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Immunocytochemical Localization of Nerve Growth Factor (NGF) in the Submandibular Gland of Adult Mice by Light and Electron Microscopy*

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Summary. Nerve growth factor (NGF) was localized in the submandibular gland of adult male mice by a direct immunocytochemical method using highly purified antibodies against NGF coupled to horseradish peroxidase. In light microscopic sections the reaction product was entirely confined to the cells of the secretory tubules. The acinar part of the gland was free of reaction product. This finding was confirmed by electron microscopy. Within the cells NGF was localized exclusively in the apical secretory granules.

No reaction was observed in the rough endoplasmic reticulum, the Golgi region or in the granules of the basal part of the cells. This observation favours the assumption that NGF is derived from a precursor molecule and that the precursor is transformed into immunologically active NGF within the secretory granules during their transport from the basal to the apical part of the tubular cells. Stimulation of the submandibular gland with carbachol (2 mg/kg) led to a massive release of the content of the secretory granules, including NGF, into the salivary duct.

Key words: Nerve growth factor – Submandibular gland mice – Immunocytochemistry.

Introduction

Since the detection of Nerve Growth Factor (NGF) (Bueker, 1948; Levi-Montalcini and Hamburger, 1951; Levi-Montalcini and Booker, 1960) its importance for the growth, differentiation and maintenance of function of adrenergic and

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sensory neurons has been well established (Levi-Montalcini and Angeletti, 1968; Levi-Montalcini, 1974). All these studies depended on the availability of sufficient amounts of NGF, which have been provided by adult male mouse submandibular gland, the richest source of NGF so far detected (Levi-Montalcini and Angeletti, 1961; Berger et al., 1975). However, it has been shown that the submandibular gland is not the exclusive site of NGF synthesis. This can be deduced from the observation that after surgical removal of the submandibular gland, the blood level of NGF recovered completely after an initial drop, in the absence of any regeneration of the submandibular gland (Hendry and Iversen, 1973). Moreover, it has been shown that a series of other tissues and also established cell lines grown in vitro are able to synthesize NGF (Levi-Montalcini and Hamburger, 1951; Burnham et al., 1972; Longo and Penhoet, 1974; Varon et al., 1974; Young et al., 1975).

In previous studies NGF was localized exclusively in the cells of the secretory tubules of the submandibular gland. The morphological differentiation of the cells and the synthesis of NGF are under the control of testosterone (Levi-Montalcini and Angeletti, 1961; Caramia et al., 1962; Caramia, 1966a; Caramia, 1966b; Goldstein and Burdman, 1965).

So far, attempts to localize NGF in specific cellular compartments have yielded contradictory results. Association with the cytoplasm, with secretory granules, with the basal part of the cell and with the nucleus have been reported on the basis of immunofluorescence studies (Levi-Montalcini and Angeletti, 1961; Goldstein and Burdman, 1965; Kumar et al., 1972). It was the aim of the present experiments to localize NGF at the ultrastructural level using highly purified NGF antibodies coupled to horseradish peroxidase.

We wish to report that NGF is localized in the secretory granules of the apical part of the secretory tubular cells and that it is released into the saliva.

Materials and Methods

Materials

Adult male mice of balb C strain were used. One group of animals received an intraperitoneal injection of 2 mg/kg of carbachol (carbamylcholine-HCl, Merck) 10 min prior to fixation.

Coupled Antibodies

Antibodies against 2.5 S NGF (NGF AB) were raised in sheep and highly purified by affinity chromatography (Stöckel et al. J. Neurochem., in press). NGF AB were coupled to horseradish peroxidase (HRP. Sigma, Type VI) by the method of Nakane and Kawaoi (1974). This procedure gives a high yield of coupled products and little inactivation of the antibody. Peroxidase activity of the coupled product (NGF AB-HRP) was controlled by the diamino benzidine (DAB. Sigma) reaction (Graham and Karnovsky, 1966) and the NGF neutralizing activity of NGF AB-HRP in a bioassay system using chicken dorsal root ganglia (Fenton, 1970).

Fixation

a) Immunocytochemistry. In preliminary experiments different fixatives were tested for their ability to retain the highest possible amount of NGF in the tissue, to preserve the highest possible antigenic

activity of NGF and to provide a good ultrastructural preservation, all at the same time. NGF antigenicity was completely abolished even by very low concentrations of glutaraldehyde. The crosslinking agent dimethylsuberimidate (20 mg/ml in 0.15 M Tris-HCl, pH 9.5, containing 0.02 M Ca^{++} recently introduced as a fixative for electron microscopy by Hassell and Hand (1974) resulted in a good preservation of the ultrastructure but again, NGF AB binding capacity was impaired.

Optimal results for our purpose were obtained with 8% formalin, freshly prepared from paraformaldehyde powder, in 0.1 M phosphate buffer, pH 7.4. All fixations were started by perfusion through the heart with a short pre-rinse of Ringer solution containing 0.1% procain and 1,000 U-USP heparin (Liquemin, Roche) followed by 8% formalin for 15 min. After dissection and trimming of tissue blocks ($2 \times 2 \times 1$ mm) from the middle part of the gland the blocks were immersed in the same fixative for a further 45 min. All solutions were kept cold and the animals were placed in ice during perfusion.

Following fixation, the tissue blocks were washed in 0.1 M phosphate buffer and embedded in polyethyleneglycol (PEG A 1,000, molecular weight 950–1,050, melting point 37–40° C, Fluka, Buchs, SG, Switzerland) according to method 2 described by Mazurkiewicz and Nakane (1972).

b) Normal Electron Microscopy. Animals were perfused through the heart with a fixative containing 2.5% glutaraldehyde (purified, Merck) and 1% paraformaldehyde in 0.1 M phosphate buffer. After soaking in phosphate buffer overnight, the tissue blocks were postfixed in 1.33% OsO_4 in s-Collidine buffer for 2 h, washed in the same buffer, block-stained with uranyl acetate for 1 h, dehydrated and embedded in Epon 812.

Immunocytochemistry

5 μ sections were cut and floated for 15 min on a 5 % glycerol-PBS (phosphate buffered saline) solution to remove the PEG and transferred to microscope slides. These slides were pretreated as follows. Clean slides were coated with a thin layer of silicon (dimethyldichlorosilane, 1% solution in benzene. Bio Rad Laboratories, Richmond, Ca.), dried and dipped into filtered hot gelatin solution (10 g purified pigskin gelatin (Eastman Kodak, Rochester, N.Y.) dissolved in 500 ml cold water, add 500 ml boiling water and 100 ml 0.5% potassium sulphate) and dried at 60° C. This procedure gave a good adhesion of the sections to the slides and allowed easy removal of the Epon embedded sections from the glass slides by dipping them for a few seconds into liquid nitrogen.

After preincubation for 15 min with PBS, the sections were exposed to NGF AB-HRP (8 μg/100 ml) for 30 min at room temperature, followed by a wash in PBS for 30 min. For the visualization of HRP activity the procedure of Graham and Karnovsky (1966) with modifications described by Mazurkiewicz and Nakane (1972) was applied. A preincubation in 0.03% DAB (without H_2O_2) for 30 min was followed by 0.03% DAB containing 0.01% H_2O_2 for 30 min (Streefkerk and Van der Ploeg, 1973) and a final wash in PBS.

For light microscopy the sections were dehydrated and mounted in Eukitt (Kindler, Freiburg, West Germany). For electron microscopy the sections were postfixed in 1.33% OsO_4 and dehydrated in a graded series of ethanols. Embedding was achieved by inverting a gelatine capsule filled with Epon 812 over the tissue section and polymerizing for 3 days at 60° C. The blocks were removed from the microscope slides by dipping them into liquid nitrogen.

Two types of control experiments were performed. In the first the sections were directly incubated with DAB with omission of the NGF AB-HRP step (endogenous peroxidase activity). In the second, H_2O_2 was omitted from the reaction mixture (autoxidation of DAB and non-specific adsorption).

Electron Microscopy

Thin sections were cut with a diamond knife on an LKB Ultrotome III. Unstained sections or sections contrasted with uranyl acetate for 3 min, or with uranyl acetate followed by lead citrate (Reynolds, 1963) for 13 min were examined in an AEI EM 801 or a Zeiss EM 10, with an accelerating voltage of 60 or 80 kV.

Results

Light Microscopy

In normal light microscopic sections, the serous secretory tubules are characterized by typical pyramidal cells arranged around a well defined duct lumen. The cells display an accumulation of secretory granules in their apical portion. In the mucous acini the lumen is often not visible, the shape of the cells is not typically pyramidal and no polar accumulation of secretory granules can be recognized.

After immunocytochemical treatment, the reaction product is localized exclusively in the cells of the serous secretory tubules. Within these cells the reaction product is apparently concentrated in the apical secretory granules whereas the basal part of the cell is free of reaction product (Fig. 1).

In control sections with either omission of NGF AB-HRP or omission of H_2O_2 from the reaction mixture containing diaminobenzidine, no formation of reaction product could be observed, even after prolonged incubation of up to 4 h.

Electron Microscopy

With the fixation procedure chosen, a generally good ultrastructural preservation of the gland tissue was achieved. The only damage observed was at the duct lumen and the most apical parts of the cells in the region of the secretory

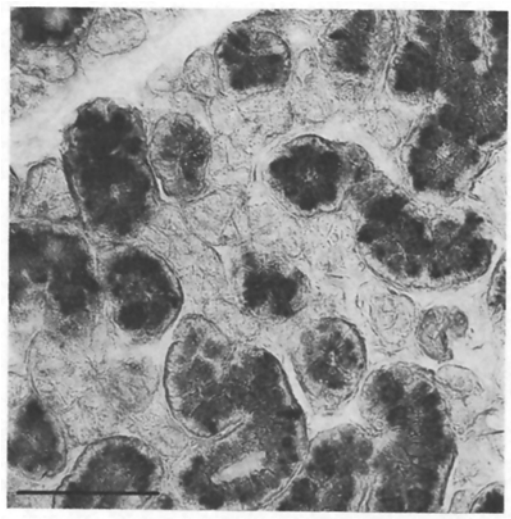

Fig. 1. Light microscopic section through the submandibular gland of an adult male mouse, treated for 30 min with NGF AB-HRP and reacted for visualization of HRP activity. The cells of the secretory tubules are heavily labeled; the reaction product is concentrated in the apical part of the cells. Scale marker 100 μ. × 190

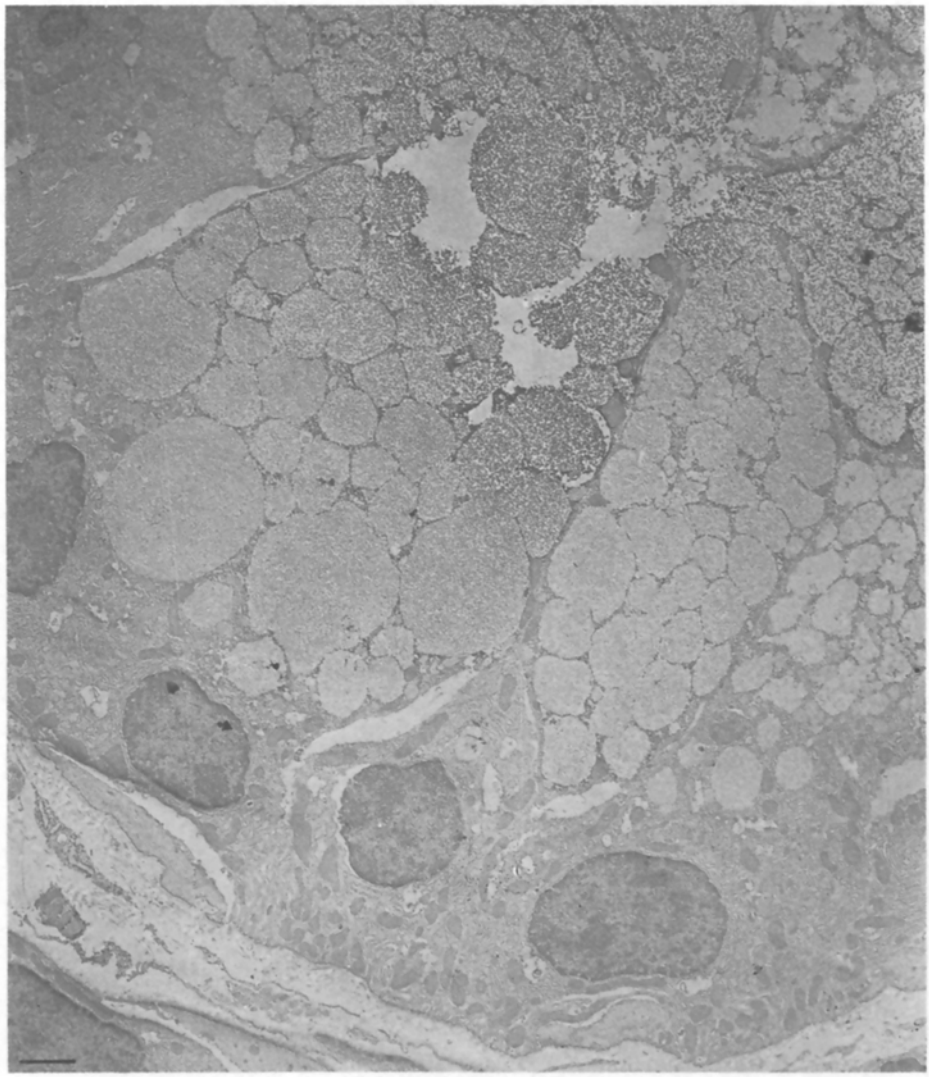

Fig. 2. Electron micrograph of secretory tubular cells treated for 30 min with NGF AB-HRP and reacted for visualization of HRP activity. NGF positive reaction is confined to secretory granules in the apical part of the cells and the secretory duct. Scale marker 2 μ. ×4,000

tubules. These cells are known to secrete proteases and since during perfusion fixation the fixative reaches the basal parts of the cells first, it is possible that the apical parts suffer proteolytic damage during the fixation procedure. All ultrastructural components typical of the male mouse submandibular gland (Caramia, 1966a, b) could be distinguished.

Treatment with NGF AB-HRP and subsequent visualization of peroxidase activity resulted in the accumulation of reaction product only in secretory tubular cells and exclusively within the secretory granules (Fig. 2). Rough endoplasmic

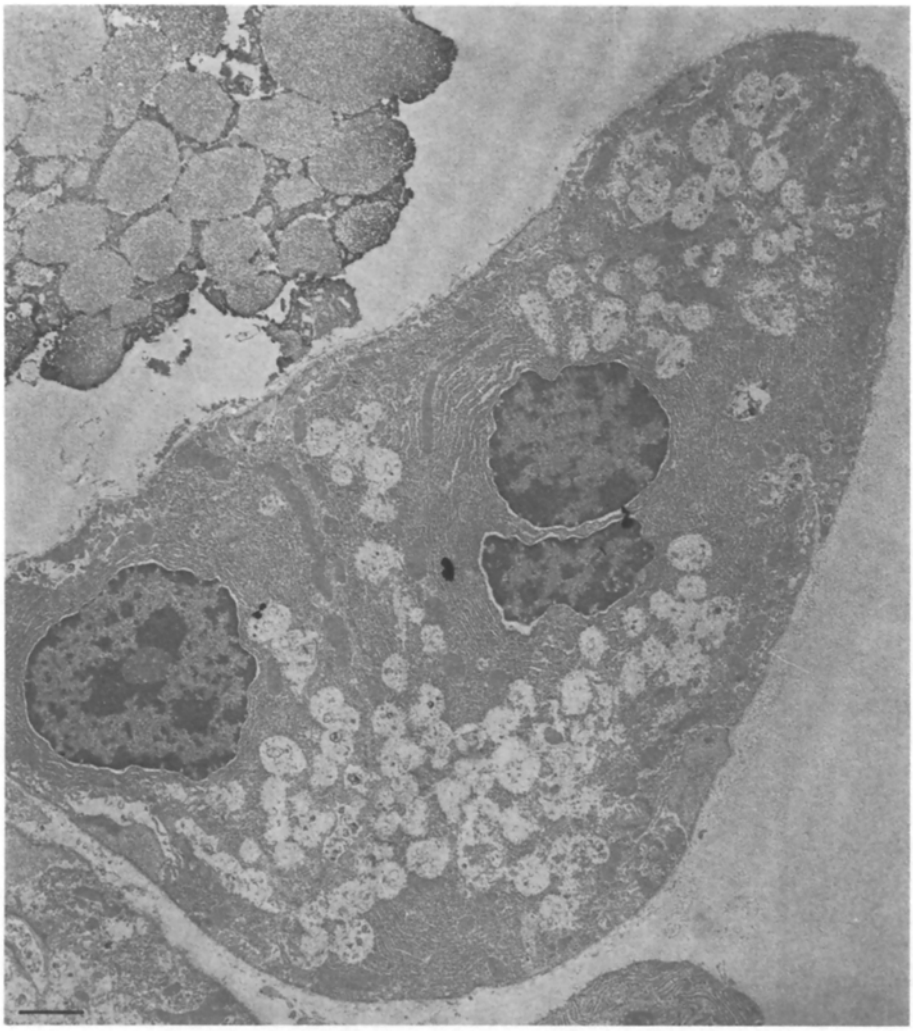

Fig. 3. Electron micrograph of acinar cells from the same section as Figure 3. No reaction product can be found in this cell type. Scale marker 2 μ. ×4,840

reticulum and the Golgi fields showed no reaction product. However, not all the granules were reactive. There was a consistent gradient from mostly unreactive granules in the basal and the middle parts of the cells to highly reactive granules in the apical part of the cells and the duct lumen (Fig. 2). Acinar cells showed no reaction at all (Fig. 3).

Incubation of the sections in a reaction mixture of diamino-benzidine without H_2O_2 or omission of the incubation step with NGF AB-HRP completely prevented the formation of reaction products, confirming the specificity of the reaction.

The intraperitoneal injection of carbachol (2 mg/kg) resulted in markedly enhanced salivation. The corresponding electron microscope pictures showed

a massive release of secretory granules into the excretory duct, the lumen of which was greatly widened as previously described by various authors (Amsterdam et al., 1969; Martinez-Hernandez et al., 1972; Maurs et al., 1974). Immunocytochemical preparations confirmed that NGF was also released into the salivary duct.

Discussion

Control experiments without NGF AB-HRP have shown that no or only very small amounts of endogenous peroxidase are present in the male mouse submandibular gland which were not detectable with procedures used in the present experiments. Endogenous peroxidase has been demonstrated in the salivary and lacrimal glands of several species (Thomson and Morell, 1967; Bloom et al., 1970, 1974; Herzog and Miller, 1970; Strum and Karnovsky, 1970) but ultrastructural studies have shown that it is preferentially localized in acinar cells, especially in cisternae of the rough endoplasmic reticulum (Bloom et al., 1970, 1974; Herzog and Miller, 1970; Strum and Karnovsky, 1970). Thus, the reaction products observed in this study can be attributed entirely to NGF.

The exclusive localization of NGF in the cells of the secretory tubules confirms the results obtained by immunofluorescence methods (Levi-Montalcini and Angeletti, 1961; Goldstein and Burdman, 1965; Kumar et al., 1972). Indirect evidence for the association of NGF with this cell type evolved from the parallelism in the time-course of development of the secretory duct cells and of high levels of NGF in the gland; castration abolished both phenomena and injection of testosterone into females or castrated males provoked a simultaneous differentiation of the secretory tubules and a massive increase in the NGF level (Caramia et al., 1962; Caramia, 1966a, b; Goldstein and Burdman, 1965).

Although previous immunofluorence studies had provided contradictory results with respect to the intracellular localization of NGF (Levi-Montalcini and Angeletti, 1961; Goldstein and Burdman, 1965; Kumar et al., 1972) our experiments have shown an exclusive localization of immunoreactive NGF in secretory granules. These findings are in agreement with the biochemical studies of Pasquini et al. (1974) who have isolated two types of secretory granules from adult male mouse submandibular glands. One type showed a high amylase content while the other type contained large amounts of NGF and epidermal growth factor (EGF). On the basis of these data and additional morphological criteria (Pasquini et al., 1974) it is possible to conclude that the amylase containing granules originate from acinar cells and the NGF containing ones from secretory tubular cells. The fragility of the large granules would explain the relatively large proportion of NGF found in the low density fraction after density gradient centrifugation.

A surprising finding was the restriction of the immunocytochemical reaction to secretory granules located in the apical part of the cells, whereas the granules in the basal part of the cells, the rough and smooth endoplasmic reticulum, the Golgi region and the condensing vacuoles showed no NGF antibody binding capacity. It can be postulated that the synthetic pathway of NGF leads from

the rough endoplasmic reticulum presumably via the Golgi region to its final packing into the secretory granules, since this is the case for many other secretory products (Jamieson and Palade, 1967a, b; Castle et al., 1972; Lillie and Han, 1973; Flickinger, 1974a, b). NGF is isolated from the submandibular gland as 7 S NGF consisting of 3 subunits (Varon et al., 1967). The antibodies used in the present experiments are directed against the β-subunit (2.5 S NGF) which mediates the full range of biological activities of NGF as exerted on adrenergic and sensory neurons. Another subunit, γ, shows proteolytic activity (Greene et al., 1968). In vitro studies suggest a continuous equilibrium between the aggregated 7 S form and the free subunits depending on the pH of the solution (Perez-Polo and Shooter, 1974). In an immunoassay using antibodies against 2.5 S NGF, 7 S NGF showed an identical behavior to 2.5 S NGF (Stöckel, unpublished results).

The significance of the 7 S complex in the submandibular gland is poorly understood. Active interactions during the formation of the molecule, especially the β-subunit, have been suggested (Perez-Polo and Shooter, 1974). Trypsin-, chymotrypsin- and renin-like proteases have also been shown to be located in the secretory duct cells of the submandibular gland and their dependence on testosterone is similar to that of NGF (Bhoola et al., 1973). In this context it may be of importance that the C-terminal of β-NGF is arginine (Angeletti and Bradshaw, 1971), and that in fact the trypsin-like protease isolated from adult male mouse submandibular gland is specific in cleaving peptide and ester linkages involving arginine or lysine. Our observation of an exclusive NGF antigenic reaction in apical secretory granules, which are ready for release, could suggest a process of physiological conversion of a proprotein to the immunoreactive NGF, probably under the influence of a protease. Also the occurrence of slight cell damage in the apical part of the cells could point to an effect of a protease in exposing an NGF antigenic site during tissue fixation as a post-mortem artifact due to the relatively slow action of the fixative.

The biological significance of the high concentration of NGF in the submandibular gland of adult male mice is unknown. Hendry and Iversen (1973) have shown that the level of NGF in the blood drops after removal of the submandibular gland, but a restoration of the original level occurs in the absence of gland regeneration. Our results have shown that NGF is released into the secretory duct, i.e. into the saliva. The fact that no NGF activity is found in the saliva indicates a rapid and massive breakdown in the saliva. Evolutionary similarity of NGF to insulin and proinsulin is suggested on the basis of amino acid sequences (Frazier et al., 1972; Boyd et al., 1974). Also, EGF which occurs together with NGF shows structural similarities to the trypsin-inhibitor protein in the secretory granules of the pancreas (Hunt et al., 1974). Like NGF, EGF is associated with a further subunit which is a protease. Consideration of the close structural and functional similarities of most exocrine glands of the gut system, including pancreas and salivary glands, might indicate a function of NGF in relation to protease inactivation during storage or protease synthesis and completion, unrelated to its effects on nervous tissue.

The extremely high concentration of NGF in the submandibular gland is a unique feature of adult male mice. Administration of high doses of NGF antise-

rum over several days during puberty have been shown to inhibit the typical differentiation of secretory tubular cells and to specifically damage this cell type. The same treatment is without effect if applied to neonatal or adult mice (Caramia et al., 1962). However, this testosterone dependent phenomenon is not exclusive for NGF: protease levels, the high concentration of glucose-6-phosphate dehydrogenase, and the specific ultrastructure of the secretory duct cells are also regulated in the same way (Caramia, 1966a, b; Bhoola et al., 1973; Nakamura et al., 1974).

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