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EXPERIMENTAL STUDY

Proapoptotic and antiapoptotic stimuli alternation in myocardial infarction experimental model

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ABSTRACT

Myocardial infarction is a life-threatening complication of the coronary artery disease - the leading cause of premature death worldwide. The severity of this condition is the result of cellular death following the myocardial ischaemia, which occurs via several mechanism including apoptosis. For the research of this condition, animal models are often employed. We established isoprenaline-induced rat model of myocardial infarction, focusing on the immunohistochemical analysis of the expression of antiapoptotic and proapoptotic proteins BCL-2 and BAX, respectively. Apoptosis (based on BAX-positivity) was activated in cardiac muscle cells within the first day, later on day 8 also in fibroblasts of the forming scar tissue. Antiapoptosis in cardiac muscle cells was weak to moderate on the day 1 and 2, on the day 8 macrophages were strongly positive for BCL-2. The results confirmed that programmed cell death as well as mechanisms of antiapoptosis contribute to the pathogenesis of myocardial infarction. Previous research demonstrated that by experimentally affecting proapoptotic and antiapoptotic signals, it is possible to influence various aspects of myocardial infarction including: infarction size, cardiac remodelling and prognosis of the heart failure. Future research is warranted to fully elucidate the role of this process during myocardial infarction, which will result in refined diagnostic and therapeutic strategies *(Tab. 1, Fig. 1, Ref. 21)*. Text in PDF *www.elis.sk*

KEY WORDS: myocardial infarction, isoprenaline, apoptosis, necrosis, BCL-2, BAX.

Introduction

The statistics of the coronary artery disease (CAD) continue showing an unfavourable outcome. It is notorious for being in the forefront of preventable deaths all over the world for the past decades despite an enormous progress in the understanding of its pathogenesis and state-of-the-art therapeutic approaches (1). The etiopathogenesis of CAD is complex and includes genetic predisposition and environmental factors causing pathological atherosclerotic changes in the wall of the coronary arteries. Narrowing of the arterial lumen will clinically manifest in different ways depending on the onset and extent of the occlusion, including acute myocardial infarction and sudden cardiac death (2). The principal pathogenetic process – atherosclerosis – is a chronic inflammatory disorder, characterized by endothelial dysfunction and progressive structural and functional changes in the wall of the affected artery (3–5). If the lesion is unstable, i.e. prone to erosion, rupture of the plaque can be complicated by thrombosis, which in turn can cause total blockage of the arterial lumen. The seriousness of the following ischaemia depends upon the size of the occluded artery and other confounding factors (6, 7). Acute coronary syndrome is a medical emergency and requires a prompt management before irreversible pathological changes develop in the heart muscle. For a long time, the sole pathological change after prolonged ischaemia was thought to be necrosis. In the past decades, it became evident that the role of apoptosis has been underestimated (8). Moreover, it has been shown that necrosis and apoptosis do not occur as independent processes, but they are interrelated. On top of that, apoptosis has been observed not only following the ischaemia, but also during reperfusion (9). The exact role of apoptosis during ischaemia/reperfusion remains an object of continuous investigation. Recent research endeavours have demonstrated that other mechanisms such as: necroptosis and pyroptosis may contribute to the complex picture (10).

The thorough understanding of these processes is still a timely topic, which is essential for the refinement of diagnostic and therapeutic approaches to myocardial infarction. The present study of the experimentally induced myocardial infarction indicate diverse proapoptotic and antiapoptotic signalling activities in different cell types of the ischemic lesion.

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Material and methods

The animal model of myocardial infarction

We established an *in vivo* rat model of myocardial infarction by administration of isoprenaline to the Wistar rats, which induced ischaemia in the heart muscle. The experimental animals aged 19 weeks, were kept in a quarantined animal holding room (ambient temperature 20–24 °C, relative humidity 45–65 %, light/dark regimen – 12/12). The rats were given *ad libitum* standard pelleted animal food and drinking water in controlled amounts one to two times per day. The experiment was approved by The Ethics Committee of the Faculty of Pharmacy, Comenius University in Bratislava and The State Veterinary and Food Administration of the Slovak Republic. During the experiment, we administered 5 mg/kg of isoprenaline intraperitoneally in one dose. We used 0.9 % solution of sodium chloride with 0.05 % ascorbic acid as the pharmaceutical vehicle of isoprenaline. The experimental animals were divided into the groups of 6 animals, euthanised in CO_2 chamber after 0.5, 1, 2, 4, 8 hours after a single isoprenalin administration and after 1, 2, 4, 8 days of daily isoprenaline administration. The control group was kept without intervention. Subsequently, we harvested the hearts and fixed the tissue samples for histological analysis in 10 % formalin (4 % aqueous solution of formaldehyde).

Histology and immunohistochemistry

The apical heart tissue specimens were processed using the routine Formalin-Fixed Paraffin-Embedded technique. Slices 5 µm thick were stained with hematoxylin and eosin (HE) and Mallory´s phosphotungstic acid-haematoxylin (PTAH) and evaluated in light microscopy.

For immunohistochemistry, after deparaffinization, the specimens were processed 30 minutes for antigen retrieval in PT Link (DAKO, Glostrup, Denmark) with citrate buffer pH 5 for BAX and TRIS buffer pH 8 for BCL-2. Next, the activity of endogenous peroxidase was blocked using the treatment by Peroxidase-Blocking Reagent of the EnVision FLEX system (DAKO) for 5 minutes. Immunohistochemical staining was performed in the AutostainerPlus (DAKO) with polyclonal rabbit anti-human BAX (dilution 1:250) and monoclonal mouse anti-human BCL-2 oncoprotein (dilution 1:150) with application of the EnVision FLEX kit (DAKO) according to the manufacturer´s instructions. The reaction product was developed with 3,3‘-diaminobenzidine (DAKO) and counterstained with haematoxylin and the slides were mounted in acrylic resin.

The extent of ischemic myocardial injury was assessed in slides stained with HE and PTAH. The signs of apoptosis were evaluated by morphometric analysis of immunohistochemically stained slides (BCL-2, BAX) using a semiquantitative evaluation by the light microscope Leica DM 2000 (Wetzlar, Germany). Staining intensity was assessed as weak, moderate and strong.

Results

The experimental conditions were well tolerated, none of the rats died during the experiment after a single isoprenaline injection, while mortality was about 20 % until day 8. The administration of isoprenaline led to myocardial ischaemia and consequent signs of myocardial infarction.

In HE stained slides taken from the animals euthanised after two hours, there was an apparent loss of cross-striation in groups of cardiomyocytes. These changes were accentuated after PTAH staining. In rats euthanised after four hours, we observed more intense focal changes, a progressive deterioration of cardiomyocytes, in some we noticed a pronounced pyknosis of nuclei and intense cytoplasmic eosinophilia. After eight hours, we observed myocardial cells without nuclei. These changes were accentuated after PTAH staining (Fig. 1). On the day 2, when necrosis was progressing, in HE stained slides, we observed advanced reparative processes, dead cells were scavenged and progressively replaced by vascularized connective tissue with numerous histiocytes. In PTAH-stained slides, newly-formed connective tissue was stained light red-brown. On the day 8, we observed a well-formed vascularized scar tissue. In PTAH-stained slides, brown-stained mature collagen fibres were clearly visible (Fig. 1).

In slides stained for the presence of BAX and BCL-2, BAX positivity was the highest on the first day, which displayed as a moderate to strong reaction in extensive apoptotic loci within cardiac muscle cells, and after eight days, due to a massive apoptosis of fibroblasts in the scar tissue. On the second day, when the scar tissue was forming, the BAX positivity in the myocytes disappeared. Considering BCL-2, fibroblasts

Tab. 1. BCL-2 and BAX expression from 0.5 hours to 8 days of isoprenaline administration.

	BCL-2	BAX
control	negative	negative
0.5 h	weak positivity	scarce sarcoplasmic positivity
1 h	weak fine granular sarcoplasmic positivity	scarce sarcoplasmic positivity
2 h	weak fine granular sarcoplasmic positivity	small areas of weak sarcoplasmic positivity
4 h	very weak sarcoplasmic positivity	larger groups with sarcoplasmic positivity, scattered sarcoplasmic weak to moderate positivity
8 h	weak perifocal sarcoplasmic positivity	strong granular sarcoplasmic positivity, extensive foci
1 day	moderate positivity of fibroblasts	only remnants of weak sarcoplasmic positivity
2 days	moderate positivity in fibroblasts	formation of scar tissue, no sarcoplasmic BAX positivity
4 days	moderate positivity in fibroblasts	weak positivity in fibroblasts of the forming scar tissue
8 days	strong positivity in macrophages, fibroblasts with weak to missing positivity	strong positivity of scar tissue fibroblasts

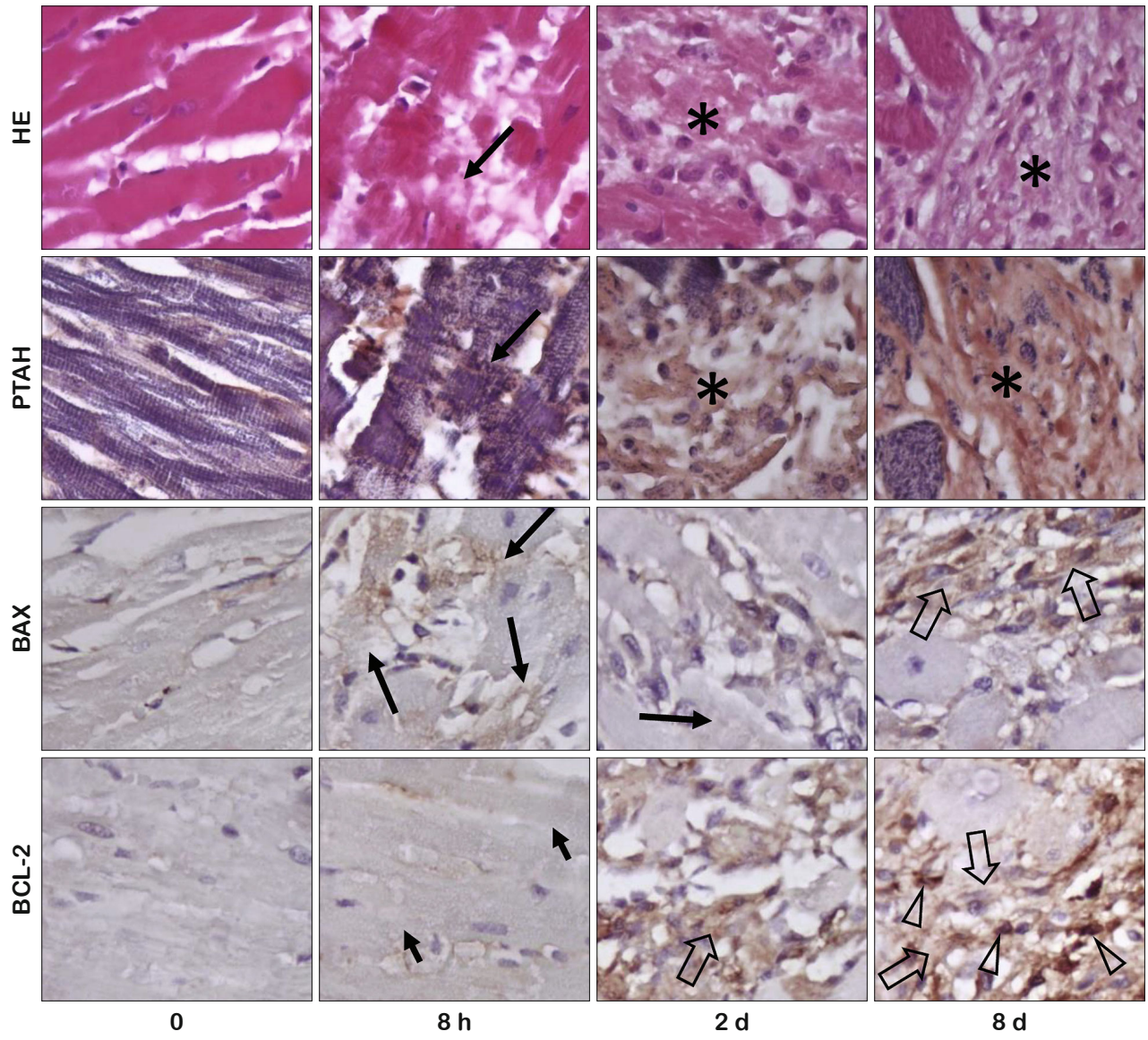

Fig. 1. Time sequence of morphological changes after induction of myocardial infarction by isoprenalin administration. After 8 hours there was decomposition of myocyte sarcoplasm (arrow) well visible as loss of crosstriation in PTAH staining. There was intense BAX expression in the affected myocytes (arrow) and only weak expression of BCL-2 in the myocardium around the affected area (short arrow). On day 2 there was formation of immature scar tissue (*) stained light brown by PTAH, with intense BCL-2 expression (open arrow) and weak BAX immunoreactivity in surviving myocytes (arrow). On day 8 the maturity of the scar tissue was documented by intense brown color (*) in the PTAH staining, the fibroblasts showed strong BAX and weak BCL-2 expression, with intense BCL-2 staining of the macrophages (arrow head). Hematoxylin and eosin(HE); phosphotungstic acid hematoxymin (PTAH); immunoperoxidase technique, diaminobenzidine; 400x.

were moderately positive on the second day, and were losing this positivity after eight days, while macrophages expressed a high level of BCL-2 protein (Fig. 1).

In summary, apoptosis (based on BAX-positivity) was activated in cardiac muscle cells within the first day. Later, on day 8 it was activated in fibroblasts of the newly formed scar tissue. Antiapoptotic activity in cardiac muscle cells was moderate on day 1 and 2, on day 8 the strongest expression of BCL-2 was in macrophages dispersed in the forming scar tissue. For clarity, the results are summarized in the Table 1.

Discussion

BCL-2 and BAX are antiapoptotic and proapoptotic proteins, respectively, which represent useful immunohistochemical markers of apoptosis. This process of programmed cell death is necessary

for a proper embryonic development, tissue homeostasis, cellular turnover, but also contributes to the pathogenesis of various diseases, including diabetes mellitus (11), cancer (12), as well as myocardial infarction (13), to name but a few. Focusing on the lastly mentioned condition, it has been shown that apoptosis plays an important role in determining various outcomes of myocardial infarction including infarction size, cardiac remodelling and prognosis of future cardiac failure (14). Our results demonstrated that BAX positivity in cardiomyocytes was the strongest after 8 hours of the isoprenaline-induced myocardial infarction, indicating that apoptosis is a significant process leading to cellular death in the first stages of ischaemic damage of the affected heart muscle. Similarly, Zhou et al (15) described that a small amount of BAX protein expression could be detected in the ischemic myocardium of rats 6 hours after acute myocardial infarction. To expose the effects of BAX mutation under the conditions of surgically-induced myocardial infarction, Hochhauser et al (16) established a BAX gene (–/–) murine knockout model. The authors concluded that mice without a functioning BAX gene developed less serious ischaemic injury and also had better prospects related to post-infarction structural changes and function.

Apoptosis occurs not only as a reaction to the myocardial ischaemia, but also during reperfusion, contributing to reperfusion injury. With regard to myocytes expression of BCL-2 in our experiment, the most significant positivity was observed on the second day. This may indicate that in prolonged ischaemic conditions, there is a tendency to counteract the proapoptotic stimuli. BCL-2 was previously also studied after myocardial conditioning. Moreover, myocardium, which had been previously conditioned by slowly alternating ischaemia with reperfusion, leading to myocardial adaptation to ischemic conditions showed that the adaptation was cardioprotective by the means of BCL-2 upregulation (17).

Liao et al (18) authored an experimental study where they attempted to reduce the apoptosis in cardiomyocytes of rats after myocardial infarction using berberine – an active ingredient of Chinese medicinal plant *Coptis chinensis*. After its administration, the authors found out that berberine increased the expression of BCL-2 and decreased the expression of BAX what resulted in the inhibition of apoptosis in cardiomyocytes. These results showed that by influencing the pro- and antiapoptotic signals in cardiomyocytes, it is possible not only to decrease the severity of the infarction size, but also to influence future unfavourable remodelling of the heart muscle, potentially resulting in heart failure.

Apoptosis regulation in myocytes of myocardial infarction affected heart muscle had been reported in several experimental works (14–17). Less attention has been devoted to the apoptotic process in other cell types participating at the myocardial infarction lesion development. Apart from cardiomyocytes as a principal cell population of interest when studying myocardial infarction, fibroblasts are cells which should not be overlooked. They have the cardinal role in the repair and remodelling of the affected heart muscle (19). Our results showed that fibroblasts were largely positive for BCL-2 after two days of isoprenaline administration, indicating that fibroblasts were active and protected from apoptotic signals. On the other hand, positivity for proapoptotic BAX was strongly induced in fibroblasts of the forming scar after eight days of isoprenaline administration. These results are in line with the knowledge that during the process of scar tissue maturation, the number of fibroblasts decreases via different mechanisms, including apoptosis (20). A proper understanding of the dynamic processes occurring in fibroblasts during myocardial infarction, including the temporal aspects of their proliferation and apoptosis, is vital for fibroblast-targeting studies, which aim at alleviating the seriousness of myocardial changes following the infarction (21).

Conclusion

The present study confirmed that cellular death as the result of myocardial infarction occurs via different mechanisms, including apoptosis. The expression of BCL-2 and BAX visualised by immunohistochemistry revealed that apoptosis set in at the initial stages of myocardial infarction, indicating that this process is a prominent driving force of cell death from the onset of ischaemia. The exact role of programmed cell death during myocardial infarction is still not completely understood, so perhaps future research will fully elucidate its significance during ischaemic/reperfusion injury, resulting in more effective diagnostic and therapeutic strategies.

Learning points

- apoptosis is an important mechanism of cellular death during myocardial infarction
- BCL-2 and BAX are suitable immunohistochemical markers for studying apoptosis
- isoprenaline-induced animal model of myocardial infarction is appropriate for its study
- proper understanding of the role of apoptosis during myocardial infarction may lead to refined diagnostic and therapeutic strategies

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