Cardiomyocyte Death Induced by Myocardial Ischemia and Reperfusion
Measurement With Recombinant Human Annexin-V in a Mouse Model

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Introduction—Phosphatidylserine (PS) externalization is regarded as one of the earliest hallmarks of cells undergoing programmed cell death. We studied the use of labeled human recombinant annexin-V, a protein selectively binding to PS, to detect cardiomyocyte death in an in vivo mouse model of cardiac ischemia and reperfusion (I/R).

Methods and Results—I/R was induced in mouse hearts by ligation and subsequent release of a suture around the left anterior descending coronary artery. Annexin-V (25 mg/kg) fused to a marker molecule was injected intra-arterially 30 minutes before euthanasia. After 15 minutes of ischemia followed by 30 minutes of reperfusion, 1.4 ± 1.2% (mean ± SD) of the cardiomyocytes in the area at risk were annexin-V positive (n = 6). This increased to 11.4 ± 1.9% after 15 minutes of ischemia followed by 90 minutes of reperfusion (n = 7) and to 20.2 ± 3.3% after 30 minutes of ischemia followed by 90 minutes of reperfusion (n = 7). In control mice, including those injected with annexin-V at the binding site of PS, no annexin-V–positive cells were observed. DNA gel electrophoresis showed typical laddering starting after 15 minutes of ischemia followed by 30 minutes of reperfusion, suggesting activation of the cell death program. Intervention in the cell death program by pretreatment with a novel Na+-H+ exchange inhibitor substantially decreased annexin-V–positive cardiomyocytes from 20.2% to 2.2% in mice after 30 minutes of ischemia followed by 90 minutes of reperfusion.

Conclusions—These data suggest that labeled annexin-V is useful for in situ detection of cell death in an in vivo model of I/R in the heart and for the evaluation of cell death–blocking strategies. (Circulation. 2000;102:1564-1568.)

Key Words: apoptosis ■ cells ■ myocardial infarction ■ reperfusion ■ proteins

To define the therapeutic window of cell death–blocking strategies after ischemia and reperfusion (I/R) of the heart, detailed information on the time frame of cell death is needed. Most of the studies evaluating cell death in the heart after I/R used detection methods based on the occurrence of DNA fragmentation, such as the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay and DNA laddering.1-4 However, because TUNEL and DNA laddering do not detect the early stages of cell death, these techniques are not ideal to assess the time frame of cell death in the heart after I/R.4 In addition, in vivo detection of cell death is not possible with TUNEL and/or DNA gel electrophoresis.

One of the earliest events after the triggering of cell death is the externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane of the cell.5-8 In a viable cell, PS is expressed only on the inner leaflet of the cell membrane because of the active transport of PS from the outer to the inner cell membrane by an aminophospholipid translocase.9 However, after activation of the cell death program, PS is externalized rapidly to the outer leaflet of the cell membrane.10 PS externalization is considered to be in close relation to the activation of key players in the cell death program and can be induced by a variety of cell death triggers.5-7,11-15

Detection of PS exposure can be easily achieved by the phospholipid binding protein annexin-V.5-7 We have demonstrated in a number of in vitro and in vivo studies that annexin-V is a specific marker for the early and late stages of cells undergoing programmed cell death and that annexin-V is also suitable for the in situ detection of cell death.5-8,16
Therefore, labeled annexin-V provides a tool for in situ detection of cell death in vivo in animals and also, at least potentially, in the clinical setting. In the present study, we evaluated the potential of labeled annexin-V to detect cell death in situ induced by I/R in the murine heart. Furthermore, we determined the time frame of cell death induction in the early phase of I/R of the heart. In addition, we tested whether labeled annexin-V is useful in evaluating the effect of cell death–blocking strategies after I/R.

**Methods**

**I/R of the Murine Heart In Vivo**

Two-month-old male Swiss mice were anesthetized with pentobarbital (100 mg/kg IP), and the trachea was intubated perorally with a stainless-steel tube. The animals were mechanically ventilated with room air. After left thoracotomy and exposure of the heart, the left anterior descending coronary artery (LAD) was ligated with 6-0 polypropylene just proximal to its main branching point. The suture was tied over a 1-mm polyethylene tube (PE-10) that was left in place during the planned period of ischemia (15 or 30 minutes). Blood flow was then reestablished by removal of the tube. The occurrence of reperfusion could be assessed by the observation of blood flow in epicardial coronary arteries through the operation microscope. For the delineation of the area at risk (AR), colloidal ink (1 mL) was injected just before euthanasia of the animal after the planned reperfusion time (30 to 90 minutes) after reinsertion of the tube under the ligature around the LAD. Then the heart was removed for analysis. For each condition, at least 6 successfully operated animals were used. Sham procedures were identical, with the exception of the actual tying of the polypropylene suture. A subgroup of animals (n = 7) received a single intra-arterial bolus of a Na’-H’ inhibitor (Eniporide, 3 mg/kg, E. Merck) before ischemia.

**Detection of Cardiomyocyte Death With Labeled Annexin-V**

Biotinylated annexin-V (Apoptest-Biotin) and annexin-V–Oregon green (Apoptest-Oregon-Green) were provided by NeXins Research BV (Kattendijke, the Netherlands). Biotinylated annexin-V (25 mg/kg) was injected into the carotid artery 30 minutes before excision of the heart. Annexin-V was always injected during reperfusion. Biotinylated annexin-V was used for quantification studies. After excision of the heart and fixation in HEPES-buffered formalin with 2 mM/L Ca2+ added, routine processing, and paraformaldehyde fixation, 4-µm-thick sections were cut perpendicular to the long axis of the heart. This was followed by staining with a standard avidin-biotin complex kit (ABC kit, Vector Laboratories). Diaminobenzidine was used as the chromogen. Sections were counterstained with hematoxylin. Control experiments were performed with the use of biotinylated annexin-V mutated for its binding site to PS in mice after 30 minutes of ischemia followed by 90 minutes of reperfusion (I/R 30/90 mice, n = 6). The kidney was used as a positive control, because annexin-V is cleared by the kidney and always shows labeling in successfully injected animals. The total area of annexin-V–positive cardiomyocytes was determined by computerized morphometry (Leica Quantimet 570). Quantitative data are expressed as the percentage (mean ± SD) of the AR staining positive for annexin-V (annexin-V/AR×100%). In a subset of I/R 30/90 animals (n = 6), Oregon green–labeled annexin-V was used for the detection of cardiomyocyte death by fluorescence microscopy according to a protocol similar to biotinylated annexin-V injection.

**TUNEL Assay**

The TUNEL assay (Boehringer-Mannheim) was used for detection of DNA strand breaks in the hearts. Paraffin-fixed sections were pretreated with 3% H2O2, and end-labeling was performed with TdT (0.06 U/µL) in TdT buffer containing 40 µmol dig-11-dUTP for 1 hour. Staining with a standard ABC kit was followed by incubation with dianaminobenzidine. Nuclei were considered apoptotic by the presence of dark brown staining. As a positive control, we used mouse intestine.

**DNA Gel Electrophoresis**

The presence of nucleosomal laddering in hearts was investigated with a commercial ligase-mediated polymerase chain reaction assay kit (Apoalert, Clontech). Briefly, DNA was isolated from tissue samples previously frozen at −70°C by using a commercial DNA purification kit (Wizard, Promega) according to the manufacturer’s instructions. DNA purity and concentration were determined by electrophoresis through an 0.8% agarose gel containing ethidium bromide, followed by visualization under UV illumination as well as by measuring absorbance at 260/280 nm. Dephosphorylated adapters were ligated to 5’ phosphorylated blunt ends with T4 DNA ligase (during 16 hours at 16°C) and served as primers in a ligase-mediated polymerase chain reaction under the following conditions: hot start (72°C for 8 minutes), 25 cycles (94°C for 60 seconds and 72°C for 180 seconds), and postcycling (72°C for 15 minutes). To confirm that equal amounts of DNA were used for polymerase chain reaction, an internal control using En-2 primer pairs was performed. Amplified DNA was subjected to gel electrophoresis on a 1.2% agarose gel containing ethidium bromide.

**Immunostaining for Intracytoplasmic IgG**

Immunostaining for IgG was performed to investigate the presence of plasma cell membrane leakage.17,18 The hearts were removed and processed as described for annexin-V–biotin detection. Incubation with a rabbit anti-mouse IgG antibody (1:100) was followed by incubation with biotinylated goat anti-rabbit as the secondary antibody. Routine staining with a standard ABC kit was followed by incubation with dianaminobenzidine. As a positive control, mouse hearts subjected to 24 hours of ischemia were used. Quantitative data are expressed as the percentage of AR staining positive for IgG (IgG/AR×100%).

**Immunoelectron Microscopy**

For analysis by electron microscopy, murine hearts from I/R 30/90 mice were used (n = 3). The hearts were fixed after annexin-V–biotin labeling of the heart with 2% paraformaldehyde and 0.2% glutaraldehyde in Apoptest binding buffer. After sectioning, the samples were sucrose-infiltrated for at least 24 hours. The sucrose-infiltrated samples were cut into ultrathin sections (70 nm) by using a Reichert-Jung Ultracut, followed by immunolabeling (at ambient temperature) as follows: grids were placed on drops of PBS with 50 mmol/L glycine for 15 minutes, transferred to blocking buffer (PBS containing 5% [wt/vol] BSA, 10% [vol/vol] newborn calf serum, 0.1% [vol/vol] cold water fish skin (CWFS) gelatin (Sigma) and 1% [vol/vol] goat normal serum) for 30 minutes, washed 3 times on drops of labeling buffer (PBS containing 0.1% [wt/vol] BSA and 0.2% [wt/vol] BSA–C), incubated with rabbit anti-biotin IgG (Chemicon International) for 60 minutes, washed 6 times for 5 minutes on drops of labeling buffer, incubated with goat anti-rabbit IgG (Aurion) conjugated with 10 nm gold diluted at 1:20 for 60 minutes, washed 6 times for 5 minutes on drops of labeling buffer, washed 5 times for 5 minutes on drops of plain PBS, postfixed for 5 minutes on drops of PBS containing 2.5% glutaraldehyde, washed once with PBS for 5 minutes, washed 5 times for 2 minutes on drops of Milli Q water (Millipore) and contrasted with 15% uranyl acetate in 50% ethanol for 10 minutes, followed by a 5-minute lead citrate staining. The labeled sections were air-dried and examined in a Philips CM 10 microscope at 80 kV.

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Data Analysis

Data are presented as mean±SD. To compare differences between groups, the Student t test (Bonferroni correction) was performed by using SPSS software.

Results

Detection of Cell Death With Biotinylated Annexin-V

In hearts from mice subjected to 15 minutes of ischemia and 30 minutes of reperfusion (I/R 15/30 mice), 1.4±1.2% (n=6) of the cardiomyocytes were annexin-V positive (Figure 1A and Figure 2). The percentage of annexin-V–positive cardio-

myocytes in the AR increased to 11.4±1.9% for mice subjected to 15 minutes of ischemia and 90 minutes of reperfusion (I/R 15/90 mice) and to 20.2±3.3% for I/R 30/90 mice (Figure 1B and 1C and Figure 2). Intervention in the cell death program by pretreatment of the mice with the novel Na+–H+ exchange inhibitor Eniporide resulted in a substantial decrease of annexin-V–positive cardiomyocytes in the I/R 30/90 group from 20.2% to 2.2% (P<0.01, Figure 2).

Annexin-V–positive cells could easily be identified as cardiomyocytes by their characteristic morphology. Annexin-V–positive cardiomyocytes exhibited selective staining at the cell membrane (Figure 1D [cell 1] and 1E and Figure 3A through 3C). Some punctuated staining could also be observed, which likely reflects the staining of the T tubules.
Intervention in the cell death pathway by a novel Na\(^+\)-H\(^+\) exchange inhibitor, Eniporide, decreased the number of annexin-V–positive cardiomyocytes in the AR to 20.2%. In mice that were pretreated with Eniporide, some background laddering was observed in cardiac tissue obtained from sham-operated animals (controls). Detection of DNA fragmentation in situ with TUNEL showed no staining of cardiomyocyte nuclei.

**Annexin-V–Biotin: Electron Microscopy**

For electron microscopic analysis of cell death in I/R 30/90 mouse hearts (n=3), we used small tissue sections obtained from the AR. Sampling of these tissue specimens was guided by biotinylated annexin-V–stained sections from serial sections of the same heart that were analyzed by light microscopy. Immunogold staining with an antibody against biotin demonstrated specific staining of the cell membranes of cardiomyocytes with an oncotic morphology, which had bound annexin-V–biotin during the experiment (Figure 5A and 5B, circles).

**Discussion**

The present study demonstrates that labeled annexin-V provides an alternative method to detect cell death in situ in an I/R model of the heart in mice. In addition, these data suggest that annexin-V may be a useful tool to evaluate cell death–blocking strategies to prevent I/R-induced injury in the heart. After 15 minutes of ischemia followed by 30 minutes of reperfusion, annexin-V–positive cardiomyocytes could already be observed in the AR. Because PS expression and subsequent binding of annexin-V are, at least in vitro, downstream from the activation of executioner caspases, such as caspase 3 (YAMA/CPP32), our data suggest that activation of the cell death program beyond the point of no return may already have occurred in these cells. Extending the reperfusion time to 90 minutes resulted in a marked increase in annexin-V–positive cardiomyocytes. Further extension of the ischemic period to 30 minutes increased the percentage of annexin-V–positive cardiomyocytes in the AR to 20.2%. Intervention in the cell death pathway by a novel Na\(^+\)-H\(^+\) exchange inhibitor, Eniporide, decreased the number of annexin-V–positive cardiomyocytes substantially. These data suggest that labeled annexin-V detects cells that have turned on an active cell death program, which can be inhibited. Activation of the cell death program within cardiomyocytes is also indicated by fragmentation of DNA in multiples of 200 bp, as shown with DNA gel electrophoresis. However, electron microscopic analysis never revealed cells with the classic apoptotic morphology as originally described for

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**Figure 4.** DNA gel electrophoresis. DNA laddering showing bands at multiples of 180 to 200 bp in hearts subjected to I/R. Intensity of bands increases with more extensive periods of I/R. DNA laddering returned to control levels in group pretreated with Eniporide. Controls show some background.

**Figure 5.** Electron microscopy of I/R 30/90 mice hearts. A. Annexin-V immunogold staining. B, Enlargement of box in panel A. Clear staining of intact plasma membrane can be seen in cardiomyocyte with oncotic morphology (circles) (2). Neighbor cardiomyocyte has normal morphology (1).
thymocytes. The annexin-V–positive cardiomyocytes appeared to have an oncotic morphology. Immunostaining for intracellular IgG, indicative of plasma cell membrane leakage, showed substantially lower values than the values found with annexin-V staining. Pretreatment of I/R 30/90 mice with Eniporide resulted in the complete absence of IgG-positive cells, which suggests that the cardiomyocytes with extensive cell membrane leakage are a result of an active cell death program. Together, our data suggest that rapid and massive cell death occurs in the heart after I/R, which is the result of an active cell death program, despite the absence of cardiomyocytes with the classic apoptotic morphology. Therefore, our data support the findings of Buja and Entmann and Ohno et al., who found TUNEL-positive cardiomyocytes and DNA laddering, indicative of an active cell death program, in the absence of cardiomyocytes with the classic apoptotic morphology. The present study supports the concept that apoptotic and oncotic mechanisms proceed at the same time within cardiomyocytes after I/R.

A point of debate is the exclusive use of morphological criteria and internucleosomal fragmentation of DNA to study apoptosis in in vivo models of cardiac injury. Possibly, the morphology of programmed cell death in relative large cells, such as cardiomyocytes and neurons, may differ from the classic morphological appearance of smaller and rapidly dividing cells, such as Jurkat cells. The limitation of using morphological criteria is also suggested from in vitro studies, which showed a change from Bax-induced apoptotic cell death to membrane-permeability cell death, when caspase activation blockers are used. In addition, necrotic cell death and apoptotic cell death may share common biological pathways. Therefore, for development of cell death–blocking strategies in the heart, it may be more efficient to focus on the understanding of biochemical pathways of cell death rather than to study the morphology of dying cells.

In conclusion, our data show that labeled annexin-V is a valuable marker for the in situ detection of cell death induced by I/R of the mouse heart and is useful in the evaluation of cell death–blocking strategies. Our data obtained with annexin-V–Oregon green support the idea that in vivo imaging with labeled annexin-V is possible. In a limited number of mice, detection of fluorescent annexin-V was performed in vivo, with the use of an operation microscope with fluorescence equipment. Annexin-V–positive areas were observed in the AR (Figure 3D). The specific and massive annexin-V staining of cardiomyocytes in the AR of the mouse hearts subjected to I/R suggests that in vivo detection of cell death with labeled annexin-V in patients suffering from an acute myocardial infarction may be possible. An interesting option may be the use of technetium-labeled annexin-V and detection with nuclear imaging. The data in the present study provide essential information as to the time of injection of labeled annexin-V and the time course of binding of annexin-V–Oregon green support the idea that in vivo imaging of annexin-V–positive areas were observed in the AR (Figure 3D). The specific and massive annexin-V staining of cardiomyocytes in the AR of the mouse hearts subjected to I/R suggests that in vivo detection of cell death with labeled annexin-V in patients suffering from an acute myocardial infarction may be possible. An interesting option may be the use of technetium-labeled annexin-V and detection with nuclear imaging. The data in the present study provide essential information as to the time of injection of labeled annexin-V and the time course of binding of annexin-V to PS in the acute phase of myocardial I/R. Preliminary data on cell death detection with technetium-labeled annexin-V have shown promising results.

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