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Original Article

Inhibition of apoptosis by the intrinsic but not the extrinsic apoptotic pathway in myocardial ischemia-reperfusion

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ABSTRACT

Summary: The detailed molecular mechanisms following activation of apoptosis in ischemia-reperfusion injury are unknown. This study using different transgenic mouse models provided first evidence that apoptosis in myocardial ischemia-reperfusion injury is rather linked to the mitochondrial pathway than to death receptor pathway.
Introduction: There is a wealth of evidence for activation of apoptosis in ischemia-reperfusion injury. However, the understanding of detailed molecular mechanism is lacking.
Methods: The extent of myocardial infarction after ligation of the left anterior descending artery in mice carrying different transgenes for inhibition of either the intrinsic or the extrinsic or a combination of both apoptotic cascades was evaluated. The extent of myocardial damage was assessed by echocardiographic determination of left ventricular (LV) ejection fraction, LV hemodynamics, troponin T, and histology. The rate of apoptosis was analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase-3 staining.
Results: Highest perioperative rate of death was observed in the dominant-negative form of a truncated Fas-associated death domain (FADD-DN) group. Infarction size by 2,3,5-triphenyltetrazolium chloride (TTC) staining was smaller in the Bcl-2, but not in the other groups as compared to wild-type mice. This was accompanied by lower troponin T values in Bcl-2 transgenic mice as compared to the all other groups. Troponin T correlated well with macroscopic extent of myocardial infarction by TTC staining. A lower decline of LV ejection fraction was seen in the Bcl-2 as compared to wild-type or FADD-DN mice. A smaller number of TUNEL- and caspase-3-positive myocyte nuclei were observed in the Bcl-2 and FADD-DN group as compared to wild-type mice.
Conclusions: We provide first evidence for protective effects on the myocardium in a transgenic mouse model of myocardial ischemia-reperfusion due to inhibition of the Bcl-2, but not the FADD pathway despite that reduced apoptotic cells were observed in both groups as compared to wild-type mice.

1. Introduction

In the past, increasing evidence has accumulated that apoptosis of cardiomyocytes contributes to the pathogenesis in several myocardial disease states [1]. This is especially true for ischemic heart disease where apoptotic cardiomyocytes have been shown in postmortem studies of human myocardial infarction, experimental myocardial infarction after coronary ligation, global ischemia in isolated perfused hearts, and myocardial hibernation [2–4]. For instance, molecular magnetic resonance imaging technique revealed large areas of apoptotic but viable myocardium in the mid-myocardium early after an ischemic injury [5]. Thus, myocardial salvage strategies after ischemic injury are of major clinical interest. Nevertheless, most of these studies did not provide evidence for the molecular apoptotic mechanisms necessary to obtain potential specific therapeutic approaches.

Molecular pathways of apoptosis involve either activation of the Fas-associated death domain (FADD) as a part of the “extrinsic” death receptor pathway on the cell surface or the “intrinsic” transition of mitochondria into a pro-apoptotic state. Induction of apoptosis by activation of the death receptor family, e.g., TNFR1, TRAIL, or Fas, by its specific ligands results in an activation of aspartate-specific proteases (caspases) finally executing cell death by selective proteolysis [1,6,7].

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Apoptosis initiated by a mitochondrion-dependent pathway results in the release of cytochrome *c* that is regulated by proteins of the Bcl-2 family. Pro-apoptotic Bcl-2-like proteins, such as Bax and Bak, initiate apoptosis by inducing the release of cytochrome *c* into the cytosolic compartment whereas apoptosis is inhibited by the anti-apoptotic regulatory protein Bcl-2 [7]. As activation of the extrinsic pathway induces the pro-apoptotic Bcl-2 family [7], interaction of both pathways may therefore potentiate anti-apoptotic effects in ischemia-reperfusion of cardiomyocytes.

This underlines the necessity for an interventional methodological approach to assess the significance of the diverse apoptotic pathways during myocardial ischemia and reperfusion. Thus, transgenic mouse models with different anti-apoptotic features under the control of the myocyte-specific α-myosin heavy chain promoter, either expressing the Bcl-2 gene or a dominant-negative form of a truncated FADD (FADD-DN), were used. The truncated part of 80 amino acids at the N-terminal represents the death effector domain that plays a key role in downstream signaling of death receptor pathway finally activating caspase-8. However, in the FADD-DN mice, the remaining truncated FADD binds to the receptors but is incapable to activate apoptosis [8]. A third mouse line is a cross-breeding of both models.

2. Methods

2.1. Transgenic mice

We used transgenic mouse lines that express either Bcl-2 or the FADD-DN transgene under the control of the murine α-myosin heavy chain promoter allowing for cardiac myocyte-specific expression of the transgene as previously characterized in detail [9]. Generation of transgenic mice (FVB/N strain) was performed according to routine protocols in the Transgenic Core Facility at the University of Heidelberg. The experimental protocols were approved by the Standing Committee on Animals of the Heidelberg Medical Area and German government agencies and were in accordance with accepted institutional policies.

2.2. Characterization of transgene expression

For Western blot analysis of tissue lysates, antibodies against Bcl-2 (1:1000; BD Bioscience Pharmingen, San Diego, CA, USA) and/or FADD-DN (1:2000, M2; Sigma-Aldrich, Seelze, Germany) were used. Bands were visualized with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Nümbrecht, Germany).

2.3. Coronary artery ligation

The investigation conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the authorities of the Regierungspräsidium Karlsruhe, Germany (AZ 35-9185.81/G-93/04). A transmural anterior myocardial infarction was produced in modification of a method previously described [10].

Five different groups of mice were studied: (1) wild-type mice without surgery (CON; $n=10$), (2) sham-operated (SH) wild-type mice (SH; $n=8$), (3) Bcl-2 mice after ligation of the left anterior descending (LAD) artery (Bcl-2; $n=11$), (4) FADD-DN mice after LAD ligation (FADD-DN; $n=16$), and (5) FADD-DNxBcl-2 mice after LAD ligation (FADD-DNxBcl-2; $n=8$).

Male mice 10–12 weeks of age were anesthetized by intraperitoneal injection of ketamine (120 mg/kg body weight) and xylazinehydrochlorid (16 mg/kg body weight). An oral endotracheal tube was inserted, and mechanical ventilation with room air was instituted (tidal volume, 200 μl; 100/min). A left-sided thoracotomy was performed, and the proximal LAD was ligated in situ for 40 min. Each myocardial infarction was confirmed by inspection immediately after LAD ligation (paleness and/or hypokinesia of left anterior ventricular myocardium). After this period of ischemia, ligation was removed and myocardium reperfused. Groups were studied 24 h after ligation. Mice appointed to the SH groups were subjected to the same procedure except LAD ligation.

3. Quantification of myocardial infarction size

3.1. Echocardiography

Transthoracic echocardiography (HDI 5000 Ultrasound; ATL, Phillips, Bothell, WA, USA) was performed after anesthesia by intraperitoneal injection of ketamine (70 mg/kg) and xylazine (3 mg/kg). In the two-dimensional mode in the parasternal short-axis view, the M-mode cursor was positioned perpendicular to the ventricular septum and left ventricular (LV) posterior wall. Digital images were analyzed by using an HDI Laboratory (ATL, Phillips, Bothell, WA, USA). M-mode measurements of LV dimensions were averaged from at least three cycles, using the leading edge-to-leading edge convention adopted by the American Society of Echocardiography. The investigator who conducted the echocardiography was blinded for the treatment status.

3.2. LV hemodynamics

Hemodynamic measurements were performed under continuation of anesthesia and mechanical ventilation as described above. The left ventricle was catheterized retrograde via the right carotid artery using a 1.4-F micro manometer catheter (Millar Instruments Inc., Houston, TX, USA). Data were recorded with a sampling rate of 1000 Hz using a standard data acquisition and analysis system (LabChart; AD Instruments, Colorado Springs, CO, USA). LV end diastolic pressure (LVEDP) and LV systolic pressure were calculated offline.

3.3. Histology

A 1-ml blood sample was taken before the entire heart was excised, separated, and weighed. LV was frozen in Tissue Tek® O.C.T. compound at −20°C until used for quantification of myocardial infarction size the next day. The hearts were cut into six transverse slices using a tissue chopper (Fig. 1). All tissues were incubated in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC, pH 7.4) for 20 min at 37°C. After 2 h of fixation in 10% formaldehyde, the slices were digitally photographed. The infarcted tissue stains a characteristic white color, whereas the viable tissue stains red. A planimeter (NIH image V1.63) was used to obtain the length of the entire endocardial circumference and that segment of the endocardial circumference made up by the infarcted portion from each of the slices of the LV.

3.4. Cardiac troponin T

Cardiac troponin T measurement was done using the 4th generation assay (Roche Diagnostics, Mannheim, Germany) on an ELECSYS 2010 automated analyzer with chemiluminescence technology. The lower limit of detection for the assay is 0.01 ng/ml. The inter-assay coefficients of variation were 20% at 0.015 μg/L, 10% at 0.03 μg/L, and 5% at 0.08 μg/L.

3.5. Quantification of apoptosis

3.5.1. Induction of apoptosis in ischemia-reperfusion by isolated perfused mouse hearts

A modified Langendorff technique was used for global ischemia-reperfusion in order to evaluate the extent of apoptotic cardiomyocytes.