EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Neuroendocrine Cells in the Dental Pulp in Health and Disease

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Neuroendocrine cells were identified in human dental pulp by immunohistochemical method using monoclonal antibodies. A population of neuroendocrine cells positively reacting to neuron-specific enolase, synaptophysin, chromogranin A, and stained with paraldehyde-fuchsin, was detected in the subodontoblastic layer of the pulp. Changes in their count, morphology, and function in caries and pulpitis concomitant with periodontitis were proven.

Key Words: *dental pulp; neuroendocrine cells; neuron-specific enolase; synaptophysin; chromogranin A*

One of the basic assumptions of classical biology that the hormonal function is a specialized function of endocrine cells was revised when biogenic amines and peptide hormones were detected in the APUD systems cells of organs and tissues, united later under the name of diffuse endocrine system [2]. It is now universally acknowledged that cells of the diffuse endocrine system located virtually in all organs and producing bioactive substances regulate homeostasis through endo- and paracrine mechanisms [1]. Therefore, they can be regarded as an indispensable component and a part of the universal system of control and defense, while the hormonal substances released by them can be regarded as signal molecules for local coordination of cell-cell interactions.

The presence of neuroendocrine cells (NEC) in the dental pulp is still disputed. Immunohistochemical studies of normal human dental pulp with streptavidin-biotin-peroxidase detected neuronal and neural crest-linked markers (neuron-specific enolase (NSE), chromogranin A, S-100 protein, synaptophysin, glial fibrillary acidic protein, and neurofilaments) only in nerve fibers [5,7]. Negative staining of the pulp cells with neuroendocrine markers can be explained by investigation of just one stage of cell differentiation during the development of the pulp tissue. The pulp of a developing tooth contains cells originating from the neural crest, which produce nerve growth factor, glial neurotrophic factor, and cerebral neurotrophic factor, and regulate the growth of dopaminergic nerve fibers into the pulp during the development of dental pulp innervation [6]. Synaptophysin was detected not only in nerve fibers, but also in structures of the paraodontoblastic zone of the pulp [4].

The aim of this study was to detect NEC in the pulp by immunohistochemical methods in health and disease.

MATERIALS AND METHODS

The dental root pulp of intact, carious teeth, and teeth with pulpitis concomitant with periodontitis was obtained by extirpation for orthodontic and therapeutic indications. The pulp from a total of 418

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frontal teeth of 294 patients of both sexes aged 23-49 years was extirpated. The patients were divided into groups. After extirpation the pulp was fixed in 10% neutral formaldehyde, dehydrated in ascending alcohols, and embedded in paraffin. Pulp sections (10 μ) were sliced on an MPS-2 paraffin rotator microtome. After deparaffinization and rehydration in descending alcohols, dental pulp sections were plunged in restoring citrate buffer (pH 6.0) and heated for 30 min in water bath at 90-95°C for demasking of the target antigens in tissues. The method is based on the so-called thermal induction of epitope return, providing recovery of the antigen structure before immunostaining.

After inhibition of endogenous peroxidase with 3% H_2O_2 in methanol, immunohistochemical reaction was carried out by three-step indirect EIA with primary monoclonal antibodies (MAb; Dako) to NEC antigenic markers (synaptophysin, NSE, chromogranin A in 1:100 dilution) according to manufacturer's instruction. One of two serial sections placed on the slide and treated with control antibodies instead of the first antibodies served as negative control in each immunohistochemical test. Bound first MAb were visualized using LSAB-2 kit (Dako). To this end, the pulp sections were first incubated in serum solution containing secondary anti-species anti-immunoglobulin biotin-labeled antibodies. In order to detect biotin label, the sections were then treated with horseradish peroxidase-conjugated streptavidin selectively binding biotin. Peroxidase activity was histochemically detected by chromogen-substrate mixture on the basis of 3-amino-9-ethylcarbasole. As a result of enzymatic reaction, the substrate was converted into insoluble reddish product coinciding by location with the location of the target antigen. The sections were thoroughly washed with Tris-phosphate buffer after each stage of immunohistochemical analysis in order to rule out nonspecific staining. The specificity of the target antigen expression in experimental samples of dental pulp tissue was also confirmed by its absence in control sections, not treated with the first MAb. For the final stage the sections were post-stained with hematoxylin and embedded in glycerol and gelatin.

In addition, the sections were stained with paraldehyde-fuchsin after Gomori (1 ml concentrated HCl, 1 ml paraldehyde, 100 ml 0.5% basal aqueous fuchsin) and left at ambient temperature for 24 h until the mixture developed a dark violet color. Since the solution is unstable, it had to be used during several days after maturation. Quantitative distribution of positive cells was evaluated by counting them in 10 visual fields (×400) with subsequent estimation of the arithmetic mean for each case.

The data were statistically processed using Student's *t* test.

RESULTS

In the subodontoblastic layer of the dental pulp of control patients Gomori staining revealed, in addition to the typical connective tissue elements, solitary paraldehyde-fuchsin-positive cells with dark purple granules in the cytoplasm. Parallel immunohistochemical study with MAb to NEC antigenic markers (chromogranin A, synaptophysin, and NSE) detected solitary positive cells only in the subodontogenic layer of the dental pulp. Chromogranin A is an acid protein widely expressed in nerve tissues and secretory granules in cells of many endocrine glands and APUD system. Synaptophysin is an integral membrane glycoprotein, a molecular marker of presynaptic neuronal vesicles. It is normally present in cells with neuroendocrine differentiation. Neuron-specific enolase is a glycolytic enzyme, catalyzing the reaction between 2-phosphoglycerate and phosphoenolpyruvate. It is known that enolase is a dimer including 2 of 3 known subunits (α, β, γ); γγ- and αγ-enolase isoforms are neuron-specific and are located mainly in the nerve tissue and NEC [7]. Hence, we identified cells positive in these reactions as NEC. Against the background of negatively stained mesenchymal structures of the pulp, NEC were characterized by larger size, polygonal irregular shape, and presence of reddish-brown lumpy heterogeneous granules in the cytoplasm often masking the nucleus. Round eccentrically positioned nuclei stained blue with hematoxylin were seen in some cells. Immunopositive cells in some areas closely contacted with nerve fibers containing synaptophysin and NSE. Chromogranin A detected in cells of the subodontoblastic layer of the pulp was not detected in nerve fibers.

Comparison of cell populations positively stained for different markers showed their similar morphology. However, the appurtenance of these cells to different populations can be determined only using combined staining detecting mutual location of the markers.

Just solitary immunopositive NEC were detected in the control group, while in caries their number increased reaching the maximum in deep caries with concomitant mild periodontitis. The highest concentration of these cells was observed in the vicinity of negatively stained disoriented and thinned layer of vacuolated odontoblasts in the region

TABLE 1. Counts of NEC in Dental Pulp Detected by Different Staining Methods in Health, Caries, Pulpitis, and Periodontitis, ($M \pm m$)

Nosological entity	*n*	Chromo-granin	Synapto-physin	NSE	Aldehyde-fuchsin
Control group	33	1.3±0.1	1.5±0.1	1.7±0.2	2.2±0.1
Superficial caries concomitant with mild periodontitis	36	2.6±0.1	2.8±0.3	1.9±0.2	3.1±0.9
Medium-deep caries with mild periodontitis	38	4.2±0.3*	3.7±0.4	3.8±0.5	4.7±0.4*
Deep caries with mild periodontitis	34	12.5±1.2***	11.6±3.2***	11.3±1.2***	11.4±1.1***
Acute focal pulpitis with moderate periodontitis	42	9.5±0.8**	8.2±0.7**	9.1±0.7**	10.3±0.9**
Acute diffuse pulpitis with moderate periodontitis	37	7.4±0.9**	6.8±0.5*	7.2±0.6**	6.9±0.5*
Chronic fibrous pulpitis with severe periodontitis	38	2.3±0.2	3.1±0.4	2.8±0.3	3.6±0.2
Chronic gangrenous pulpitis with severe periodontitis	36	0.5±0.1	1.4±0.1	1.6±0.1	0.9±0.1

Note. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to the control group.

corresponding to the focus of lesions. The overwhelming majority of cells were detected by antibodies to chromogranin A. These cells were round or oval. The cytoplasm contained evenly distributed granules stained reddish-brown, cell nuclei were not seen.

The number of nerve fibers containing synaptophysin and NSE increased in the pulp after destruction of hard dental tissues.

In pulpitis combined with medium severe periodontitis, the count of NEC decreased, but did not reach the basal values (Table 1). It seems that the cells were squeezed out by inflammatory polymorphonuclear infiltration. In acute pulpitis, the immunopositive nerve fibers closely reacting with inflammation cells and penetrating into vast infiltration areas along plethoric vessels grew around inflamed tissue. The number of APUD system cells decreased in chronic pulpitis against the background of depleted cell composition of the dental pulp and growth of sclerotic cords.

It can be hypothesized that NEC of the pulp located near the pulp-dentin interface and receiving information from the environment release peptide hormones producing local (paracrine) and distant (endocrine) effects. These cells regulate microcirculation, metabolism, fibroblast proliferation, and functional activity of immunocompetent cells in the focus of immune inflammation [3].

The detected increased incidence of nerve fibers in caries and pulpitis can be explained by their involvement in the regulation of the inflammatory process in parallel with the APUD system cells.

During disease, when tissue homeostasis is disordered in caries concomitant with periodontitis, NEC induce significant changes in the functioning of the adjacent pulp cells. Their count starts changing during the initial involvement of hard dental tissues and precedes the development of acute and chronic inflammatory process in the pulp. Weak reaction of NEC in intact pulp to nerve crest markers can be explained by partial loss of their capacity to express these antigens during cytodifferentiation. According to our data, this capacity is retained and stimulated under pathological conditions.

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