Structural changes of the human superior cervical ganglion following ischemic stroke

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Summary. Objective. The sympathetic nervous system participates in the modulation of cerebrovascular autoregulation. The most important source of sympathetic innervation of the cerebral arteries is the superior cervical ganglion. The aim of this study was to investigate signs of the neurodegenerative alteration in the sympathetic ganglia including the evaluation of apoptosis of neuronal and satellite cells in the human superior cervical ganglion after ischemic stroke, because so far alterations in human sympathetic ganglia related to the injury to peripheral tissue have not been enough analyzed.

Materials and methods. We investigated human superior cervical ganglia from eight patients who died of ischemic stroke and from seven control subjects. Neurohistological examination of sympathetic ganglia was performed on 5 µm paraffin sections stained with cresyl violet. TUNEL method was applied to assess apoptotic cells of sympathetic ganglia.

Results. The present investigation showed that: (1) signs of neurodegenerative alteration (darkly stained and deformed neurons with vacuoles, lymphocytic infiltrates, gliocyte proliferation) were markedly expressed in the ganglia of stroke patients; (2) apoptotic neuronal and glial cell death was observed in the human superior cervical ganglia of the control and stroke groups; (3) heterogenic distribution of apoptotic neurons and glial cells as well as individual variations in both groups were identified; (4) higher apoptotic index of sympathetic neurons (89%) in the stroke group than in the control group was found.

Conclusions. We associated these findings with retrograde reaction of the neuronal cell body to axonal damage, which occurs in the ischemic focus of blood vessels innervated by superior cervical ganglion.

Introduction
The sympathetic nervous system, the predominant innervation to the cerebral arteries, participates in the modulation of cerebrovascular autoregulation by controlling the intracranial pressure, blood volume, and cerebrospinal fluid production and protects cerebral blood flow and blood-brain barrier integrity by limiting cerebrovascular dilation during severe arterial hypertension, arterial hypoxia, and hypercapnia (1). Abundant perivascular nerve plexuses composed of the sympathetic, parasympathetic, and sensory perivascular nerves have been identified in the wall of human major cerebral arteries at the base of the brain and the pial arteries (2, 3). The most important source of sympathetic innervation of the cerebral arteries is the superior cervical ganglion (SCG). The pathway from SCG to all basal cerebral arteries lies along the internal carotid and vertebral arteries (4).

Acute cerebral ischemia and traumatic brain injury cause perivascular nerve fiber damage and thus contributes to vascular abnormalities in cerebral circulation (5, 6). The studies on experimental animals revealed that the cerebral arterial occlusion by extraluminal electrocoagulation induced a marked decrease in the perivascular innervation – including the catecholamine-containing nerve fibers – of the occluded middle cerebral artery (6). Aging also results in the decrease of cerebrovascular nerve density, particularly in the internal carotid artery (2). Thus, impairment of peripheral target pathway or axotomy evokes a characteristic retrograde reaction in the neurons of origin accompanied by marked alterations in the satellite glial cells (7). The cellular response to axonal injury begins as...
central chromatolysis and might evolve as apoptotic cell death along a synchronous time course (8).

Since SCG is the main source of sympathetic innervation of the cerebral arteries, we proposed a hypothesis that a stroke damaging the integrity of cerebral arteries and the structure of perivascular nervous plexus may cause distal axonal damage and indirectly contribute to defects in axonal transport and thus cause axotomy changes in the same SCG. Experimental investigations of rat cardiac sympathetic neurons following myocardial infarction revealed that infarction altered both the distribution and noradrenergic properties of cardiac sympathetic neurons. Apparent sympathetic denervation of the heart after myocardial infarction is accompanied by the loss of norepinephrine uptake and the depletion of neuronal norepinephrine (9, 10). Another experimental study revealed that target deprivation produced by visual cortex ablation caused the death of geniculate neurons in the dorsal lateral geniculate nucleus by an apoptotic process after axotomy (11).

The alterations of human sympathetic ganglia related to the injury to peripheral tissue have not been analyzed yet. The effect of sympathetic nervous system is realized through neurons of the superior cervical ganglion whose sympathetic nerve fibers innervate cerebral arteries. Therefore, the aim of the present study was to evaluate the influence of ischemic stroke on the morphology of SCG and to investigate signs of neurodegenerative alteration including apoptosis of neuronal and glial cells in the human superior cervical ganglion following ischemic stroke.

Materials and methods

Materials. Post mortem material of superior cervical ganglia was obtained from 15 persons (8 men, 7 women) at the age of 60 to 93 years in accordance with the ethical requirements of Kaunas University of Medicine (state permission No. 152/2004). The subjects were divided into two groups: control and stroke. Eight of them, aged from 64 to 93 years, were included in the stroke group. This group was composed of patients who died from ischemic stroke. The control group consisted of seven persons aged from 60 to 75 years who died from diseases not related to heart and/or brain disorders. In the control group, the causes of mortality were mainly associated with traumas and suicides.

SCG were collected within 12–18 hours after biological death. The ganglia were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 48 hours, dehydrated, immersed in xylene, and embedded in paraffin. Further, they were cut in 5-µm-thick sections and stained with cresyl violet. For TUNEL we used apoptosis kit, in situ Cell Death Detection, POD Cat. No. 1684817, Roche Diagnostics DNase I (Roche), in accordance to technique described by Negoescu et al. (12). Paraffin-embedded 5-µm-thick sections were prepared for the TUNEL method. Deparaffinized sections were preincubated for 10 minutes at room temperature in phosphate-buffered saline (PBS) containing 0.25% TritonX-100, and after blocking of endogenous peroxidase activity with 3% hydrogen peroxide for 30 min, slides were washed (3×5 min) in a phosphate-buffered saline (PBS). Sections for antigen retrieval were placed in citrate buffer and kept in microwave oven for 10 min, then cooled at room temperature. Sections were treated with DNase to induce strand breaks. After this procedure, sections were washed in PBS and were blocked for 10 min in 0.1% bovine serum albumin in PBS. The sections were incubated with TUNEL mix (Tdt-terminal deoxynucleotidyl transferase and a mixture of digoxigenin-labeled nucleotides) for 60 min at 37°C in a humidified chamber. After rinsing in PBS, sections were incubated in POD for 30 min at 37°C. Slides were then covered with diaminobenzidine in chromogen solution for 7 min for peroxidase detection, gently rinsed in distilled water, and counterstained with hematoxylin and eosin.

The histological sections of the superior cervical ganglion were observed at ×400 magnification using Leica DM RB microscope. Ten randomly chosen non-overlapping fields were examined from each SCG area. A total of 700 and 750 neurons with clearly visible nuclei and nucleoli were analyzed in the control and stroke groups, respectively. All TUNEL-positive neurons and glial cells in 10 visual fields of each ganglion were counted, and the number of positive and negative cells was calculated. Apoptotic index (the ratio of TUNEL-labeled neurons to the number of all neurons) was determined as well (13). The number of apoptotic and normal glial cells around normal neurons as well as intensively and weakly stained apoptotic neurons in human sympathetic ganglia were calculated. All data are reported as mean ± standard error (SE). Statistical analysis was conducted using SPSS 12.0 for Windows software package. One-way analysis of variance (ANOVA), followed by a post hoc test was used. Significance was accepted at the level of P<0.05.
Results

Morphological peculiarities of sympathetic neurons and glial cells in the human superior cervical ganglia

Differences in the distribution and morphology of ganglion cells were observed in the human SCG in the control and stroke groups. Staining with cresyl violet showed a selection of various size and shape neurons in the human superior cervical ganglion. Sympathetic neurons were distributed in small groups of varying sizes or scattered diffusely. In some old sympathetic ganglia of the control group, the vast majority of neurons demonstrated an apparently normal cytoplasmic morphology. The ganglion neuron profiles were circular or – more commonly – oval shaped and surrounded by 2–4 glial cells which processes formed the neuron glial capsule. The nucleus was usually eccentrically located and contained prominent nucleoli. In other old human SCG of the control group, many neurons were shrunken and contained many vacuoles or showed hypertrophy. In the old sympathetic neurons, pyknotic nuclei, diffusely or “clumpy” organized tigroid in the neuronal cytoplasm, and prominent inclusions of lipofuscin were detected (Fig. 1a). Meanwhile, in the human SCG of old stroke subjects, numerous shrunken neurons with microvacuoles in the cytoplasm as well gliocyte proliferation were found (Fig. 1b). The increased affinity of the neuron cytoplasm to basic dyes and lymphocytic infiltrates were also detected in the sympathetic ganglia of stroke subjects (Fig. 1b). The typical signs of nerve cell death (intensely stained and deformed neuron bodies and vacuoles in the cytoplasm) and neuron regeneration (swollen cell body containing microvacuoles and lightly stained cytoplasm) were found in the sympathetic ganglia of stroke subjects. This mixed view of neuron death and regeneration signs was detected in the ganglia of patients who survived 3 months after stroke. Most neurons with the signs of nerve cell death were found in other ganglia of patients who survived one week after stroke.

Apoptosis of the human sympathetic ganglionic neurons and glial cells

Heterogenic and non-uniform distribution of apoptotic neurons and apoptotic glial cells and individual variations were observed in the human SCG of the control and stroke groups (Fig. 2). Additionally, many neurons possessed lipofuscin, vacuolization, and shrunken cytoplasm.

In the sympathetic ganglia, apoptotic neurons and glial cells showed regional distribution. In some ganglia of the control group, diffusely distributed apoptotic neurons and apoptotic glial cells were found with nonapoptotic neurons localized among them (Fig. 3a). Other ganglia showed various, heterogenic view of clusters: some clusters consisted of all apoptotic neurons and apoptotic glial cells, while other contained shrunken, vacuolated, but nonapoptotic ganglion cells. Meanwhile, a cluster of numerous enlarged and only apoptotic neurons was found in the ganglion of stroke subject...
who survived 3 months after stroke (Fig. 3b). The majority of TUNEL-positive neurons in the old control ganglia preserved normal neuronal structure and the entire membrane of cytoplasm, and other apoptotic neurons contained vacuoles in the cytoplasm (Fig. 3c). Meanwhile, almost all ganglia of stroke subjects consisted of shrunken TUNEL-positive neurons and TUNEL-negative glial cells (Fig. 3d). Apoptotic neurons had abnormal nuclei exhibiting chromatin condensation or nuclear fragmentation. TUNEL-positive neurons had round or shrunken nucleus with condensed chromatin (Fig. 3a). Slightly swollen nuclei with chromatin margination and fragmentation were detected in other sympathetic apoptotic neurons (Fig. 3b). Glial cells with abnormal, intensely stained, irregular or ring-shaped nuclei were frequently seen (Fig. 3a, b).

The number of apoptotic ganglionic cells in the control group varied from 5.6±0.6 to 14.3±1.6 with the mean value being 11.3±0.9 per visual field. The number of apoptotic glial cells in this group varied from 10±1.6 to 107±6.0 with the mean value being 68.9±8.4 per visual field. In the old human SCG, 67% of apoptotic neurons of all neurons were found. On the average, five glial cells were found around normal neurons, and 65% of them were nonapoptotic (Table).

In the stroke group, almost all ganglia demonstrated apoptotic cells (Fig. 3b, d). The vast majority of apoptotic neurons were shrunken and vacuolated, and their nuclei with marginated chromatin were localized eccentrically. In addition to this, pyknosis was characteristic of most of these neurons. In some ganglia, very small sympathetic neurons with unchanged structure or some hypertrophic neurons were found.

The number of apoptotic ganglionic cells in the stroke group varied from 5.6±0.4 to 13±0.7 with the mean value being 10.4±0.4 per visual field. The number of apoptotic glial cells in the stroke group varied from 24±3.8 to 114±9.6 with the mean value being 69±3.6 per visual field. In the old human SCG, apoptotic neurons accounted for 89% of all neurons. On the average, five glial cells were found around normal neurons, and 46% of them were nonapoptotic (Table).

Commonly, SCG in the control and in the stroke patients demonstrated apoptotic morphology, such as shrinkage of cell bodies and fragmentation and condensation of nuclei. The apoptotic index was higher in old stroke ganglia as compared to the old control group, but the number of apoptotic neurons per visible field showed any significant differences in above mentioned groups. Interestingly, approximately two-thirds of the investigated apoptotic ganglionic cells showed intensive staining for apoptosis in nuclei, but one-third demonstrated weak staining for nuclei in both stroke and control SCG.

Discussion

The present neurohistological investigation revealed the signs of neurodegenerative alteration such as darkly stained neurons and deformed neurons with vacuoles, lymphocytic infiltrates, gliocyte proliferation and...
Fig. 3. TUNEL-positive cells of the human superior cervical ganglion in the old control (a, c) and in the old stroke groups (b, d)

Diffusely distributed TUNEL-positive neurons (open arrowheads) and TUNEL-negative neurons among them were detected in the old control group (a). The boxed area (a) is enlarged on the right side and shows apoptotic neurons (open arrowheads) and apoptotic glial cells (black arrows), and nonapoptotic neurons (black arrowheads) surrounded by apoptotic glial cells. The cluster of numerous enlarged apoptotic neurons (open arrowheads) and apoptotic gliocyte (black arrows) were found in the ganglion of stroke subject (b). The boxed area (b) is enlarged on the right side, and TUNEL-positive ganglionic cells show apoptotic morphology, such as nucleus condensation and fragmentation. Many apoptotic (white arrowheads) and some grossly vacuolated neurons (black asterisks) and a single nonapoptotic neuron (black arrowhead) among them were seen in the old control sympathetic ganglion (c). Meanwhile, almost all apoptotic and shrunken sympathetic neurons and apoptotic glial cells were found in the SCG of stroke subject (d).

apoptotic cell death in human superior cervical ganglia following ischemic stroke. These changes were more pronounced in the ganglia of stroke subjects. In the present study, the brain stroke was chosen as a model of an indirectly evoked damage to axons. The alterations of human sympathetic ganglia related to the injury to peripheral tissue have not been sufficiently analyzed so far. In the present study, we made the hypothesis that programmed cell death may be also involved in the death of sympathetic neurons occurring in human SCG following ischemic stroke. Apoptosis plays an important role in the development of the nervous system.

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and in the maintenance of tissue homeostasis in adult body (14). Apoptosis, the morphological expression of programmed cell death, is a genetically regulated process and is essential for the elimination of unnecessary and damaged cells (15).

To identify dying cells by apoptosis, we used TUNEL method, which allows for the detection of DNA fragmentation in apoptotic cells. The main apoptotic parameters such as shrinkage of the cytoplasm and condensation of the nucleus were detected in SCG, but apoptotic bodies and membrane blebbing were not found. A more precise confirmation of apoptosis might be done by using electron microscopy and DNR electrophoresis (16). Artifacts due to postmortem delay were not encountered in the specimens of the present study, since only material with a postmortem delay of 18 hours was used.

Experimental research of the apoptosis of retrogradely degenerating neurons in the adult rats revealed that target deprivation produced by visual cortex ablation caused the death of geniculate neurons in the dorsal lateral geniculate nucleus by an apoptotic process following axotomy (11). According to the previous reports, sympathetic postganglionic nerve fibers branch from the superior cervical ganglion and form perivascular plexuses of cerebral arteries (17) that constrict in response to cervical sympathetic stimulation and dilate when these fibers become interrupted (18). Since SCG is the main source of sympathetic innervation of the cerebral arteries, we proposed the hypothesis that the arterial sympathetic fibers presented in a stroke region are possibly affected by an ischemic process and thus may indirectly contribute to defects in axonal transport and in the same SCG. As a result, pathologic axotomy develops suggesting about process of apoptosis in sympathetic ganglion.

Both aging and neurodegenerative diseases are known to decrease the sympathetic nerve density of the intrinsic nerve plexuses of the basal cerebral arteries (17). This may also have a reversible effect on SCG. Similar alterations as decreased number of neurons and density of nerve fibers in old age were found in the human intracardiac nerve plexuses (19) and in the intracardiac ganglia (20). However, there are only limited experimental data concerning structural changes of SCG following damage to the peripheral target (21).

Human superior cervical ganglion is complicated object for analysis since this kind of ganglion shows the neuronal internal and external structural polymorphism, heterogenesis, and various manifestations of damage. The TUNEL analysis performed in the human SCG ganglion from the subjects who experienced brain stroke and those obtained from the control group revealed that the distribution of apoptotic neurons and apoptotic glial cells was not uniform. Moreover, neurons demonstrated clear and similar morphological characteristics of apoptosis: fragmentation and condensation of the nucleus and shrinkage of neuronal cytoplasm. Apoptotic index (89% of apoptotic cells) was significantly higher in the ganglia of stroke subjects as compared to old human sympathetic ganglia of the control group, but the number of apoptotic neurons and apoptotic glial cells per visible field did not show any significant differences. Numerous apoptotic ganglionic cells (apoptotic index 67%) were detected in the old sympathetic ganglia of the control group. The functional significance of the apoptosis in the aging process is not well defined. In recent years, accumulating evidence indicates that dysregulation of the apoptotic process may be involved in some aging process; however, it is still debatable whether aging suppresses or

| Variable                | Old control group | Old stroke group |
|-------------------------|-------------------|------------------|
|                        | mean±SE range     | mean±SE range    |
| Apoptotic neurons       | 11.3±0.9 6–14     | 10.4±0.4 6–13    |
| Normal neurons          | 5.3±0.6* 3–7      | 2±0.2* 0.4–4     |
| Apoptotic index         | 0.67±0.1* 0.2–1   | 0.89±0.1* 0.8–1  |
| Apoptotic glial cells   | 68.9±8.4 10–107   | 69±3.6 24–114    |
| Apoptotic gl/n          | 1.6±0.1 0.2–3     | 2.8±0.2 2–4      |
| Normal gl/n             | 2.96±0.1 1.7–3    | 2.4±0.2 0–6      |

*Statistically significant differences between groups (P<0.05).

Apoptotic gl/n – apoptotic glial cells around nonapoptotic neuron; normal gl/n – normal glial cells around nonapoptotic neuron. TUNEL method.
enhances apoptosis in vivo. However, it is known that apoptosis plays an important role in the aging process (22). Experimental investigation of old dogs showed that the number of apoptotic cells in the brain of aged dogs was slightly increasing with age. Morphological apoptotic features characterized by condensation of nuclear chromatin and swollen cytoplasm were observed in neurons and glial cells of the brain of old dogs (23). Beside this, apoptosis is also known to play an important role in age-related neuronal loss in human and in canine brains (24).

Interestingly, approximately two-thirds of the investigated apoptotic ganglionic cells showed intensive staining for apoptosis in nuclei, but one-third demonstrated weak staining for nuclei in both stroke and control SCG.

In the sympathetic ganglia from old specimens, isolated nonapoptotic neurons were distributed among apoptotic neurons and glial cells. It might be related to the different neuronal subpopulations in the sympathetic ganglion, which are known to innervate different peripheral targets (25). Meanwhile, almost all apoptotic shrunken neurons were detected in the human SCG of stroke subjects, except for the ganglia from a patient who survived 3 months after stroke. In these sympathetic ganglia, hypertrophic nonapoptotic neurons were found among apoptotic ganglionic cells.

First morphological changes occurring shortly after the brain stroke seem to affect SCG glial cells more than neurons and it might be explained by the fact that these cells serve as the first barrier of defense and by other functions for gangliocytes. Commonly, apoptosis in SCG seems to be an unequal process due the both aging and stroke. However, an increase in the number of affected neurons in stroke subjects exceeding that in old patients might be explained by individual variations and/or some additional unknown pathogenetic compensatory mechanisms activated by the stroke.

One of the most important mechanisms inducing the death of sympathetic neurons is a deficiency of nerve growth factor (NGF) (26). Since nerve growth factors are synthesized by peripheral target, its injury during axotomy or removal results in the deficiency of nerve growth factor and the expression of apoptotic signals, which leads to the neuronal cell death (26, 27). Studies on experimental animals ascertained that neuron apoptosis is evoked by axotomy and damage to axons (28, 29). Axonal damage can occur following compression, trauma, inflammation, or other types of lesion. Axonal injury leads to a marked retrograde reaction of the neuronal cell body and satellite cells to axon damage (14).

Damage to axons and the reduced level of NGF cause changes in neuropeptide expression and peptide phenotype of axotomized neurons (30). Axotomy of sympathetic neurons in the SCG increases the levels of vasoactive intestinal peptide, galanin, and substance P and decreases mRNA levels of neuropeptide Y and tyrosine hydroxylase (30).

We assume that stroke possibly caused damage to distal axons in cerebrovascular plexuses of our patients and thus partially induced changes in the human superior cervical ganglion. We proposed the hypothesis that our findings such as apoptotic neurons and apoptotic glial cells, proliferation of satellite cells, and lymphocytic infiltrates in the stroke ganglia are related to pathological axotomy. We have not found any reports on the investigation of the relationship between the neuropathological signs in sympathetic ganglia and ischemic stroke.

Therefore, further experimental studies are needed to verify these results and to analyze alterations in sympathetic ganglia related to the injury of the peripheral tissue as well as the precise mechanisms of neuronal death and their relation to age-related plasticity of the autonomic nervous system.

Conclusions
The present investigation revealed that signs of neurodegenerative alteration (darkly stained and deformed neurons with vacuoles, lymphocytic infiltrates, and gliocyte proliferation) were markedly expressed in the ganglia of stroke patients. Apoptotic cell death was observed in the human superior cervical ganglion in both the control and the stroke groups and seems to be unequal and complex process, because of heterogenic and not uniform distribution of apoptotic neurons and apoptotic glial cells and individual variations in both groups. Relatively higher apoptotic index was found in the stroke group than in the control group.

This study suggests that ischemic stroke is related to neurodegenerative alteration of neurons and glial cells of the human superior cervical ganglion. These changes might be associated with pathological axotomy.

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Žmogaus viršutinio kaklo mazgo struktūriniai pokyčiai po išeminio insulto

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Raktažodžiai: žmogaus viršutinis kaklo mazgas, simpatinis neuronas, apoptozė, išeminis insultas, TUNEL metodas.

Santrauka. Simpatinė nervų sistema autoreguliacinius mechanizmайs moduluoją smegenų kraujagyslių funkciją. Svarbiausias smegenų kraujagyslių simpatinės inervacijos šaltinis yra simpatinio kamieno viršutinis kaklo mazgas. Darbo tikslas. Iš��gūrę žmogaus viršutinio kaklo mazgo neurodegeneraciniai pokyčiai ir įvertinti neuronų bei glėsų ląstelių apoptozės požymius po išeminio insulto. Žmogaus simpatinių mazgų pokyčiai, susiję su inervuojamo audinio pažeidimu, kol kas nepakankamai išﬁgūrę.

Tyrimo medžiaga ir metodai. Išﬁgūrę aštuonių žmonių, mirusių nuo išeminio insulto, ir septynių kontrolinės grupės žmonių viršutinio kaklo mazgai. Šių mazgų neurohistologinis įvertinimas atliktas 5 mikronų storio parafininiuose pjaūviose, dažytuose krezilviole. Simpatinių mazgų apoptozinės ląstelės tirtos taikant TUNEL metodą.

Rezultatai. 1. Neurodegeneraciniai pokyčių požymiai (tamsiai nusidūžę ir deformavęs neuronai su vakuolėmis, limfocitų inﬁltracija, glėsų ląstelių proliferacija) vyrauja insulto grupės mazguose. 2. Daug apoptozinių neuronų ir apoptozinių glėsų ląstelių rasta žmogaus viršutinio kaklo mazguose tiek senyvo anžiuos kontrolinėje, tiek insulto grupėse. 3. Heterogeniškas ir netolygus apoptozinių neuronų ir glėsų ląstelių pasiskirstymas bei individualių variacijų aptikta kontrolinės ir insulto grupės žmonių viršutiniuose kaklo mazguose. 4. Insulto grupės simpatiniuose mazguose rastas didesnis apoptozinis indeksas (89 proc. apoptozinių neuronų) palyginti su kontrolinės grupės mazgais.

Išvada. Šio tyrimo duomenis mes siejame su neuronų kūno retrogradine reakcija į aškonų pažeidimą, kuris vystosi viršutinio kaklo mazgo inervuotų kraujagyslių iššmieniame žūdyje.

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