Bcl-2 and Bax expression in adult rat hearts after coronary occlusion: age-associated differences

Lixin Liu, Gohar Azhar, Wei Gao, Xiaomin Zhang, and Jeanne Y. Wei

Division on Aging, Harvard Medical School and Gerontology Division, Beth Israel-Deaconess Medical Center, Boston, Massachusetts 02215

ABSTRACT

It has been reported that programmed cell death (apoptosis) occurs during myocardial infarction. The influence of age on programmed cell death or DNA fragmentation after coronary occlusion has not been extensively characterized. To test the hypothesis that there are age-related differences in susceptibility to DNA fragmentation during ischemia-infarction, we studied DNA fragmentation in young adult and old male F344 rat hearts after acute coronary artery occlusion. Hearts were studied at 1, 3, and 5 h after coronary ligation. The percentage of apoptotic cells was determined by the in situ end-labeling technique, and internucleosomal fragmentation (DNA laddering) pattern was also analyzed. Our results show that 1) DNA fragmentation began earlier and peaked earlier in the old compared with young adult hearts during infarction; 2) there was heightened expression of both Bcl-2 and Bax in the old hearts at baseline; and 3) the Bcl-2-to-Bax ratio was higher in the older heart after coronary ligation. These results suggest that, compared with the young adult heart, the aged heart may be more susceptible to ischemia-induced DNA fragmentation.

Methods

Anesthesia was induced by using ketamine hydrochloride (40 mg/kg ip) and xylazine (10 mg/kg ip). Endotracheal intubation was performed under direct visualization. Controlled ventilation with room air was provided by using a rodent ventilator (Harvard Apparatus model 638). The heart was exposed through a left intercostal thoracotomy when the animals were killed. The viable myocardium was identified by injection with 1% Evans blue dye into the aortic root. The appearance of cyanosis and bulging of the anterior left ventricle demonstrated successful coronary occlusion. A total of 80 animals were included in the study: 60 animals (30 young adult, 30 old) underwent LAD occlusion and 20 underwent sham operation. Animals were killed 1, 3, and 5 h and 7 days after coronary occlusion. Twenty (10 young adult, 10 old) sham-operated animals (with thoracotomy but without coronary ligation) served as controls.

Histology. After coronary occlusion, the experimental animals were killed at the time points listed above. Sham-operated controls (operated on, but ligature was not tied) were also killed at the same time points. The heart was quickly removed and washed in ice-cold PBS. Parts of the free
wall of the left ventricle including the infarcted and peri-
infarcted regions were fixed in 10% buffered neutral formala-
dehyde solution overnight at 4°C and then embedded in paraf-
fin. Sections were cut to a 5-µm thickness. The slides were
stained with hematoxylin and eosin. The sections of hearts
from animals that were killed at 7 days were also stained
with Masson trichrome.

Intrunucleosomal DNA fragmentation assay. Intrunucle-
osomal DNA fragmentation assay (DNA ladder pattern analy-
sis) was performed. Briefly, tissues were homogenized in 5 ml
of lysis buffer containing 10 mM Tris·HCl at pH 8.0 and 100
mM EDTA (TEA), SDS (0.5 ml), and RNase (Promega) and
incubated at 37°C for 60 min. A second incubation was
performed at 50°C for 3 h after the addition of protease K (100 µg/ml, Promega). The final incubation was completed in
1 M NaCl overnight at 4°C. The solution was then spun at
12,000 rpm for 20 min, and the supernatant was extracted
twice with phenol and chloroform-isopropanol (49:1). DNA
was precipitated in cold ethanol at −20°C. DNA (20 µg) was
then loaded onto 1.6% agarose gel containing 0.5 µg/ml
ethidium bromide, electrophoresed in 1 × TBE, and visual-
ized under ultraviolet illumination.

In situ identification of nuclear DNA fragmentation in rat
hearts. After being deparaffinized, the tissue sections were
stained with anti-Deoxyribonuclease-I (Promega) and the
tissue sections were stained by using the in situ terminal
deoxyribonuclease-I transfection-mediated Nick end-labeling (TUNEL) method with the
in situ ApopTag detection kit (Oncor, Gaithersburg, MD) to
identify cells showing nuclear DNA fragmentation. The pro-
dure is based on the method described by Schmitz et al. (28). Residues of digoxigenin nucleotide are catalytically added to
the DNA by terminal deoxyribonuclease-I transforan
server, an enzyme that catalyzed a template-independent addition of
deoxyribonuclease-I to triphosphate to the 3'-OH ends of double-
and single-stranded DNA. The anti-digoxigenin antibody frag-
ment carries either a fluorescein or peroxidase antibody to the
reaction site.

For negative controls of DNA fragmentation, sections were
stained without terminal deoxyribonuclease-I transference. The
sections of fluorescein-labeled tissue were also mounted
under glass cover slips with a drop of staining buffer contain-
ing propidium iodide-Antifade (1:4, Oncor) to visualize all the
nuclei (apoptotic and apoptotic). The sections stained for
DNA fragmentation labeled with peroxidase were used for
quantitative analysis. In a subset of six animals (3 adult, 3
old), double staining with mouse monoclonal anti-α-sarco-
meric actin antibody (1:500 for 30 min at room temperature)
was performed to identify the myocytes and to confirm that
the DNA fragmentation occurred in the cardiac myocyte
nuclei.

The percentage of ApopTag-labeled cells was determined
with a microscope containing an eyepiece grid (×200 magnifi-
cation). Ten microscopic fields per section were selected from
within the ischemic region and twenty fields per section from
the nonischemic region. In each field, 100 cells were counted in
the ischemic region, and 200 cells were counted in the
nonischemic region. The percentage of apoptotic cells was
calculated for the ischemic and the nonischemic regions. A
minimum of 5 slides per ischemic region and 25 slides per
nonischemic region were used. The results from three differ-
ent experiments were used to determine the means ± SD at
each time point for each age group.

Protein extraction. The heart tissues were washed in cold
PBS. They were then homogenized in lysis buffer (20 mM
HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM
EDTA, and 1.0 mM DTT) with protease inhibitors (2 µg each
of aprotinin, leupeptin, pepstatin A, and 0.5 mM phenylmeth-
ysulfonfyl fluoride), incubated on ice for 30 min, and centri-
fuged at 10,000 g at 4°C for 20 min. The supernatants were
stored at −70°C. Lysate protein was quantitated by using a
commercial assay (Bio-Rad) with BSA as a reference stan-
dard.

Western blotting. Proteins (30 µg/sample) were separated
by SDS-polyacrylamide gel (7.5–12%) under denaturing
conditions and electrotransferred onto nitrocellulose (Bio-
Rad) for 1 h at 100 V. The membranes were blocked with 5%
nonfat milk in Tris-buffered saline with 0.1% Tween 20
(TBST) overnight at 4°C. Primary antibodies were used in a
1:1,000 concentration in TBST with 5% nonfat milk for 2 h at
room temperature. The Bcl-2 monoclonal antibody was ob-
tained from PharMingen (San Diego, CA). The Bax antibody
was polyclonal and was obtained from Oncogene (Cambridge,
MA). Horseradish peroxidase-conjugated secondary antibo-
dies were added in a 1:2,000 concentration for 1 h at room
temperature. Films were developed by using the enhanced
chemiluminescence method (Amersham, Arlington Heights,
IL). β-Actin controls were used for all Western blots, and
densitometric results were adjusted accordingly.

Statistics. Data were collected from adult and old rats that were subjected to LAD ligation and killed at the
time points of 1, 3, and 5 h and 1 and 7 days after the
procedure. At each time point, six young adult and six old
animals were studied in the experimental group. There were
20 (10 young adult, 10 old) animals in the control group.
The percentage of cells with DNA fragmentation in the ischec-
mixed area was determined within each group. ANOVA
was performed across the time points for the two age groups.
For comparison at a single time point, the P value was
cauculated by use of the unpaired t-test. Bonferroni correction
was applied to multiple comparisons. The Mann-Whitney
nonparametric test was applied to infarct size for significance
testing. P ≤ 0.05 was considered to be significant.

The means ± SD of the densitometric results of the
proteins were used in a separate analysis. Mean control
densitometric values within each age group were normalized
to 1. Mean Bcl-2 and Bax normalized values for young and old
were calculated for each time point. These values were then
calculated to use a Bcl-2-to-Bax ratio for each age group.
The control Bcl-2-to-Bax ratio was normalized to 1. ANOVA
was applied to the protein ratios to assess age-related differences
over time.

RESULTS

As shown in Fig. 1, LAD ligation resulted in an
anterior wall infarction in the left ventricle. In all
hearts of the experimental groups, there were clear
histological features of recent ischemia and infarction,
including neutrophil infiltration, eosinophilia, coagula-
tion necrosis, and cellular edema. The estimated infarct
size at 7 days, as percentage of the left ventricular
myocardium, was 13.8 ± 3.7 in the young adult and
24.1 ± 2.3 in the old animals. The infarct size was
significantly larger in the old compared with the young
adult animals (P < 0.05).

At baseline, there was a very low level of DNA
fragmentation in the hearts of both young adult (Y) and
old (O) rats (Y = 1.9 ± 0.5 × 10−6; O = 4.1 ± 0.4 × 10−6,
P < 0.05), with the level being higher in the old hearts.
After LAD occlusion, the DNA fragmentation in the
nonischemic regions of the experimental hearts did not
change significantly over time and remained similar to
the baseline values within each age group (ANOVA: Y
nonischemic across time, P = 0.13; O nonischemic

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across, \( P = 0.14 \). The age-time interaction for the nonischemic controls was not significant (ANOVA: \( P = 0.39 \)).

In the ischemic regions of the experimental hearts, DNA fragmentation was demonstrated to be increased in both young adult and old hearts (Figs. 2 and 3). Within each age group, there was a significant effect over time (ANOVA: \( Y, P < 0.04; O, P < 0.01 \)). The age-time interaction was also significant (ANOVA: \( P < 0.001 \)). At 3 h (\( Y = 3.7 \pm 1.1; O = 7.8 \pm 0.8; P < 0.002 \)), the percentage of DNA fragmentation cells was higher in the old than in the young adult. In the old, it reached a peak at 3 h and remained elevated at 5 h (Figs. 2 and 3). In contrast to the old, the young adult heart sustained peak DNA fragmentation later, at 5 h (\( Y = 14.3 \pm 2.1; O = 8.0 \pm 3.2; P < 0.02 \)). The degree of DNA fragmentation in both age groups was much lower at 1 day and was close to baseline at 7 days. There was no significant age-associated difference in DNA fragmentation at 1 or 7 days.

A DNA laddering pattern characteristic of DNA fragmentation was distinctly visible in the infarcted tissue in both age groups from 3 to 24 h after LAD ligation (Fig. 4). It was interesting that the fluorescent in situ end-labeling staining of cells with DNA fragmentation was visible in the heart as early as 1 h after LAD ligation. This was also detectable with DNA electrophoresis as demonstrated by a visible DNA laddering pattern at 1 h. However, a more intense DNA ladder was seen at 3 and 5 h.

Quantitative analysis of DNA fragmentation was performed by using the TUNEL method at the single-cell level. As the DNA was not radiolabeled, the DNA electrophoresis was not used for quantitative analysis but only to confirm the characteristic DNA fragmentation pattern associated with the apoptotic process. The variation in the intensity of the DNA ladder appeared to correlate with the quantitative estimation of DNA fragmentation as determined with the TUNEL technique.

At baseline, both Bcl-2 and Bax proteins were higher in the old than in the young adult hearts (\( P < 0.05, \) Figs. 5 and 6). Within each age group, the effect across time on Bcl-2 levels was only significant in young adult (ANOVA: \( Y, P < 0.02; O, P < 0.09 \)). When the values at the various time points were normalized to the control values, Bcl-2 protein response to ischemia-infarction tended to be higher in the hearts of young adult compared with old rats after coronary occlusion (Fig. 7). Bax protein levels, however, were substantially lower in the old compared with young adult hearts during ischemia-infarction (\( P < 0.001 \), age-time interaction, repeated-measures ANOVA; Figs. 5–7). Within each age group, the effect across time on Bax protein levels was significant (ANOVA: \( Y, P < 0.05; O, P < 0.001 \)).

The ratio of Bcl-2 to Bax protein expression was determined at each time point by using values that were normalized to the control protein levels within each age group (Fig. 7). The normalized Bcl-2-to-Bax ratio was significantly higher after coronary occlusion in the old than in the young adult hearts (\( P < 0.02 \)).

**DISCUSSION**

There are three major findings in this study. First, DNA fragmentation occurs within the first several hours after coronary occlusion and apparently peaks earlier in the old than in the young adult heart. Second, the basal levels of Bcl-2 and Bax are higher in the old than in the young adult heart. Third, ischemic stress results in higher anti- to pro-apoptotic protein ratios in the old compared with young adult heart.

A recent study of autopsy specimens from the human hearts after death due to acute myocardial infarction included patients between 58 and 93 yr old, but there was no age comparison per se (10). The infarcts were also >12 h old at autopsy, so that the study probably missed the period of acute evolution of the infarct, which occurs within the first 6–7 h. Other studies of human hearts during acute myocardial infarction sug-
Fig. 2. Detection of DNA strand breaks in myocyte nuclei by biotinylated dUTP labeling of left ventricular free wall (magnification ×200). A: young adult, 3 h postligation; B: young adult, 3 h postligation, double-stained with propidium iodide (red); C: young adult, 3 h postligation, double-stained with α-sarcomeric actin antibody; D: old, 3 h postligation; E: old, 3 h postligation, double-stained with propidium iodide (red); F: old, 3 h postligation, double-stained with α-sarcomeric actin antibody; G: young adult, 5 h postligation; H: young adult, 5 h postligation, double-stained with propidium iodide (red); I: young adult, 5 h postligation, double-stained with α-sarcomeric actin antibody; J: old, 5 h postligation; K: old, 5 h postligation, double-stained with propidium iodide (red); L: old, 5 h postligation, double-stained with α-sarcomeric actin antibody.
suggest that apoptosis does occur and that it may provide a new target for designing cardioprotection during infarction (22, 24, 26, 27). One animal study investigated apoptosis vs. necrosis at different time points during infarction in adult rats and found that peak apoptosis occurred at around 4.5 h, whereas peak necrosis occurred at 24 h (14).

In the present study, the findings of DNA fragmentation being maximal in the young adult heart at 5 h are in agreement with that study (14). It is possible that transiently ischemic cardiomyocytes may undergo preferential apoptosis compared with cells that are supplied by completely occluded arteries, which tend to die some time later by necrosis (2, 5, 14, 27). These studies are noteworthy in that they demonstrate that not only can apoptosis occur extremely rapidly but that it may precede necrosis by several hours after coronary occlusion.

It is interesting that, in the present study, the percentage of apoptotic cells at 5 h was quantitatively greater in the young adult than in the old heart. One possible explanation for this finding might be that the hearts of old animals had higher apoptosis at baseline, a previously reported finding (15). In addition, it is likely that because the old heart is chronically exposed to some degree of ischemic stress at baseline, as reflected by higher levels of Bax protein at baseline, the cells in the older heart could have been preconditioned...
to undergo apoptosis earlier at 3 h (22). We were interested in focusing our study on apoptosis in young adult and old hearts because these cells may be potentially salvageable through targeted intervention. Moreover, the ratio of cells with apoptosis to necrosis after infarction has been well studied by others (14, 15).

In the present study, we were able to confirm the early onset of DNA fragmentation after acute LAD occlusion (2, 5, 14, 27). Our findings also demonstrated a highly significant age difference in the time course of DNA fragmentation after acute myocardial infarction. This may have potential bearing on the notion of the period of optimal clinical intervention in elderly patients with acute myocardial infarction. It is important to keep in mind the fact that other stimuli can also induce an apoptotic window, although on a different time frame. These stimuli include pressure overload in myocardial infarction (31) as well as hypertensive heart disease (8). It is therefore quite possible that a myocardial infarction would have a different effect on the DNA fragmentation process as well as the expression of pro- and anti-apoptotic genes in the hearts of older persons or animals due to the concomitant presence of atherosclerotic and/or hypertensive or other heart diseases.

The finding that peak DNA fragmentation occurred earlier in the old heart signifies perhaps a greater susceptibility to acute ischemic stress with advancing age. Other work in our laboratory involving the study of acute stress due to hypoxia-reoxygenation on the brain has shown that, compared with the brain of the young adult, the brain of the old animal is also more susceptible to DNA fragmentation after hypoxia-reoxygenation (18).

It is possible that increased protein expression of Bcl-2, an inhibitor of apoptosis, or decreased expression of Bax, an inducer of apoptosis, in the heart during ischemia or hypoxic injury could serve to reduce the extent of DNA fragmentation (13, 22). In the present study, both Bcl-2 and Bax protein levels at baseline were higher in the heart of the old compared with the young adult rat, perhaps indicative of a greater level of chronic stress in the old. This might explain the lower level of peak DNA fragmentation that occurred earlier in the older hearts. Despite a heightened stress response, the older hearts underwent earlier peak DNA fragmentation than that in the young adult. It is possible that a higher basal level of pro-apoptotic proteins such as Bax in the old heart might indicate prior ischemic stress and it might also have set the stage for earlier DNA fragmentation after cellular injury. It is also possible that the pro-apoptotic stimuli of increased Bax protein expression at baseline in the old heart could lead to a parallel increase in basal expression in the anti-apoptotic protein Bcl-2 (4).

In an effort to better understand the basis of the age differences in Bcl-2 and Bax expression, we sought to isolate the myocytes from the hearts of the young adult and old rats. However, with each isolation only a very few cardiomyocytes from the old hearts survived, and they were from the nonischemic areas of the heart. Therefore a comparison using isolated myocytes from young adult vs. old hearts was not done. Nevertheless, we believe that our findings of age-associated differences using
whole heart tissues from ischemic-peri-infarct regions of young adult vs. old hearts do make a contribution to the literature and are reflective of the pathophysiology of acute myocardial infarction in vivo.

It is known that the absolute levels of pro- or anti-apoptotic proteins do not necessarily correlate with cell death or survival. Rather, it is the ratio of pro- to anti-apoptotic proteins in a cell that probably determines its fate (22, 27). It is interesting that in the old rat heart the level of Bax expression is so significantly suppressed at 3 h, whereas Bcl-2 rises above basal levels at that time. The reasons for this are not entirely clear but may involve the production of other anti-apoptotic factors that may repress Bax expression. Future studies will help to elucidate these facts.

It was interesting that the ratio of pro- to anti-apoptotic proteins is clearly in favor of Bcl-2 in the old hearts at 3 h. The fact that DNA fragmentation in the old hearts appeared to peak at 3 h when the Bcl-2-to-Bax ratio is also maximal, can be explained by the fact that a lag time exists between the ratio of Bcl-2 to Bax expression and DNA fragmentation. The DNA fragmentation that was measured is indicative of cumulative DNA fragmentation between 1 and 3 h, and the process of DNA fragmentation usually occurs later than that of Bcl-2 and Bax expression. Furthermore, it is quite possible that the reason the DNA fragmentation peaked at 3 h and did not rise further at 5 h in the old heart was because of the elevated Bcl-2-to-Bax ratio which peaked at 3 h and thereby served to limit the DNA fragmentation in the old hearts.

There are several limitations in this experiment. The focus of the present study was to evaluate in vivo age-associated changes in whole heart tissue in response to acute ischemic stress. We did not study the cell type-specific responses. Further efforts to define how age differences in myocardial response are reflective of changes in myocyte vs. fibroblast activity would be of interest. It is possible that a different ischemic stress threshold might exist for the different cell types in the heart, with cardiomyocytes being more sensitive than fibroblasts. However, demonstration of this difference will require separate in vitro and in vivo studies. It should be noted also that the in situ end-labeling method of DNA labeling has its limitations and preferentially detects double-strand DNA breaks in cells undergoing apoptosis. It is possible that a percentage of single-strand DNA breaks in preapoptotic cells were missed. The present study was focused on the protein expression of Bcl-2 and Bax because of the tight association and ratio that these two proteins have in mediating cellular apoptosis. A number of other proteins that are also important in cellular apoptosis, including p53, p21WAF, and c-Myc, were not characterized in this study (1, 6, 12, 19). Future studies to pursue these other proteins would probably be informative. Further work to evaluate possible age-related differences in neutrophilic response to injury will also be of interest (3, 7, 20, 23, 25).

Perspectives

The underlying reasons for a greater degree of cellular stress in the old heart are likely to be multifactorial and will require detailed studies. It is conceivable, however, that one might be able to potentially alter the ratio of pro- and anti-apoptotic proteins to thereby increase the resilience of the older heart during ischemic stress. The molecular mechanisms of the differential rate of DNA fragmentation between the young adult and old hearts merit further investigation. Needless to say, an understanding of these mechanisms will probably help in the design of future therapies intended to reduce the consistently higher morbidity and mortality associated with acute myocardial ischemia-infarction in the elderly population.

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Address for reprint requests: J. Y. Wei, Gerontology Div., Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, MA 02215.

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