Effect of Endothelin-1 on Apoptosis, Proliferation, and Protein Synthesis in Cardiomyocytes of Newborn Albino Rats

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Five intraperitoneal injections of endothelin-1 (100 μg/kg) to newborn albino rats on days 2-6 of life did not change the number of nuclei expressing PCNA in the left-ventricular myocardium. The number of nucleoli, area and perimeter of cardiomyocytes (isolated by the method of alkaline dissociation) increased. The number of myocyte nuclei in the state of apoptosis (evaluated by the TUNEL method) increased significantly. Presumably, partial loss of cardiomyocytes as a result of apoptosis activation after treatment with endothelin-1 is compensated by increased size and transcription activity of remaining cardiomyocytes.

Key Words: *endothelin; apoptosis; proliferation; myocardial morphogenesis*

Our previous studies showed different reactions of the myocardium to endothelin-1 (ET-1). The type of reaction depended on the peptide dose and number of injections, as well as on animal age [3-5]. In our further investigation of the effect of ET-1 on morphogenetic processes in rat myocardium we studied the intensity of apoptosis of myocardial cells after repeated injections of the agent. Apoptosis is a regular and obligatory process accompanying the formation of the heart. Disorders in spatial and temporal characteristics of programmed cell death in cardiomyocytes (CMC) underlie many cardiovascular diseases [7,8].

MATERIALS AND METHODS

Experiments were carried out on 26 newborn albino rats intraperitoneally injected with ET-1 (100 μg/kg) on days 2-6 of life. The agent was kindly provided by Prof. Zh. D. Bespalova, M. D. (Laboratory of Peptide Synthesis, Cardiology Research and Production Complex, Ministry of Health of Russian Federation). Controls were injected with an equal volume of 0.9% NaCl. Morphological characteristics of the myocardium were studied in 7-day-old rats (24 h after the last injection of ET-1). The heart was removed and fixed in 10% neutral formalin in phosphate buffer for 3 days and embedded in histowax routinely. Sections (5-μ) were mounted onto poly-L-lysine-coated slides.

For isolation of CMC, a fragment of left-ventricular myocardium was fixed in formalin for at least 2 weeks, and after alkaline dissociation the cytological preparations were made as described previously [1] and stained with azur and eosin.

Immunohistochemical detection of proliferating cell nuclear antigen (PCNA, clone PC10) was carried out using standard streptavidin-biotin method with restoration of the antigenic structure in a water bath using Dako reagents. The nuclei were post-stained with Mayer hematoxylin. The index of labeled nuclei in the left-ventricular myocardium was estimated and

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expressed in percents of the total number of examined nuclei (at least 2000 per preparation).

The intensity of apoptosis was evaluated by the TUNEL method (labeling of terminal DNA fragments). The number of TUNEL$^+$ CMC nuclei was expressed in percents of the total number of nuclei [5]. The study was carried out in the University of Turku (Finland) [7,8].

For detection of transcription activity in CMC, morphometrical parameters of nucleolar organizer regions (NOR) were studied using staining with 50% silver nitrate [2].

Geometrical parameters of CMC and NOR were studied using a MEKOS-C image analysis system. The areas and perimeters of CMC, number and areas of NOR were measured.

The results were statistically processed using Pierson's correlation coefficient (r) and Student's t test. The differences between the control and experimental groups were considered significant at $p<0.05$.

RESULTS

The index of CMC nuclei labeled with anti-PCNA antibodies virtually did not change after ET-1 treatment in comparison with the control (Table 1). It is noteworthy that binding of anti-PCNA antibodies to CMC nuclei several times surpassed the corresponding parameter for thymidine. The mechanisms of these differences were discussed previously [3].

Changes in geometrical parameters of isolated CMC after administration of 100 μg/kg ET-1 were in general universally directed in comparison with the effects of 50 μg/kg ET-1 studied previously [5]. The areas and perimeters of isolated CMC significantly increased, which was paralleled by an increase in the number of NOR (Table 1). No significant changes in the nucleolar area were detected. Morphometrical parameters of NOR reflect plastic (protein synthesizing) function of CMC. On the other hand, we cannot rule out the possibility that during the studied ontogenetic period (neonatal) these parameters characterize not only the synthesis of contractile proteins, but also proliferative activity of CMC, as it was described for rapidly proliferating cell populations [10]. However, the absence of changes in the index of labeled nuclei, increased area of isolated CMC, and increased number of NOR after ET-1 treatment suggest that in this case the geometrical parameters of NOR largely reflect heterosynthetic processes of protein production.

The number of TUNEL$^+$ CMC nuclei in the left-ventricular myocardium increased (Table 1) indicating intensification of programmed cell death. The studied stage of postnatal morphogenesis of the myocardium is characterized by intensive cell division and apoptosis with a tendency to their gradual decrease. We cannot compare the absolute values of PCNA labeled nuclei index and the number of apoptotic nuclei because of methodological difficulties and the duration of these processes in the cell. We therefore compared Pierson's coefficients of correlation between the number of PCNA$^+$ and TUNEL$^+$ nuclei and between CMC size and number of nuclei with signs of apoptosis. A stable positive correlation (r=0.68) was detected only for the second pair. In other words, activation of apoptosis in some CMC correlated with increase in the geometrical sizes of the other CMC population.

The data on the effects of ET-1 on apoptosis and DNA synthesis in the myocardium are contradictory: both stimulatory and suppressing effects of this peptide were reported [12]. The majority of scientists consider that ET-1 has an antiapoptotic effect on CMC. It is believed that ET-1 is a physiological antagonist of proapoptotic C-type natriuretic peptide in intact myocardium [11]. Antagonism between these vasoactive peptides was demonstrated for DNA and protein synthesis in rat myocardium [3]. On the other hand, in our study the number of nuclei with terminal DNA breaks characteristic of apoptosis far surpassed the control (Table 1).

Our previous studies showed that injections of ET-1 activated free-radical oxidation in the heart [4].

TABLE 1. Effects of 5 Injections of ET-1 in a Dose of 100 μg/kg on the Morphometrical Parameters of Left-Ventricular Myocardium of Newborn Albino Rats ($M\pm m$)

Parameter	Control	ET-1
Index of nuclei labeled with anti-PCNA antibodies, %	27.36±1.07	27.52±2.02
Number of TUNEL$^+$ nuclei, %	0.044±0.01	0.12±0.02*
CMC area, μ^2	685.75±57.32	894.17±31.94*
CMC perimeter, μ	142.72±5.4	161.23±3.87*
Number of nucleoli	2.47±0.05	2.69±0.08*
Nucleolus area, μ^2	1.21±0.06	1.06±0.06

Note. *$p<0.05$ compared to the control.

Dysfunction of CMC mitochondria, which is paralleled by increased expression of ET-1 in the heart [14] and activation of CMC apoptosis [13], is observed under conditions of oxidative stress.

Contradictory reports about the effects of ET-1 on apoptosis can be explained by age-specific features in myocardial reaction to the peptide injection. Changes in ET-1 receptor populations in the kidneys during the rat ontogeny were described [9]. We previously showed that the dose of the peptide is significant for manifestation of its morphogenetic effects. Stimulation of DNA synthesis in the myocardium of newborn rats was observed only after administration of 10 μg/kg ET-1 [4,5].

Presumably, the influences on the heart associated with systemic effects of ET-1 predominate after administration of this agent in a dose of 100 μg/kg. In our experiments the general hemodynamic effect (spasm of peripheral vessels) was seen from pale skin of newborn rats 5-7 min after intraperitoneal injection of the peptide.

Hence, repeated injection of ET-1 in a dose of 100 μg/kg under conditions of stable proliferative activity stimulated apoptosis and transcription activity of the newborn albino rat CMC.

The following interpretation of our findings seems to be most realistic. Activation of apoptosis and partial loss of CMC population and ongoing active growth of the heart under the effect of hemodynamic load require compensation for lost cells. This compensation can be attained via two routes: increase in the number of cells or DNA production in them or enlargement of existing CMC. Our results suggest that the second way is more possible in newborn rats injected with ET-1. This mode of regeneration (hypertrophy without appreciable cell hyperplasia) in various pathological treatments is characteristic of adult mammalian myocardium [6].

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