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Title: Participation of the Fas and Fas ligand systems in apoptosis during atrophy of the rat submandibular glands

Running title: Fas systems in salivary gland atrophy

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Summary

Most acinar cells and some duct cells undergo apoptosis during atrophy of the submandibular gland. The present study was designed to elucidate whether Fas and its receptor ligand (FasL) are involved during apoptotic atrophy of the gland. The excretory duct of the right submandibular gland of rats was doubly ligated with metal clips from 1 to 14 days for induction of gland atrophy. Control rats were untreated. Fas and FasL expression in the atrophied submandibular gland was detected using immunohistochemistry (IHC) and Western immunoblot. Expression of activated caspase 8 and activated caspase 3 was also detected with IHC. Fas-positive acinar and duct cells and FasL-positive duct cells increased in the atrophic glands at 3 and 5 days after duct ligation when apoptotic cells were commonly observed. Thereafter, Fas- and FasL-positive cells declined in number. Patterns of expression of Fas and FasL using Western immunoblots concurred with the IHC results. Activated caspase 8-positive cells were present at every time interval but peaked at 3 and 5 days following duct ligation. The cells showing immunoreaction for activated caspase 3 first appeared on day 3, with the peak in apoptosis, after which they decreased. The results indicate that the Fas/FasL systems likely play an important role in apoptotic pathways during atrophy of the submandibular gland.

Keywords Fas, Fas ligand, apoptosis, submandibular gland, atrophy

Introduction

Obstructive sialadenitis in the human is an atrophic salivary gland disease with histological characteristics such as marked acinar cell loss, prominence of ducts and fibrosis (Matthews & Dardick 1988). The molecular pathways that control induction of apoptosis in such a disease are still, for the most part, unknown. Atrophy of the salivary glands after duct ligation in experimental animals shows a histology comparable to that of the human disease (Cummins et al. 1994). Although acinar cell disappearance in the atrophic glands had been interpreted previously as resulting from acinar cell de-differentiation to simpler rest cells which were cuboidal or flattened squamous-like cells, acinar cell loss due to apoptosis has now been described during atrophy of parotid gland (Walker & Gobe 1987), the submandibular gland (Takahashi et al. 2000) and the sublingual gland (Takahashi et al. 2002). Some duct cell apoptosis also takes place, although more often duct cells proliferate during glandular atrophy (Walker & Gobe 1987; Takahashi et al. 2000, 2002).

Apoptosis is triggered by a variety of extrinsic and intrinsic signals. The signals activate signal cascades, and this results in cytoskeletal disruption, cell shrinkage and membrane blebbing (Thompson 1995). However, it still remains unclear what signals induce apoptosis in atrophy of the salivary gland. One of the key receptor-activated apoptotic pathways involves Fas and its receptor ligand, FasL. Fas, also called Apo-1 or CD95, is a 45kDa type-I transmembrane protein with a signal sequence at the extracellular N terminus and a sequence containing death domain in the cytoplasmic region and belongs to the tumor

necrosis factor (TNF) receptor/nerve growth factor superfamily. FasL is a 40kDa TNF-related type-II transmembrane protein (Maher et al. 2002). Binding of FasL and Fas or agonistic Fas antibodies and Fas induces apoptosis in Fas-expressing cells through activation of caspases including caspase 8 and caspase 3 (Scaffidi et al. 1998; Stennicke et al. 1998). Fas expression has been identified in various tissues such as thymus, heart, lung, liver and ovary (Watanabe-Fukunaga et al. 1992), suggesting that the Fas/FasL systems are involved in many aspects of apoptosis in normal development, homeostasis, and tumor regression (Suda & Nagata 1994).

The contribution of the Fas/FasL systems in the pathogenesis of salivary gland atrophy is still controversial. Only Shibata and colleagues (2002) have reported on their involvement, in human sialolithiasis of minor salivary glands, as part of Sjögren's syndrome (SS), an autoimmune disorder with severe oral and ocular dryness. The aim of the present study was to elucidate whether the Fas/FasL systems have altered expression during atrophy of the submandibular gland induced by excretory duct ligation and especially during increased apoptosis. Expression of selected caspase pathway proteins, activated caspase 8 and activated caspase 3, was also investigated.

Methods

Animals

Seven-week-old male Wistar rats (Hokudo Co. Ltd., Sapporo, Japan), weighing 190-220g, were used in the present study. The rats were housed with free access to pellet food and tap water during the experimental period. The experimental protocol was approved by the Animal Ethics Committee and carried out in accordance with the Guide for the Care and Use of Laboratory Animals of Hokkaido University Graduate School of Dental Medicine.

Induction of submandibular gland atrophy

The animals in the experimental group were anesthetized with inhalation of ether, and the right submandibular gland and its excretory duct were exposed through a ventral incision in the neck. The right excretory duct was doubly ligated with Ligaclips (Ethicon Endo-Surgery Inc., Cincinnati, OH, USA) near the hilum for induction of submandibular gland atrophy, and then the skin closed with silk sutures. The experimental animals (N = 9 per experimental time) were killed at 1, 3, 5, 10 or 14 days after duct ligation. Untreated animals (N = 9) served as the controls. In the rodent submandibular and sublingual glands, it is considered that atrophy is caused both by duct obstruction and by damage to the corda tympani running on the excretory duct (Harrison & Garrett 1972; Harrison et al. 2001)

Immunohistochemistry (IHC) for Fas and FasL

The fresh right submandibular glands for Fas and FasL IHC were excised immediately after the animals were killed by deep inhalation of ether (N = 4 per treated or control groups). The samples were embedded in Tissue Tek OCT compound (Miles Scientific, Naperville, IL, USA) and frozen in liquid nitrogen. Cryostat sections of 5 μ m thickness were obtained, fixed in pure acetone for 10 min at 4°C and immersed in 0.3% hydrogen peroxide to inhibit endogenous peroxidase. The prepared sections were reacted with anti-Fas rabbit polyclonal antibody (M-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-FasL rabbit polyclonal antibody (C-178, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Then the sections were incubated with biotinylated anti-rabbit swine polyclonal antibody (DakoCytomation, Kyoto, Japan) and next with streptavidin-biotin peroxidase complex (ABC) (DakoCytomation, Kyoto, Japan). The immunoreaction of Fas and FasL was visualized with 3, 3'-diaminobenzidine (DAB). The immunostained sections were lightly counterstained with hematoxylin. Normal rabbit serum was substituted for both primary antibodies in negative control.

IHC for activated caspase 8 and activated caspase 3

The animals (N = 4 per treated or control groups) were perfused with 4% paraformaldehyde buffered at pH 7.4 with 0.1M phosphate buffer under sodium pentobarbital general anesthesia. The right submandibular glands were immersed in the same fixative for 24 hours, and then prepared routinely for paraffin sections. The deparaffinized sections of 4 μ m thickness were used for activated caspase 8 and activated caspase 3 or stained with hematoxylin and eosin

(HE) for histology. For IHC, the endogenous peroxidase was inhibited with 0.3% hydrogen peroxide. As primary antibodies, anti-caspase 8 rabbit polyclonal antibody (Ab-4, LAB VISION, Fremont, CA, USA) recognizing activated caspase 8 and anti-activated caspase 3 rabbit polyclonal antibody (4-1-18, Cell Signaling Technology, Beverly, MA, USA) were used. Before incubation with the antibodies, the sections were boiled in 10mM citrate buffer at pH 6.0 (ChemMate Target Retrieval Solution, DakoCytomation, Kyoto, Japan) for 40 mins. The bound primary antibodies were detected using LSAB+Kit/Peroxidase (DakoCytomation, Kyoto, Japan), developed by DAB and lightly counterstained with hematoxylin. Negative control sections were incubated with normal rabbit serum in the absence of both primary antibodies.

Quantification

In the HE sections, apoptosis of acinar cells and duct cells were counted at a magnification of x400 in five randomly selected fields. The main histological characteristics of apoptosis were cellular shrinkage and blebbing into apoptotic bodies, and nuclear chromatin compaction with occasional crescents of dense chromatin against the nuclear membrane. In the sections immunostained with activated caspase 8 or activated caspase 3, the positive cells were counted in the same way. The average of the five fields was calculated and used as a representative value for that animal. Expression levels of Fas and FasL were determined by immunohistochemical localization. The whole area of the immunostained sections was observed at x200 and the indices of five grades were recorded as follows: 0=no positivity;

1=to 10% labeling; 2=10 to 20% labeling; 3=20 to 30% labeling; 4=over 30% labeling. The means and standard error of the mean (SEM) of all data were calculated for four experimental animals at each time point and for four control animals. One-way analysis of variance (ANOVA), followed by the Fisher's protected least significant difference (PLSD) post-hoc test, were performed with StatView 4.5 statistical software (Abacus Concepts, Berkeley, CA, USA). Values of $p < 0.05$ were considered statistically different.

Western blot analysis for Fas and FasL

Experimental animals at 1, 3, 5 or 14 days after duct ligation and untreated controls (N = 1 per group) were used in Western blot analysis. The fresh glands were obtained in the same way as described in Fas and FasL immunohistochemistry and were frozen in liquid nitrogen. The frozen tissues were homogenized in ice-cold TRIZOL reagent (Life Technologies, Grand Island, NY, USA) and protein was extracted as instructed by the manufacturer. Equal amounts of protein from each group were determined by Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and were applied to 10% SDS-PAGE gel electrophoresis. As positive controls, mouse thymus extract (SC-2406, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for Fas and whole cell lysates of human chronic myelogenous leukemia (K-562, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for FasL were also electrophoresed. The separated proteins were transferred onto Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA) and incubated with anti-Fas antibody or anti-FasL antibody overnight at 4°C. Biotinylated anti-rabbit swine polyclonal antibody and ABC were used to detect the

bound primary antibodies. The antibodies and ABC used in Western blot analysis were same ones in Fas and FasL immunohistochemistry. The signals were visualized by using ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, Arlington Heights, IL, USA).

Results

Histology

Detailed aspects of the histological changes during atrophy of submandibular glands over the time scale used in the current paper have been reported in our previous paper (Takahashi et al. 2000). We verified the alterations in apoptosis in the current experiments and these results are presented in Table 1. Histologically, the control glands were normal (Figure 1a). In the experimental glands, the glandular tissue became edematous and ducts dilated at 1 day following duct ligation (Figure 1b). At day 3, apoptosis of many acinar cells and some duct cells was identified and was significantly increased over control tissues ($p < 0.05$), and acinar cells rapidly decreased in number (Figure 1c). At 10 days, most acinar cells had disappeared, leaving residual ducts (Figure 1d).

Immunohistochemistry for Fas and FasL

Negative control sections for Fas and FasL immunohistochemical staining showed no positive reactions. Quantification of Fas and FasL expression patterns is shown in Table 2. In this model of tissue atrophy, the FasL-positive lymphocytes infiltrating in the atrophic glandular tissue were few in number. In submandibular gland tissue from control animals, several intercalated duct cells were positive for Fas (Figure 2a), and some cells of the large interlobular ducts showed positive reaction for FasL (Figure 3a). Other cells were negative for both antibodies. In the submandibular glands from experimental animals on day 1 after duct

ligation, the immunoreactions against both antibodies were similar to those in the control.

From day 3 to day 10, many acinar cells and duct cells showed positive reaction to Fas (Figure 2b). On days 3 and 5, many FasL-positive cells were observed in small ducts (Figure 3b) as well as interlobular ducts. Thereafter, the positive reactions for both antibodies decreased. Fas- or FasL-positivity remained in the infrequent parenchymal cells identified in the atrophic tissue at day 14 (Figure 2c, 3c).

IHC for activated caspase 8 and activated caspase 3

Quantitative analysis showed that the activated caspase 8-positive cell number of day 3 and 5 were statistically significantly different from that of the control and that the peak was identified at 3 days (Table 3). The activated caspase 3-positive cell number at 3 days was the highest compared with all other groups (Table 3). No activated caspase 8-positive cells were identified in the control submandibular glands (Figure 4a). In the duct ligated glands, there were activated caspase 8-positive reactions at every time interval, and most of them were acinar cells. Many activated caspase 8-positive cells were observed at 3 days (Figure 4b), after which they decreased in number. At 14 days, a few activated caspase 8-positive reactions were found (Figure 4c). The positivity for activated caspase 3 was not observed in the submandibular glands from control animals (Figure 5a) and the experimental animals at 1 day following duct ligation. There were many activated caspase 3-positive acinar cells at 3 days (Figure 5b), but they were less frequent at later time points (Figure 5c). Negative control sections for activated caspase 8 and activated caspase 3 showed no reaction.

Western blot analysis for Fas and FasL

A clear band of Fas was detected at 45 kDa in the positive control. The same band was also identified at 5 days post-obstruction, while there were no reactions in the fractions of controls and other timepoints (day 1 and day 14 are demonstrated) (Figure 6). In the positive control of FasL, the immunoreaction was clearly identified at 40 kDa. In the glandular tissue, there was a band at day 5 post-obstruction, however no reactions were detected in the fractions of control and other time points (day 1 and day 14 demonstrated) (Figure 7).

Discussion

In our previous study examining the same experimental model of atrophy of the salivary gland for apoptotic morphology, and verification with TUNEL and transmission electron microscopy, apoptotic acinar cells were identified much more than apoptotic duct cells during the pathogenesis of the atrophy. TUNEL labeling indices of acinar and duct cells increased between days 3 and 7 after duct ligation (Takahashi et al. 2000). The present investigation confirmed these morphological results and found that expression patterns of the Fas/FasL systems coincided with the pattern of apoptosis, showing indirectly that Fas is a mediator of apoptosis in submandibular gland atrophy.

Although a wide range of normal tissues express Fas, FasL expression is often restricted to immune cells such as natural killer cell and activated T cell (Suda et al. 1993) and immune-privileged tissues such as the eye (Griffith et al. 1995), testis (Bellgrau et al. 1995) and intervertebral disc (Takada et al. 2002). Recently, more FasL-expressing cells have been demonstrated in several other tissues under certain conditions, for example, hepatocytes in alcoholic liver damage (Galle et al. 1995), tubular epithelial cells in chronic renal failure (Schelling et al. 1998), oocytes in ovarian follicle atresia (Hakuno et al. 1996) and others. In these cases, FasL mediates apoptosis by interacting with the Fas receptor on neighboring cells. FasL may be released from the cell surface by metalloproteinases like TNF as a soluble form of FasL (Kayagaki et al. 1995; Tanaka et al. 1995), which mediates apoptosis in an autocrine or paracrine fashion (Galle et al. 1995). In the present study, FasL expression was identified

within the same period as increased occurrence of apoptosis (Takahashi et al. 2000) by both immunohistochemistry and Western blotting. FasL-positive lymphocytes infiltrating in atrophic glandular tissue were few in number, and remaining duct cells often showed positivity against FasL. Shibata et al. (2002) reported that duct and acinar cells express FasL in human sialolithiasis of minor salivary glands, as part of SS. Taking our data and this report together, it would appear that the Fas/FasL systems play important roles for apoptosis in salivary gland atrophy. It has also been reported that Fas/FasL systems are associated with apoptosis induction in chronic pancreatitis (Yasuda et al. 1999; Kornmann et al. 2000; Hasel et al. 2001). However, it is still controversial whether FasL from T cells interacts with Fas on the parenchymal cells (Hasel et al. 2001) or FasL from parenchymal cells interacts with Fas on these same cells (Kornmann et al. 2001).

There are two different cell types involved in Fas and FasL system-mediated apoptosis (Scaffidi et al. 1998; Barnhart et al. 2003). In type I cells, Fas triggering strongly activates caspase 8 at the death-inducing signal complex (DISC) (Curtin & Cotter 2003), and then activated caspase 8 leads to cleavage of pro-caspase 3 and activation of caspase 3 (Stennicke 1998). In type II cells, very little DISC and small amounts of active caspase 8 are formed, leading to the activation of the mitochondrial pathway, and finally activation of caspase 8 and caspase 3 (Scaffidi et al. 1998; Barnhart et al. 2003). The activated caspase 3 in both cell types cleaves various cellular substrates, and finally induces the morphological changes of apoptosis in cells (Thompson 1995) and the degradation of chromosomal DNA into nucleosomal units (Sakahira et al. 1998; McIlroy et al. 1999; Nagata 1999). Thus,

activated caspase 8 as initiator caspase and activated caspase 3 as executor caspase are essential in Fas and FasL system-mediated apoptosis regardless of cell types (Hua et al. 2005). In our investigation, we have used the IHC detection of activated caspase 8 and activated caspase 3 as indirect evidence of participation of the Fas/FasL systems in apoptotic cell death. We have described many activated caspase 8-positive cells on days 3 and 5, and activated caspase 3-positive cells were commonly observed on day 3. Thus we suggest that the Fas/FasL systems trigger off apoptosis in the atrophic submandibular glands.

The histological characteristics of SS, a disorder with severe ocular and oral dryness, are mononuclear cell infiltration and destruction of parenchymal tissue in salivary and lacrimal glands (Manganelli & Fietta 2003). Although Ohlsson et al. (2001) mentioned that Fas-induced apoptosis is a rare event in SS, many investigators have considered that the Fas/FasL systems are involved in apoptosis in SS, since Fas expression and apoptosis of acinar and duct cells are identified in SS of patients (Kong et al. 1997; Matsumura et al. 1998; Shibata et al. 2002) and experimental animals (Saito et al. 1999; Hayashi et al. 2003). This idea and the results of the present study suggest that the parenchymal cell apoptosis in salivary glands under various pathological conditions may be induced by Fas and FasL system regardless of the pathological conditions, although further studies will be needed for better understanding of the mechanism of apoptosis in SS.

In conclusion, the present study demonstrated that the Fas and FasL systems are closely linked to induction of apoptosis in the atrophy of the rat submandibular glands, and by inference with results from other investigations, these apoptotic pathways may occur more

broadly in destruction of the salivary gland from multiple causes. By understanding the complex mechanisms of apoptotic induction in atrophy of the salivary gland, the development of novel therapeutic strategies designed to protect salivary function may become possible.

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Legends

Figure 1 Histology (HE). (a) Control. Normal submandibular gland. (b) Day 1. Ducts dilate. (c) Day 3. Many apoptosis in acini (arrowheads). (d) Day14. Most acini disappear, leaving many ducts. Bars=50 μ m

Figure 2. Fas immunohistochemistry. (a) Control. Several intercalated duct cells are positive (arrowheads). (b) Day 5. Many acinar cells show positive reaction. (c) Day 14. The positivity for Fas (arrowheads) decreases in the atrophic submandibular gland. Bars=50 μ m

Figure 3. FasL immunohistochemistry. (a) Control. The positivity for FasL is identified in a large interlobular duct (arrowheads). (b) Day 5. FasL-positive cells are observed in small ducts. (c) Day 14. A few FasL-positive reactions are observed in small ducts (arrowheads). Bars=50 μ m

Figure 4. Activated caspase 8 immunohistochemistry. (a) Control. The normal submandibular gland shows no reaction. (b) Day 3. Activated caspase 8-positive acinar cells are commonly identified (arrowheads). (c) Day14. The positive reaction (arrowhead) decreases in number. Bars=25 μ m

Figure 5. Activated caspase 3 immunohistochemistry. (a) Control. No immunoreaction is

identified in the normal gland. (b) Day 3. There are many positive reactions against activated caspase 3. (c) Day 14. The number of FasL-positive cells (arrowhead) is small. Bar=25 μ m

Figure 6. Western blot analysis of proteins from different intervals of submandibular glands for Fas. P; positive control (mouse thymus extract). C; control (normal submandibular gland). 1d; 1 day after duct ligation. 5d; day 5. 14d; day 14. The positive reactions are identified in positive control and day 5 at 45 kDa.

Figure 7. Western blot analysis for FasL. P; positive control (whole cell lysate of human chronic myelogenous leukemia). C; control (normal submandibular gland). 1d; 1 day after duct ligation. 5d; day 5. 14d; day 14. The immunoreactions are identified at 40 kDa in positive control and day 5.

Table 1. The numbers of apoptosis are counted at x400 in five randomly selected fields and are averaged.

Table 3. The numbers of the positive cells are counted at a magnification of x400 in five randomly selected fields and are averaged.

Table 1

The number of apoptosis of acinar cells and duct cells during atrophy of submandibular glands (\pm SEM)

	N	Acinar Cells	Duct Cells
Control	4	0.00 \pm 0.00	0.00 \pm 0.00
Day 1	4	0.10 \pm 0.06	0.00 \pm 0.00
Day 3	4	22.10 \pm 4.70*	0.95 \pm 0.28*
Day 5	4	7.45 \pm 0.95*	1.65 \pm 0.40*
Day10	4	2.40 \pm 0.41	0.95 \pm 0.38*
Day14	4	1.90 \pm 0.98	0.60 \pm 0.26

*p<0.05 compared with the control

Table 2

Grades of Fas and FasL expression during atrophy of submandibular glands (\pm SEM)

	N	Fas	FasL
Control	4	1.25 \pm 0.25	1.00 \pm 0.00
Day 1	4	1.50 \pm 0.29	1.00 \pm 0.00
Day 3	4	2.75 \pm 0.25*	2.50 \pm 0.29*
Day 5	4	3.50 \pm 0.29*	2.75 \pm 0.25*
Day10	4	3.25 \pm 0.25*	1.50 \pm 0.29
Day14	4	1.00 \pm 0.00	1.00 \pm 0.00

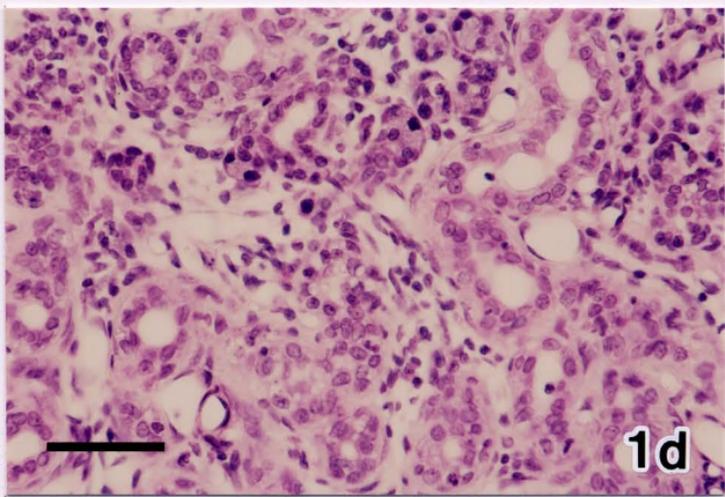
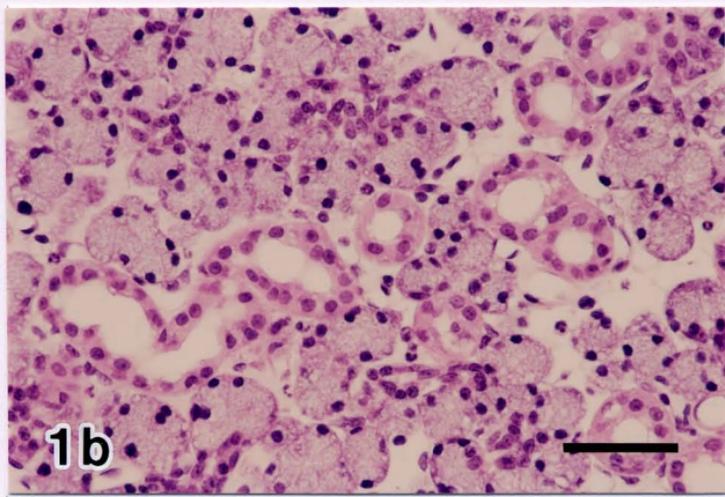
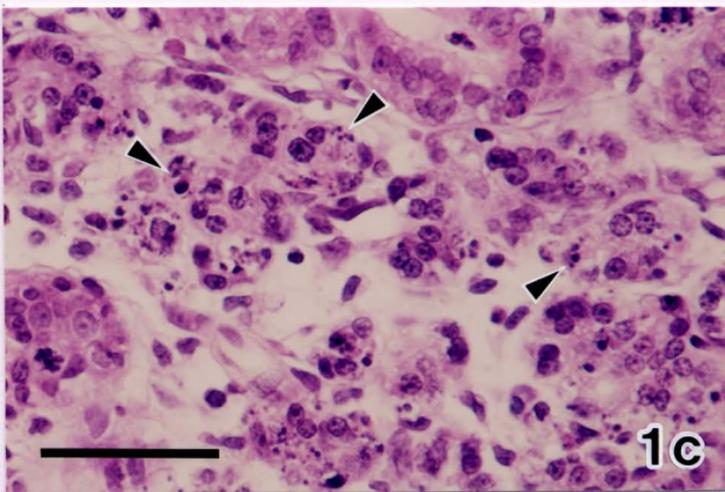
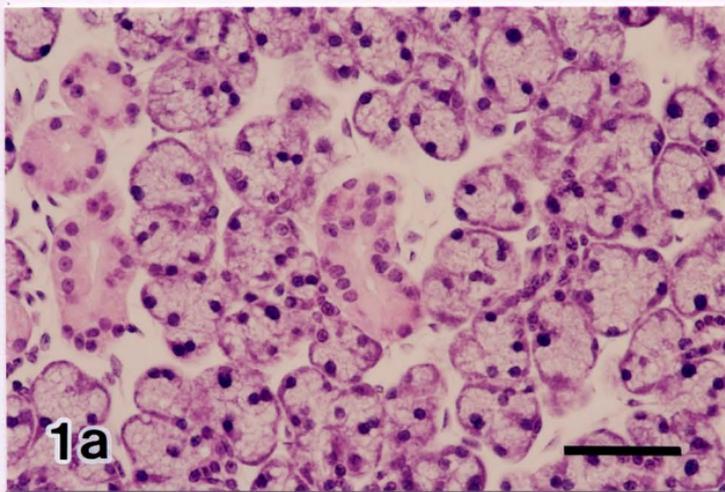
*p<0.05 compared with the control

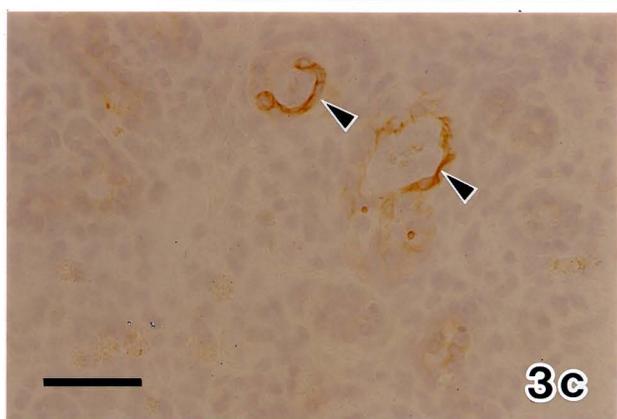
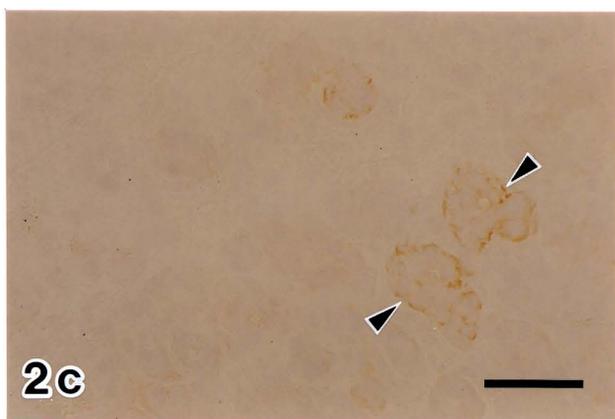
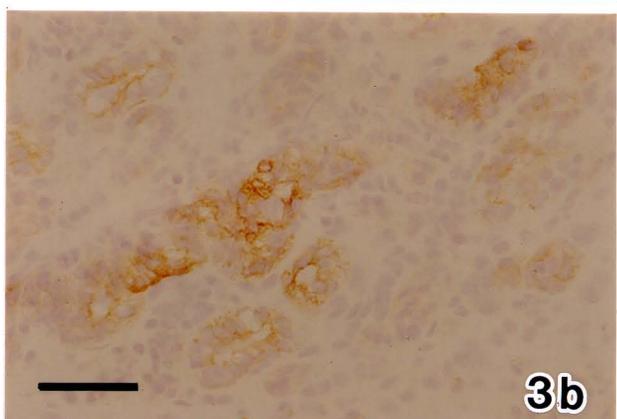
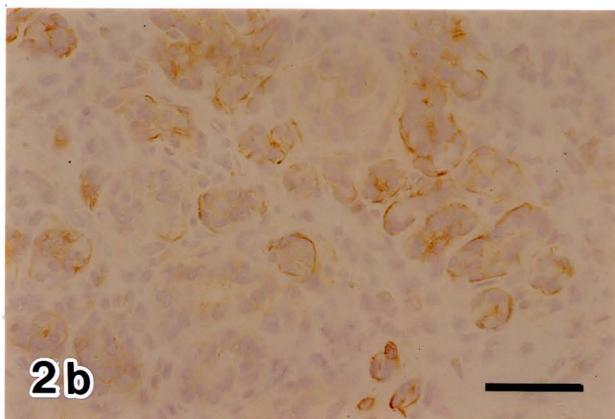
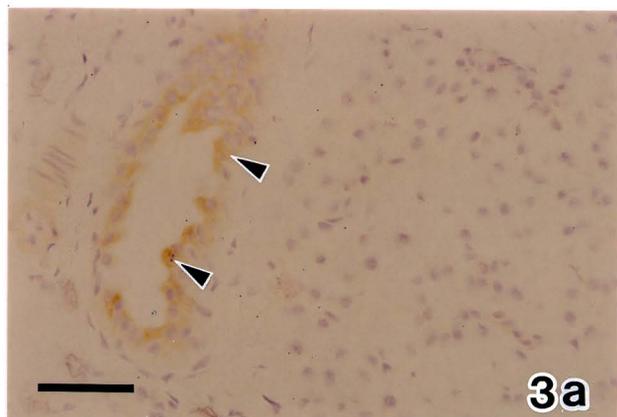
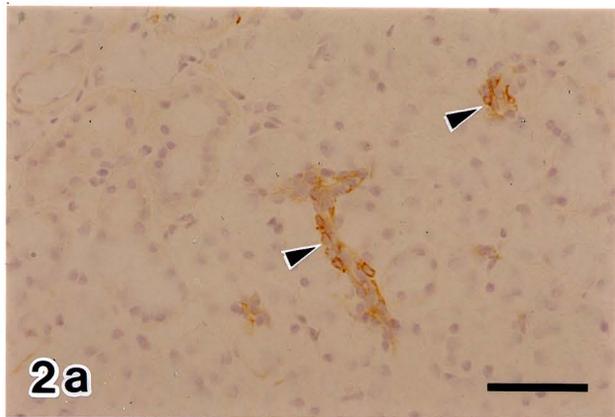
Table 3

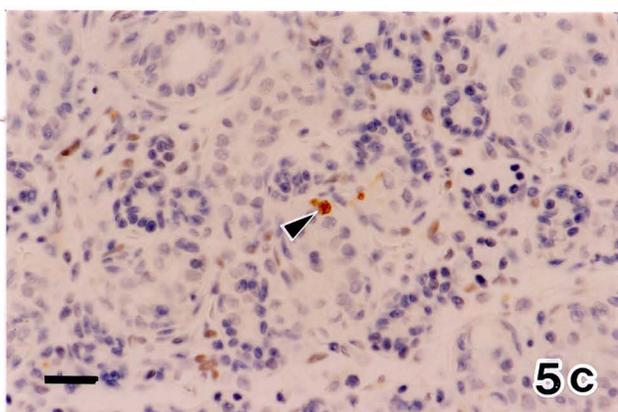
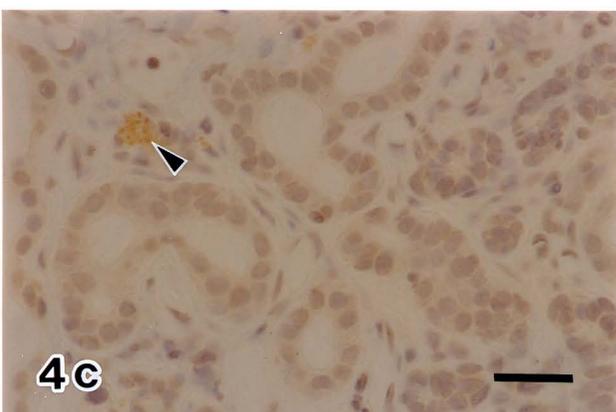
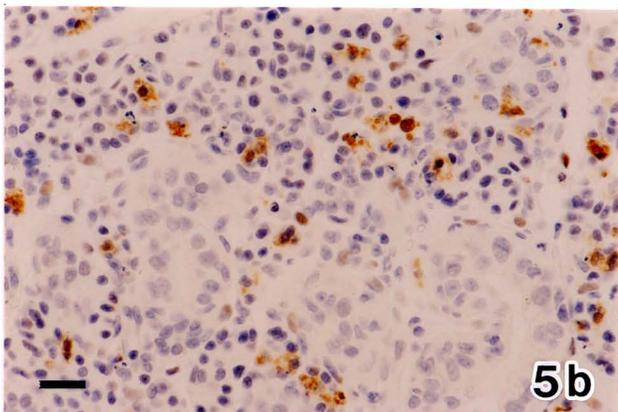
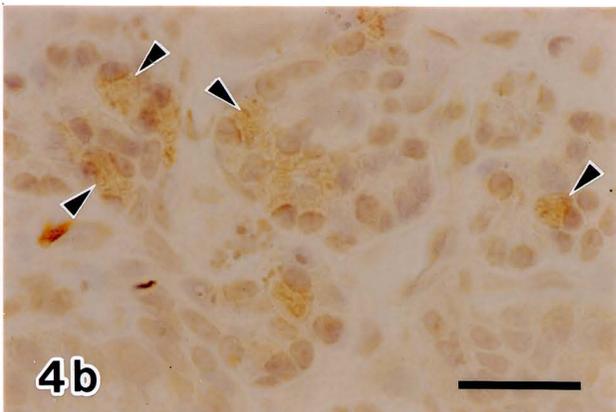
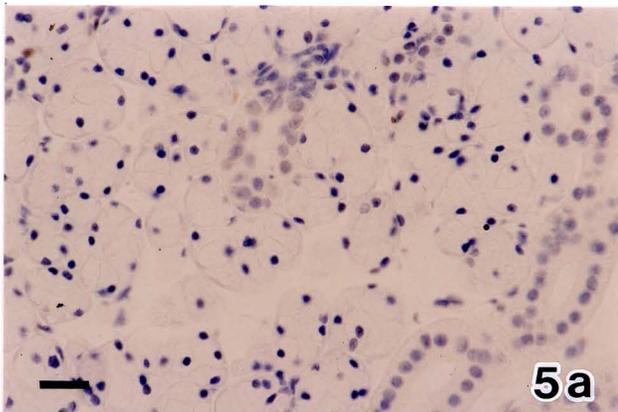
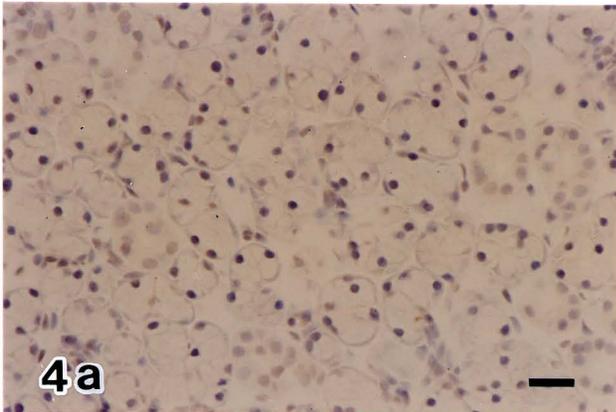
The number of activated caspase 8- and activated caspase 3-positive cells during atrophy of submandibular glands (\pm SEM)

	N	Activated Caspase 8	Activated Caspase 3
Control	4	0.00 \pm 0.00	0.00 \pm 0.00
Day 1	4	1.95 \pm 0.74	0.00 \pm 0.00
Day 3	4	9.10 \pm 1.29*	41.65 \pm 21.63*
Day 5	4	4.10 \pm 1.58*	8.30 \pm 3.76
Day10	4	2.35 \pm 0.68	8.40 \pm 4.65
Day14	4	0.45 \pm 0.15	6.30 \pm 1.95

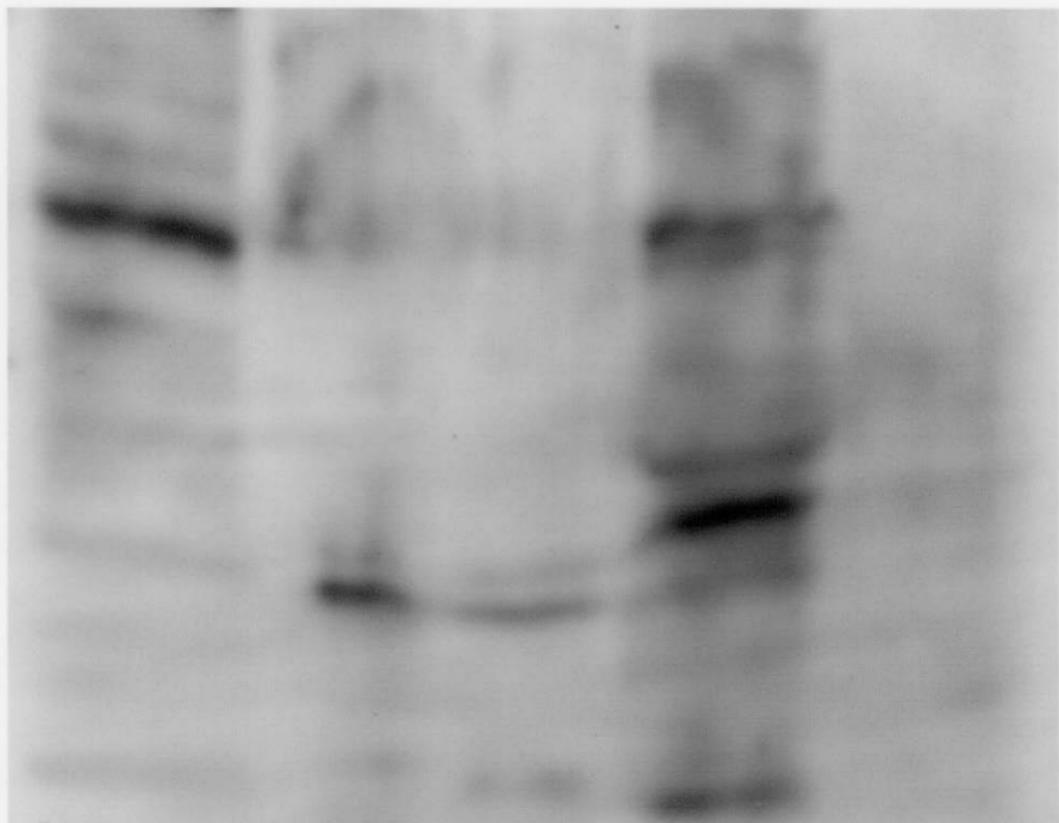
*p<0.05 compared with the control







45kD →



P

C

1d

5d

14d

40kD →



P

C

1d

5d

14d