DISTRIBUTION OF NITRIC OXIDE SYNTHASE ISOFORMS IN PERIORAL EXOCRINE GLANDS IN RATS

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Abstract

흰쥐 구강주위 외분비선에서 산화질소 합성동위효소의 분포

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내인성 산화질소는 산화질소 합성효소에 의하여 합성되며 여러 분비선에서 다양한 기능을 하리라 추측되고 있다. 구강주위 외분비선은 형태적으로 유사하나 분비물의 성분과 분비량은 서로 달라 이들 조직에서 산화질소 동위효소의 분포와 기능을 추론함은 흥미 있는 일이다. 또한 구강주위 외분비선과 분비선의 지배신경의 산화질소동위효소의 분포에 관한 보고는 희박하다. 본 연구는 흰쥐 구강 주위 외분비조직, 즉 3대 타액선, 혀의 소타액선, 누선 그리고 구강점막의 피지선과 지배신경 및 신경절에서 eNOS와 nNOS의 분포를 면역조직화학 방법에 의하여 관찰하여 다음의 결과를 얻었다.

nNOS는 악하선신경절, 대타액선의 분비도관 주위의 신경절후신경섬유, 혀의 소타액선 주위의 신경절후섬유, 누선에서 강한 양성반응을 보였다. nNOS는 대타액선과 근상피세포에서 중등도의 양성반응을 보였고 이중 이하선에서 반응이 가장 약하였으며, 피지선의 분비관에서 약한 반응을 보였다. 그러나 상교감신경절과 삼차신경절, 소타액선의 분비관 및 대,소 타액선의 선포에서는 반응이 매우 미약하거나 관찰되지 않았다.

eNOS는 혈관의 내피세포와 대타액선의 분비관, 누선의 분비관 및 선포에서 강한 양성 반응을 보였고, 근상피세포에서 중등도의 반응을, 피지선에서 약한 반응을 보였다. 모든 신경절과 신경섬유에서 eNOS의 반응은 음성이었고 타액선의 일부 선포에서는 미약한 면역반응을 보였다.

이상의 결과 eNOS에 의해 합성된 NO는 악안면영역의 외분비선에서 혈류량의 조절과 분비도관의 기능 조절에 관여하고, nNOS에 의한 NO는 외분비선의 자율신경계에서 신경전달물질로의 기능과 분비도관에서의 분비기능 조절에 관여함을 시사하였다.

Ⅰ. Introduction

Nitric oxide (NO), a gas which diffuses readily across the membrane, has been recognized to act as a signalling molecule which binds directly to an enzyme inside the target cells. NO is made by NO synthase (NOS) through deamination of the amino acid, L-arginine, which was first found in the vascular endothelium. The enzyme was isolated, purified, cloned and characterized (Bredt et al, 1990, 1991).

It has become clear that there are at least three isoforms of NOS : Type I NOS or nNOS (constitutive form found in nervous tissue), Type II or iNOS (inducible form in macrophages and hepatocytes) and Type III or eNOS (constitutive form in vascular endothelial cells). nNOS, a 155 kDa protein, was first purified (Mitchell et al., 1991; Bredt and Snyder, 1992) and cloned (Bredt et al., 1991) and highly expressed in the central nervous system and also in the peripheral nerves and the skeletal muscle. nNOS isoenzyme is also widely expressed in other tissues (Knowles and Moncada, 1994). eNOS is a 135 kDa calcium dependant protein, being located in the membrane fraction (Pollock et al., 1991). It was found in vascular endothelial cells and activates soluble guanylate cyclase to relax vascular smooth muscle (Griffith et al., 1984). The inducible form plays an important role in tumor growth (Klotz et al., 1998) and angiogenesis and affects cytotoxicity and tumor induced immunosuppression.

Perioral exocrine glands consist mainly of the 3 major salivary glands, lacrimal, sebaceous glands and minor salivary glands. These glands are similar one another in their secretory function and histological findings, but different in their secretory nature. The functional roles of NOS in exocrine glands were documented for insulin secre-

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tion in the pancreatic secretion (Ember et al., 1997; Xu et al., 1997). The distribution of NOS in the salivary gland was also reported by Lohinai et al. (1995). They focused on ducts and nerve fibers around them in the feline submandibular gland. NO in salivary glands was also reported to control blood flow to the glands by releasing vasoactive intestinal polypeptide (VIP) from the parasympathetic nerve ending (Edwards and Garrett, 1993), kallikrein-kinin system (Damas, 1994) or by mediating Ca^{++} concentration via cGMP pathway (Xu et al., 1997). The distribution and function of NOS in the lacrimal, sebaceous and minor salivary glands have not yet been determined. Nor has it been reported whether perioral exocrine gland is related with the sympathetic superior cervical, parasympathetic submandibular and trigeminal sensory ganglia. This study was performed to find eNOS and nNOS distribution in perioral exocrine glands and their innervating ganglia and nerve fibers.

Ⅱ. Materials and Methods

Sprague-Dawley rats weighing about 250gm were used. The abdominal cavities were opened under pentobarbital sodium anesthesia (50mg/kg, IP) for perfusion fixation via the abdominal aorta. Phosphate buffered saline (10mM phosphate, pH 7.5, 0.15M sodium chloride) was irrigated to take out blood via the inferior vena cava, and 4% paraformaldehyde in PBS was perfused for fixation.

The sublingual gland, submandibular, parotid, lacrimal glands, the tongue including the minor salivary glands and the sebaceous glands in buccal mucosa were removed. The submandibular ganglion with the submandibular gland, the trigeminal and superior cervical ganglia were also excised and fixed in 4% paraformaldehyde for 2~3 hours. The tissues were dehydrated, paraffin-embedded and cut into sections 5um thick. All the tissues were H-E stained for the morphological characterization and Periodic acid Schiff (PAS) and hematoxylin-counterstained to find the mucus or serous nature in the salivary and lacrimal glands.

Immunohistochemical staining was carried out using Vectastain Elite ABC Kit (Vector LAb. Burlingame, CA, U.S.A.). Purified monoclonal mouse anti-endothelial and anti-neuronal NOS (Transduction Lab., Lexington, KY, U.S.A.) were used as primary antibodies. Normal horse serum was used for negative control instead of primary antibodies.

The sections were deparaffinized with xylene and rinsed in PBS. Endogenous peroxidase was blocked by incubation in 3% H_2O_2 in water for 5 minutes. The tissues were incubated in 10% horse serum to block non-specific reactions for 10 minutes and reacted in primary antibody diluted to 1:400 with 0.3% bovine serum albumin in PBS overnight. The tissues were washed with PBS and incubated in biotinylated anti-mouse IgG secondary antibody for 10 minutes. The sections were incubated in streptavidin/peroxidase complex for 5 minutes and developed with 3-amino-9-ethylcarbazole for 6 minutes. The sections were counterstained with Mayer's hematoxylin and mounted with canada balsam for photography in light microscope.

Ⅲ. Results

1. Histological Findings

The salivary glands in rats were encapsulated and divided into lobes or lobules by connective tissue septa. The parenchyma of the salivary glands consists of the excretory ducts and acini. The ducts consist of the excretory, striated and intercalated portions of varied length and size and have cuboidal and columnar epithelia. The ducts were abundant and well branched in the submandibular and sublingual glands compared with those in the parotid gland. The sublingual gland was purely mucous in secretion, which was noted by pink stained acini in PAS. The nuclei of the acini were located near the base of the acini cells and appeared angular and compressed (Fig. 1). The parotid gland was serous in secretion and PAS negative. The serous cells were cuboidal and formed a small tubular lumen (Fig. 2). The submandibular gland was mixed in secretion. Many serous cells were displaced to the terminal portion of the acinus to form the demilunes (Fig. 3). The minor salivary glands in the tongue were located in the posterior part of the tongue, where the mucous glands were further posterior. The ducts were not many compared with those of the major salivary glands. Von Ebner's gland ducts, ducts of serous gland in the tongue, were opened into the sulcus around the circumvallate papilla (Fig. 5).

The lacrimal gland was of the tubular type and its terminal portions were relatively large and irregular (Fig. 4). The sebaceous glands were sac-like and opened into hair follicles.

The ganglion cells in the submandibular, superior cervical, and trigeminal ganglia were large and bright in H-E stain. The submandibular and superior cervical ganglia were encapsulated by fibrous tissue and rich in nerve fibers. The submandibular ganglion was observed as dispersed accumulations of ganglionic cell bodies in varying numbers close to the main excretory ducts. The trigeminal ganglion cells were dispersed in the ganglion with nerve fibers.

2. Immunohistochemical Findings (Table 1, 2)

The glands and their ganglia were stained using immunohistochemistry for e-NOS and n-NOS. Negative controls were not stained in all the glands.

3. Endothelial nitric oxide synthase (Figs. 6~13)

eNOS-immunoreactivity was strongly demonstrated in the vascular

Table 1. Immunoreactivity of NOS isoforms in rat perioral exocrine glands

	nNOS						eNOS					
	SL	SM	PT	LA	SE	MT	SL	SM	PT	LA	SE	MT
excretory ducts	++	++	+	++	+	-	+++	+++	++	+++	+	-
Acini	-	-	-	+++	+	-	+	+	-	+++	-	-
Myoepithelial cells	++	++	++	?	NS	?	++	++	++	?	NS	?
Vascular endothelium	-	-	-	-	-	-	+++	+++	+++	+++	NS	+++

- : negative, + : weak, ++ : moderate, +++ : strong reactivity.

SL : sublingual gland, SM : submandibular gland, PT : parotid gland, LA : Lacrimal gland,

SE : Sebaceous gland, MT : minor salivary gland in tongue, NS : No structure, ? : questionable.

Table 2. Immunoreactivity of NOS isoforms in the ganglia and their fibers which innervate rat perioral exocrine glands

	nNOS	eNOS
Trigeminal ganglion	-	-
Superior cervical ganglion	-	-
Submandibular ganglion	+++	-
Parasympathetic postganglionic fibers	+++	-
Trigeminal sensory fibers	+/-	-
Trigeminal motor fibers	-	-

- : negative, +/- : weak or negative, +++ : strong reactivity.

endothelial cells in all the perioral glands. Most of the excretory, striated and intercalated ductal epithelia also were strongly stained in the 3 major salivary glands. Although the stain intensities of the excretory ducts were varied, they were strongest in the sublingual gland, and the submandibular and parotid gland in this order. On the contrary, the excretory ducts of the minor salivary glands in the tongue showed no immunoreactivity. Some acini of the sublingual and submandibular glands were occasionally very weakly stained, but those of the parotid gland were seldom stained. The myoepithelial cells were scattered between the acini lining cells and the basement membrane in all the major salivary glands. They were found to have moderate intensites.

Many of the ducts and acini in the lacrimal gland showed strong reaction. Relatively weak reactivity was observed in the sebaceous gland ducts and secretory cells. The superior cervical, trigeminal and submandibular ganglia and their preganglionic and postganglionic fibers did not show immunoreactivity.

4. Neuronal nitric oxide synthase (Figs. 14～23)

Strong nNOS-immunoreactivity was seen in a large number of neuronal cell bodies of the submandibular ganglia. nNOS immunoreactivity was diffusely spread over the cytoplasm and around the dark nuclei. From the cell bodies, axonal processes protruded and ran between the perikarya, and formed coarse nNOS immunoreactive nerve fibers from the ganglia. Most of the cell bodies showed various reaction intensities, while some of the cells displayed none. Immunoreactivity was also observed in the nerve fibers adjacent to the excretory ducts.

On the other hand, no immunoreactivity could be seen in the cell bodies of the superior cervical and trigeminal ganglia. Between the cell bodies of the trigeminal sensory roots some extremely fine nerve fibers displayed weak reactivity. No immunoreactivity could be demonstrated in the motor root of the trigeminal ganglia.

nNOS-immunoreactivity was found in some nerve fibers near the minor salivary glands of the tongue and the lingual papillae. The reactive fibers were very tiny and thin. Some thick fibers were observed to be reactive in a portion of the whole fibers, which might be mixed in function.

nNOS-immunoreactivity was also seen in the ducts of the major salivary glands and acini, and ducts of the lacrimal and sebaceous glands. The intensities were strong in the lacrimal gland, moderate in the sublingual, and submandibular glands and weak in the parotid and sebaceous glands.

Moderate immunoreactivity was observed in the myoepithelial cells in the major salivary glands. No nNOS immunoreactivity could be demonstrated in the acini of the salivary glands and vascular endothelium.

Ⅳ. Discussion

NOS isoforms contain two consensus binding sequences for NADPH-d (Nicotinamide adenine dinucleotide phosphate diaphorase), two for FAD (Flavin adenine nucleotide), one for

FMN(Flavin mononucleotide), one for calmodulin and one consensus sequence for phosphorylation by cyclic AMP-dependant protein kinase (PKA). NADPH-d as a cofactor of NOS (Forstermann et al., 1991; Hope et al., 1991) and co-distributor in the central and peripheral nervous tissue (Dawson et al., 1991; Soinila, et al., 1996) has been used for the localization of NOS for its simplicity in method. The localization using NADPH-d histochemistry and NOS-immunoreactivity was in accordance in several ganglia such as neuronal cell bodies in major pelvic, nodose and dorsal root ganglia. On the contrary, a discrepancy in distribution was also reported in the brain (Kharazia et al., 1994) and the stellate and mesenteric ganglia (Santer and Symons, 1993; Alm et al, 1995). Thus, immunohistochemical reactions were used to find NOS isoform localization in the exocrine glands in the present study.

The parasympathetic submandibular and otic ganglia are important sources for the innervation of the submandibular, sublingual and parotid glands, respectively. The pterygopalatine ganglion is also a parasympathetic ganglion for the secretion of the lacrimal gland. This study demonstrated that many nNOS-immunoreactive ganglion cells and postganglionic fibers were abundant in the submandibular ganglion. The intensity was, however, varied among cells and some of the cells did not show any reactivity. Depletion of nNOS or different neuropeptides contained in those cells may be explained by the diversity of intensities, although a detailed functional study is needed.

The parasympathetic ganglia responsible for the exocrine secretion in the head and neck regions are also innervated by the sympathetic and sensory nerve fibers. The sensory fibers in the parasympathetic submandibular, lacrimal and otic ganglia originate from the mandibular and maxillary nerves, whose cell bodies are in the trigeminal ganglion. The sympathetic fibers related to the ganglia are postganglionic from the superior cervical ganglion. The cell bodies and preganglionic and postganglionic sympathetic fibers could not be demonstrated to have any nNOS-immunoreactivity in the present study. This finding was similar to those reported in many different sympathetic ganglia (Anderson et al., 1993; Ceccatelli et al., 1994; Vanhatalo and Soinila, 1994). Moreover, the motor and sensory roots of trigeminal nerves seldom displayed nNOS immunoreactivity. This finding was similar to the previous report in that only 1% cells in sensory ganglia expressed NOS at early adulthood (Bredt and Snyder, 1994). The nNOS-immunoreactive nerve fibers close to the salivary gland ducts, ganglia and minor salivary glands in the tongue should be, therefore, parasympathetic. Their chemical nature to be cholinergic or non-adrenergic non-cholinergic (NANC) and the possible co-localization of two neurotransmitters should be further studied using other approaches such as double immunolocalization of NOS and choline acetyltransferase.

Nitric oxide has been regarded as a putative NANC neurotransmitter. NO may also act as NANC neurotransmitter in the salivary glands, which is substantiated by the reports that NOS immunoreactive nerve fibers were found in the respiratory epithelium, gastrointestinal tract and penile corpus spongiosum as NANC fibers and NO could control VIP secretion in the parasympathetic nerve endings (Fisher et al., 1996; Belvisi et al., 1995). It was also found in the trachea and bronchiolar tracts (Rosbe et al., 1996), where it might function to dilate the respiratory tract by increasing cGMP concentration in the respiratory smooth muscle (Ward et al., 1995). NO may also control intracellular calcium levels through bradykinin and ATP (Sakai et al, 1995) and mucin secretion in tracheal epithelium (Adler, 1995).

The intercalated, striated and excretory ducts were shown to have eNOS and nNOS-immunoreactivities in all the major salivary and lacrimal glands, especially in the sublingual and submandibular glands. The possibility that NO, therefore, plays a role in controling the lumen of the ducts or electrolyte balance of the cells in the salivary and lacrimal gland ducts is suggested as exhibited in the other glands. The ducts in the minor serous and mucus glands in the tongue and the Von Ebner's gland duct, where salivation is relatively minor compared with the major glands, did not show any immunoreactivity on both eNOS and nNOS. These findings suggest that the diameter of the ducts and secretory volume can be regulated by NO in the major salivary glands. eNOS was strongly stained in the vascular endothelial cells in the perioral glands. NO in the salivary glands can also diffuse across cell membranes and results in vasodilation by increasing cGMP (Edward and Garret, 1993; Knowles and Moncada, 1994).

Interestingly most acini cells in the salivary glands were seldom stained in contrast to the excretory ducts in those glands. The myoepithelial cells in the major salivary glands and the acini of the lacrimal gland showed strong or moderate immunoreactivity. This suggests that NO helps acinar secretion by the contraction of myoepithelial cells in the salivary glands. Furthermore NO released by excretory ducts may diffuse into acini cells and act on the cells directly to secrete watery and organic saliva (Kim, 1995) since NO as a gas can easily diffuse into neighboring cells and work locally to be converted to nitrates and nitrites by oxygen and water.

In summary, NOS isoform localizations in various ganglia and secretory epithelium may reflect different functional roles of NO in the neural transmission in the ganglia and excretory functions of the effector cells. It is suggested that eNOS-synthesized NO is implicated in the regulation of blood flow and secretion in perioral exocrine glands, whereas nNOS-synthesized NO is involved in the regulation of secretion in the glands, acting as a neurotransmitter in their innervating parasympathetic ganglia.

Ⅴ. Conclusion

Endogenous nitric oxide (NO) synthesized by NO synthase (NOS) has been reported to play a role in the secretion of exocrine glands. Little has been found, however, on the distribution of NOS isoforms in perioral exocrine glands and their innervating ganglia. This study was carried out to investigate the distribution of endothelial (eNOS) and neuronal NOS (nNOS) in the 3 major salivary glands, minor salivary glands in the tongue, lacrimal and sebaceous glands in the buccal mucosa and their innervating ganglia and nerve fibers. Monoclonal mouse anti-endothelial NOS and anti-neuronal NOS were used as primary antibodies for immunohistochemistry.

nNOS immunoreactivity was strongly demonstrated in the submandibular ganglion and postganglionic fibers in the submandibular and minor salivary glands in the tongue and in the lacrimal gland. nNOS was also moderately stained in the myoepithelial cells and excretory ducts of the major salivary glands, where the ducts in the parotid gland stained weakest. The reactivity was weak in the sebaceous gland and hardly demonstrated in the superior cervical ganglion, trigeminal ganglion, excretory ducts of the minor salivary glands and acini of all the salivary glands.

A strong eNOS immunoreactivity was observed in the vascular endothelial cells, excretory ducts of the major salivary glands and the lacrimal gland. The eNOS immunoreactivity was moderate in the myoepithelial cells in the major salivary glands. On the contrary, a very weak reactivity was seen in some acini of the major salivary glands and the sebaceous gland, and no reactivity demonstrated in the ganglia and nerve fibers.

These results suggest that eNOS-synthesized NO is implicated in the regulation of blood flow and secretion in perioral exocrine glands, whereas nNOS-synthesized NO is involved in the regulation of secretion in the perioral exocrine glands and may act as a neurotransmitter in their innervating parasympathetic ganglia.

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사진부도 설명

Fig. 1. All of mucous acini in the sublingual gland are stained pink with PAS, on the contrary the ducts and surrounding connective tissue are blue with hematoxylin. PAS-Hematoxylin stain, ×100
Fig. 2. All of serous acini in the parotid gland are not stained with PAS. PAS-Hematoxylin stain, ×100
Fig. 3. Around half of acini in the submandibular gland, which are stained with PAS, are mucous. PAS-Hematoxylin stain, ×100
Fig. 4. The lacrimal gland is entirely negative to PAS stain. The ducts and secretory portions of the lacrimal gland are tubular shape. PAS-Hematoxylin stain, ×100
Fig. 5. A posterior part of the rat tongue shows the anterior serous gland (SM) and posterior mucous gland (MG). The serous gland ducts (von Ebner's gland duct) opens into the sulcus of circumvallate papilla (CP). H-E stain, ×80
Fig. 6. Strong eNOS immunoreactivities are seen in many ducts of the sublingual gland. On the contrary, any immunoreactivity can not be seen in acini. ×150
Fig. 7. Strong to moderate eNOS immnoreactivities are demonstrated in the submandibular gland ducts and vessels (arrows). ×200
Fig. 8. The parotid gland ducts are eNOS-immunostained moderately compared to the sublingual and submaxillary glands ducts. ×200
Fig. 9. The lacrimal gland shows strong eNOS immunoreactivities on both acini and ducts. ×200
Fig. 10. The sebaceous glands open into hair follicles. Their acini shows weak eNOS immunoreactivities. ×100
Fig. 11. Myoepithelial cells (arrows) around acini and ducts in sublingual gland have eNOS immunoreactivities. ×150
Fig. 12. nNOS immunoreactivities are also found in myoepithelial cells (arrows) in the parotid gland. ×200
Fig. 13. Any eNOS immunoreactive cells can not be demonstrated in acini of the tongue. ×180
Fig. 14. Strong nNOS immunoreactivity are seen in the submandibular ganglia (G) and nerve fibers (arrows) around the excretory duct (ED). ×200
Fig. 15. No nNOS immunoreactivities can be demonstrated in ganglion cell bodies and nerve fibers of the superior cervical ganglion. ×200
Fig. 16. nNOS immunoreactive cell bodies in the trigeminal ganglion are few. ×300
Fig. 17. Weak nNOS immunoreactivity are demonstrated in some sensory fibers (SF) of the trigeminal ganglion in comparison with motor fibers (MF) which have no the reactivity. ×80
Fig. 18. Moderate nNOS immunoreactivity are seen in the sublingual gland ducts. No acini show the reactivity. ×100
Fig. 19. Low magnification of the submandibular gland shows nNOS immunoreactivities in the ducts. Plenty of the reactive cells are seen. ×60
Fig. 20. Weak nNOS immunoreactivity is observed in the parotid gland ducts, on the contrary the relatively stronger reactivity also is seen in nerve fibers (arrows) around vessels and ducts. ×150
Fig. 21. The lacrimal gland shows strong eNOS immunoreactivities in both acini and ducts. ×250
Fig. 22. Very weak staining with nNOS is seen in the sebaceous gland. ×100
Fig. 23. Many strong nNOS reactive nerve fibers (arrows) are demonstrated near the minor salivary gland of the tongue. On the contrary any reactivity can not be seen in acini of the gland. ×120

사진부도 1

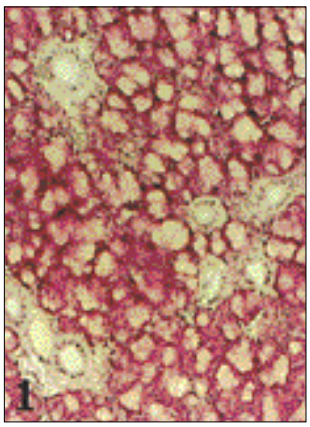


Fig. 1

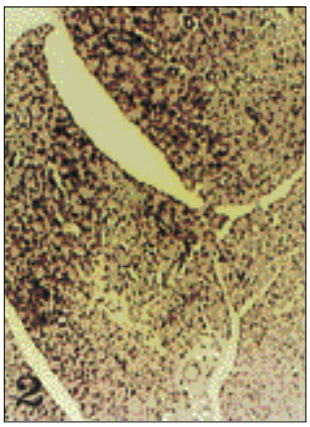
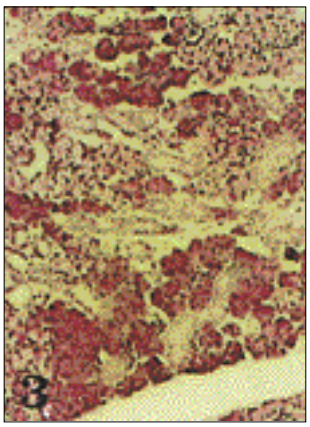


Fig. 2



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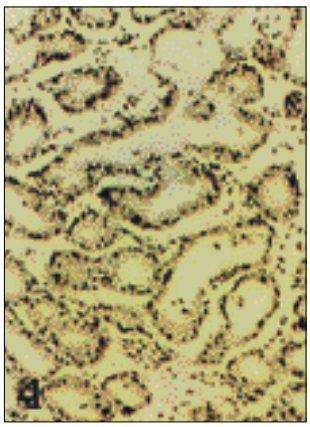

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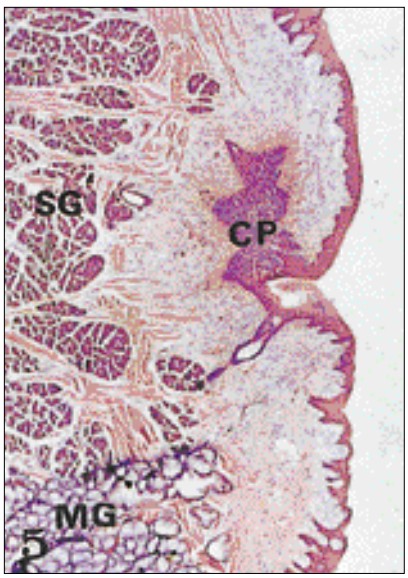

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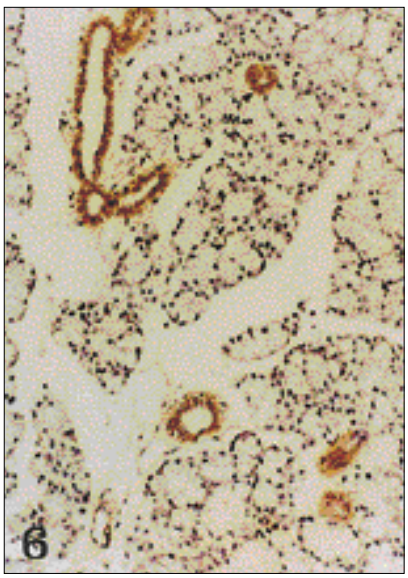

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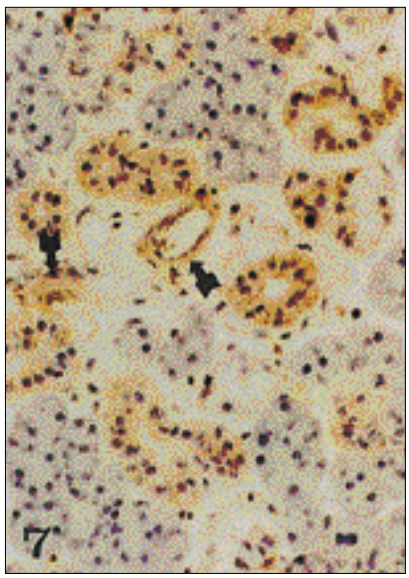

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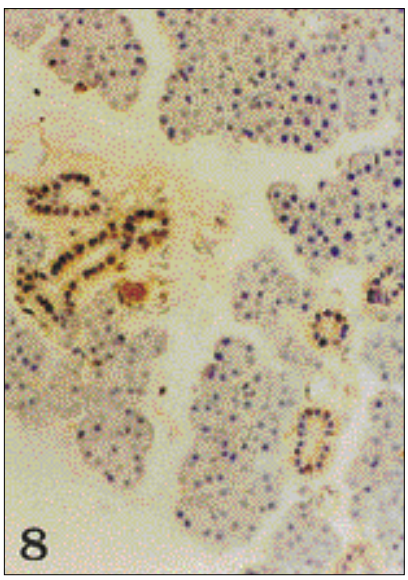

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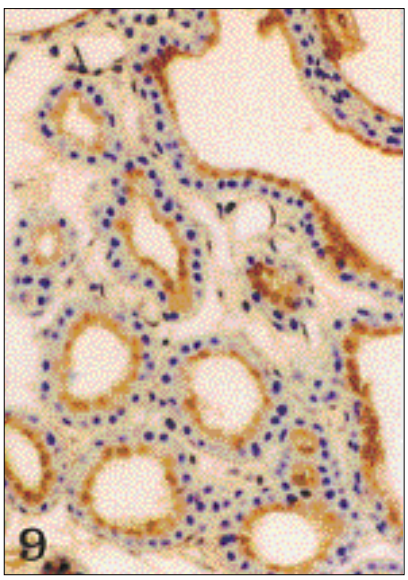

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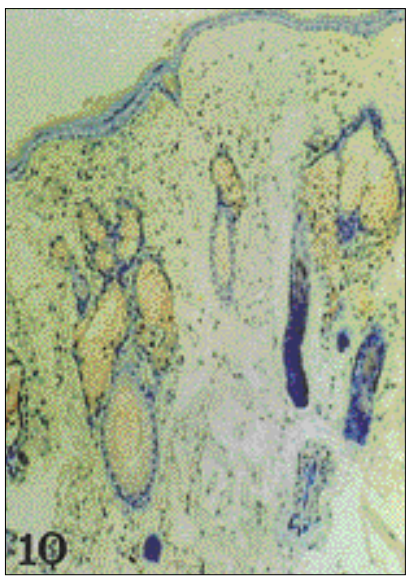
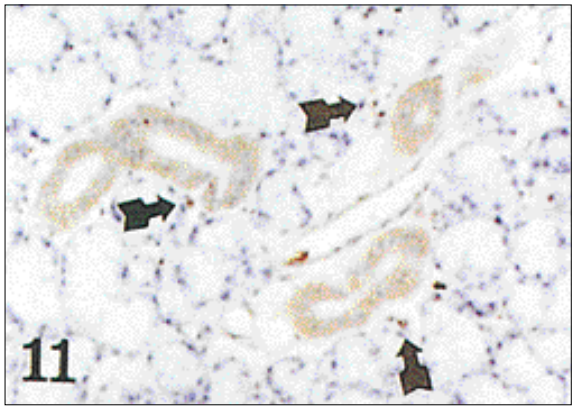


Fig. 10

사진부도 2



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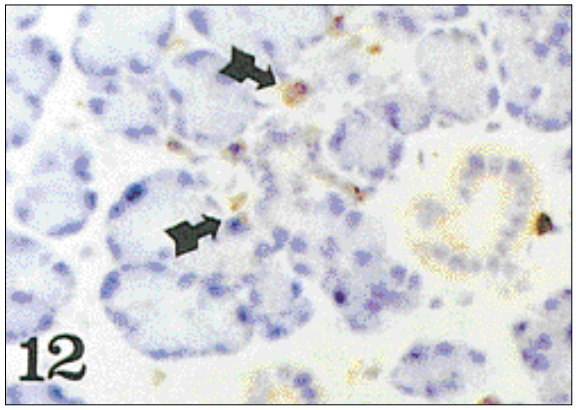
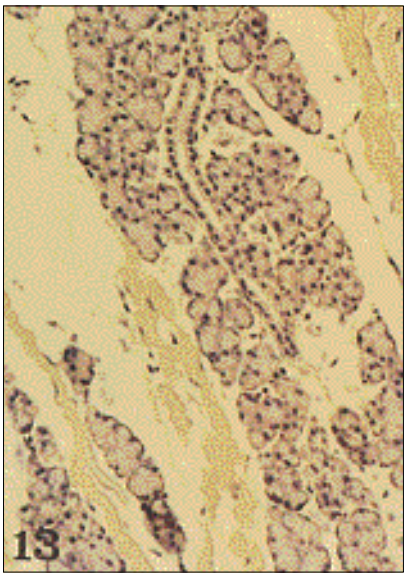


Fig. 12



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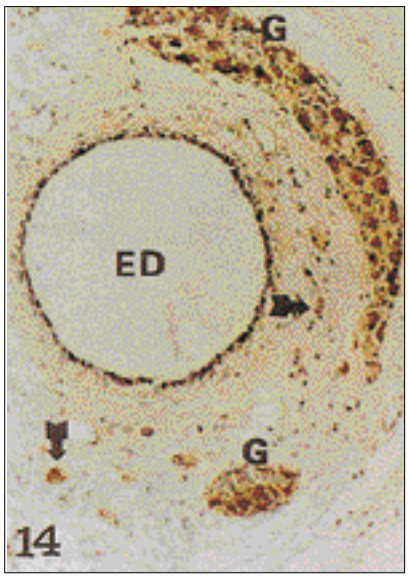

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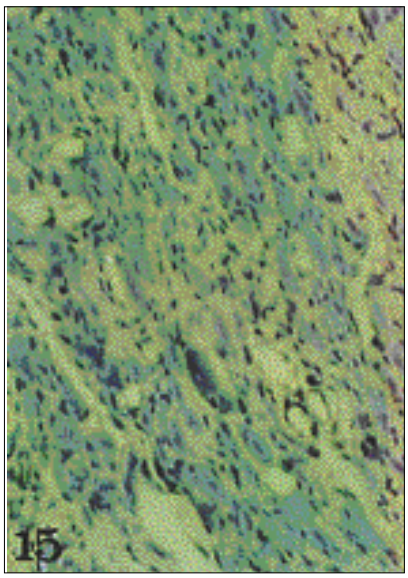

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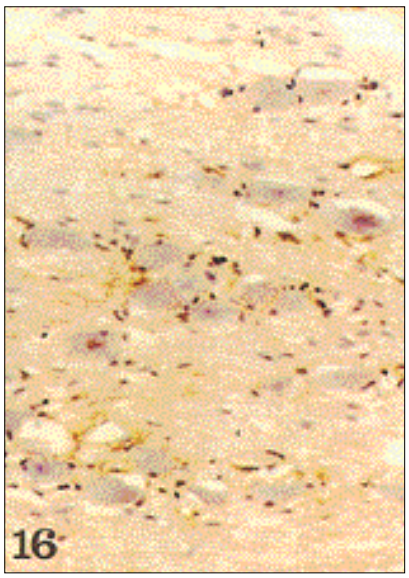

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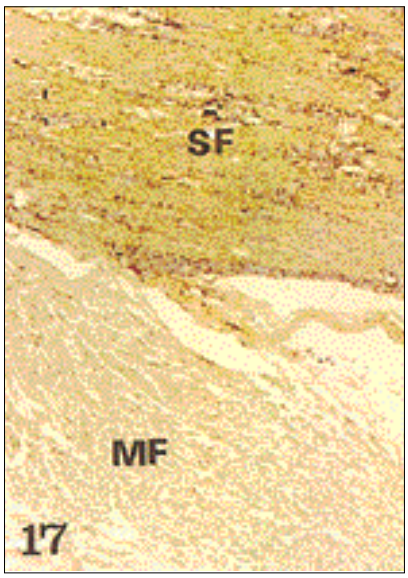


Fig. 17

사진부도 3

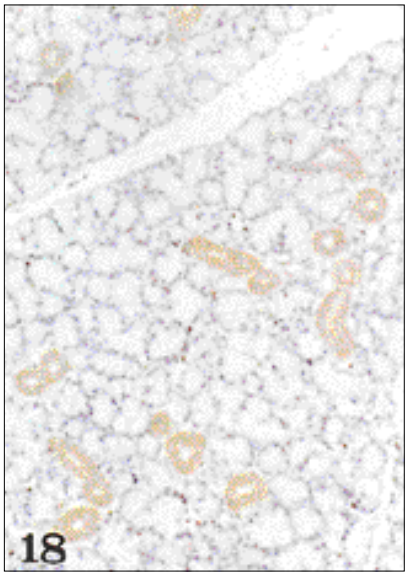
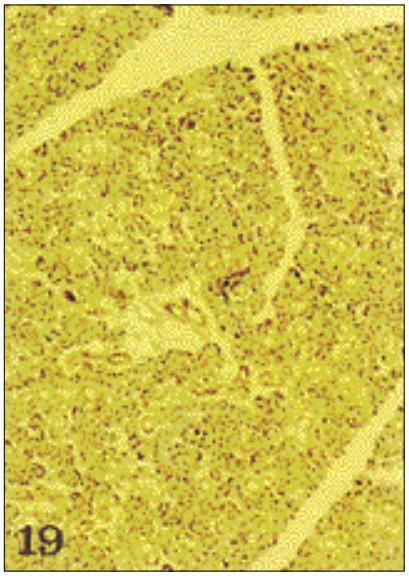


Fig. 18



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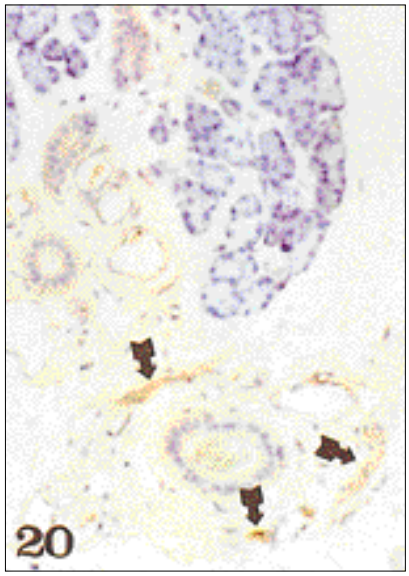

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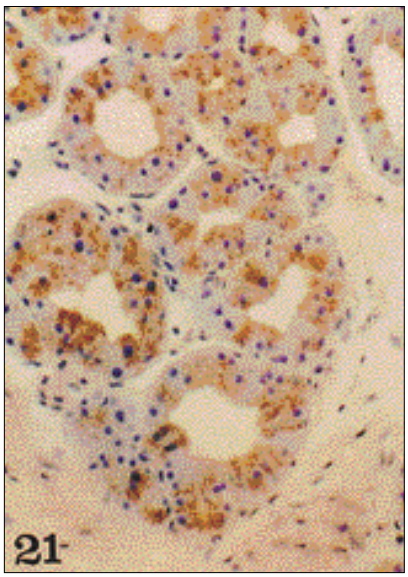

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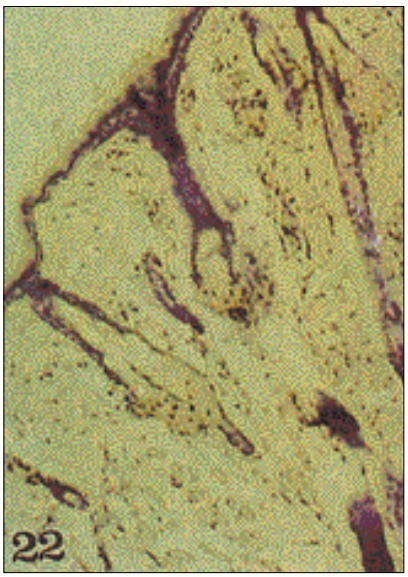

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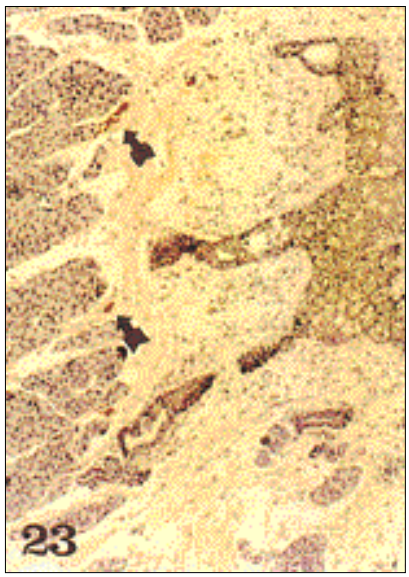

Fig. 23