Involvement of CD95/Apo1/Fas in Cell Death After Myocardial Ischemia

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Methods and Results—To study the role of CD95/Fas/Apo1 for induction of postischemic cell death, we used an ischemia/reperfusion model of isolated rat and mouse hearts in Langendorff perfusion. In this model, caspase-dependent apoptosis occurred during postischemic reperfusion. Moreover, soluble CD95 ligand/Fas ligand was released by the postischemic hearts early after the onset of reperfusion. In addition, this ligand was synthesized de novo under these circumstances. Similar findings were observed for other “death-inducing” ligands, such as tumor necrosis factor (TNF)-α and TNF-related apoptosis-inducing ligand. In primary adult rat myocyte culture, hypoxia and reoxygenation caused a marked increase in sensitivity to the apoptotic effects of CD95 ligand. Isolated hearts from mice lacking functional CD95 (lpr) display marked reduction in cell death after ischemia and reperfusion compared with wild-type controls.

Conclusions—These data suggest that CD95/Apo1/Fas is directly involved in cell death after myocardial ischemia. The CD95 system might thus represent a novel target for therapeutic prevention of postischemic cell death in the heart. (Circulation. 2000;102:915-920.)

Key Words: ischemia □ reperfusion □ apoptosis □ genes

After occlusion of a coronary artery, deprivation of oxygen and energy causes severe damage in the affected tissue and mediates cell death. Moreover, reperfusion of the occluded artery may aggravate cardiac dysfunction (myocardial stunning) and increase infarct size. Because the acute myocyte loss caused by ischemia/reperfusion is irreversible, it determines chronic organ dysfunction and development of heart failure.

Cell death occurring after ischemia/reperfusion in the heart is largely attributed to necrosis caused by energy depletion (ATP), calcium overload, acidosis, and oxidative stress. Necrotic cell death is characterized by cell swelling and rupture initiating an inflammatory response in the tissue. Interestingly, disappearance of myocytes without inflammatory response occurs after ischemia in the heart, as well as in, for example, neuronal tissue. This loss of myocytes is effected by apoptosis, or programmed cell death, which induces DNA cleavage and autolysis. In contrast to necrosis, apoptosis is a highly selective process controlled and tightly regulated by intracellular signal transduction that may be initiated by cell stress, such as metabolic injury, or by ligand-receptor binding. Three members of the family of death-inducing ligands (DILs) have been characterized so far: CD95 ligand (CD95L), TNF-related apoptosis-inducing ligand (TRAIL), and tumor necrosis factor-α (TNF-α). DILs are membrane-bound proteins that can be cleaved into a soluble form by metalloproteinases, cysteineproteinases, or TNF-α–converting enzyme in the case of CD95L, TRAIL, and TNF-α, respectively, although the soluble proteins appear to be less potent than membrane-bound DILs. CD95L, TRAIL, and TNF-α signal apoptosis on specific interaction with their receptors CD95, TRAIL-receptors 1 and 2, and TNF receptor (TNFR) I, respectively. Multimerization of the receptor by DILs recruits adapter molecules such as Fas-associated death domain protein or TNFR-associated death domain protein, which mediate activation of the downstream apoptosis effectors, eg, the caspase cascade. Sequential cleavage of caspases then causes DNA fragmentation and cell death. Apoptosis/programmed cell death is an important control mechanism for organ development and tissue homeostasis in the heart as well. Evidence for apoptotic cell death during hypoxia, ischemia, and reperfusion in the heart was obtained in animal studies, in experiments with primary cell culture, and in human autopsies. Apoptosis occurs preferentially in the border zone around the necrotic core. As yet, it is not completely clear to what extent apoptosis contributes to total cell loss after myocardial infarction during
both ischemia and reperfusion. Postischemic apoptosis can be attenuated by preconditioning, an effect dependent on caspases and vacuolar proton ATPase. In addition, a contribution of death receptor systems to cell death in the heart was suggested by the finding of increased TNF-α, TNFRs, and CD95 in the postischemic myocardium. Thus, it was tempting to assume that DILs might be involved in triggering postischemic myocardial cell death. To investigate this hypothesis, experiments were performed using perfusions of isolated rat and mouse hearts as well as primary cultures of adult rat cardiac cells.

**Methods**

**Materials**

The caspase inhibitor zVAD-fmk was obtained from Enzyme System Products. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay was performed with terminal deoxynucleotidyl transferase purchased from Boehringer Mannheim. CD95L antibodies from Transduction Laboratories and p62 from M. Hahn/J. Tschopp were used for Western blotting with identical results; immunohistochemistry for CD95L was performed with N50-1 antibody (PharMingen). TRAIL antibody was purchased from Santa Cruz, and TNF-α antibody was purchased from Sigma. The TRAIL protein was produced in a recombinant expression system using the yeast *Pichia pastoris*. In brief, *P. pastoris* was transformed by cloned His-tagged TRAIL DNA under the AOX-1 promoter. Expression protein was induced by addition of methanol. Thereafter, cells were lysed, and TRAIL protein was purified by the nickel-histidine interaction.

**Isolated Hearts in Langendorff Perfusion**

The care of animals and all experimental procedures conform with the Guide for the Care and Use of Laboratory Animals. After male Wistar rats (200 to 300 g) or 8-week-old C57 BL/6 mice were anesthetized, the aorta was quickly cannulated, and Langendorff perfusion was performed as described previously with modified Krebs-Henseleit buffer (in mmol/L: NaCl 116, CaCl2 1.6, KH2PO4 1.2, KCl 3.8, MgCl2 1.2, NaHCO3 23.2, mannitol 16, glucose 11.5, and pyruvate 2, plus insulin 5 U/L), either at constant volume rates (rat hearts, 5 mL/min) or at constant perfusion pressure (mouse hearts, 55 cm H2O). Global ischemia (25 and 20 minutes in rats and mice, respectively, 37°C) was applied by fluid interruption, followed by reperfusion at preischemic conditions. Transudates were collected at 15-minute intervals during the whole course of the experiment as interstitial/tissue fluid extruded from the surface of the heart. Where indicated, zVAD-fmk (50 mmol/L) was added to the buffer during the first 30 minutes of reperfusion. At the end of the experiments, hearts were snap-frozen in nitrogen.

Apoptotic cell death of cardiac cells was estimated by TUNEL staining, which was performed in ventricular tissue sections as described. In brief, fixed tissue sections were permeabilized and incubated with TdT and biotin-16-UTP for 30 minutes. Anti-UTP antibody was visualized with the horseradish peroxidase (HRP)-coupled biotin-streptavidin system and DAB for color reaction. TUNEL-positive cells were counted in 15 sections per heart under magnification. Alternatively, the percentage of TUNEL-positive cells in tissue sections was estimated with an automated image analysis system (Leica Q600). Sections were scanned automatically by a computer-directed microscope stage under magnification. Color and size were defined either for TUNEL-positive or for Nissl-positive cells. Then 12x12 fields were analyzed under stable relative illumination. At least 5000 (rat hearts) or 15 000 (mouse hearts) cells were quantified. The percentage of apoptotic cells was defined as number of TUNEL-positive cells per total number of cells. All sections of either mouse or rat experiments were analyzed in 1 session of size-oriented image processing and evaluated by the same independent and blinded operator.

**Primary Culture of Adult Rat Cardiomyocytes**

Primary adult rat cardiomyocytes were prepared as published before. Cells were seeded on laminin-pretreated dishes in medium 199 for 4 hours. For induction of ischemia, cells were placed in glucose-free medium in a chamber continuously gassed with humidified 95% N2, 5% CO2 at 37°C for 6 hours. With this approach, oxygen tensions <10 mm Hg are achieved after 30 minutes, as assessed by gas chromatography–mass spectrometry, and pH decreased from 7.4 to 7.2 within 4 hours. Reoxygenation was performed by addition of glucose (4.5%) and changing of the atmosphere to 95% air, 5% CO2 for another 6 hours. Directly after onset of reoxygenation, primary adult cardiomyocytes were stimulated with anti-CD95 antibody Jo2 (PharMingen, 5 µg/mL) or recombinant CD95L (Alexis, 5 µg/mL), recombinant TRAIL produced in *P. pastoris* (6 µg/mL), or recombinant rat TNF-α (R&D systems, 600 ng/mL) in the presence of cycloheximide (100 ng/mL). CD95-mediated cell death was induced equally by an agonistic anti-CD95 antibody (Jo-2) and by recombinant CD95L (data not shown). Control cells were left in 95% air, 5% CO2 at 37°C normal glucose concentration for 6 hours and were stimulated with DILs in the presence of cycloheximide without previous ischemia. Cell survival was estimated by counting trypan blue–excluding cells per field under light microscopy. Specific cell survival (%) was calculated with cells without stimulation by DILs used as controls. Specific cell death (%) was calculated as 100–specific cell survival (%).

**Measurement of DILs**

For soluble DILs, transudates of each isolated organ were divided into aliquots for a sampling time of 15 minutes, each aliquot containing the total protein amount coming off the surface of the heart in this interval and therefore being comparable to each other. Subsequently, aliquots were concentrated by use of membrane exclusion columns (Grenier), either at total volume of 60 µL. Aliquots of 10 µL per time point were subjected to Western blot analysis for each soluble DIL. To assess DIL tissue concentration within the hearts, tissue sections were lysed and total protein was extracted. Immunoprecipitation was performed in the case of TNF-α and TRAIL. α-Tubulin was used as loading control. Western blot analysis of transudate or tissue sample was performed as described. In brief, samples were separated on a 12% polyacrylamide gel, blotted onto a nitrocellulose membrane, and incubated with a specific antibody. HRP-conjugated secondary antibody was visualized with luminol. Immunohistochemistry was performed as described. In brief, fixed sections were permeabilized and incubated with specific antibody, which was visualized by HRP-conjugated secondary antibody with DAB.

**Statistical Methods**

The results are given as mean±SEM. Statistical analysis was performed with 1-way ANOVA. Whenever a significant effect was obtained with ANOVA, we performed multiple comparison tests between the groups, using Bonferroni’s test (Figure 1) or Dunnett’s post hoc test comparing variable groups to 1 control group (Figure 4). For comparison of wild-type and *pr*–mutant mice, we used the Wilcoxon rank sum test (Figure 5). Differences between groups were considered significant at a value of *P*<0.05. The SPSS statistical software package was used throughout the analysis.

**Results**

**Induction of Caspase-Dependent Cell Death by Ischemia/Reperfusion**

After ischemia/reperfusion, cardiac cells die by necrosis and/or apoptosis. To directly study the role of apoptosis systems for postischemic cell death in the heart, we performed transient global ischemia in isolated, saline-perfused rat hearts, followed by reperfusion for various time intervals (Langendorff hearts). Because saline perfusion eliminates (for example) leukocytes from the perfused heart, this model allows specific study of cardiac processes. Apoptotic cell

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death was detected by TUNEL staining of cells undergoing DNA fragmentation (see Methods of Reference 22). Twenty-five minutes of global ischemia followed by 4 hours of reperfusion induced a significant increase in TUNEL-positive cells (Figure 1A). This finding suggests that postischemic cell death in cardiac cells occurs independently of blood-derived cells or factors. To study whether cell death detected by TUNEL is susceptible to apoptotic signal transduction, we used zVAD-fmk, a broad-spectrum inhibitor of most caspases. Activation of caspases is specifically linked to apoptotic but not to necrotic cell death. Addition of zVAD-fmk completely inhibited the increase of TUNEL-positive cells during postischemic reperfusion (Figure 1B). These data suggest that caspases contribute to induction of death in cardiac cells after ischemia/reperfusion.

Release of Soluble DILs From Postischemic Langendorff Hearts During Reperfusion

Induction of apoptosis in response to cellular stress has been shown to involve activation of apoptosis-inducing ligand/receptor systems such as CD95. We therefore studied local production of DILs after ischemia/reperfusion by collecting transudates from the surface of the heart, which represent interstitial and lymphoid fluid. Aliquots were compared for absolute amount of DIL protein over time. Western blot analysis of concentrated transudates (×200) revealed that CD95L, TNF-α, and TRAIL are released or cleaved into the extracellular fluid early after onset of reperfusion (Figure 2). In time-matched transudates of control hearts not subjected to ischemia, no DILs were detected in Western blot analysis (data not shown). In the same experiments, no measurable DIL proteins were found in the coronary venous buffer, which had passed through the isolated heart (data not shown). Proteolytic cleavage as a mechanism of CD95L release was suggested by the size of the protein detected (31 kDa), which was smaller than that of the membrane-bound forms (37 kDa).

Production of DILs During Reperfusion in Postischemic Langendorff Hearts

In addition to DIL proteins secreted into the extracellular fluid, the cardiac content of membrane-bound DIL is relevant as a store of apoptotic signal carriers. In unstimulated cardiac tissue, no DIL proteins can be detected by Western blot analysis (Figure 3A). However, de novo production of DIL proteins in cardiac cells is found within a few hours of reperfusion after ischemia. Maximum CD95L production is obtained as soon as 2 hours after onset of reperfusion (Figure 3A). Similar results were obtained by immunohistochemistry (Figure 3B). These experiments show that cells within the heart may produce and release DILs on ischemia/reperfusion, suggesting that cardiac cells in the affected area of the heart may be exposed to increased concentrations of DILs after an ischemic insult.
Induction of Cell Death by DILs in Cardiomyocytes After Simulated Ischemia

To further examine the functional importance of DILs produced and released in situ, we examined a cell culture model of simulated ischemia in adult rat cardiomyocytes. These data deal with the ability of DILs to induce cell death in cardiomyocytes. For stimulation with DILs, we used cell survival as readout in the experiment rather than TUNEL staining because of practical issues associated with quantification of adherent and nonadherent cells.

Normoxic control adult rat cardiomyocytes display constitutive resistance against DIL-induced cell death, even in the presence of cycloheximide, a protein synthesis inhibitor that usually sensitizes cells to TNF-α–induced cell death (Figure 4, open bars). During the simulated ischemia protocol, cardiomyocytes die over time so that 33% of all adherent primary adult rat cardiomyocytes are dead within 6 hours (data not shown). No additional cell death is found during reoxygenation at any time point. In contrast, exposure to exogenous DILs during reoxygenation induces further cell death (Figure 4, solid bars, normalized to cells subjected to simulated ischemia and reoxygenation but no DILs). The most prominent effect was induced by CD95. These results suggest that simulated ischemia/reoxygenation in cardiac myocytes increases the sensitivity of these myocytes to DIL-induced cell death.

Attenuation of Ischemia/Reperfusion-Mediated Cell Death by Dysfunctional CD95

We specifically studied the role of the CD95/Apo1/Fas system for cell death during postischemic reperfusion in the heart by use of Langendorff perfusion of mouse hearts in which the CD95 death pathway is nonfunctional because of mutations in the CD95 receptor. Isolated hearts of lpr mice were compared with those of wild-type controls, both on C57 BL/6 background. In Langendorff perfusion, 20 minutes of global ischemia was followed by 4 hours of reperfusion in both groups. Longer periods of ischemia and/or reperfusion resulted in cardiac arrhythmias and a greater variability between organs. TUNEL staining was performed and evaluated by manual counting (data not shown) or computer-assisted analysis (Figure 5), which yielded similar results. The amount of apoptotic cell death measured by TUNEL staining was significantly (P<0.0002) lower in lpr hearts than in strain-matched controls (Figure 5). Because no statistically significant differences were detected in coronary perfusion pressure and heart rate between lpr hearts and
chemic apoptosis have not yet been identified. In the wild-type mice (wt, n=9) or death by dysfunctional CD95. Hearts were isolated from wild-type controls (Table), the decrease of TUNEL-positive cells in lpr hearts appears to be due to the lack of functional CD95.

**Discussion**

Earlier concepts about cell death during postischemic reperfusion attributed the loss of living and functional cells largely to necrosis. However, apoptotic cell death was also recently observed during postischemic reperfusion in the heart. The molecular mechanisms causing postischemic apoptosis have not yet been identified. In the present study, we examined the impact of apoptosis systems, in particular the CD95 system, on cell death after ischemia and reperfusion.

In the present study, TUNEL positivity was used as a parameter for the detection of apoptotic cardiac cells. The specificity of TUNEL assay in measurement of apoptosis has been discussed because of differences of apoptotic cell numbers detected under comparable conditions and possible overlaps with the detection of necrotic cells displaying breaks of double-strand DNA hours after the onset of the insult. In our study, however, the rate of TUNEL-positive cells was susceptible to interventions known to influence exclusively apoptosis but not necrosis. Thus, experiments with the broad-spectrum caspase inhibitor zVAD-fmk as well as in mice lacking a functional CD95 receptor both revealed a decrease in the number of TUNEL-positive cells. Therefore, at least in the isolated organs used and with respect to the substantial number of cells analyzed by computer-assisted evaluation (5000 cells per heart and 15 000 cells per organ for calculation of DIL-specific reoxygenation period, surviving cells were measured by counting trypan-negative cells per field. For calculation of DIL-specific cell death, postischemic cells without additional stimulation by DILs were used as controls. Open bars show specific, DIL-induced apoptosis (6 hours) on non-postischemic cardiomyocytes using untreated cells as reference. Similar results were obtained in 3 independent experiments. Data are mean of duplicates±SEM. *P<0.001 vs unstimulated control.

**Figure 4.** Induction of cell death by DILs in cardiomyocytes after simulated ischemia. Primary adult rat cardiomyocytes were exposed to 6 hours of ischemia followed by 6 hours of reoxygenation (solid bars). DILs were present during reoxygenation in the presence of cycloheximide. After end of reoxygenation period, surviving cells were measured by counting trypan-negative cells per field. For calculation of DIL-specific cell death, postischemic cells without additional stimulation by DILs were used as controls. Open bars show specific, DIL-induced apoptosis (6 hours) on non-postischemic cardiomyocytes using untreated cells as reference. Similar results were obtained in 3 independent experiments. Data are mean of duplicates±SEM. *P<0.001 vs unstimulated control.

**Figure 5.** Attenuation of ischemia/reperfusion-mediated cell death by dysfunctional CD95. Hearts were isolated from wild-type mice (wt, n=9) or lpr mice lacking functional CD95 (n=11). Ischemia for 20 minutes was followed by 4 hours of reperfusion. Each point represents percentage of TUNEL-positive cells obtained in 1 organ, as analyzed by automated image processing. Gray bars indicate mean obtained in each group±SEM. *P<0.0002.

<table>
<thead>
<tr>
<th>Coronary Flow and Heart Rate in Mouse Hearts Before and After Ischemia</th>
<th>Wild-Type</th>
<th>lpr Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preischemic CF, mL/min</td>
<td>1.90±0.09</td>
<td>2.07±0.28</td>
</tr>
<tr>
<td>Postischemic CF 15 min, mL/min</td>
<td>1.75±0.08</td>
<td>1.85±0.23</td>
</tr>
<tr>
<td>Postischemic CF 4 h, mL/min</td>
<td>1.55±0.21</td>
<td>1.21±0.16</td>
</tr>
<tr>
<td>Preischemic HR, bpm</td>
<td>355±46</td>
<td>370±35</td>
</tr>
<tr>
<td>Postischemic HR 15 min, bpm</td>
<td>268±27</td>
<td>298±33</td>
</tr>
<tr>
<td>Postischemic HF 4 h, bpm</td>
<td>199±23</td>
<td>224±48</td>
</tr>
</tbody>
</table>

CF indicates coronary flow; HR, heart rate. Data are mean±SEM of 9 (wild-type) and 11 (lpr) experiments.
become prominent sources of DILs (Figures 2 and 3), although in vivo, white blood cells may contribute to a further increase of apoptosis-inducing factors.

Interestingly, however, the presence of DILs alone does not suffice to induce cell death (Figure 4). Nonischemic control cells do not display a constitutive sensitivity for cell death induction by CD95L, TRAIL, or TNF-α. Furthermore, reoxygenation after simulated ischemia as used in our model did not induce cell death on its own. These results differ from earlier observations by Karwatowska-Prokopczuk et al., who found increased cell death of neonatal cardiomyocytes after severe metabolic inhibition, including cyanide exposure mediated by severe acidification. The milder form of metabolic injury used here, by N₂/O₂ exposure, leads to a moderate drop of pH. The protocol does not lead to spontaneous cell death during reoxygenation but rather to increased sensitivity for DIL-induced cell death in cardiomyocytes. Apoptosis sensitivity may also be modified by alterations of signal proteins downstream of the receptor–ligand interaction. Thus, Fas-associated death domain–like interleukin 1β-converting enzyme inhibitory protein (FLIP), which disables signal transduction from CD95 receptor to caspases, is abundantly present in normoxic cardiac tissue but is degraded after ischemia and reperfusion. Therefore, downregulation of FLIP, among other intracellular regulatory proteins, may represent an important mechanism determining enhanced apoptosis sensitivity of cardiomyocytes after ischemia and might cause altered apoptosis sensitivity, eg, after preconditioning.

The CD95 system plays an important role in the regulation of physiological homeostasis in the immune system. In addition, apoptosis induction by CD95 has been shown to participate in various types of stress-induced apoptosis, eg, in drug-induced apoptosis or postischemic apoptosis in the brain. The data presented here show for the first time that a functional CD95 system contributes to cell death in cardiac cells in response to ischemia/reperfusion injury. Experiments that compared CD95 receptor mutant lpr mice with wild-type controls revealed a significant difference in the occurrence of TUNEL-positive apoptotic cells. Therefore, the CD95 system might play an important role in cardiac autodestruction during ischemia/reperfusion.

In summary, we demonstrate here that DILs, in particular CD95L, are induced during postischemic reperfusion and that enhanced cell death after myocardial ischemia is dependent on a functional CD95 ligand-receptor interaction. Because inhibition of caspase activation and disruption of the CD95 receptor signaling represent 2 approaches to decrease postischemic cell death of the heart, further studies will be conducted to assess the therapeutic potential of these interventions aiming at the reduction of myocardial reperfusion injury.

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References

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