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Cardiomyocyte Autophagia and Morphological Alterations in the Left Ventricular Myocardium during Acute Focal Ischemia

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In experiments on rabbits we evaluated the intensity of cardiomyocyte autophagia by the level beclin-1 protein and morphology of the left ventricular myocardium on days 1, 3, and 5 after the onset of focal ischemia caused by ligation of the descending branch of the left coronary artery. The morphological alterations in the left ventricular myocardium were accompanied by intensification of cardiomyocyte autophagia, which attained maximum on postligation day 1.

Key Words: *autophagia; beclin-1; cardiomyocyte; myocardium; focal ischemia*

In the study of chronic heart disease, much attention is paid to the possibility of controlling programmed cell death with various types of pharmacotherapy. It is a common knowledge that in the complex of degenerative alterations in the myocardium, a prominent role is played by activation of cardiomyocyte apoptosis [2]. An alternative mechanism promoting degradation and loss of cells under the effect of a number of unfavorable factors is autophagy, *i.e.*, engulfment of individual organelles and cytoplasmic fragments by autophagosomes surrounded by double layer membrane leading to their destruction by hydrolases [4]. Autophagy is triggered by various inducers of cell stress such as deficiency of nutrients, hypoxia, ROS, and damage to DNA and/or organelles [7].

It should be stressed that in some cases, autophagy can manifest itself as a concomitant mechanism accompanying cell death, while in others, it is a part of the reparative process responsible for cell survival via mobilization of its resources [8]. In light of this, a concept of the optimum for this reparative process was elaborated, which assumes that down-regulated autophagy promotes cell aging, while up-regulated autophagy kill them [1].

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Some papers advanced a hypothesis that intensification of autophagy is a key mechanism protecting the myocardium against the consequences of ischemia-induced lesions [9,10]. However, there are no data on activity of the processes mediating autophagy of cardiomyocytes during structural alteration provoked by acute myocardial ischemia. Our aim was to fill this gap.

MATERIALS AND METHODS

The experiments were carried out on male Chinchilla rabbits weighing 3.0-3.5 kg. The animals were maintained, operated, and examined in strict adherence to Order No. 75 of USSR Ministry of Health 5 (August 12, 1977) and European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The rabbits (n=16) were randomized into 4 equal groups: one control (intact) and 3 experimental groups that comprised rabbits with modeled acute focal ischemia of the left ventricle (LV) examined on postsurgery days 1, 3, and 5. Focal ischemia was produced in narcotized rabbits by ligation of the descending branch of the left coronary artery between its middle one-third and lower one-third subdivisions.

On respective postsurgery days, thoracotomy and heart extirpation were performed under general anesthesia. The specimens of macroscopically vital LV

myocardium bordering upon the necrotic focus were fixed for 72 h in 4% neutral paraformaldehyde, processed routinely, and embedded into paraffin. Histological sections (5 μ) were prepared on a Slidt-2003 microtome and mounted on poly-L-lysine-coated and routine microscope slides for immunohistochemical and morphometric examinations, respectively. The sections were deparaffinized in xylene and passed through a series of decreasing alcohol concentrations.

Activity of cardiomyocyte autophagy was assessed according to the content of beclin-1 (bcl-1) protein assayed in immunochemical reaction with polyclonal primary antibodies raised in rabbits (Santa Cruz Biotechnology). bcl-1 plays the major role in autophagy by securing sequestration of the removed organelles [3,5]. The immunochemical reaction was visualized using UltraVision Detection System kit (Thermo Scientific). The specimens were post-stained with Mayer's hematoxylin. The reaction was documented as positive after appearance of the brown color. In each specimen, 30 fields of view were examined at 400× with Avtandilov's eyepiece graticule. The percentage ratio of the number of equidistant points in positively stained cardiomyocyte cytoplasm to the total number of points occupied by this cytoplasm was calculated.

The morphometric analysis of myocardial specimens was carried out in histological sections stained with hematoxylin and eosin. Light microscopy with Avtandilov's eyepiece graticule analyzed 30 visual fields at 400× in each specimen to determine the volume percent (v/v %) of myofibrils, cardiomyocyte nuclei, the destruction and infiltration sites, and the volume of extracellular space. The nuclear-cytoplasmic ratio was calculated as the ratio of the volume occupied by cardiomyocyte nuclei to that of myofibrils.

The data were analyzed statistically using Student's t test at $p<0.05$.

RESULTS

On day 1 after the onset of focal ischemia in LV, the level of bcl-1 in cardiomyocyte cytoplasm increased form the control value of 3.0±0.5 to 18±3% ($p\leq0.05$). On the postischemic days 3 and 5, the level of bcl-1 gradually decreased to 14±3 and 8±2%, respectively, but significantly exceeded the control level ($p\leq0.05$). Visual inspection of myocardial sections revealed mosaic and uneven positive immunohistochemical reaction. There was a rising trend in the number of positively stained myofibrils when approaching the area immediately verging on the myocardial necrotic focus. At the same time, the incidence of positive staining in all specimens correlated with the density of vascular network: the autophagia processes were most prominent in the myocardial layers with more developed circulation.

The specimens demonstrated uneven immunohistochemical staining of the cardiomyocytes, which probably reflected various degree of autophagy.

On postischemic day 1, the structural alterations in LV myocardium were rather moderate manifesting themselves by homogenization of cardiomyocyte cross striation, focal wavy course of the myofibrils, and individual hemorrhagic sites with the blood formed elements. In comparison with the control group, ischemia significantly diminished the volume of myofibrils and expanded the extracellular space (Table 1), which could result from the development of extracellular edema and/or a decrease in the size of myofibrils. These alterations were accompanied by a significant decrease in the volume of cardiomyocyte nuclei in LV and a dramatic increase in the area of destructed tissue.

On postischemic day 3, the boundaries of myofibrils became blurred in comparison with those observed on day 1. Moreover, orientation of myofibrils was disturbed, some myofibrils were overcontracted,

TABLE 1. Morphometry of Histological Sections of LV Myocardium on Days 1, 3, and 5 after Simulation of Acute Focal Ischemia ($M\pm m$)

Structural elements	Control	LV ischemia		
		day 1	day 3	day 5
Myofibrils, v/v %	88.04±0.37	72.81±2.05*	59.14±3.22*+	51.69±3.72*
Nuclei, v/v %	5.48±0.21	4.16±0.24*	3.17±0.24*+	3.17±0.35*
Nuclear-cytoplasmic ratio	0.060±0.003	0.060±0.003	0.050±0.004*	0.040±0.004*
Vessels, v/v %	1.01±0.77	0.30±0.13	1.50±0.45+	1.64±0.53
Destruction sites, v/v %	0.29±0.06	6.21±1.24*	21.94±2.98*+	29.76±3.80*
Extracellular space, v/v %	6.19±0.32	16.14±1.34*	14.03±1.00*	13.67±1.11*

Note. $p<0.05$ in comparison with *control and +previous examination day.

and the hemorrhagic areas became more expanded. When compared to the morphometric data obtained on postischemic day 1, the volume occupied by myofibrils decreased, while the number of destructed sites rose. In addition, there was further drop in the volume occupied by the cardiomyocyte nuclei. However, the volume of extracellular space remained at the level observed on the first postischemic day.

On postischemic day 5, the pathological alterations in myocardium were even more pronounced: homogenization of myofibrils increased, eosinophilic infiltration appeared, and the boundaries of many cells became unclear. In comparison with postischemic day 3, there was a decreasing trend for the volume of myofibrils and an increasing one for the number of destruction sites. However, the volume occupied by nuclei did not change in comparison with that observed on postischemic day 3.

On postischemic days 3 and 5, the nuclear-cytoplasmic ratio decreased slightly but significantly in comparison with the control value reflecting a rather pronounced destruction of the nuclei and a drop in the number of viable cardiomyocytes.

Taking into account growing character of structural alterations in the myocardium provoked by ischemia, one can hypothesize that gradual down-regulation of cardiomyocyte autophagia relatively to the peak value observed on postischemic day 1 results from a decrease of energy used by the cells for the adaptive and accommodation processes. This study corroborates the view that autophagia is a mechanism protecting the cardiomyocytes against the death and preventing expansion of the necrotic area during myocardial infarction [6].

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