Apoptosis as a Mechanism of Maxillary Sinus Mucosa Injury in Rats after Experimental Transection of the Maxillary Nerve

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Transection of the maxillary nerve initiates apoptosis of the maxillary sinus mucosa cells in rats. Significant activation of apoptosis and proapoptotic factor p53 was found in the epithelium during week 1 after nerve transection. In delayed period after injury, apoptotic cells predominated in the submucosa against the background of Bcl-2 hypoexpression.

Key Words: *apoptosis factors; p53; Bcl-2; regeneration; deafferentation*

Apoptosis is initiated by gene induction modifying metabolic conditions of the nearest microenvironment [1,7]. Molecular factors p53 and Bcl-2 regulating activity of proliferation and apoptotic death of cells make an important contribution to these processes [3,8,14]. These processes can be triggered by CNS fiber injury [4,5]. For example, apoptosis of the acoustic nuclei neurons after acoustic nerve transection is observed during the first 24 h after the injury [8], while transection of the optic nerve in mice stimulates apoptosis of more than 90% retinal ganglionic neuronal population [1,2]. The mechanisms of programmed cell death in response to peripheral nerve injury are less studied.

We studied apoptosis and expression of pro- and antiapoptotic factors in the maxillary sinus mucosa in rats in response to maxillary nerve transection.

MATERIALS AND METHODS

All experiments were carried out in accordance with regulations for humane handling of laboratory animals, developed by the Ethic Committee of Vladivostok State Medical University. The material was obtained from 30 outbred male rats (200-300 g) kept under standard vivarium conditions. The animals were intravenously anesthetized with sodium pentobarbital (30 mg/kg). Deafferentation of the maxilla and sinus was attained by unilateral transaction of the maxillary nerve. Apoptosis was studied on sections of the maxillary sinus mucosa on days 1, 3, 7, 14, and 21 after nerve transection. Intact rats served as controls. The animals were decapitated, the mucosa was removed and fixed for 4 h in 4% buffered paraformaldehyde (pH 7.2) at 4°C. The specimens were then washed for 24 h in 0.1 M phosphate buffer (pH 7.2) and 30% sucrose solution.

Apoptosis was studied by the immunocytochemical TUNEL method (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling), based on the detection of fragmented DNA. Specimens of the mucosa were plunged in 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) and fixed for 3-4 h at 4°C. After fixation, the material was washed for 24 h in 0.1 M phosphate buffer, pH 7.4 (the solution replaced with fresh portions 7-8 times) and then plunged in 15% buffered sucrose solution. The sections (20 μ) were prepared in a cryostat, postfixed in cold ethanol-acetic acid solution for 5 min at -20°C, after which washed 2×5 min in phosphate buffer. TUNEL-stained structures were detected using ApopTag Direct In Situ Apoptosis Detection Kit (Chemicon). After washing from the first antibodies, the preparations were plunged in solution of peroxidase-conjugated antidigoxigenine, prepared according to the instruction. Vector NovaRed substrate (Vector Laboratories) served as the chromogen. Cell nuclei were poststained

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with 1% methylene green solution. The sections were dehydrated, clarified in xylene, and embedded in balm. Some sections were incubated in solution of Fap fragment of FITC-conjugated second porcine antibodies to rabbit Ig (Dako), 1:100. The sections were washed in phosphate buffer, mounted on slides, embedded in glycerol, and examined under a Polyvar microscope using a FITC filter (B1 450-490 nm).

Immunocytochemical identification of Bcl-2 and p53 was carried out as follows. The sections were incubated with rabbit polyclonal first antibodies to Bcl-2 (Chemicon) and mouse monoclonal antibodies to p53 (Chemicon) diluted 1:200 in solution phosphate buffer and normal goat serum. After washout (1 h), the sections were incubated with the corresponding second biotinilated goat antibodies (1:100, Vector Laboratories) and then with avidin-peroxidase complex (Vectastain ABC Kit, Vector Laboratories, BA-2000) for 1 h. The final step of the treatment was detection of peroxidase ABC complex with 0.03% diaminobenzidene and 0.01% hydrogen peroxide for 10-20 min, after which the sections were dehydrated and embedded in balm.

For quantitative evaluation of the results, a section of standard thickness (20 μ) was selected in each preparation. The sections were examined under a light microscope with an ocular grid consisting of equal squares and allowing counting of all cells interacting with substrates in this section. The content of Bcl-2- and p53-immunoreactive cells was estimated as the difference between the sum of cells stained with methylene green. TUNEL-positive nuclei were counted using an ocular morphometric grid in mucosa sites of 100×100 μ. The apoptotic index (AI) was estimated as the proportion of total count of TUNEL-positive nuclei (N_{TUNEL}) to the count of toluidine blue stained cells with visible nonpyknotic nucleus (N_t), by the formula $AI=(N_{TUNEL}\times100)/N_t$. The results of quantitative analysis were statistically processed using Student's *t* test ($p<0.05$).

RESULTS

TUNEL-positive cells were unevenly scattered in the mucosa layers. During the immediate period after the injury (days 1-7), they predominated in the epithelial layer amd migrating in a gradient-like manner to the submucosa (Fig. 1, *a*). On days 14-21, the majority of labeled cells were located in the loose connective tissue, where they were located mainly in the perivascular spaces (Table 1). Importantly, the microvessel walls did not react with TUNEL. It was impossible to identify the type of apoptotic cells. Intense fluorescence of their nuclei stained by the TUNEL method showed signs of DNA fragmentation. They looked like fluorescent dots (apoptotic bodies), which fused to form rings, semi rings, and homogenous conglomerations (Fig. 1, *b*). They were seen in sites of the nuclei location and were evenly distributed in the cytoplasm, shifted to the cytoplasmic membrane, or grouped by one of the cell soma poles. Fluorescence of the nuclei of solitary fibroblast-like cells, forming clusters, was seen in the submucosa.

The location of p53 and Bcl-2 immunoreactivity in the maxillary sinus mucosa did not coincide and depended on the period elapsed after the injury (Table 2). On day 1 after maxillary nerve transaction, solitary p53-immunoreactive cells were detected in the epithelial layer. Later their number increased and peaked on days 14-21 after injury (Fig. 2, *a*). The distribution of Bcl-2-immunoreactive cells exhibited an opposite trend. They were found in fact everywhere on days 1-3, mainly in the epithelial layer and perivascularly in the submucosa (Fig. 2, *b*). The location of Bcl-2-immunoreactive cells did not change much during later periods after maxillary nerve transection, but the intensity of immunoreactivity increased significantly in the submucosa and mucous membrane (Table 2). Presumably, Bcl-2 expression during delayed period of deafferentation limited the number of apoptotic cells and created favorable conditions for proliferation, survival, and active functioning of the respective mucosa cells.

The main target for Bcl-2 molecule is mitochondrion, and hence, we can hypothesize mainly mitochondrial pathway of apoptosis after deafferentation of the maxillary sinus mucosa. p53 molecule plays an important role in modification of the mitochondrial membrane stability and is directly involved in triggering of programmed cell death [3,10]. The protein (p53AIP1)

TABLE 1. Apoptotic Index of Rat Maxillary Sinus Mucosa Cells after Unilateral Deafferentation of the Maxillary Nerve

Mucosa structures	Control	Time after nerve transection				
		day 1	day 3	day 7	day 14	day 21
Epithelium	-	300.5±29.9	116.8±42.1	89.2±24.1	59.6±18.5	38.54±22.6
Lamina propria cells	-	53.95±5.95	49.12±9.40	92.4±11.2	108.6±32.1	33.1±9.1
Submucosa cells	-	26.58±2.5	77.80±12.28	88.13±5.90	55.8±13.4	47.12±11.63

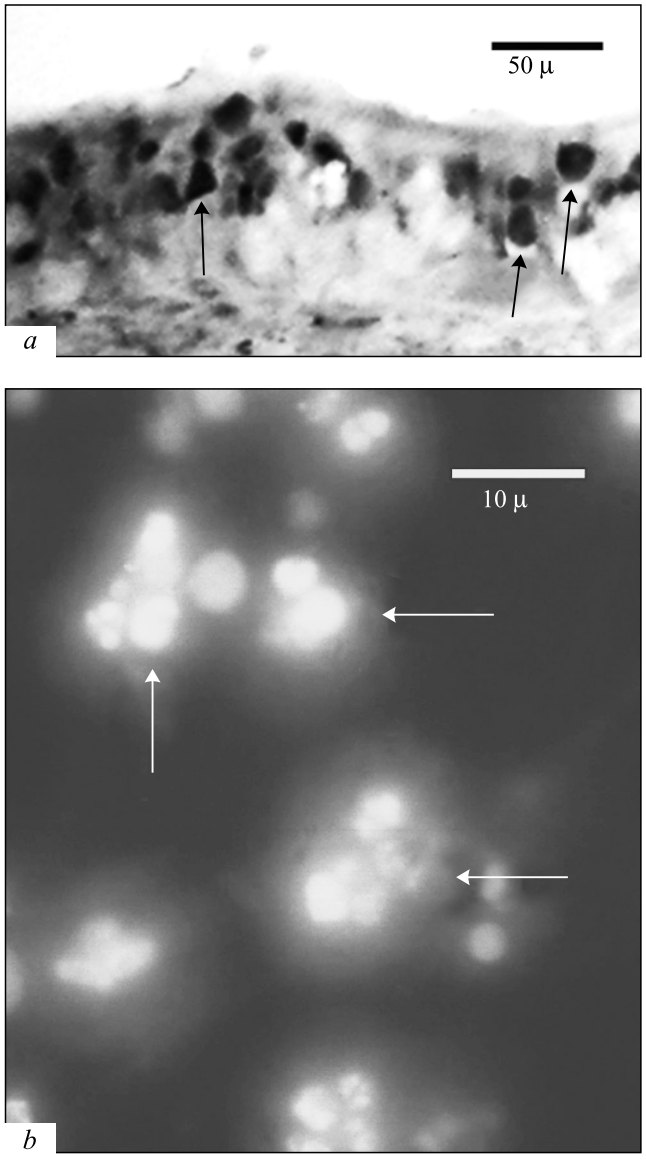

Fig. 1. TUNEL-positive cells in rat maxillary sinus mucosa after unilateral transection of the maxillary nerve. *a*) accumulation of TUNEL-immunoreactive epitheliocytes (arrows), day 3; *b*) cell nuclei in the lamina propria with signs of apoptotic destruction (arrows) are labeled as intensely fluorescing dots fusing into homogenous agglomerations shifting to the nucleus periphery; day 14.

mediating the p53-independent route of apoptosis was detected in mitochondrial membrane [9]. Some authors previously noted that p53 repressed the Bcl-2 promotor gene [10] and that Bcl-2 suppressed the p53 proapoptotic function in nerve fiber injury [5].

The morphological heterogeneity of TUNEL-immunoreactive cells is presumably determined by different mechanisms of their programmed death. Alternative approaches to apoptosis triggering were therefore hypothesized [6,12]. One them (mitochondrial) is induced by the production of p53 transcription factor; its deficiency leads to tumor-like proliferation

TABLE 2. Distribution of p53- and Bcl-2-Immunoreactive Cells in Rat Maxillary Sinus Mucosa in Maxillary Nerve Deafferentation

Mucosa structures	p53-immunoreactive cells					Bcl-2-immunoreactive cells				
	control	day 1	day 3	day 7	day 14	control	day 1	day 3	day 7	day 14
Epithelium	-	57.8±2.1	76.2±4.1	29±3	18.0±2.6	12.0±3.1	35.0±2.9	67.0±4.2	83.0±1.3	103.0±2.6
Lamina propria cells	-	42±4	89.2±4.1	59.6±3.5	38.54±2.1	7.4±2.9	63.0±2.1	89.2±2.1	59.6±1.5	38.54±2.1
Submucosa cells	14.0±2.9	84±2	97.0±2.1	137.0±1.8	176.0±2.6	11.0±2.1	24.0±4.2	32.0±2.4	27.0±2.2	17±2

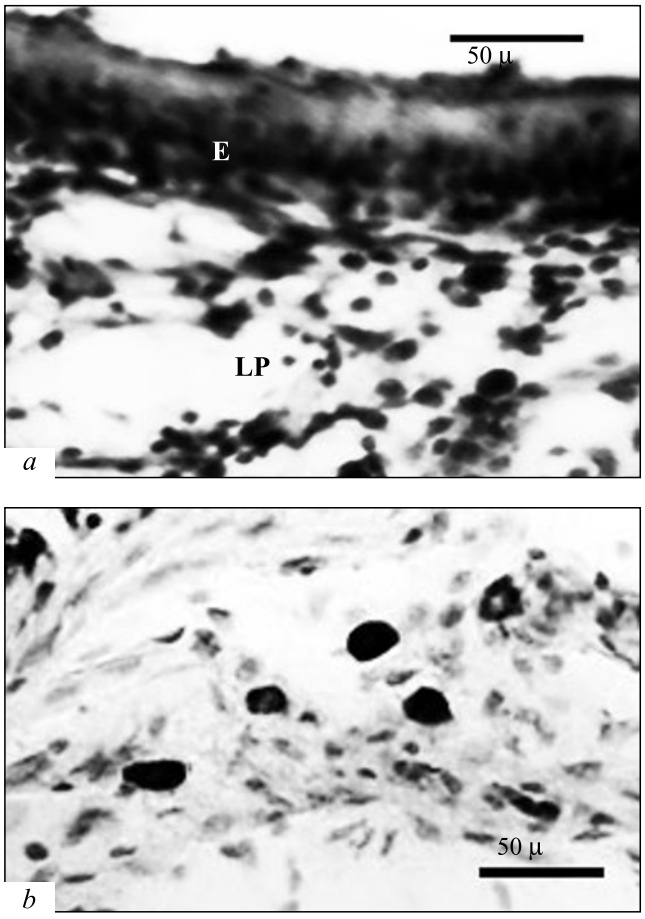

Fig. 2. Expression of p53 and Bcl-2 in rat maxillary sinus mucosa at different terms after maxillary nerve trauma. *a*) p53-immunoreactive cells of the epithelium (E) and lamina propria (LP), day 14; *b*) Bcl-2 location in lamina propria, day 3 after nerve transection.

of epitheliocytes [13]. Another mechanism of apoptosis develops in lymphocytes and is blocked by Bcl-2 factor [10].

Early development of apoptosis is inhibited by trophic factors, which are transported in an anterograde mode via afferent fibers. By ceasing the contacts with the first afferents, the cells loose trophic support and die [11]. The initial period of apoptosis is induced by expression of nerve growth factor which, reacting with p75 receptor, triggers the cell death mechanisms [1,3]. Irreversible activation of this process in nerve transection is mediated through proapoptosic enzymes – caspases and p53 [7]. Obviously, imbalance between cell proliferation intensity and apoptosis is essential for the regeneratory processes efficiency in injury. Our data indicate that intense expression of Bcl-2 during the early period after maxillary nerve injury is associated with low expression of p53 and hence, low apoptotic index. However, the expression of p53 increases sharply during delayed period after the injury, while the expression of Bcl-2 critically drops, which is paralleled by intense apoptosis of mucosa cells.

Hence, transection of the maxillary nerve initiates apoptotic death of mucosa cells in the rat maxillary sinus after its deafferentation, which correlates with the p-53 and Bcl-2 immunoreactivity balance. Posttraumatic regeneration of the mucosa is associated with a decrease in the apoptotic epitheliocyte density and increase of the apoptotic index in the submucosa during delayed periods of deafferentation.

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