On the presence and localization of epidermal and nerve growth factors in human whole saliva

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Using antibodies to mouse submandibular epidermal growth factor (EFG) and nerve growth factor (NGF), immunohistochemical studies were performed on sections of flash-frozen human whole saliva in both light and transmission electron microscopy. Light microscopy showed the presence of overall network-like immunoreactions in both the EGF and NGF antibody-treated sections. Electron microscopy showed clearly detectable ultrastructural reaction patterns for both growth factor antibodies. The individual structural elements were more distinct for the EGF antibody-treated sections, in which the reaction elements had approximate diameters of 0.05 μm. In the NGF antibody-treated sections the corresponding approximate diameters were 0.02 μm. In both the EGF and NGF antibody-treated sections heavily stained bacteria-like particles were also frequently observed. □ *Electron microscopy; immunochemistry; light microscopy*

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Both light and conventional electron microscopic studies of sections of flash-frozen human saliva have recently shown this body fluid to have highly complex internal structures (1–4). In sections approximately 10 μm thick a continuous, probably partly colloidal network with integrated, dispersed, lipoid droplets was thus initially identified both in whole saliva and saliva fractions from subjectively healthy donors (1–3). Furthermore, transmission electron microscopic examinations of thin (90–100 nm) saliva sections demonstrated a range of additional ultrastructural components within the major network structure (4). These architectural components included an outer, lightly stained, foam-like zone and an inner core consisting of a moderately electron-dense, reticulated phase, with multiple granules of different size, shape, and electron density.

As it was considered possible that especially the inner core could represent a phase with specific biochemical activity, and since the peptide epidermal growth factor (EGF) has been identified in studies of human and animal saliva and salivary glands (5–7), it was considered worthwhile to use our recently developed method for freeze-sectioning of saliva to initiate a study to identify the presence and possible localization of EGF in human whole saliva. As it was considered likely that another peptide, nerve growth factor (NGF), is also present in human whole saliva, this was also included in the study.

Materials and methods

Two to three milliliters of stimulated whole saliva was donated during empty mouth chewing movements by a 30-year-old female donor and a 51-year-old male donor. Both donors were subjectively healthy and were fully dentate and in good oral health at general dental examinations.

The saliva samples were handled in accordance with the methods described in detail by Glantz et al. (1–3). Subsequently, the saliva samples were flash-frozen by

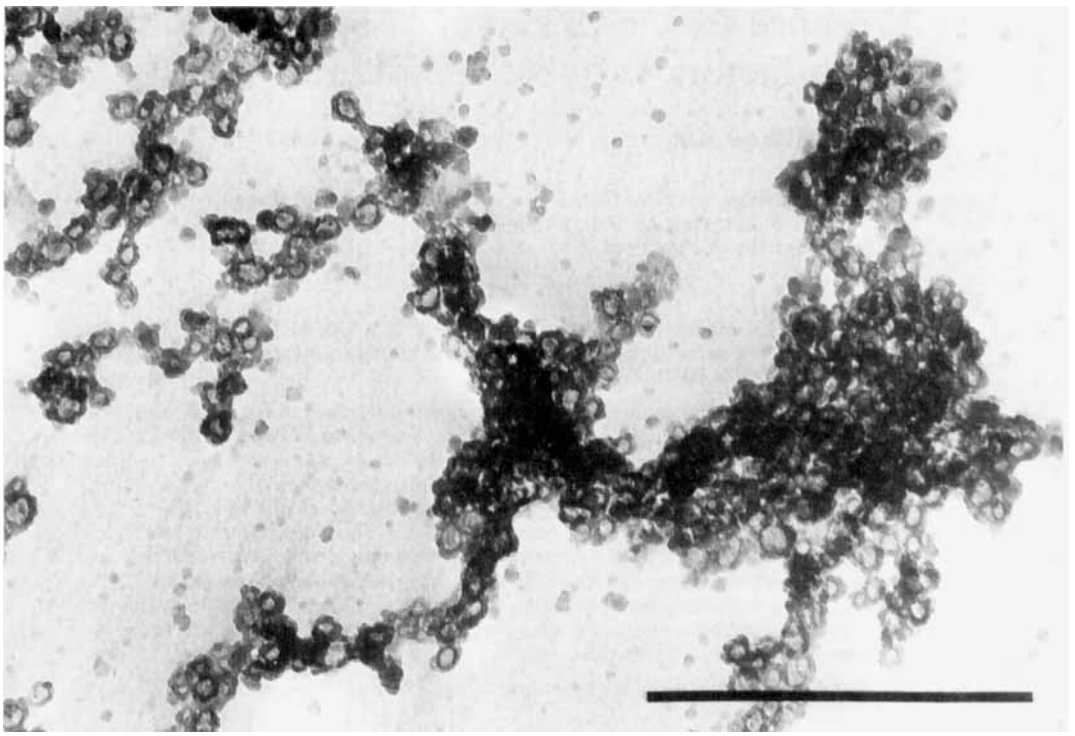

Fig. 1. Transmission electron micrograph of EGF antibody-treated saliva specimen from a subjectively healthy 51-year-old male donor. The spherical, granular-like reaction structures are composed of an electron-dense periphery with an electron-lucent central core and are arranged in chains and/or clusters. Length of bar = 1 μm.

immersion in liquid nitrogen and then mounted, sectioned, and stained or otherwise treated before microscopic examination.

In this study the fresh-frozen saliva samples were sectioned to a thickness of about 10 μm for both light microscopy (2) and transmission electron microscopy (TEM) (3). Some of the sections were also pre-treated with a 10% aqueous solution of hydrogen peroxide and methanol to inactivate possible endogenous salivary peroxidases. Finally, sections, except the controls, were then treated for 1 h with a 0.05% solution (1:20 dilution) of either EGF or NGF mouse antibodies to submandibular EGF/NGF.

Thereafter, all sections were treated for 30 min with a 5% solution of horseradish peroxidase-conjugated swine anti-rabbit IgG, followed by staining with a 0.005% solution of diaminobenzidine (Graham–Karnousky's solution). Finally, the TEM sections were transferred to grids coated with Cu-Butvar 98 and carbon (3–4). Light microscopy and TEM were performed in accordance with standard methods.

Results

The examinations of the antibody-treated sections showed that several detectable reactions had taken place. Few, if any, differences were, however, observed between the sections pretreated with hydrogen peroxide/methanol and those that had not undergone such pretreatments. Further, no appreciable reactions could be observed on control sections. Finally, although basically

of the same kind, somewhat more pronounced reactions had taken place in the sections from the male donor.

At the light-microscopic level, the presence of a stained network with structural details and variable dimensions could be identified for both the EGF and NGF antibody-treated sections. Within the major structural components of the saliva sections, multiple, comparatively heavily stained rounded structures could be observed, often in bundle-like arrangements. Multiple, heavily stained microorganism-like particles were also identified, frequently in large concentrations, as were large, less heavily stained objects with sizes and shapes similar to those of epithelial cells.

TEM examination of the antibody-treated sections showed the presence of several ultrastructural differences both for EGF and NGF (Figs. 1 and 2).

Thus, in the EGF antibody-treated sections, a comparatively distinct ultrastructural reaction pattern was observed, in which many spherical structures were arranged in chains or clusters. The individual structural elements measured approximately 0.05 μm in diameter (Fig. 1). Around the peripheries of these granular elements, four to six small, rounded extensions or buds per element were frequently noted. The granules consisted of an electron-lucent core with a thin electron-dense peripheral cover or shell.

In the NGF antibody-treated sections the

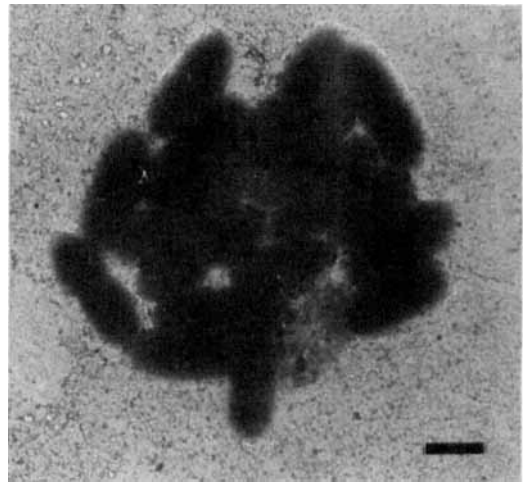

Fig. 3. Transmission electron micrograph of NGF antibody-treated saliva specimen from a subjectively healthy 51-year-old male donor. Multiple, heavily stained, bacterial-like particles are noted, surrounded by diffusely stained or clear zones. Length of bar = 1 μm.

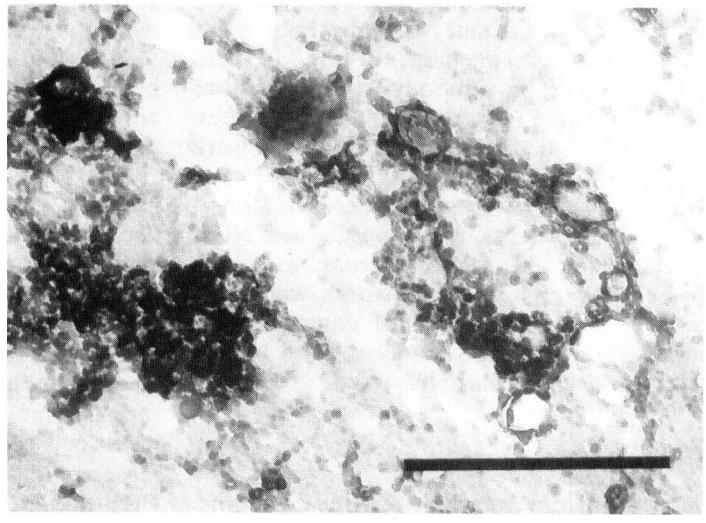

Fig. 2. Transmission electron micrograph of NGF antibody-treated saliva specimen from a subjectively healthy 51-year-old male donor. Small, electron-dense, spherical reaction structures are observed either individually or in sheets resembling paracrystalline configurations. Note also the presence of multiple rounded areas without staining. Length of bar = 1 μm.

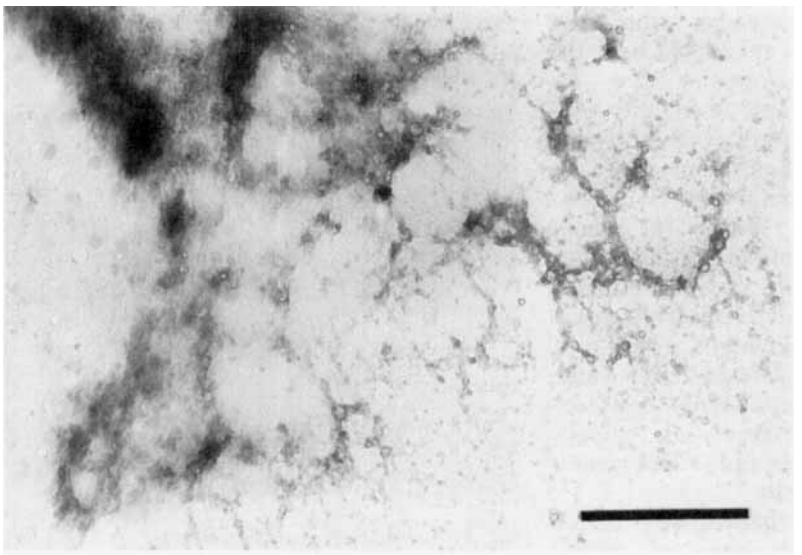

Fig. 4. Transmission electron micrograph of EGF antibody-treated saliva specimen from a subjectively healthy 30-year-old female donor. Diffusely stained fibrillar networks are observed. In addition, foci of moderately stained, spherical bodies of variable diameter are also occasionally noted. Length of bar = 1 μm.

reaction patterns were less distinct, with smaller stained elements measuring approximately 0.02 μm in diameter. In many instances they were arranged either individually or in sheets or resembling paracrystalline configurations (Fig. 2). These granules differed from EGF in that they were smaller and completely electron-dense in reactivity. In many of the NGF antibody-treated sections, circular areas with minimal or no staining could also be noted. Most of these areas had approximate individual diameters of about 0.1 μm and were occasionally arranged in clusters. In both EGF and NGF antibody-treated sections heavily stained bacterial-like particles, often surrounded by diffusely stained zones, were also noted (Fig. 3).

In addition to these rather well-defined structural elements, in both EGF and NGF antibody-treated sections areas with diffusely stained fibrillar networks were also identified (Fig. 4). Foci of comparatively large, rounded, moderately stained bodies were also occasionally observed.

Discussion

The recent development of a new method for microscopic studies of body fluids has made it possible to identify specifically a microarchitecture in human saliva (1–3). Transmission electron microscopic examinations of thin sections of such saliva have also shown the presence of a granular structure inside the major structural components (4). As some of these structures could represent areas of specific biochemical activity, it was considered worthwhile to perform certain immunocytochemical procedures to substantiate these activities. The peptide epidermal growth factor (EGF) was selected for identification and localization, as it has been shown to be present in human saliva and human salivary glands (8, 9). A similar peptide known as nerve growth factor (NGF) has not been reported to be secreted by human salivary glands but was selected because it was considered likely that NGF is also present in human saliva.

The immunocytochemical labeling techniques used in this study are well established and proven to be useful tools in a wide range of experimental situations. Therefore, even though unexpected cross-reactions could have occurred, especially when the differences in the reaction patterns between EGF and NGF and the negative results of

the control experiments are taken into consideration, it is likely that the positive reaction patterns were created mainly by salivary EGF- and NGF- or EGF/NGF-containing complexes, respectively.

The antibodies used had been developed from mouse submandibular EGF and NGF, which have been demonstrated to possess close biochemical similarities with human EGF and NGF (10). On the basis of this fact and the above discussion, it was concluded that the methods applied were valid and accurate for the study of the presence and localization of EGF and NGF activity in human whole saliva.

When results of this study were examined, it was evident that both EGF and NGF were present in the whole saliva samples (Figs. 1 and 2). Although the presence of EGF confirms previous reports (7–9), NGF has not been previously reported to be present in human whole saliva.

The light microscopic examinations showed that EGF and NGF activities were restricted to seemingly coinciding network structures in the whole saliva sections. When these structures were compared with conventional histologic staining of human whole saliva (2), the similarities were so clear that these were also concluded to reflect the same type of structure.

The use of thin sectioning TEM techniques showed that the major structural components in whole saliva—that is, those with a diameter above about 1 μm—had inner ultrastructural features indicating areas with specific biologic activity (4). In addition to the above, TEM examination of thick EGF and NGF antibody-treated sections showed reaction patterns resembling the 'core' granules described above in thin section of whole saliva. It was therefore concluded that at least some of the ultrastructural reactive features inside major salivary structural components were created by elements with EGF or NGF activities.

When the NGF antibody-treated sections were examined, multiple rounded areas without staining were also observed throughout the sections (Fig. 2). The similarities in shape and size between these unstained areas and the EGF antibody reaction elements indicate that they may be identical. Therefore, the fact that no clear type of similarly unstained NGF areas could be observed in the EGF antibody-treated sections could simply mean that the smaller dimensions and more dispersed arrangements of the NGF-containing structures were masked by or coincided with the background structures of the specimens with resin coatings.

It is hoped that further studies, which are in progress, will identify other specific salivary activities localized within the network components of this highly complex body fluid. From an energy preservation perspective, a concentration of salivary-specific biologic activity appears logical, as the other barrier of fine network structures, observed by means of thin-section ultrastructural techniques, could protect the active substances at least temporarily from reacting with or being inactivated by outside electrolyte components. The noted microstructures indicate that a release of active material is taking or could easily take place. The recent finding (11) that mouse saliva prevents B-nerve growth factor-dependent neurite formation in cell cultures may be due to spontaneous protective encapsulation of growth factor substances, probably by amphiphilic salivary structural elements. Thus, at least on a short-term basis, with their observed location the identified growth factors could be preserved for reaction with organic substances, which would be carried into the inner components of the salivary network to be biochemically altered, possibly in conjunction with blood exposed to saliva. Dagogo-Jack et al. (8) have, for example, recently demonstrated human salivary EGF activity at room temperature over periods of up to 10 days.

Even though other explanations are possible from a theoretical point of view, the noted locations of multiple microorganism-like particles both at light and electron microscopic levels inside the major components of the salivary network is a significant finding and substantiates our previously made observations (2, 3). Preliminary data also suggest the presence of some type or types of interaction, probably between the antibodies and at least certain oral microorganisms, most likely either in the form of

antimicrobial activity by the structures with growth factor activities or EGF/NGF-like activity by certain oral microorganisms. As both EGF and NGF have binding proteins with arginine esterase activities, an antimicrobial activity could very well be due to actions by these binding proteins.

From a general biologic point of view, the results of this study provide a rational basis for the instinct among both humans and animals to suck and lick wounds on their skin. By doing so, a number of microorganisms are probably removed from the wound area and transported into the inner salivary structures to be attacked by antimicrobial substances. In addition to and at the same time, a salivary network is also donated, which contains factors that promote or exert an influence on wound healing. The presence of EGF- and NGF-containing complexes in saliva may therefore also greatly contribute to the well-known comparatively rapid healing of intraoral wounds.

Epidermal growth factor has been demonstrated to have important gastric cytoprotective functions (10, 12), and it is possible that nerve growth factor also has a biologic function stretching beyond the oral cavity. The described relatively protected localizations of salivary EGF and NGF could contribute in preserving such functions.

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References

1. Glantz P-O, Vaughan C, Natiella JR. On structures in human mixed saliva. J Dent Res 1988;67(spec iss A):133.
2. Glantz P-O, Natiella JR, Vaughan CD, Meyer AE, Baier RE. Structural studies of human saliva. Acta Odontol Scand 1989;47:17–24.
3. Glantz P-O, Wirth SM, Baier RE, Wirth JE. Electron microscopic studies of human mixed saliva. Acta Odontol Scand 1989;47:7–15.
4. Glantz P-O, Friberg SE, Wirth SM, Baier RE. Thin-section transmission electron microscopy of human mixed saliva. Acta Odontol Scand 1989;47:111–116.
5. Cohen S. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new born animal. J Biol Chem 1962;237:1555–62.
6. Carpenter G, Cohen S. Epidermal growth factor. Ann Rev Biochem 1979;48:193–216.
7. Elder JB, Williams G, Lacy E, Gregory H. Cellular localization of humanurogastrone/epidermal growth factor. Nature 1978;271:466–7.
8. Dagogo-Jack S, Atkinson S, Kendall-Taylor P. Homologous radioimmunoassay for epidermal growth factor in human saliva. J Immunoassay 1985;6:125–36.
9. Ohmura E, Emoto N, Tsushima T, Watanabe S, Takeuchi T, Kawamura M, Shigemoto M, Shizume K. Salivary immunoreactive human epidermal growth factor (IR-hEGF) in patients with peptic ulcer disease. Hepatogastroenterol 1987;34:160–3.
10. Daughaday WH, Heath E. Physiological and possible clinical significance of epidermal and nerve growth factors. Clin Endocr Metabol 1983;13:207–26.
11. Lakshamanan J. Mouse saliva prevents the B-nerve growth factor-dependent neurite formation in PC-12 cells. Neurosci Res 1987;4:211–27.
12. Skov Olsen P, Seier Poulsen S, Kirkegaard P, Nexø E. Role of submandibular saliva and epidermal growth factor in gastric cytoprotection. Gastroenterology 1984;87:103–8.