—原著—

Expression of Immunoreactivities for Manganese and Copper/Zinc Superoxide Dismutases (Mn- and Cu/Zn-SODs) During Development of the Rat Submandibular Gland

Mikiko Arimatsu[1,2], Takeyasu Maeda[1], Yoshiro Kawano[1]

[1]*Division of Oral Anatomy (Chief: Prof. Takeyasu Maeda), Department of Oral Biological Science, Niigata University Graduate School of Medical and Dental Sciences,*
[2]*Division of Pedodontics, (Chief: Prof. Tadashi Noda), Department of Oral Health Science, Niigata University Graduate School of Medical and Dental Sciences*

Received 15 October ; Accepted 15 October, 2002

Key words : superoxide dismutase, submandibular gland, development, immunohistochemistry, rat.

Abstract: Manganase- and copper-zinc superoxide dismutases (Mn- and Cu/Zn-SODs) are representative enzymes for scavenging superoxide radicals, which are considered to cause aging and cell damage. Our recent study has demonstrated the localization of Mn- and Cu/Zn-SOD proteins as well as the expression of Mn- and Cu/Zn-SOD mRNAs in all types of duct cells – and not in acinar cells – in the submandibular gland of adult rats. However, the expression of these two enzymes remains unclear in rat developing submandibular gland. Thus, the present study examined the expression of Mn- and Cu/Zn-SODs in the submandibular gland of rats aged from embryonic 18 days to postnatal 8 weeks by an immunohistochemical technique using specific antisera. The deparaffinized sections were processed for the avidin-biotin complex method. On the prenatal 18th day, a small number of epithelial duct cells and cells in acini exhibited Cu/Zn-SOD-immunoreactivity, but they did not show any immunoreactions for Mn-SODs. From postnatal 1 day to 1 week, Mn- and Cu/Zn-SOD-immunoreactivities were found in both the duct cells and the cells in acini. This drastic change in the expression of Mn- and Cu/Zn-SOD-immunoreactivities between prenatal and postnatal periods was believed to relate to the commencement of pulmonary respiration due to oxygen exposure. After 2 weeks, however, the cells in the acini lost Mn- and Cu/Zn-SOD-immunoreactions, though the duct cells retained them, suggesting that the duct cells in the mature submandibular gland exhibit greater resistance against oxidative stress than do the acinar cells.

INTRODUCTION

Active oxygen species and free radicals play crucial roles in protection against bacterial infection and scavenging by antioxidant enzymes. Active oxygen species and antioxidant enzymes are considered to be implicated in a variety of pathologic processes including aging[1, 2] and cell damage[3-6] in spite of their favorable balance in a healthy organism. Superoxide disumtase (SOD), also known as superoxide oxidoreductase, is a key enzyme that protects cells against oxidative injury: it resolves superoxide into H_2O_2 and O_2, and H_2O_2 is subsequently broken down by catalase and glutathione peroxidase into H_2O and O_2. Biochemical analyses have revealed at least three different SOD enzymes in mammalian tissues. They are classified into two forms of intracellular SOD[7] and one extracellular SOD[8]. The intracellular SODs include manganese SOD (Mn-SOD) and copper-zinc SOD (Cu/Zn-SOD). The Mn-SOD is found predominantly in the mitochondrial matrix while Cu/Zn-SOD is uniform throughout the cytoplasm[9].

The formation of salivary gland is initiated by the proliferation of oral epithelial cells and their down-growth into the underlying mesenchyme[10-12]. During

development, interactions of oral epithelial cells with mesenchymal cells and extracellular matrix components induce the branching of the epithelial rudiment, the formation of ducts, and cytodifferentiation. The salivary glands are most likely protected by a specific defense system against free radicals and reactive oxygen species because the acini are connected through their ducts to the oral cavity, which is usually exposed to various kinds of free radical-formative substances[13-15]. Furthermore, bacteria inducing periodontitis have been shown to produce free radicals and reactive oxygen species[16].

Many immunohistochemical and ultrastructural studies have reported the existence of SOD in various tissues including submandibular gland[17-24]. Our recent studies using immunocytochemistry and *in situ* hybridization histochemistry have demonstrated the localization of Mn- and Cu/Zn-SOD proteins and the expression of Mn- and Cu/Zn-SOD mRNAs in the submandibular gland of mature rats, suggesting a different resistance against oxidatives between acinar and duct cells[25,26]. However, details of the developmental aspects of SOD-expressions have remained unclear in the submandibular gland because information on the expression of SODs in the salivary gland has been limited for fetal and neonatal animals[17-19].

In the present study, therefore, alterations in expression of Mn- and Cu/Zn-SODs were investigated in the submandibular glands of developing and mature rats by immunohistochemical techniques using specific antisera.

MATERIALS AND METHODS

A total of 24 Wistar rats were used in this immunohistochemical observation. Animals at embryonic 18 days of gestation, postnatal 1 and 3 days (n=3 each) were decapitated under deep anesthesia by an intraperitoneal injection of 8% chloral hydrate (400mg/kg), and the decapitated heads including the submandibular glands were immersed in 4% paraformaldehyde in a 0.1M phosphate buffer (pH7.4). After immersion fixation overnight, the specimens were decalcified with 10% EDTA solution at 4°C for a few days. The rats aged postnatal 1, 2, 3, 4 and 8 weeks (n=3 each) were deeply anesthetized in the same manner as described above, and fixed by a transcardiac perfusion with 4% paraformaldehyde in a 0.1M phosphate buffer (pH7.4). After perfusion fixation, the submandibular glands were removed *en bloc*, and immersed in the same fixative for an additional 24 hours at 4°C. Both the decalcified heads and the excised submandibular glands were dehydrated through an ascending series of ethanol, and embedded in paraffin. The paraffin-embedded sections were serially cut at a thickness of 5 μm with a microtome.

For immunohistochemistry, deparaffinized sections were processed for the avidin-biotin-complex (ABC) method according to Hsu et al. (1981)[27]. These sections were immersed in absolute methanol containing 0.3% hydrogen peroxide for the inhibition of endogenous peroxidase activities. After rinsing in 0.01M phosphate buffered-saline (PBS), they were incubated with 2.5% normal goat serum (Vector Laboratories, Burlingame, CA, U.S.A) for 30 min to reduce background staining. The sections were then subsequently reacted with polyclonal antisera against either Mn- or Cu/Zn-SOD (StressGen Biotechnologies Corp., Victoria, Canada) diluted to 1:1000 overnight. Following a thorough rinse in 0.1M PBS, they were reacted with two consecutive incubations with a biotinylated anti-rabbit IgG (1:100, Vector Laboratories) and avidin-biotin complex (Vector ABC kit, Vector, Laboratories) at 60 min each. Chromogen reaction sites were made visible by incubating sections with 0.04% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemicals Co. Ltd., St. Louis, MO, U.S.A.) in the presence of 0.003% hydrogen peroxide in a 0.05M Tris-HCl buffer (pH7.6). These immunohistochemical incubations were performed in a moister chamber at room temperature. The immunostained sections were counter-stained with 0.3% methylene blue, and examined with a Nikon FX microscope (Nikon Co. Ltd., Tokyo, Japan). For histological observation, deparaffinized sections were stained with hematoxylin and eosin, or with 1% toluidine blue.

The specificity of the primary antisera against Mn- and Cu/Zn-SODs was checked by a preabsorption test. The primary antisera were preabsorbed with excess corresponding antigens (StressGen Biotechnologies) at 4°C overnight. Sections stained with the preabsorbed antisera did not show any specific immunoreaction. Furthermore, immunohistochemical controls were performed by: 1) replacing the primary antisera with non-immune serum or PBS; and 2) omitting the anti-

rabbit IgG or the avidin-biotin complex. These control sections also revealed no immunoreaction, leading us to consider the immunoreactions to be specific.

RESULTS

Embryonic 18 days

The formation of submandibular glands already had begun at this stage. The submandibular glands were composed of acini and a duct system and had rich interstitial connective tissues (Fig. 1 a). It was easy to distinguish acini from epithelial duct cells. The acini were composed of columnar cells with basally located nuclei and a rich cytoplasm which contained intense eosinophic granules (Fig. 1 b). The salivary ducts, which consisted of cuboid cells, had a scanty cytoplasm. Although the salivary ducts were observed to connect with the acini, no formation of striated and granular ducts was recognizable at this stage.

A small number of duct cells and the cells in the acini showed Cu/Zn-SOD-immunoreactivity (Fig. 1 c). These immunoreactions were diffusely distributed either throughout the cytoplasm, or restricted to their nuclei. However, no specific immunoreaction for Mn-SOD was found in either acinar cells or epithelial duct cells (Fig. 1 d).

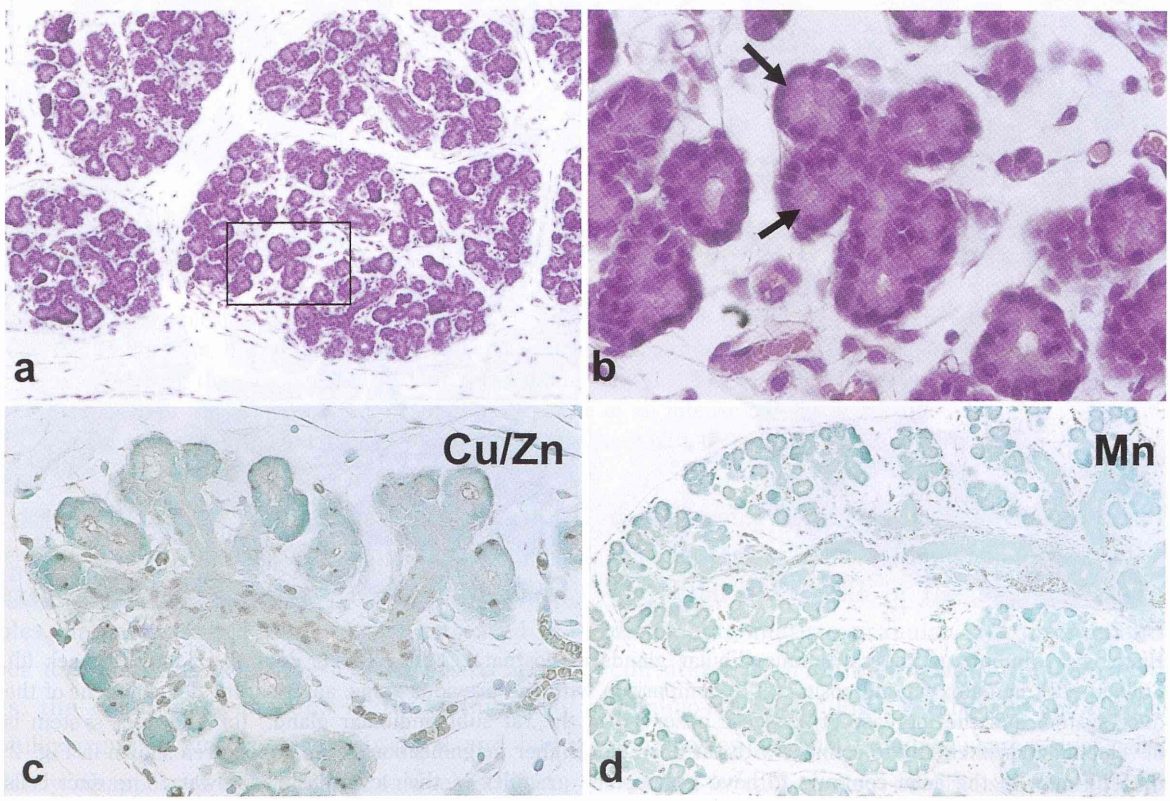

Fig. 1
Histological (a, b) and immunohistochemical (c, d) findings of the rat submandibular glands at embryonic 18 days. (a) The submandibular gland is observed to contain developing acini and a duct system among rich intestinal connective tissue. (b) Higher magnification of the boxed area shown in Fig. 1 a. The cells in the acini are filed with eosinophilic granules in their rich cytoplasm (arrows). Stained with hematoxyin and eosin. (c, d) Immunoreactions for Cu/Zn- (c) and Mn- (d) SODs. The epithelial duct cells and a few cells in the acini are positive for immunoreactions (c), but no Mn-SOD-immunoreaction is found on embryonic 18th day. Immunoreactivities for Cu/Zn-SOD are localized either the nuclei or cytoplasm. a; ×78, b; ×375, c; ×250, d; ×62.5.

Postnatal 1 day to 1 week

The formation of the acini and duct system had proceeded further in the rat submandibular glands than at the previous stage: the number and volume of acini appeared to increase daily (Fig. 2 a, b, d). Almost all cells in the acini continued to exhibit intense eosinophilic granules in their cytoplasm (Fig. 2 c). The salivary ducts also extended their branches throughout the rat submandibular gland (Fig. 2 b, d). However, typical intercalated cells and granular cells

were not recognizable, but the connection of the salivary ducts with acini was frequently observed at this stage.

Immunohistochemistry for Mn- and Cu/Zn-SODs was able to demonstrate both immunoreactions in the cells in acini and the epithelial duct cells with variable immuno-intensities (Fig. 3 a, b). These immunoreactions were diffusely distributed throughout the cytoplasm (Fig. 3 c), although Mn-SOD-immunoreactivity has been reported as mitochondrial SOD in adult tissues[9, 25]. These immuno-intensities in the acini appeared unchanged from postnatal 1 and 3 days, but they showed a tendency to decrease through postnatal 3 days to 1 week (Fig. 3 d). However, the duct cells retained the same immuno-intensity as that at postnatal 1 day to 1 week.

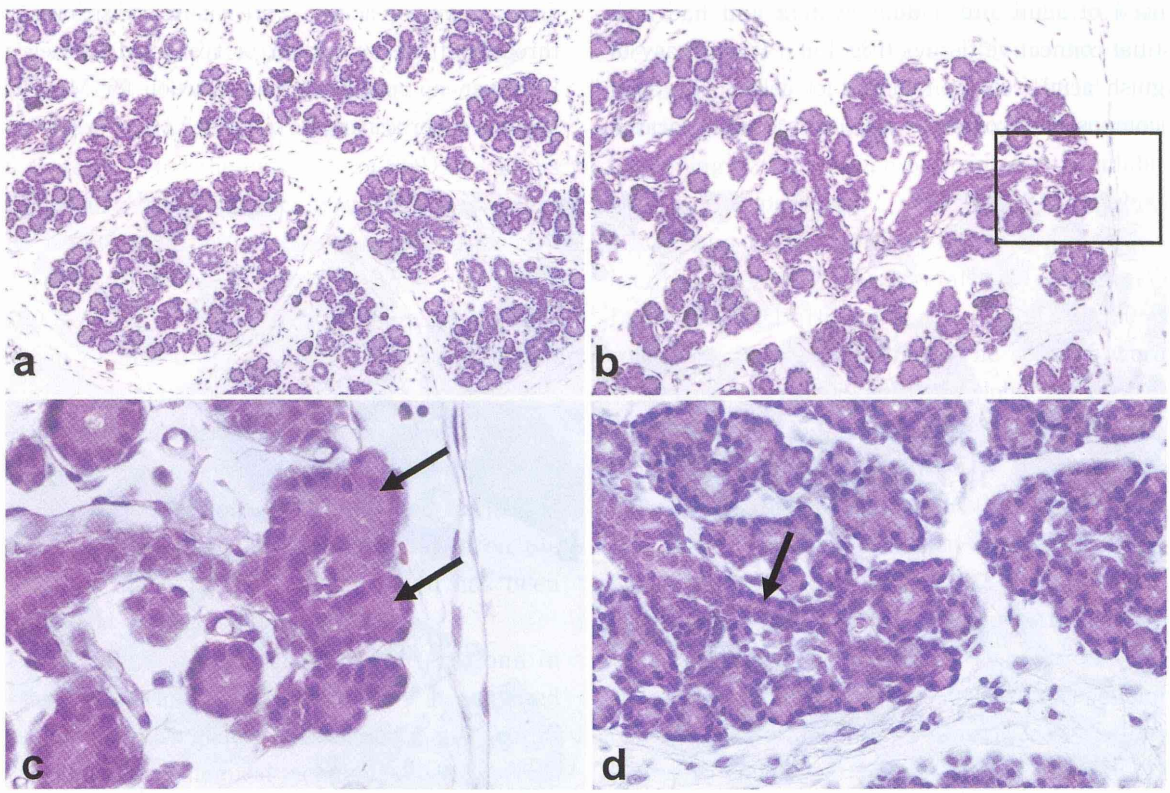

Fig. 2
Histological findings of the rat submandibular glands at postnatal 1 day (a), 3 days (b, c) and 1 week (d). Stained with hematoxyin and eosin. (a) In comparison with the previous stage, an apparent development of the duct system and the increase of acini are observed in the rat submandibular gland. (b) The duct system is developing and extending to connect with the acini. (c) Higher magnification of the boxed area shown in Fig. 2 b. The cells in the acini continue to have eosinophilic granules in their cytoplasm (arrows). The duct cells connecting with the acini show a cuboidal appearance, different from the typical intercalated cells seen in mature submandibular glands. (d) The duct systems are observed to elongate more than at the previous stages (arrow), but the duct cells connecting with the acini still show a cuboidal appearance. a; ×78, b; ×94, c; ×375, d; ×250.

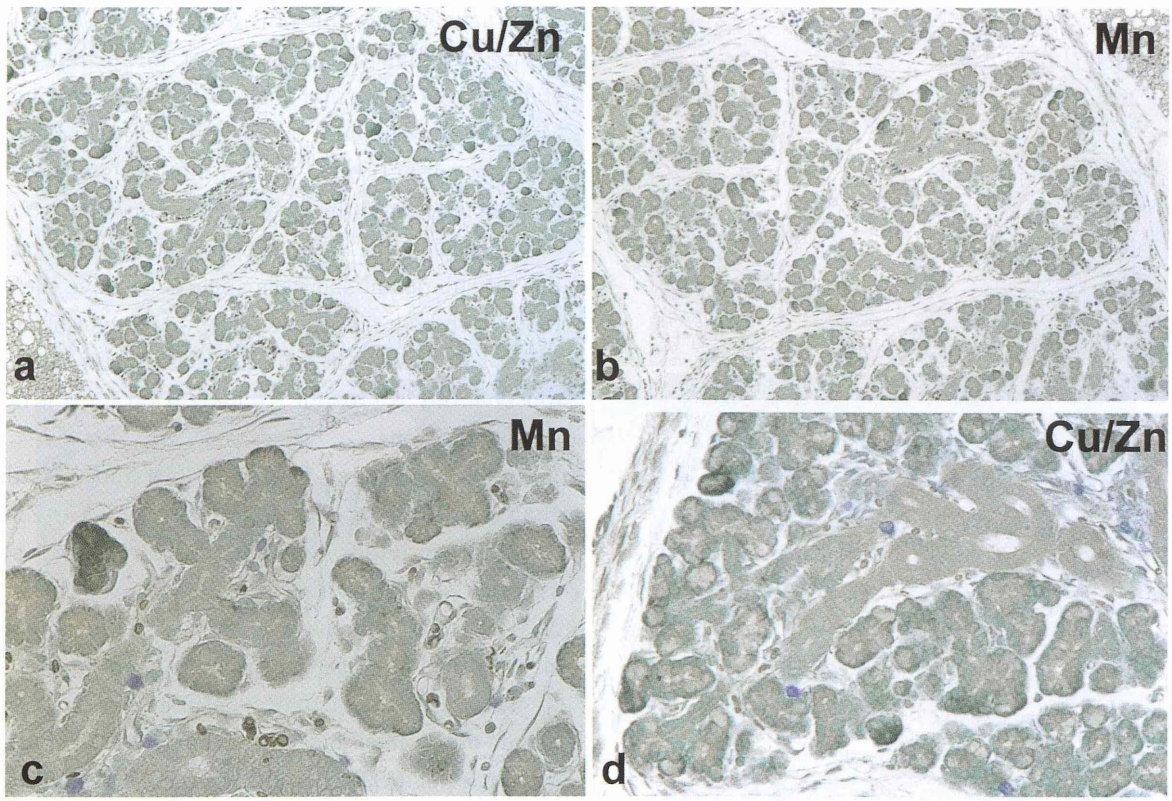

Fig. 3
Immunohistochemical findings at postnatal 1 day (a-c) and 1 week (d). Immunoreactions for Cu/Zn- (a, d) and Mn- (b, c) SODs. (a, b) Both the cells in the acini and epithelial duct cells show immunoreactivities. (c) Highly magnified view of the submandibular gland on postnatal Day 1. The Mn-SOD-immunoreaction is recognized in the cytoplasm of the acini and duct system. (d) In spite of an intense immunoreaction in the duct system, the cells of the acini appear to decrease in immuno-intensity. a; ×62.5, b; ×62.5, c; ×250, d; ×94.

From postnatal 2 to 8 weeks

The cells in the acini with eosinophilic granules decreased in number; instead, those with clear granules (hereafter called acinar cells) increased in number to occupy the acini (Fig. 4). At postnatal 3 weeks, the occurrence of cells in the acini with basophilic granules was considerably rare, and had completely disappeared by postnatal 4 weeks (Fig. 4 b). On the other hand, the granular ducts, which contained eosinophilic granules in the supranuclear region, had differentiated from the epithelial duct cells around postnatal 3 weeks. In addition to striated and granular ducts, the formation of intercalated ducts had begun near the acini around postnatal 2 weeks (Fig. 4 a). The intercalated ducts consisted of cuboid cells with a poor cytoplasm. Thus, the acini were observed to connect with the granular ducts through the intercalated ducts. No specific morphological change in the rat submandibular glands was found after postnatal 4 weeks.

Immunohistochemistry for Mn- and Cu/Zn-SOD demonstrated numerous immunoreactive cells in the duct system of the rat submandibular gland. Many epithelial duct cells in the excretory, striated, developing/mature granular and intercalated cells contained both immunoreactivities with various immuno-intensities (Fig. 5). The Mn- and Cu/Zn-SOD-immuno-positive and -immuno-negative duct cells intermingled in each duct of the rat submandibular gland (Fig. 5 b, c). In Mn-SOD-immunohistochemistry, the immunoreaction showed a granular appearance in the infranuclear region of the cytoplasm (Fig. 5 a, c, f). On the other hand, Cu/Zn-SOD-immunoreactions were usually localized in the cytoplasm of the duct cells (Fig. 5 b, d, e). In some cases, a few positive duct cells had immunoreactions both in the nucleus and cytoplasm (data not shown).

In contrast, all acinal cells were found to become negative in immunoreactions for Mn- and Cu/Zn-SODs postnatal after prenatal 2 weeks (Fig. 5).

The alterations in the expression of Mn- and Cu/Zn-SODs-immunoreactions are summarized in Table 1.

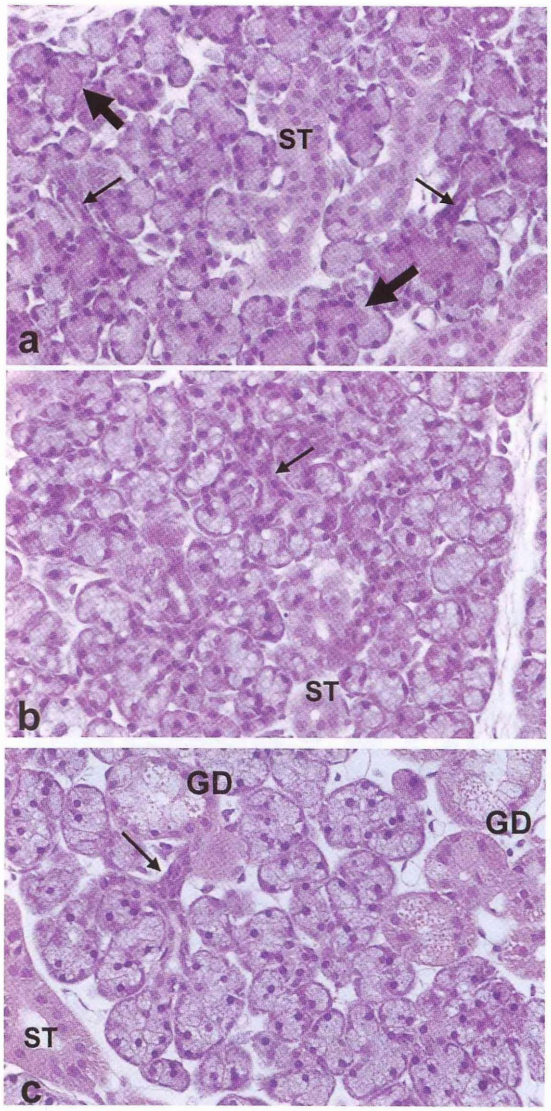

Fig. 4
Histological observations of the rat submandibular glands at postnatal 2 (a), 4 (b) and 8 (c) weeks. Stained with hematoxylin and eosin. The ratios of the cells with clear granules in the acini appear to increase chronologically. By postnatal 8 weeks (c), all cells in the acini have been replaced by cells with eosinophilic granules (thick arrows). The intercalated ducts (arrows) consisting of flat cells with a poor cytoplasm first appeare around postnatal 2 weeks. They consist of flat cells with poor cytoplasm. In addition to a striated duct (ST) and intercalated duct (arrows), the granular ducts (GD), which are characteristic of granules in the cytoplasm, are found in the rat submandibular gland at postnatal 8 weeks. a-c; ×100.

DISCUSSION

The present immunohistochemical study was able to demonstrate different expression patterns of Mn- and Cu/Zn-SOD-immunoreactivities during the development of the rat submandibular gland. The localization of immunoreactions for Mn- and Cu/Zn-SODs in the submandibular gland -- except for prenatal early stages -- was consistent with the previous findings that Mn- and Cu/Zn-SODs are mitochondrial and cytosolic SODs, respectively[7,8]. Since these two SOD-immunoreactions (this study) and - mRNAs[25,26] were co-localized in the same types of submandibular cells, the rat submandibular cells apparently have the ability to produce both mitochondrial Mn- and cytosolic Cu/Zn-SODs. However, there is no possible explanation for unexpected cytosolic localization of Mn-SOD-immunoreactions.

A most interesting finding in this study is a drastic change in the expressions of Mn- and Cu/Zn-SOD-immunoreactivities between prenatal and postnatal stages: the cells in the acini and the epithelial duct cells of submandibular glands were immuno-negative at the prenatal stage, but immuno-positive after birth. Identical expression patterns in the submandibular glands of the fetal and neonatal rats were reported by Munim et al. (1992)[19]. These findings readily lead us to suppose the involvement of pulmonary respiration in this change. Previous studies have reported lower levels of antioxidant enzymes in various tissues in the fetus than in the full-term neonate[19,28-32]. This has been explained by a low demand for cellular antioxidant capacity in the fetus due to the low oxygen concentration in tissues during intrauterine life[33] and by the increased production of superoxide radicals due to an increase in oxygen concentration in tissues after birth[34]. An *in vitro* study has demonstrated that exposure to atmospheric oxygen is toxic to early-stage embryos, and further showed that this toxicity decreased with the addition of exogenous SOD into the culture medium[35]. It is reasonable to consider that Mn- and Cu/Zn-SOD-expressions after birth account for the necessity of the rat submandibular glands for protection against oxygen exposure.

The present observation of 2- to 8-week-old rats showed Mn- and Cu/Zn-SOD-immunoreactivities in all types of epithelial duct cells including excretory, striated, granular and intercalated ducts, but never in the acinar cells, consistent with our previous findings[25,26]. According to developmental studies[36-40], the immature acini contain three types of secretory cells such as acinar, proacinar and terminal tubular cells at the different stages of differentiation and/or maturation. However, the terminal tubular and proacinar cells

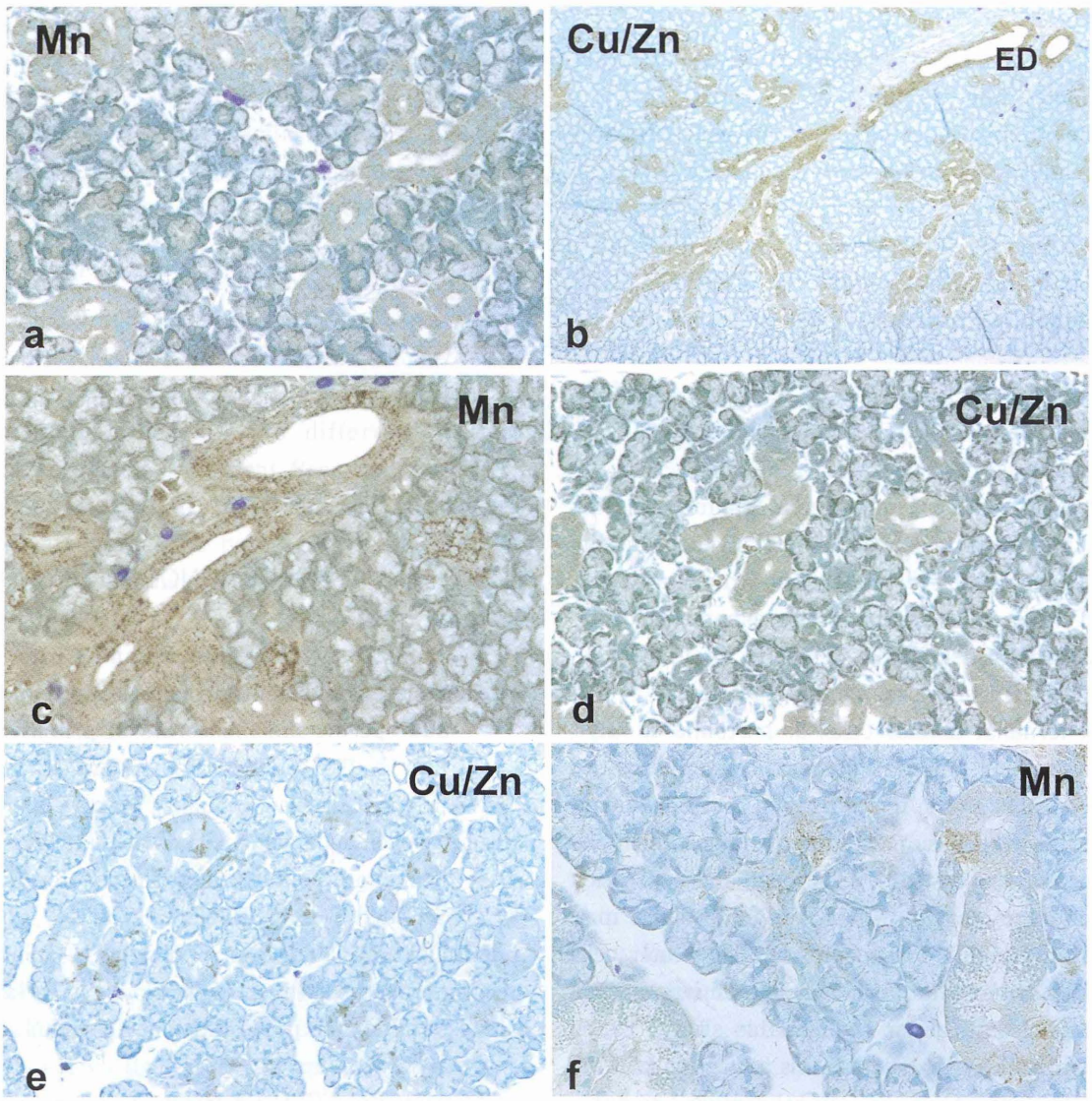

Fig. 5
Mn- (a, c, f) and Cu/Zn-SOD (b, d, e) -immunoreactions at postnatal 2 (a), 3 (b, c), 4 (d) and 8 (e, f) weeks. All the kinds of epithelial duct cells including the intercalated, granular, striated and excretory duct cells (ED) retain both Mn- and Cu/Zn-SOD-immunoreactivities in various immuno-intensities, although the acini completely lose immunoreactions after postnatal 2 weeks. The Mn-SOD-immunoreaction is found in the cytoplasm while Cu/Zn-SOD-immunoreactivity shows a granular appearance in the cytoplasm. a; ×180, b; ×62.5, c; ×180, d; ×180, e; ×125, f; ×250.

(Type I cell and Type III cell called by Cutler and Chaudhry[36], respectively), disappear by postnatal 4 and 2 weeks, respectively[37, 38]. Thus, the acini at postnatal 8 weeks can be regarded as mature acini which are composed of only acinar cells. On the other hand, the differentiation of epithelial cells of all ducts has been reported to conclude by postnatal 4 weeks in the rat submandibular gland[41]. Taken together, these findings show that the Mn- and Cu/Zn-SOD-immunoreactivities indicate the different levels of resistance against reactive oxygen species and antioxidant enzymes between duct and acinar cells after cytodifferentiation of the submandibular gland.

It is interesting that the acinar cells lost their Mn- and Cu/Zn-SOD-immunoreactivities during postnatal stages. Previous investigators have paid attention to the expression and localization of SOD-proteins and mRNAs in mature[25, 26] as well as fetal and neonate rats[19], but no definitive information on this has been provided to date. In spite of the loss of Mn- and Cu/Zn-SOD-immunoreactivities in the acinar cells, the epithelial cells of salivary ducts retained both immunoreactions. One possible explanation for this finding is the maturation and/or differentiation process

		E18D		P1D	P3D	P1W	P2W	P3W	P4W	P8W
ACINI		-*	±**	+	+	+	-	-	-	-
DUCT	ID	NOT DEVELOPED					+	+	+	+
	GD	NOT DEVELOPED						+	+	+
	ST	NOT DEVELOPED		+	+	+	+	+	+	+
	ED	-*	±**	+	+	+	+	+	+	+

Table 1 .
Changes in immunoreactions for Mn- and Cu/Zn-SODs in the rat submandibular gland. ID : intercalated duct. GD : granular duct, ST : striated duct, ED : excretory duct. * : Mn-SOD, ** : Cu/Zn-SOD.

of acini in the rat submandibular glands. In this process, apoptosis has been shown to occur in the acini, in particular the terminal tubular region: TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) analysis revealed that TUNEL-positive cells reached a maximum level (about 14% of the total terminal tubular cells) at postnatal 23 days, after which their numbers decreased progressively[42] (Hayashi et al., 2000). Active oxygen and antioxidant enzymes have been suggested to be involved in the occurrence of apoptosis[43-45] . The postnatal expression of immunoreactions for Mn- and Cu/Zn-SODs may serve as an inhibitor for apoptosis. The acinar cells lose SOD-enzymes after terminal differentiation when apoptotic cells are never seen in this study. Taken together, it is likely that the acini is a weak link in the defense against apoptosis. Furthermore, diabetes mellitus in the rat induces the destruction of acinar cells, *i.e.* xerostomia by apoptosis[49] . Indeed, experimental studies including radiation[45, 46] , duct obstruction[47] , and the administration of ethanol ingetion[48] place the induction of apoptosis in acinar cells, not in duct cells, of mature rat submandibular glands.

ACKNOWLEDGEMENTS

The authors thank Prof. Tadashi Noda, Division of Pedodontics, for his encouragement throughout this study. They also thank the staff of the Division of Oral Anatomy, Department of Biological Science, Niigata University Graduate School of Medical and Dental Sciences, and especially Mr. Kiichi Takeuchi for his excellent technical assistance. This study was supported by a Grant to Frontier Science from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

1) Tolmasoff, J. M., Ono, T. and Cutler, R. G. : Superoxide dismutase : correlation with life-span and specific metabolic rate in primate species. Proc. Natl. Acad. Sci. USA, 77 : 2777-2781, 1980.
2) Honda, S., Ishii, N., Suzuki, K. and Matsuo, M. : Oxygen-dependent perturbation of life span and aging rate in the nematode. J. Gerontol. A Biol. Sci. Med. Sci., 48 : B57-B61, 1993.
3) Ohkuma, N., Kajita, S., Iizuka, H. : Superoxide dismutase in epidermis : its relation to keratinocyte proliferation. J. Dermatol., 14 : 562-568, 1987.
4) Mullarkey, C. J., Edelstein, D. and Brownlee, M. : Free radical generation by early glycation products; a mechanism for accelerated atherogenesis in diabetes. Biochem. Biophys. Res. Commun., 173 : 932-939, 1990.
5) Kim, C. D. and Hong, K. W. : Preventive effect of rebanipide on gastric lesions induced by ischemia-reperfusion in rat. J. Pharmacol. Exp. Ther., 275 : 340-344, 1995.
6) Chen, S. H. and Pan, S. : Sources of superoxide radicals involved in the pathogenesis of diethyldithiocarbamate-induced gastric antral ulcer in rats. J. Formos. Med. Assoc., 97 : 131-134, 1998.
7) Weisiger, R. A. and Fridovich, I. : Mitochondrial superoxide dismutase. Site of synthesis and intramitochondrial localization. J. Biol. Chem., 248 : 4793-4796, 1973.
8) Marklund, S. L. : Human copper-containig

superoxide dismutase of high molecular weight. Proc. Natl. Acad. Sci. USA, 79 : 7634-7638, 1982.
9) Slot, J. W., Geuze, H. J., Freeman, B. A. and Crapo, J.D. : Intracellular localization of the copper-zinc and manganese superoxide dismutases in rat liver parenchymal cells. Lab Invest., 55 : 363-371, 1986.
10) Redman, R. S. : Development of the salivary glands. In The Salivary system, ed. Sreebny, L. E., p. 2-17, Boca Raton, CRC Press, 1987.
11) Cutler, L. S. : The role of extracellular matrix in the morphogenesis and differentiation of salivary glands. Adv. Dent. Res., 4 : 27-33, 1990.
12) Denny, P. C., Ball, W. D. and Redman, R. S. : Salivary glands : a paradigm for diversity of gland development. Crit. Rev. Oral Biol. Med., 8 : 51-75, 1997.
13) Pryor, W. A. and Dooley, M. M. : Inactivation of human alphal-proteinase inhibitor by cigarette smoke : effect of smoke phase and buffer. Am. Rev. Resp. Dis., 131 : 941-943, 1985.
14) Steinberg, D., Parthasarathy, S. and Carew, T. E. : Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. New Engl. J. Med., 302 : 915-924, 1989.
15) Sagai, M., Saito, H., Ichinose, T., Kodama, M. and Mori, Y. : Biological effects of diesel exhaust particles. I. In vitro production of superoxide and in vivo toxicity in mouse. Free Ragic. Biol. Med., 14 : 37-47, 1993.
16) Battino, M., Bullon, P., Wilson, M. and Newman, H. : Oxidative injury and inflammatory periodontal diseases : the challenge of anti-oxidants to free radicals and reactive oxygen species. Crit. Rev. Oral Biol. Med., 10 : 458-476, 1999.
17) Thaete, L. G., Crouch, R. K., Schulte, B. A. and Spicer, S.S. : The immunolocalization of copper-zinc superoxide dismutase in canine tissues. J. Histochem. Cytochem., 31 : 1399-1406, 1983.
18) Thaete, L. G., Crouch, R. K. and Spicer, S. S. : Immunolocalization of copper-zinc superoxide dismutase. II Rat. J. Histochem. Cytochem., 33 : 803-808, 1985.
19) Munim, A., Asayama, K., Dobashi, K., Suzuki, K., Kawaoi, A. and Kato, K. : Immunohistochemical localization of superoxide dismutase in fetal and neonatal rat tissues. J. Histochem. Cytochem., 40 : 1705-1713, 1992.
20) Nonogaki, T., Noda, Y., Narimoto, K., Shiotani, M., Mori, T., Matsuda, T. and Yoshida, O. : Localization of Cu/Zn-superoxide dismutase in the human male genital organs. Hum. Reprod., 7 : 81-85, 1992.
21) Muse, K. E., Oberley, T. D., Sempf, J. M. and Oberley, L. W. : Immunolocalization of antioxidant enzymes in adult hamster kidney. Histochem. J., 26 : 734-753, 1994.
22) Oury, T. D., Chang, L. Y., Marklund, S. L., Day, B. J. and Crapo, J. D. : Immunocytochemical localization of extracellular superoxide dismutase in human lung. Lab. Invest., 70 : 889-898, 1994.
23) Moreno, S., Nardacci, R. and Ceru, M. P. : Regional and ultrastructural immunolocalization of copper-zinc superoxide diamtuase in rat central nervous system. J. Histochem. Cytochem., 45 : 1611-1622, 1997.
24) Loenders, B., Van Mechelen, E., Nicolai, S., Buyssens, N., Van Osselaer, N., Jorens, P. G., Willems, J., Herman, A. G. and Slegers, H. : Localization of extracellular superoxide dismutase in rat lung : neutrophils and macrophages as carriers of the enzyme. Free Radic. Biol. Med., 24 : 1097-1106, 1998.
25) Yamamoto, H., Sasaki, J., Nomura, T. and Nawa, T. : Expression of manganese superoxide dismutase in rat submandibular gland demonstrated by in situ hybridization and immunohistochemistry. Ann. Anat., 181 : 519-522, 1999.
26) Yamamoto, H., Kawano, Y., Nozawa-Inoue, K., Aita, M., Miki, Y., Sasaki, J. and Maeda, T. : The expression of copper, zinc superoxide dismutase (Cu/Zn-SOD) in the rat submandibular gland. Biomed. Res., 23 : 85-90, 2002.
27) Hsu, S.M., Raine, L. and Fanger, H. : Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem., 29 : 577-580, 1981.
28) Yam, J., Frank, L. and Roberts, R. J. : Age-related development of pulmonary antioxidant enzymes in the rat. Proc. Soc. Exp. Biol. Med., 157 : 293-296, 1978.
29) Yoshioka, T., Shimada, T. and Sekiba, K. : Lipid

peroxidation and antioxidants in the rat lung during development. Biol. Neonate, 38 : 161, 1980.
30) Tanswell, A. K. and Freeman, B. A. : Pulmonary antioxidant enzyme maturation in the fetal and neonatal rat. I. Developmental profiles. Pediatr. Res., 18 : 584-587. 1984.
31) Gerdin, E., Tyden, O. and Eriksson, U. J. : The development of antioxidant enzymatic defense in the perinatal rat lung : activities of superoxide dismutase, glutathione peroxidase, and catalase. Pediatr. Res., 19 : 687-691, 1985.
32) Hayashibe, H., Asayama, K., Dobashi, K. and Kato K. : Prenatal development of antioxidant enzymes in rat lung, kidney, and heat: marked increase in immunoreactive superoxide dismutase, glutathione peroxidase, and catalase in the kidney. Pediatr. Res., 27 : 472-475, 1990.
33) Autor, A. P., Frank, L., Roberts, R. J. : Developmental characteristics of pulmonary superoxide dismutase : relationship to idiopathic respiratory distress syndrome. Pediatr. Res., 10 : 154-158, 1976.
34) Freeman, B. A. and Crapo, J. D. : Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. J. Biol. Chem., 256 : 10986-10992, 1981.
35) Noda, Y., Matsumoto, H., Umaoka, Y., Tatsumi, K., Kishi, J. and Mori, T. : Involvement of superoxide radicals in the mouse two-cell block. Mol. Reprod. Dev., 28 : 356-360, 1991.
36) Cutler, L. S. and Chaudhry, A. P. : Cytodifferentiation of the acinar cells of the rat submandibular gland. Dev. Biol., 41 : 31-41, 1974.
37) Chang, W. W. L. : Cell population changes during acinus formation in the postnatal rat submandibular gland. Anat. Res., 178 : 187-202, 1974.
38) Alvares, E. P. and Sesso, A. : Cell proliferation, differentiation and transformation in the rat submandibular gland during early postnatal growth. A quantitative and morphological study. Arch. Histol. Jpn., 38 : 177-208, 1975.
39) Srivastava, H. C. : Development of acinar cells in the rat submandibular gland. J. Anat., 123 : 459-465, 1977.
40) Moreira, J. E., Ball, W. D., Mirels, L. and Hand, A. R. : Accumulation and localization of two adult acinar cell secretory proteins during development of the rat submandibular gland. Am. J. Anat., 191 : 167-184, 1991.
41) Pinkstaff, C. A. : The cytology of salivary gland. Int. Rev. Cytol., 63 : 141-261, 1980.
42) Hayashi, H., Ozono, S., Watanabe, K., Nagatsu, I. and Onozuka, M. : Morphological aspects of the postnatal development of submandibular glands in male rats : Involvement of apoptosis. J Histochem Cytochem., 48 : 695-698, 2000.
43) Hanada, H., Kashiwagi, A., Takehara, Y., Kanno, T., Yabuki, M., Sasaki, J., Inoue, M. and Utsumi, K. : Do reactive oxygen species underlie the mechanism of apoptosis in the tadpole tail? Free Radic. Biol. Med., 23 : 294-301, 1997.
44) Sugino, N., Takiguchi, S., Kashida, S., Takayama, H., Yamagata, Y., Nakamura, Y. and Kato, H. : Suppression of intracellular superoxide dismutase activity by antisense oligonucleotides causes inhibition of progesterone production by rat luteal cells. Biol. Reprod., 61 : 1133-1138, 1999.
45) Kannan, K. and Jain, K. S. : Oxidative stress and apoptosis. Pathophysiology, 7 : 153-163, 2000.
46) Paardekooper, G. M., Cammelli, S., Zeilstra, L. J., Coppes, R. P. and Konings, A. W. : Radiation-induced apoptosis in relation to acute impairment of rat salivary gland function. Int. J. Radiat. Biol., 73 : 641-648, 1998.
47) Walker, N. I. and Gobe, G. C. : Cell death and cell proliferation during atrophy of rat parotid gland induced by duct obstruction. J. Pathol., 153 : 333-344, 1987.
48) Slomiany, B. L., Piotrowski, J. and Slomiany, A. : Chronic alcohol ingestion enhances tumor necrosis factor-alpha expression and salivary gland apoptosis. Alcohol Clin. Exp. Res., 21 : 1530-1533, 1997.
49) Cutler, L. S., Pinney, H. E., Christian, C. and Russotto, S. B. : Ultrastructural studies of the rat submandibular gland in streptozotocin induced diabetes mellitus. Virchows Arch. A Pathol. Anat. Histol., 382 : 301-311, 1979.