Coronary Reperfusion Following Ischemia
Different Expression of Bcl-2 and Bax Proteins,
and Cardiomyocyte Apoptosis
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SUMMARY
The aim of this work was to examine factors that could be involved in the occurrence of apoptosis in rat hearts subjected to coronary occlusion followed by reperfusion. To this end, we studied the expression of the pro- and anti-apoptotic factors, bax and bcl-2, respectively, in reperfused ischemic hearts and in hearts injected with bFGF or saline. In anesthetized rats the left coronary artery was occluded for 45 min, the anesthesia withdrawn and the occlusion removed to allow reperfusion; in sham-operated rats the occlusion was omitted. After 4 hours the rats were decapitated and the heart excised. Sections from the left ventricle were stained with anti-bcl-2-antibody and anti-bax-antibody using the TUNEL method which detects apoptosis. Fragmentation of DNA isolated from reperfused ventricles was examined by agarose electrophoresis. In reperfused hearts no bcl-2 staining was observed in the discrete area in which many cardiomyocyte nuclei were stained by the TUNEL method; outside this area staining for bcl-2 was more marked than in sham-operated rats. Sections from reperfused hearts were stained for bax protein over a wide area including the apoptotic region; sham-operated hearts showed little reaction. Staining for bcl-2 was demonstrable in some nuclei in hearts from saline-injected rats; the numbers were unaffected by i. v. bFGF. Ischemia/reperfusion increases the overall expression of both bcl-2 and bax proteins, but bcl-2 is lost from the reperfused area as indicated by TUNEL staining. Accordingly, the ratio of bcl-2 to bax was reduced in the reperfused area, indicating a pro-apoptotic trend. The marked increase in bcl-2 outside the reperfused area could be a mechanism with which to salvage surviving cardiomyocytes. (Jpn Heart J 2001; 42: 759-770)

Key words: Bcl-2, Bax, Protein, Apoptosis, Ischemia/reperfusion, Immunohistochemistry

APOPTOSIS of cardiomyocytes is induced by reperfusion after transient ischemia in rabbit and rat heart.¹,²) This active gene-directed process of cell sui—
cide is controlled by pro-apoptotic and anti-apoptotic mediators. In a recent study, upregulation of the pro-apoptotic protein p53, which regulates transcription of anti-apoptotic bcl-2 and pro-apoptotic bax, was observed in rat ventricular tissue exposed to reperfusion after ischemia.

Bcl-2 protein is a cytosolic protein with a lipid-anchoring domain which allows it to target the nucleus and to inhibit apoptosis. While Bcl-2 is expressed in fetal heart, its presence in normal adult myocardium is controversial. It could not be detected even in so-called 'hibernating' human and goat hearts. The expression of bcl-2 is markedly reduced in cardiac tissues by apoptosis-inducing stresses. These include coronary reperfusion after ischemia in isolated rat heart, and anoxia-reoxygenation or angiotensin II treatment of cultured endothelial cells from coronary blood vessels and of cardiomyocytes. In isolated rat heart global ischemia followed by reperfusion caused a reduction in bcl-2 mRNA. On the other hand, bcl-2 is expressed in cytoplasm of cardiomyocytes within the area at risk in coronary-occluded rat hearts and in salvaged cardiomyocytes in infarcted and failing human hearts. There is, however, conflicting report which found that in the rabbit heart bcl-2 expression was not observed in cardiomyocytes around the infarcted area, being detectable only in the intramyocardial arteriolar walls; furthermore it was unaffected by reperfusion after ischemia.

Bax, a member of the bcl-family, homodimerizes and forms heterodimers with bcl-2 protein, reducing its anti-apoptotic effect. Overexpression of bax accelerates apoptotic death. Misao, et al reported that in human hearts bax was overexpressed in salvaged myocytes within the risk area and in long-standing myocardial infarcts. In contrast, Kajstura, et al found that expression of myocardial bax protein was high in sham-operated rat hearts and was unaffected by myocardial infarction.

Thus, the available data on expression of bcl-2 and bax proteins in reperfused hearts, and their relation to apoptosis of cardiomyocytes, seem equivocal: a further study of bcl-2 expression in cardiomyocytes is clearly important. It could, moreover, be of wider significance in view of the observation that in tumor cells and autoimmune-intolerable-lymphocytes, the over-expression of bcl-2 reduces apoptosis leading to the development of fatal diseases.

In the present work we used immunohistochemistry to study the expression of bcl-2 and bax in the area of cardiac tissue exposed to reperfusion after a transient ischemia, and in the adjacent normally perfused tissue. Tissue from sham-operated hearts was also examined. In addition, the possible relation between basic fibroblast growth factor (bFGF) and bcl-2 expression was investigated in another set of experiments.
MATERIALS AND METHODS

The experiments were all done on 8 week-old male Wistar rats. All procedures conformed with the guide for the care and use of laboratory animals published by the School of Medicine, Hokkaido University.

In the bax and bcl-2 study, 14 rats were subjected to ischemia/reperfusion and 5 to sham operation. In the DNA study, 3 rats were subjected to each procedure.

As described previously,\textsuperscript{5,16-19} in the ischemia/reperfusion study, rats were anesthetized with ethyl ether, artificially ventilated through a tracheal cannula and thoracotomized, and the left coronary artery was occluded with a ligature for 45 minutes. The ligature was then removed to allow reperfusion, the chest closed, the tracheal cannula removed, and the anesthesia withdrawn. The animals breathed room air spontaneously during the 4 hour reperfusion period. The sham-operated rats were treated identically except that the coronary ligature was not tied. At the end of the reperfusion period the rats were rapidly decapitated with a guillotine. Hearts were quickly excised, fixed with 4\% paraformaldehyde and embedded in paraffin. Sections from ventricular tissue were stained for bcl-2 and bax immunohistochemically, and apoptosis was detected by in situ nick end-labeling (TUNEL). Methyl green or hematoxylin was used as a counterstain for nuclei.

To demonstrate bcl-2 and bax proteins, tissue sections (4 \( \mu \)m thick) mounted on silane-coated glass slides were deparaffinized in xylene. After rehydration the slides were incubated in 0.3\% \( \mathrm{H}_2\mathrm{O}_2 \) in methanol for 15 minutes at room temperature to quench endogenous peroxidase. Non-specific immunoglobulin binding sites were blocked with normal goat serum. The primary antibodies, rabbit anti-rat/mouse bcl-2 antibody (Pharmigen, \# 15616E, San Diego, CA, USA) and rabbit anti-rat/mouse bax antibody (PM-13686E) were diluted to 1:100 and 1:500, respectively, and applied to the sections which were incubated overnight at 4\(^\circ\)C.

The second antibody, biotinylated goat anti-rabbit IgG antibody, was then applied and the sections incubated for 30 minutes at 37\(^\circ\)C. Subsequently sections were washed and incubated with peroxidase-conjugated streptavidin for 30 minutes at room temperature. The color was developed by a 10 minute incubation in a solution containing 3,3’-diaminobenzidine. Structures showing stained deposits were taken to be bcl-2 sensitive nuclei when their contour was filled with the deposits. Some nuclei whose contour was only partially filled were not considered to be bcl-2 sensitive, unless otherwise noted. The structures were elongated in the longitudinal sections and round in the transverse cross sections.
To study the relation between bFGF and bcl-2 expression, bFGF (PeproTech, Inc., Rocky Hill, NJ, USA) diluted in saline was injected into the tail vein of 8 rats at the dose of 16 µg/kg BW in a volume of 300 µl. A further 8 rats injected with saline alone served as controls. Rats were killed at different times after injection. Sections from left ventricles were immunohistochemically stained for bcl-2 as described above, except that the primary bcl-2 antibody was Pharmigen, #13456E used at a dilution of 1:1000. A modified Gomori-trichrome method was used to show mitochondria in sections adjacent to those stained for bcl-2.

Each section was photographed with an Olympus microscope camera with a ×4, ×40 or oil immersion ×100 objective lens. The discrete area where TUNEL-stained nuclei were distributed, and the whole cross-sectional area of the left ventricle, was measured on the micrographs by planimetry. TUNEL-and methyl green-stained nuclei were counted to provide nuclear percentages in five randomly selected visual fields of either the inner or middle muscle layer.

For analysis of DNA fragmentation by agar gel electrophoresis, three additional rats were subjected to ischemia/reperfusion and three more to the sham-operation. The upper half of the left ventricular free wall was used for electrophoresis according to the conventional method with a slight modification as described previously.\(^5\)

**RESULTS**

Low magnification micrographs in Figure 1 provide an overview of the distribution of stained cells and/or nuclei in serial cross-sections of ventricular tissue from one rat. Section A shows that TUNEL-stained nuclei were confined to the discrete area indicated by the filled arrow. This area constituted 18±3 % (mean ±SD) of the total area of the left ventricular section and will be referred to as reperfused area. In the normally perfused tissue outside the reperfused area almost no nuclei were stained by the TUNEL method.

Staining for bcl-2 protein is shown in Figure 1 B. It is noteworthy that staining was weak within the reperfused area (arrow), where TUNEL-stained nuclei were found. In sharp contrast, staining for bcl-2 was marked in the normally perfused wide area where TUNEL staining was absent. Section C shows staining for bax both within and outside the reperfused area.

Figure 2 shows the distribution of bcl-2 at a higher magnification. In the section from a sham-operated heart (Figure 2 A) some weakly stained elements are present but the cytoplasm is unstained. Figure 2 B shows a section taken from the normally perfused area surrounding the reperfused area: Many elements are strongly stained, in addition, there is a marked reaction in the cytoplasm. Disre-
Regarding the strength of the stain, the number of stained elements per mm² in the normally perfused area was 226±41 vs 127±12 in sham-operated heart (mean±SD, P<0.02). In contrast, Figure 2C shows that in the reperfused area none of the elements were stained, nor was there any reaction in the cytoplasm.

Practically no bax staining was found in sham-operated hearts even at high power (Figure 3A). Reperfusion caused an increase in staining throughout the left ventricular wall. Figure 1 C shows that bax staining was detectable in the reperfused area (arrow); the clear staining of the cytoplasm is more obvious in the high

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**Figure 1.** Low power micrographs of adjacent sections from ventricular tissue stained for nick-end labeling (TUNEL) for DNA breaks (A), for bel-2 (B) and bax (C) proteins. Arrows indicate the discrete area of TUNEL-staining in A, the lack of bel-2 staining in the corresponding area in B, and the expression of bax in the same area in C. Scale bar: 500 µm.

**Figure 2.** Micrographs of bel-2-staining from sham-operated heart (A), normally perfused area (B) and reperfused area (C). B and C were obtained from the relevant areas in Figure 1B. Scale bar: 50 µm.
power view shown in Figure 3B. Bax staining was also found over the normally perfused area (micrograph not shown).

Very few nuclei were stained by the TUNEL method either in the normally perfused area (Figure 4A) or in sham-operated hearts (micrograph not shown). As Figure 4 B shows, a number of elements were markedly stained by the TUNEL method in the reperfused area, suggesting the induction of apoptosis. The number of TUNEL-sensitive nuclei calculated as a proportion of total nuclei was markedly higher in the reperfused area than in the normally perfused area (45±4 % vs 2±2%, mean±SD; p<0.01). DNA obtained from the reperfused area exhibited a ladder formation on agarose electrophoresis (Figure 5).

Apoptotic changes in cardiomyocyte nuclei were confirmed by microscopy which revealed condensation of chromatin into V-shaped (Figure 6a) or round (Figure 6b) bodies stained by the TUNEL method. A second nucleus in Figure 6b is weakly stained but shows no clear chromatin condensation.
A comparison of sections processed for bcl-2, for nuclei and mitochondria suggests that the bcl-2 stained elongated or round elements are nuclei while mitochondria appear as small bcl-2-sensitive granules. Corresponding areas under a ×100 objective lens in stained sections are shown in Figure 7. Bcl-2 is expressed clearly in nuclei and weakly in mitochondria in cardiomyocytes that have not been subjected to surgical procedures. However, left ventricular tissue of unoperated rats injected with bFGF showed no detectable increase in bcl-2 stainable
elements at 1, 2, 3 or 5 hours compared with unoperated rats injected with saline (Figure 8).

**Figure 7.** Micrographs of representative cross-sectioned areas from sections of ventricular tissue from saline-injected rats, stained for bcl-2 and counter stained with hematoxylin (A) and for mitochondria by the modified Gomori-trichrome method (B). Light brown stained elements (some of them are marked with thin arrows) in A seem to correspond qualitatively to violet or dark red stained mitochondria in B. Thick arrows mark nuclei. Scale bar: 10 µm.

**Figure 8.** Density of nuclei whose clear contour was filled with bcl-2-stained deposits in ventricular tissue from bFGF- and saline-injected rats. Injections were made under light anesthesia using ethylether except one rat (*) which received saline injection without any anesthesia.
DISCUSSION

The observed localization of bcl-2 protein in nuclei of cardiomyocytes is in accordance with its function of repairing damaged DNA; work with a retroviral vector has shown that it is distributed mainly on the nuclear membrane.\textsuperscript{20,21} The stain of mitochondria is consistent with its residence in the outer mitochondrial membrane.\textsuperscript{21} The staining of several nuclei in sham-operated rats may be due to manipulation of the heart during the passage of the thread round the coronary artery and its presence there for over 4 hours.

An interesting finding in the present study on the effects of reperfusion is the existence of a discrete area where bcl-2 was virtually absent, while there was marked staining in the surrounding area. This marked expression of bcl-2 outside the unstained area has not previously been observed in an ischemia/reperfusion model, but seems to be partially consistent with the reported distribution of bcl-2 in salvaged cardiomyocytes in chronic ischemia.\textsuperscript{11,12} Thus, it appears that while a small amount of bcl-2 is normally expressed in cardiomyocytes, this expression is greatly enhanced when a stress is applied to the heart.

The cause of this bcl-2 upregulation remains unknown but certain observations may be relevant. First, it is known that bcl-2 is upregulated by bFGF in 24-48 hours.\textsuperscript{22,24} Second, we have shown that there is widespread expression of bFGF throughout the entire ventricular wall within a 2 hour period of reperfusion following 3 minutes of ischemia.\textsuperscript{16} Further, the addition of bFGF (4 ng/ml) to the medium caused an increase in bcl-2 in cultured vascular endothelial cells.\textsuperscript{24} The results suggest that bFGF might play a role in the increase in bcl-2 protein in ventricular tissue in response to ischemia/reperfusion. Hence we designed experiments to test whether injected bFGF could stimulate bcl-2 expression within 5 hours, a time period similar to the present reperfusion period. Since bFGF affects gene expression through a paracrine pathway and internization, it takes a relatively long time to stimulate cultured endothelial cells to increase bcl-2 protein expression. We examined the bcl-2 staining at 5 hours after bFGF injection with an expectation that bFGF may exert its positive effects more quickly in living rats than in cultured cells. The failure of relatively high intravenous doses of bFGF to induce a significant increase in the bcl-2 expression over 1 to 5 hours does not necessarily rule out a relationship: i.v. bFGF may not have reached the target.

It may be relevant that bcl-2 mRNA is increased by preconditioning.\textsuperscript{7} During left coronary occlusion the normally perfused area is subjected to an abnormal contractile load, producing an excessive stretch and oxygen debt. This might be equivalent to preconditioning and result in a subsequent increase in bcl-2 expression, when the occlusion is removed.

Transient coronary occlusion increased bax expression in both reperfused
and normally perfused areas. Bax was consistently increased in the discrete area where bcl-2 was virtually absent. It is possible that p53 induced by the transient ischemia somehow switches off bcl-2 expression and switches on bax expression within the discrete area. The upregulation of bax expression is in agreement with previous results from a model of continuous occlusion in rats.\(^25\) The reason for bax expression in the surrounding area is unknown.

No quantitative measurements were made in the present study, but the histological results suggest that the ratio of bcl-2 to bax was reduced in the reperfused area; this would accelerate the apoptosis of cardiomyocytes.\(^25\) Outside this area both bcl-2 and bax were increased, hence the ratio of anti-apoptotic to pro-apoptotic proteins may not be altered. This could account for the lack of TUNEL-staining in the normally perfused area. Thus, the increase in bcl-2 protein in the normally perfused area seems to be a biological response to protect cardiomyocytes from apoptosis that might result from the overload imposed on normal tissue during coronary occlusion.

The present findings appear to partially contradict some previous studies. Yue, et al\(^{13}\) found no significant evidence of upregulation of bcl-2 in normally perfused areas of rabbit hearts subjected to reperfusion after ischemia. It is possible that there could be a species difference in the response of cardiac tissue to ischemia. Maulik, et al\(^{7}\) reported a marked decrease in bcl-2 mRNA in a global ischemia/reperfusion model of isolated rat hearts mounted in a Langendorff apparatus and perfused with oxygenated modified Krebs-Henseleit physiological solution at 37°C. After the preparation had stabilized the perfusion was stopped for 30 minutes; this was followed by a 3 hour period of reperfusion. The interruption in perfusion resulted in global ischemia of the cardiac tissues. This global ischemia/reperfusion model seems unlikely to reveal local differences in the expression of bcl-2. Maulik, et al\(^{7}\) did not observe any alteration in bax expression in rat hearts subjected to chronic ischemia; it seems reasonable to suggest that chronic ischemia, and reperfusion after ischemia, may affect bax expression differently.

In conclusion, the stress caused by ischemia/reperfusion produced an upregulation of bcl-2 protein in the normally perfused area outside the discrete area of reperfusion. The localized reduction in bcl-2 protein and the increase in bax protein, probably resulting from the upregulation of p53, together with the expression of Fas\(^{19}\) protein, seem likely to contribute to the process of apoptosis in reperfused cardiac tissues.
REFERENCES

