Ischemia-reperfusion induced apoptosis and p53 expression in the course of rat heterotopic heart transplantation

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BRIEF COMMUNICATION

Ischemia–Reperfusion-Induced Apoptosis and p53 Expression in the Course of Rat Heterotopic Heart Transplantation

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INTRODUCTION

It is well accepted that the burst of free radical production associated with reperfusion of previously ischemic tissues is an important mediator of tissue damage (Grace, 1994). Although the cell loss in the postischemic period has been classically considered to occur via necrosis (Formigli *et al.*, 1992), it is becoming apparent that oxidative stress may also induce apoptosis (Sandstrom *et al.*, 1994). This type of cell death depends upon the activation of a peculiar genetic program of cell suicide and the specific endonucleosomal digestion of DNA (Arends *et al.*, 1990). Contrary to necrosis, apoptosis is a short-lasting phenomenon and does not induce an inflammatory response and subsequent damage to the adjacent cells. In the present study, we used a novel model of heterotopic rat heart transplantation to examine whether apoptosis could contribute to the myocardial damage after ischemia and reperfusion. Since a universal marker of apoptosis is lacking, we used *in situ* end labeling of DNA breaks in combination with ultrastructural analysis for the identification of the typical morphological features of apoptosis. Altered p53 gene expression was also evaluated in the ischemic and reperfused (I-R) myocardium to test the necessity of this protein for the induction of the apoptotic process during myocardial reperfusion syndrome.

MATERIALS AND METHODS

Surgical protocol. For heterotopic heart transplantation, 15 Wistar rats were randomly divided into three groups: control ($n = 5$), donor ($n = 5$), and recipient ($n = 5$). The animals received humane care according to the requirements of the Italian health System. As previously reported (Perna *et al.*, 1996), the hearts from the donor anesthetized animals were excised through a bilateral thoracotomy, arrested with St. Thomas solution, and stored in cold saline (4°C) solution for 2 h before reimplantation into the recipient animals. Segments of the aorta and inferior cava of the recipient animals were then exposed at the renal level by sharp dissection, separately clamped, and

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opened. The explanted hearts were reimplanted by terminolateral aortoaortic anastomoses and terminolateral pulmonary–cava anastomoses. After the anastomoses were completed, the hearts were reperfused for 30 min and then excised. Control hearts were rapidly excised from animals killed by cervical dislocation. The effects of ischemia were not considered, since previous observations of ours (Perna *et al.*, 1996) have shown that the techniques used to preserve hearts before reimplantation are able, at least for a short period, to protect the myocardium from ischemic injury.

In situ determination of apoptosis. To detect DNA fragmentation, *in situ* nick end labeling (ISEL) was performed on paraffin-embedded tissue sections from samples of the left ventricle anterior wall of control and I-R myocardium. According to Wijsman *et al.*, (1993), the sections were incubated with the Klenow labeling reaction mixture containing the Klenow fragment of DNA polymerase I and biotinylated deoxynucleotides (Klenow–FragEL DNA Fragmentation Detection Kit; Calbiochem, Cambridge) and processed following the manufacturer's instructions.

Quantitative and statistical analysis. An average of 100 random microscopic fields (3 for each tissue section) of 138,000 μm^2 each were examined in the control and I-R samples at 250× in order to determine the number of unlabeled and *in situ*-labeled myocyte and nonmyocyte nuclei. To avoid nonspecific DNA labeling, only the dark-stained nuclei were counted. The results of the analysis were indicated as mean and standard error values of total and apoptotic nuclei. Differences were statistically analyzed using the Student *t* test for unpaired values, with one tail. $P < 0.001$ was accepted as significant.

Transmission electron microscopy (TEM). Myocardial biopsies from the left ventricular wall of the control and I-R hearts were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, and routinely processed for transmission electron microscopic observation.

Immunohistochemical detection of p53. Myocardial sections obtained by paraffin-embedded specimens of the control and I-R myocardium were deparaffinized, hydrated, and heated by microwaving for 10 min before incubation with DO7 anti-human p53 monoclonal antibody (DAKO, Carpinteria, CA) for 1 h at room temperature. The reaction was revealed using the streptavidin–biotin alkaline phosphatase technique (DAKO, LSAB kit). Negative controls were obtained by substituting the primary MoAb with nonimmune mouse serum.

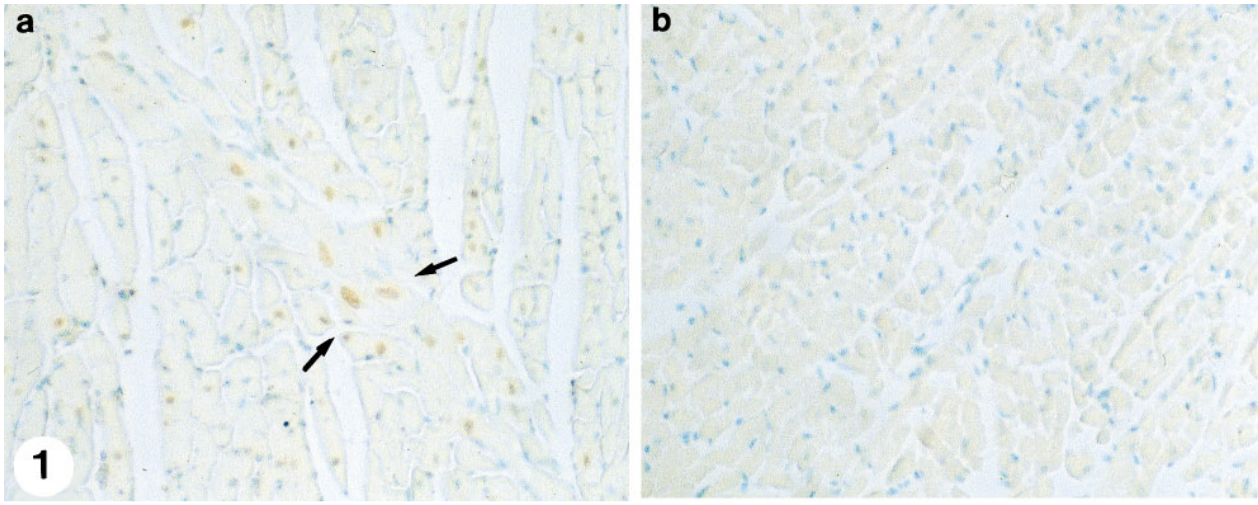
Western blotting analysis. Myocardial bioptic specimens from the left ventricle of control and I-R animals were homogenized in modified RIPA buffer, sonicated twice, and assayed for protein by Bradford's method (Bradford, 1976). To verify the expression of p53 protein, equal amounts of protein extracts (100 μg) were size-fractionated by electrophoresis on 10% SDS–polyacrylamide gel and electroblotted to nitrocellulose membrane. The membrane was then incubated at 4°C overnight with MoAb against p53 (Pab 421) (Calbiochem Corp. San Diego, CA). The immunodetection of p53–Ab complexes was carried out with peroxidase-conjugated anti-mouse IgG secondary antibody (Sigma, Ltd.) diluted 1:10,000 for 1 h. The

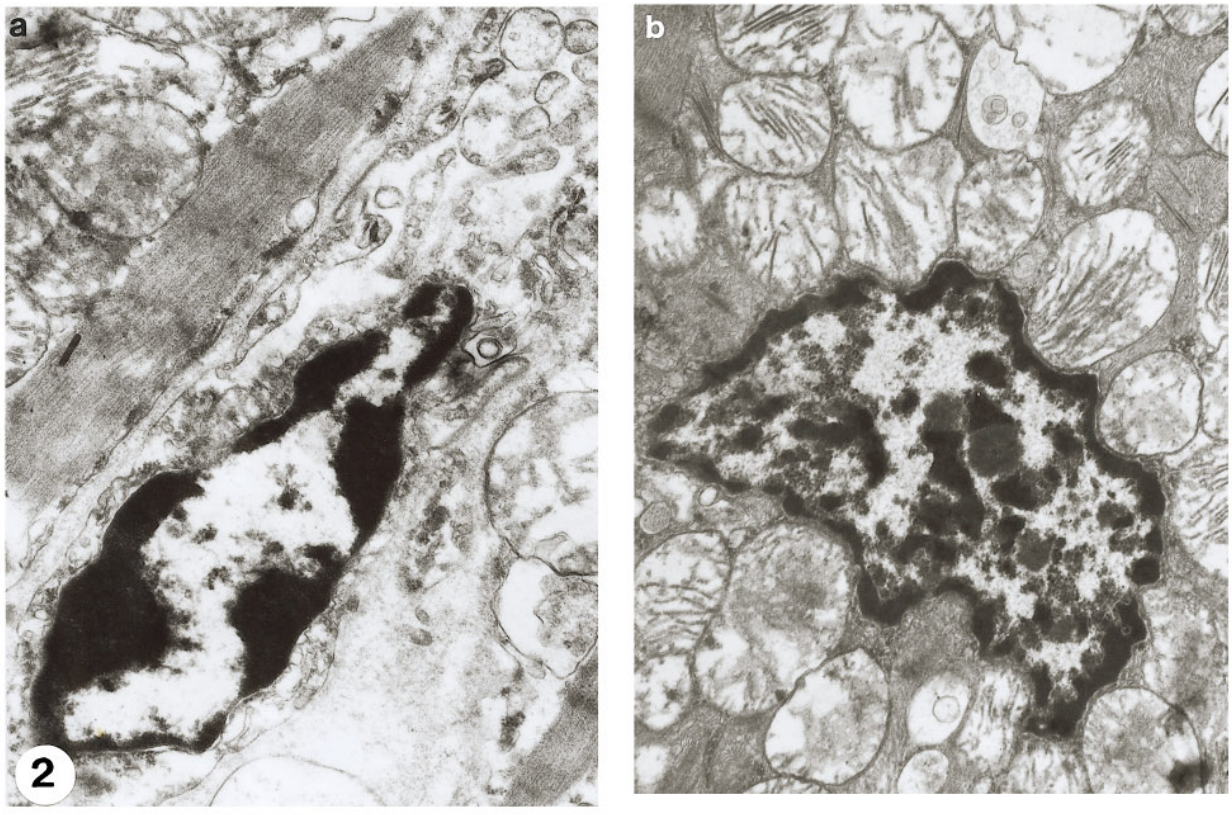
FIG. 1. Detection of DNA fragmentation of I-R (a) and control (b) myocardium as determined by ISEL assay. (a) Numerous nuclei confined to a region of the I-R myocardium show the typical nuclear positive staining (arrows) (LM × 280). (b) No appreciable nuclear staining is visible in the control myocardium (LM ×280).

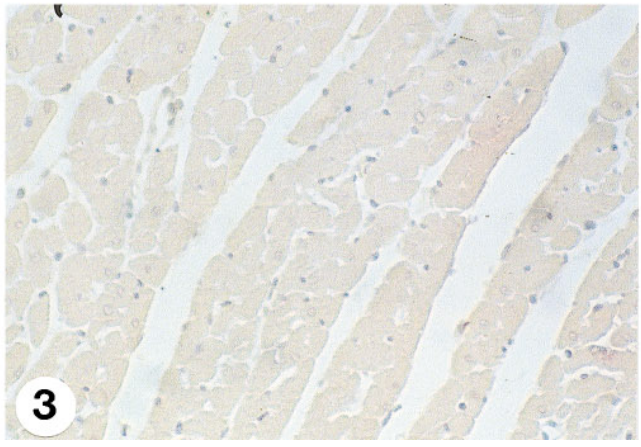
FIG. 2. Transmission electron microscopy of I-R myocardium. (a) Formation of crescent caps of chromatin against the nuclear envelope is visible in the nucleus of an endothelial cell (×17,600). (b) Variable density chromatin condensations are shown within the nucleoplasm of a myocyte nucleus (×14,500).

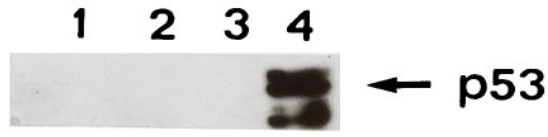
FIG. 3. Alkaline-phosphatase staining of I-R myocardium with MoAb against p53 protein. No specific reaction is visible within the tissue (LM ×280).

FIG. 4. Western immunoblot analysis of p53 protein expression in control and I-R rat myocardium. 100 μg of total protein extracts from each sample was size-fractionated by electrophoresis and electroblotted to nitrocellulose membrane. Lanes 1 and 2, I-R myocardium; lane 3, normal myocardium; lane 4, A431 carcinoma cell line (30 μg of total lysate), which overexpresses mutant p53, was used as positive control.









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blot was incubated with ECL detection reagent (Amersham Buchler, Ltd.) for 1 min and exposed to BIOMAX Light-1 film (Kodak, Inc.).

RESULTS AND DISCUSSION

In accordance with previous findings (Gottlieb *et al.*, 1994; Colucci, 1996), we have demonstrated that apoptosis may represent a critical event in the pathogenesis of myocardial ischemia–reperfusion syndrome. In fact, by ISEL staining a consistent DNA fragmentation (Fig. 1a) was found in myocytes and vascular endothelial cells of I-R myocardium. In particular, a mean of 84.3 ± SE 9.48 on a total mean number of 270 ± SE 6.32 nuclei, corresponding to approximately 31.2%, stained positively upon reperfusion. In contrast, absence or minimal staining was seen in the control sections (Fig. 1b). The nuclear staining was predominantly found in apparently normal-looking cells and typically confined to regions surrounded by areas containing almost no apoptotic nuclei. The DNA fragmentation was associated with ultrastructural changes of apoptosis in the I-R myocardium. Indeed, a considerable number of the endothelial cells of small blood vessels and myocytes showed characteristic chromatin condensations, formation of crescent chromatin caps apposed to the nuclear envelope (Fig. 2a), and nuclear and cytoplasmic fragmentation (Fig. 2b) eventually leading to the formation of typical apoptotic bodies. Necrotic myocytes and endothelial cells were also identified in I-R myocardium by TEM (data not shown).

It is well known that the aforementioned molecular and morphological alterations apparently require a specific genetic involvement, consisting in the abnormal expression of a series of apoptotic genes and/or deficiency of other genes promoting cell survival (Steller, 1995). A direct role for p53 gene expression in the induction of apoptosis has been described in several cell types and under different experimental conditions (Yonish-Rouach *et al.*, 1991; Prives, 1994). Interestingly, it was recently reported that cultured neonatal cardial myocytes exhibit increased p53 protein levels in response to severe hypoxia, and overexpression of wt p53 by adenovirus gene transfer is sufficient to trigger apoptosis in normoxic myocytes (Long *et al.*, 1997). Based on these considerations, we investigated whether the expression of p53 protein was altered in the I-R myocardium. However, no increase of p53 labeling was found in the I-R myocardium. In fact, by Western blotting and immunocytochemistry, this protein was almost undetectable in both control and reperfused samples (Figs. 3–4), suggesting that the induction of apoptosis under these experimental conditions may be independent of p53 gene expression. On the other hand, the studies on the role of p53 protein in the regulation of the cell death have been focused in actively proliferating cells, such as tumor cells and cells of developing organs. These studies have led to the concept that p53 may be considered a guardian of the genome, its activation leading to cell cycle arrest and eventually to cell death in order to prevent DNA mutations induced by genotoxic stresses from being incorporated within the nucleus of the daughter cells. Therefore, it is likely that, except for cardiomyocytes from embryonic hearts and in the early phase of postnatal development, functional p53 protein may not be required in terminally differentiated cardiomyocytes. In agreement with this hypothesis, no documentation of elevated p53 transcript and immunolabeling has been found in several models of myocardial apoptosis, such as acute myocardial infarction and cardiac hypertrophy, and the absence of p53 in knockout mice neither blocks nor delays myocyte apoptosis (Kim *et al.*, 1994; Cheng *et al.*, 1996; Bialik *et al.*, 1997).

In conclusion, our results suggest that reperfusion of previously ischemic myocardium, as occurs in the course of heart transplantation, may represent a critical event to trigger apoptosis. The mechanisms by which oxidative stress induces apoptosis in the adult I-R myocardium seem to be independent of p53 gene expression. Studies are in progress in our laboratory to delineate the genetic component of the apoptotic process elicited by ischemia–reperfusion in transplanted heart.

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