to proliferation of the acinar cells (Zajicek et al., 1985; Man et al., 2001). On the other hand, in the damaged salivary gland, the appearance and role of duct-like structures has been noticed. Takahashi et al. (1998) described that the duct-like structures originate from the intralobular ducts and supply the precusor cells of the acinar cells. On the other hand, Scott et al. (1999) and Yagishita et al. (2001) demonstrated that the duct-like structures are derived from acini which have lost their secretory granules, and which could again recover to become normal acini. The duct-like structures in the present study may also participate in the tissue recovery of the rat submandibular gland parenchyma, probably accompanied by cell proliferation, because of the

appearance of PCNA-positive cells, although it was not possible to clarify the origin of the duct-like structures.

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