Dog Cardiomyocyte Death Induced In Vivo by Ischemia and Reperfusion

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Abstract

The type of myocardial cell death induced in dog heart by *in vivo* ischemia and reperfusion and its relationship with the cellular energy state has been analyzed. The combination of electron microscopy and DNA gel electrophoresis shows that ischemia and reperfusion induce both necrotic and apoptotic death of dog myocardial cells. Nucleosomal DNA laddering has not been observed after prolonged ischemia alone indicating that necrosis mainly occurs during this treatment. Appearance of apoptotic cells correlates with the restoration of energy production and increase in the content of creatine phosphate. Ischemia and reperfusion had no effect on the expression of bcl-x, bak, bax, and calpain but induced expression of CD95 in dog myocardial cells. A possible scenario of cell injury during ischemia and reperfusion is discussed.

Key words: apoptosis, myocardial cells, ischemia, reperfusion, CD95 (Fas/APO-1).

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The occurrence of cardiomyocyte death within a brief period following acute ischemia and reperfusion leads to cardiac dysfunction. Although early reperfusion decreases heart damage, an additional and often larger population of cells die following restoration of blood flow. This observation has led to the concept that cells die from irreversible reperfusion oxidative damage presumably sustained as a consequence of the generation of reactive oxygen species. However, therapeutic strategies based upon the application of antioxidants have met only limited clinical success. Recent evidence has revealed more detail regarding the molecular basis of cardiomyocyte death and has led to the idea that ischemia/reperfusion damage may not be the direct cause. Rather, experimental observations have led several investigators to hypothesize that the molecular basis of cardiomyocyte death is through apoptosis. This would lead to the conclusion that ischemia and reperfusion oxidative damage may not be catastrophic but rather may act to signal the initiation of a programmed cell death. More importantly from a medical perspective is the fact that this hypothesis leads to a novel therapeutic strategy based upon apoptosis suppression instead of or in addition to blockade of oxidative damage.

Two distinct types of cell death, i.e. necrosis and apoptosis, are now described and intensively investigated in the recent literature [14, 28, 29, 31]. Necrosis is considered to be a catastrophic metabolic failure resulting directly from severe molecular and/or structural damage and leading to general catabolic degradation. Necrosis is commonly marked by an early increase in total cell volume and subcellular organelle volume followed by autolysis. Nuclear changes are found to be late events and are a consequence of activation of cell hydrolytic enzymes. This mode of cell death is generally considered to contribute to further regional tissue damage and inflammation.

Contrary to early thought, apoptosis is substantially more prevalent as a biological phenomenon than is necrosis. Programmed deletion and physiological cell death as well as many cases of cell death in various pathogenic processes occur through the highly regulated apoptotic pathway. Apoptosis is an active cellular response to physiological signals or damage. Therefore, cell death may be prevented

by means unrelated to signals that initiate the process. This is in contrast with necrosis which can be inhibited only by decreasing the level of sustained cell injury. Apoptosis is ultrastructurally seen to involve chromatin condensation and margination, cell shrinkage, blebbing, extensive protein crosslinking through activation of tissue transglutaminase, and nuclear fragmentation *in vivo* associated with formation of so-called apoptotic bodies. These apoptotic bodies are ultimately phagocytosed by neighboring cells and tissue integrity is not compromised. On the molecular level apoptosis is often characterized by enzymatic internucleosomal fragmentation of nuclear DNA which usually precedes loss of plasma membrane integrity. Many genes belonging to distinct gene families are involved in regulation of apoptosis but few have been directly associated with mediation of the process *per se* [31].

Apoptosis is a normal and fundamentally important process to most if not all multicellular organisms. However, it appears that in some instances where tissue organization requires maintenance of highly developed architecture, such as in the central nervous system, and neuromuscular systems, apoptotic death is likely to compromise tissue, and ultimately, organ function. The heart is such an organ where the microarchitecture largely determines organ function and phagocytosis and replacement of apoptotic cardiomyocytes is not likely. The result of acute appearance of apoptotic cardiomyocytes may be local functional failure, post-apoptotic necrosis, inflammation, and ultimately organ damage and disfunction.

Tanaka *et al.* [27] observed internucleosomal DNA fragmentation after prolonged incubation of rat neonatal cardiomyocytes under hypoxia *in vitro*. Gottlieb et al. [7] detected similar DNA fragmentation in rabbit myocardium after ischemia combined with subsequent reperfused, but not after ischemia only. They did not find apoptotic bodies in myocardium nor did transmission electron microscopy reveal typical apoptotic morphology. However, similar to observations reported by our laboratory [30], the pattern of chromatin condensation following ischemia alone differed from those after subsequent reperfusion. Using in situ DNA end labeling several other groups have reported involvement of apoptosis in the development of chronic heart failure in dogs [26], ischemia/reperfusion induced myocardial injury in rats [32], and myocardial infarction in humans [10].

The purpose of this study was to analyze myocardial cell death induced in dog heart by acute ischemia produced by artery occlusion followed by restoration of blood flow. Cell death was related to the cellular energy state and expression of specific genes which may be involved in the apoptotic pathway.

Materials and Methods

Animal preparation

Adult mongrel dogs of either sex weighing 10-15 kg were anesthetized with 35 mg/kg pentobarbital intraperitoneally (i.p.). The open chest animals were intubated and artificially ventilated with the mixture of ambient room air and O_2.

Left ventricular pressure (LVP) and its first derivative (dP/dt) as well as heart rate (HR) were measured continuously with a catheter introduced into the LV cavity through the right carotid artery [16]. The electrocardiogram (ECG) was registered in positions that correspond to the first and second standard ones.

Cardiac microdialysis technique

The microdialysis probe was inserted into the myocardium of the left ventricle wall within the perfused region to be made ischemic following arterial occlusion. Placement of the probe was determined by observing the area of the left ventricle that became cyanotic upon brief (approximately 10 sec) occlusion of a branch of the left anterior descending coronary artery (LAD).

Microdialysis probes were constructed with a single dialysis fiber (Cordis Dow, Brussels, Belgium; 0.25 mm OD, molecular weight cutoff 5000). With the aid of a bent needle the fiber was pulled through the myocardium at a maximum depth of approximately 2 mm. Each end of the fiber was inserted into inflow and outflow silicon tubes and sealed in place with cyanoacrylic glue. These connections provided rigid fixation on the fiber in the myocardium. The dialysis fiber length was approximately 20 mm. The inflow silicon tube was connected to a glass syringe of a perfusion pump, an outlet tube served for dialysate collection. The same microdialysis probe was implanted into the nonischemic area of the left ventricle. After implantation, the microdialysis probes were perfused with physiologic Ringer's solution (in mmol: 147 Na^+, 4.0 K^+, 2.3 Ca^{2+}) at a rate 3 ml/min. 30 min after the start of perfusion microdialysis samples were collected during experiment. This allowed for the metabolite diffusion to occur from the interstitial fluid to the dialysis fiber [16]. The metabolite effluent concentrations from the microdialysis probes thus represent interstitial fluid concentrations. *In vitro* experiments showed that dialysate lactate and creatine corresponded to 30 ± 1% of the interstitial metabolite concentration.

Design of experiments

Two *in vivo* protocols were followed. In the first experimental protocol (n = 4) LAD was occluded for 1 hr and reperfused for 1, 3, or 6 hrs. At the end of ischemia or reperfusion, left ventricular transmural biopsies from nonischemic and ischemic or reperfused regions were taken for metabolite analysis, microscopy and DNA isolation. In the second experimental protocol (n = 3) LAD was occluded for 4 hr. Immediately after ischemic period biopsies were taken from intact and occluded regions. Dialysate samples from non ischemic and injured areas of the left ventricle were collected every 20 min in both series of experiments.

Metabolite measurements

Biopsies from the risk and nonischemic regions of the left ventricle were frozen within 10 sec in liquid nitrogen.

The frozen tissue was pulverized in a mortar with liquid nitrogen. The acid soluble fraction was extracted by a cooled 6% perchloric acid (10 ml/g frozen tissue) and centrifuged at 2500 x g for 10 min at 4°C. Supernatants were neutralized with 5M K_2CO_3 to pH 7.4. The $KClO_4$ precipitate was centrifuged under the same conditions [20].

Concentrations of ATP, ADP, AMP, phosphocreatine (PCr), creatine (Cr) and lactate in neutralized tissue extracts were measured spectrophotometrically by enzymatic methods [1, 8, 11, 17, 18]. Concentrations of lactate and Cr in dialysate samples were determined using modified enzymatic microassays.

Electron microscopy

The tissue samples from the effected and control areas were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.6-7.8) for 12-24 hours and postfixed for 24-48 hours with osmium tetroxide in the same buffer containing 0.01% potassium bichromate. The samples were dehydrated and embedded in Epon-Araldite. After epoxy resin polymerization the samples were sectioned and the ultrathin sections were stained with uranyl acetate and lead citrate. The sections were viewed in JEM-100B (JEOL) electron microscope.

DNA isolation and electrophoresis

DNA was isolated from tissue samples frozen in liquid nitrogen by proteinase K - phenol method as described [23]. All DNA samples were treated with 100 µg/ml of DNase-free RNase, extracted twice with phenol/chloroform, precipitated with ethanol, and dissolved in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA. Electrophoresis was performed in a horizontal 1.2% agarose gel (15-20 µg DNA per lane) in 89 mM Tris-phosphate buffer, 8 mM EDTA, pH 8.0 [23]. DNA was visualized by staining with ethidium bromide (0.5 mg/ml).

RNA isolation and Northern hybridization

RNA was isolated from ischemic and non-ischemic zones of the heart as described [5]. Poly(A)+RNA was purified as described [23]. 20 µg total RNA or 5 µg poly(A)+RNA were fractionated by electrophoresis in a 1% agarose - 2.2 M formaldehyde gel. The gels were blotted onto nitrocellulose membrane, prehybridized and hybridized under high stringency to DNA probes in the presence of 40% formaldehyde at 42°C [23]. All DNA probes except Fas were full length cDNAs. Final washing condition for dog-specific Fas probe was 0.1 x SSC, 0.1% SDS at 60°C, for other probes 2 x SSC, 0.1% SDS at 55°C were used.

Results

Left ventricular pressure, its first derivative and heart rate were measured continuously using a catheter introduced into the left ventricle cavity through the right carotid artery.

Neither elimination of blood flow in one of the areas of the coronary bed (1 or 4 hours) nor subsequent restoration of blood flow (1, 3 or 6 hours) after 1 hour ischemia induced any significant changes in the total physiological parameters (data not shown). This demonstrates that in our experiments the effect of local ischemia on global cardiac function was insignificant.

It should be pointed out that there were the signs of myocardial ischemia on ECG.

Cellular energy state of ischemic and reperfused heart

1 hr ischemia and subsequent 6 hr reperfusion had no significant effect on tissue levels of high energy phosphates and Cr in nonischemic region. The mean contents of these metabolites in cardiac tissue corresponded to the normal values [12] and were 23.15 ± 2.07, 26.91 ± 3.34 and 58.8 ± 4.24 for ATP, PCr and Cr respectively. Thus, experimental conditions were adequate to maintain myocardial oxidative metabolism regardless of the duration of reperfusion.

Effects of 1 hr regional ischemia and reperfusion on myocardial contents of adenine nucleotides (AN), PCr and Cr of nonischemic and reperfused regions of the left ventricle are shown in Fig. 1.

After 1 hr ischemia, the contents of ATP and ΣAN (ATP + ADP + AMP) decreased to 55 and 67%, respectively, and additional drop was observed during 1 hr reperfusion (to 31.4 and 24% of control values, respectively). There were no significant changes in the content of ATP and ΣAN after 3 hr reperfusion. By 6 hr reperfusion, the content of ATP and ΣAN in postischemic region remained reduced but was partially restored to about 55-60% of the normal values.

The level of PCr after 1 hr ischemia decreased dramatically (to 11% of normal value) while the content of Cr increased respectively. During reperfusion, the PCr level showed gradual restoration to the initial level. However, the total Cr decreased due to partial loss of Cr from myocardial cells. This possibility was verified by measuring the changes in Cr concentration in cardiac interstitial fluid (Fig. 2a). The initial baseline dialysate Cr before ischemia was about 40-50 mM and did not differ significantly from that in nonischemic region. During ischemia, a rapid degradation of PCr led to accumulation of intracellular Cr and its release from myocardial cells, resulting in its increase in the dialysate. Restoration of coronary flow by reperfusion led to the second transient increase in dialysate Cr followed by a decrease in its concentration to the normal value at 90 min of reperfusion. This change reflects a rapid clearing of accumulated Cr from interstitial fluid.

The initial baseline lactate concentration in the dialysate before ischemia (0.8 mM) did not differ from the value found in the dialysate of nonischemic regions (Fig. 2a). The increase in dialysate lactate during ischemia corresponds to an activation of anaerobic glycolysis in myocardial tissue and a diffusion of excess lactate into the interstitial fluid. As with Cr, subsequent reperfusion diminished dialysate lactate but more gradually. During the first 3 hr of reperfusion, the extracellular concentration of lactate remained at an elevated level suggesting the persistence of anaerobic glycolysis.

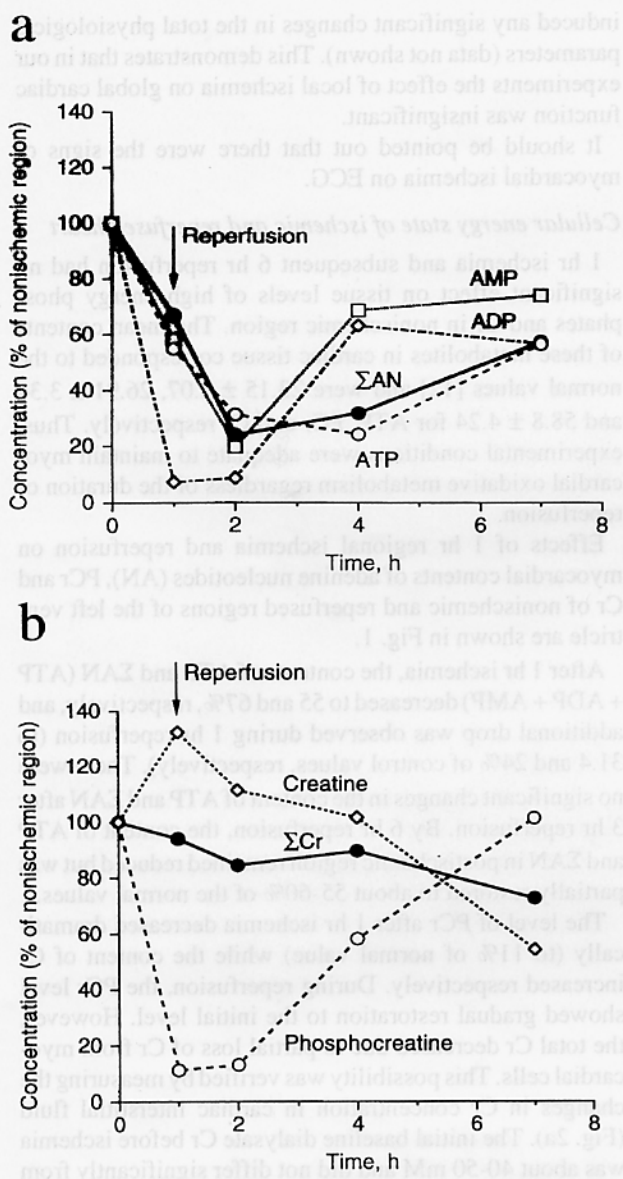

Figure 1. Changes in concentration of adenine nucleotides (a) and creatine and phosphocreatine (b) during canine heart ischemia and reperfusion. The concentration of adenine nucleotides, creatine and phosphocreatine was measured in the ischemic and control areas as described in Materials and Methods. The changes in concentration of each compound in the ischemic area are given as a percentage of the respective value in the control zone.

Myocardial contents of AN, PCr, Cr and lactate in nonischemic and ischemic regions of canine heart after 4 hr ischemia are presented in Table 1. By the end of the experiment, the contents of all metabolites in nonischemic region did not differ from normal values [12]. However in the ischemic region ATP, ADP and AMP were reduced by 70, 45 and 60% respectively. As a result the total loss of

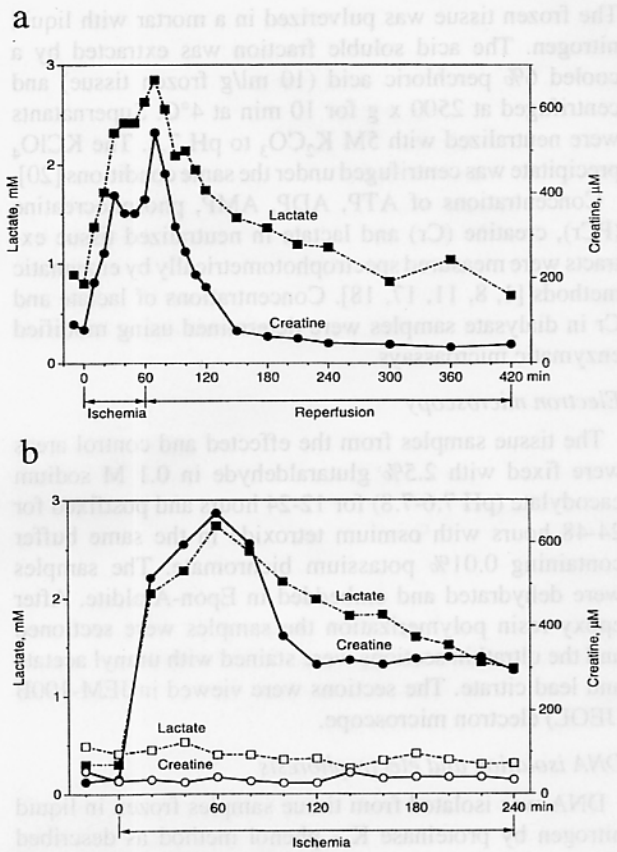

Figure 2. Changes of lactate and creatine concentration in dialysates collected from ischemic and non-ischemic regions of the left ventricle of the canine heart during 1 hr ischemia and 6 hr reperfusion (a) or 4 hr ischemia (b). Open and closed symbols correspond to non-ischemic and ischemic regions, respectively.

AN pool was about 69% of the initial value. PCr decreased more dramatically to about 14% of the normal value. Breakdown of PCr led to a rise in myocardial Cr content. The total Cr pool was decreased, however, by 20% compared to the initial level. This loss could be induced by diffusion of intracellular Cr to the interstitial space and its subsequent removal by collateral blood flow.

This is confirmed by dialysate Cr profile obtained during prolonged ischemia (Fig. 2b). As in the first series of experiments dialysate Cr reached the maximal values by 60 min of ischemia and then decreased. Such increase in interstitial Cr probably reflects rapid breakdown of PCr during the initial period of ischemia and the release of Cr from cardiomyocytes. As compared to reperfusion, the release of accumulated Cr from cells during prolonged ischemia occurred more slowly. Thus, by the end of 4 hr of ischemia, the dialysate Cr remained 3-fold higher than the normal value, while it decreased to the initial level during the first hour of reperfusion (Figs. 2a, b). This observation probably explains the more pronounced loss

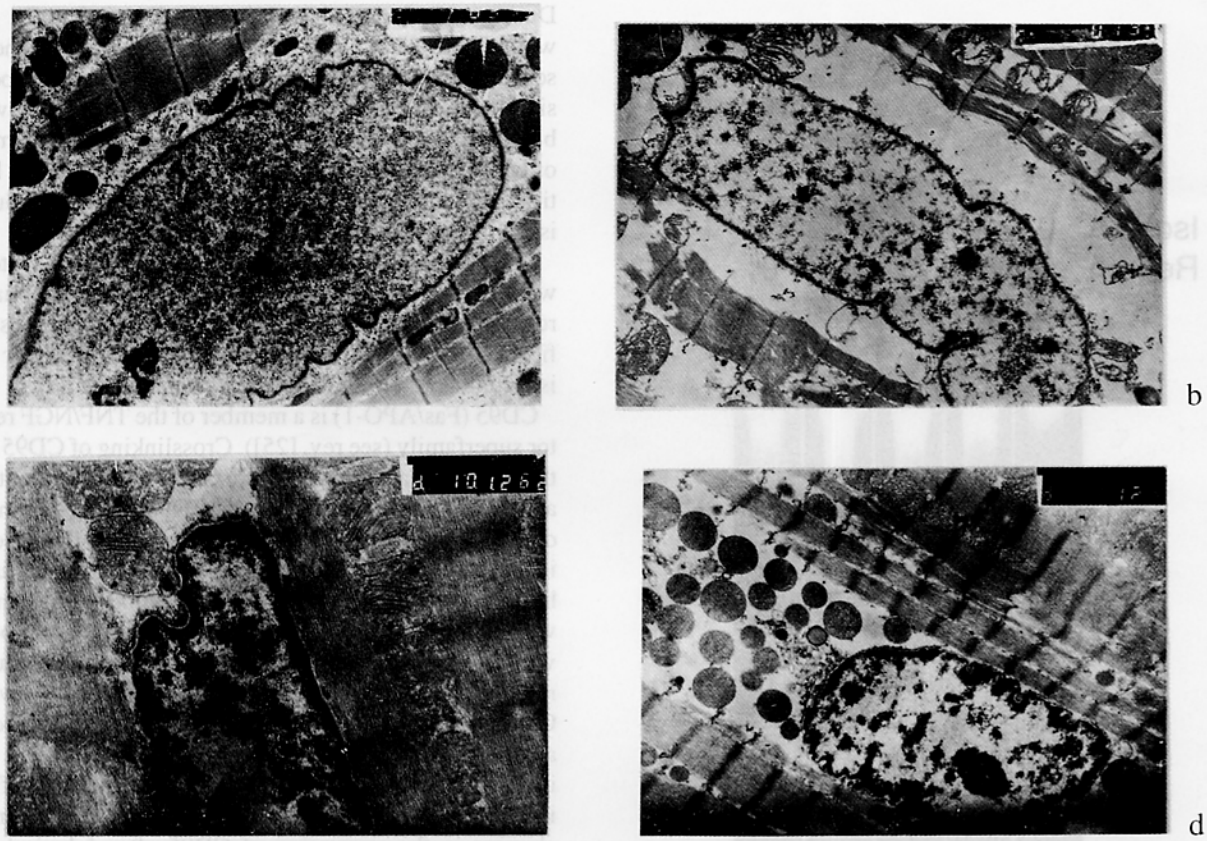

Figure 3. Transmission electron microscopy of normal (a), necrotic (b), apoptotic (c) myocardial cells and apoptotic cells with secondary necrotic changes (d). After 1 hr ischemia and 3 hr reperfusion sections prepared from non-ischemic (a) and ischemic areas (b-d) were analyzed.

of the total Cr from myocardial tissue during reperfusion in spite of better recovery of PCr.

Lactate concentration in the interstitial fluid also increased rapidly in ischemic region during the initial 60 min of experiment and reached the maximal concentration of about 2.7 mM. This value is about 10 times higher than the initial baseline lactate levels before ischemia and the average lactate concentration in the dialysate of the nonischemic region. During next 3 hr of ischemia, lactate concentration decreased gradually (remaining 4 times higher than the initial level by the end of ischemic period). The kinetics of dialysate lactate most likely corresponds to an activation of anaerobic glycolysis during the initial phase of ischemia and the subsequent reduction of glycolytic flux with an increase of ischemia duration. These data are in good accordance with an increased lactate content found in ischemic region compared to its concentration in nonischemic area.

Electron microscopy

Normal cardiomyocytes from non-ischemic areas (Fig. 3a) and those from ischemic regions were analyzed by EM after 1 hr ischemia and 3 hr reperfusion (Fig. 3b-d). In all of our experiments we found three different types of morphological changes of cardiomyocytes after ischemia and reperfusion: (i) Significant destruction of cytoplasm and organelles with some secondary changes in nuclear structure but without prominent chromatin condensation which is characteristic of necrotic cell death (Fig. 3b). (ii) Marginal and central chromatin condensation in nuclei of cardiomyocytes with almost intact cytoplasm , mitochondria and other cellular organelles (Fig. 3c). These changes indicate the activation of the apoptotic pathway although chromatin condensation is less intensive compared to the typical apoptotic pattern of thymocyte nuclei. (iii) Some cells have apoptotic morphology of nuclei with initial necrotic changes in cytoplasm (Fig 3d). Thus ischemia and reperfusion in the dog heart induce both necrosis and apoptosis of cardiomyocytes and at least some apoptotic cells undergo secondary necrotic changes. In this situation, the quantitation of cells dying by a necrotic or apoptotic mechanism based on electron microscopy seems unreasonable.

DNA degradation

DNA electrophoresis reveals appearance of chromatin degradation products (nucleosomes and their oligomers)

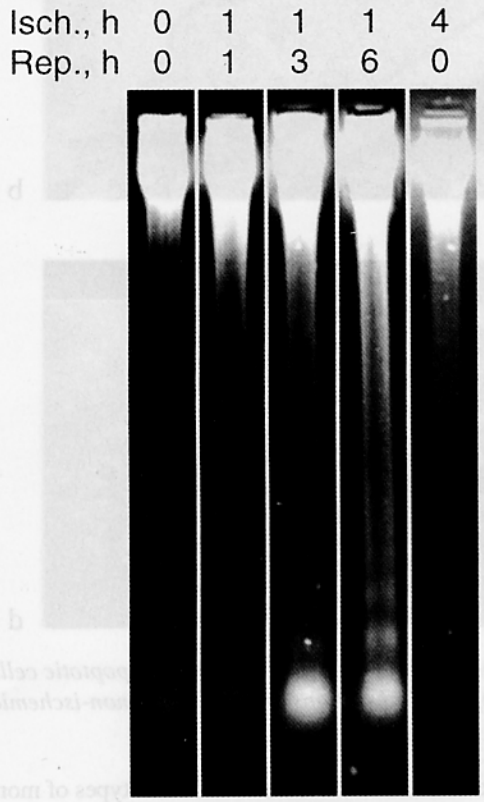

Figure 4. Agarose electrophoresis of DNA isolated from normal dog heart, after ischemia or after ischemia with subsequent reperfusion.

after one hour reperfusion. By 3 and 6 hrs their amount increases (Fig. 4). The quantity of chromatin degradation products in the "intact" zone is much lower (data not shown). 4 hr of ischemia without reperfusion does not produce a nucleosomal ladder.

Gene expression

It is well established that in different systems apoptosis is positively and negatively regulated by products of specific genes. We analyzed the effect of ischemia and reperfusion on expression of some of these genes in cardiac cells.

Bcl-2 and bcl-x prevent apoptotic cell death induced by various factors, while other members of bcl-2 family such as bax and bak possess pro-apoptotic activity [31]. RNA was isolated from cardiac cells after ischemia and reperfusion and also from untreated dog heart and gene expression was analyzed by Northern blotting. We could not detect bcl-2 mRNA in any of these RNA samples (data not shown) which is in agreement with published data [9]. During reperfusion the myocardium is infiltrated with white blood cells many of which express bcl-2. The absence of detectable signal from bcl-2 mRNA after reperfusion indicates that the contribution of mRNA from white blood cells into cardiac RNA is low. Three other members of bcl-2 family, bcl-x, bax, and bak are expressed in heart tissue but the level of expression was not changed during ischemia and reperfusion (Fig. 5a-c).

Calpain is a calcium-activated neutral cysteine protease, whose activity increases in cardiac cells upon ischemia and reperfusion [33, 34]. Nonetheless we could not find significant changes in the amount of calpain mRNA either after ischemia or after subsequent reperfusion (Fig 5d).

CD95 (Fas/APO-1) is a member of the TNF/NGF receptor superfamily (see rev. [25]). Crosslinking of CD95 with the specific Fas ligand or anti-CD95 antibodies initiates apoptosis in different cell types. CD95 expression in cardiac cells is shown although the function of this receptor in cardiomyocytes is not clear. Since during reperfusion heart tissue is infiltrated by white blood cells some of which can express CD95-ligand this receptor can be involved in initiation of apoptosis of cardiac cells during reperfusion. Recently Tanaka et al. found higher level of CD95 (Fas/APO-1) mRNA in rat neonatal cardiomyocytes after prolonged hypoxia [27]. Fig. 5e, f shows that 1 hr ischemia and 3 or 6 hr reperfusion cause a significant increase in the amount of CD95 mRNA. There were no changes in the expression of CD95 after 1 hr ischemia

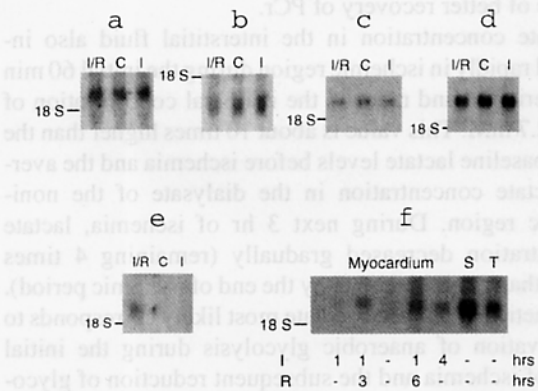

Figure 5. Northern blot analysis of gene expression in control myocardium (C), after 1 hr ischemia (I) or after 1 hr ischemia and reperfusion (I/R). Probes: a, bak; b, bax; c, bcl-x; d, calpain;, e and f, CD95. a-d and f, total RNA; e, poly(A)+RNA. a-e: I/R, 1 hr ischemia and 3 hr reperfusion. f: time of ischemia and reperfusion is indicated in figure; S and T, dog spleen and thymus RNA, respectively.

alone and a slight increase of CD95 mRNA after 4 hr ischemia.

Discussion

We subjected canine heart in situ to regional ischemia and subsequent reperfusion or to long-term regional ischemia to find out: (i) the type of cell death characteristic of each treatment, and (ii) possible relationship between energy production and the mechanism of cell death.

A combination of electron microscopy and DNA gel electrophoresis showed that ischemia and reperfusion induce both necrotic and apoptotic death of myocardial cells. Nucleosomal DNA laddering has not been observed after prolonged ischemia alone indicating that necrosis mainly occurs during this treatment. These results are in good agreement with data obtained in different systems both *in vivo* and *in vitro* [7, 10, 32]. It is not clear from the data presented if additional cell injury that occurs during reperfusion is important for the triggering of apoptosis, or if apoptosis is mainly initiated during ischemia but and partial restoration of normal metabolic processes is necessary for cells to proceed the apoptotic pathway. The second suggestion seems more probable although death of a portion of the cardiac cells can be initiated during reperfusion.

Metabolic disturbances were assessed by tissue content of high energy phosphates, Cr and lactate at the end of experiment and by temporal profile of interstitial lactate and Cr during ischemia and reperfusion. This approach is promising diagnostic tool for monitoring myocardial metabolism complementary to analysis of sequential biopsies from normal and ischemic regions, since it avoids additional damage of myocardial tissue and microcirculation. Another advantage of this technique is that serial probes of myocardial interstitial fluid containing Cr, the degradation product of PCr, and lactate, a by-product of glycolysis, can be obtained simultaneously from ischemic and nonischemic regions. Unlike lactate, readily diffuses across cell membranes, the Cr efflux from myocytes occurs most likely due to sarcolemmal disruption [22]. Thus, increased Cr concentration in the dialysate may also indicate the increased membrane permeability and the loss of cell volume regulation.

The results we obtained are in good agreement with previous reports describing effects of regional ischemia and reperfusion in canine heart on myocardial metabolism [12, 13, 22, 24, 32]. The ischemic/reperfusion injury model that used in our study demonstrates that AN pools which are depleted from the heart during 1 hr ischemia are only partially restored during 6 hr reperfusion (Fig. 1). This finding is consistent with a very low rate of *de novo* synthesis of AN and an inability of salvage pathways to restore the AN pool to the normal values [21]. A more rapid resynthesis of PCr observed after 3 hr reperfusion, despite the reduced intracellular ATP concentration, indicates recovery of energy production in mitochondria by oxidative phosphorylation. This is in accordance with a parallel decrease of interstitial lactate to the initial level (Fig. 2a). The normalization of lactate level can be also attributed to restoration of blood flow in postischemic region to values sufficient to clear accumulated lactate. These results show that if the energy source is necessary for the apoptotic pathway to proceed PCr can be used in cardiac cells.

More dramatic disturbances in myocardial metabolism are induced by 4 hr regional ischemia (Table 1; Fig. 2b). In these experiments about 70 and 86% of ATP and PCr respectively were depleted in ischemic region. ADP and AMP levels also were at least two times lower. Myocardial lactate levels were more than 20 times higher compared to control values. Severe membrane damage was confirmed by increased Cr level in the interstitial fluid combined with a significant loss of the total Cr from ischemic tissue. These metabolic changes correspond to the irreversibly injured cells [21] and indicate once more that prolonged ischemia induces primarily necrotic cell death.

Both ischemia and reperfusion had no effect on the expression of bcl-x, bak, bax, and calpain genes, which are involved in the apoptotic pathway (Fig. 5a-d). Thus, the activity of these genes is not regulated at the transcriptional level. However it does not mean that the respective proteins do not participate in apoptosis induced in this system. For example, the significant induction of calpain activity in myocardial cells by ischemia and reperfusion is well documented [33, 34], and calpain inhibitors prevent cardiomyocyte death [30] but the calpain induction probably occurs at the posttranscriptional level [34]. It is interesting that from this viewpoint the response of neuronal cells on ischemia and reperfusion is different. There are data indicating the induction of bax and inhibition of bcl-2 and bcl-x expression in neurons dying by apoptosis during ischemia and reperfusion [15].

Ischemia and reperfusion induced expression of CD95 in dog cardiac cells (Fig. 5e, f) as well as in rat neonatal cardiomyocytes after prolonged hypoxia [27]. Induction of

Table 1. Effect of 4 hr ischemia on the content of adenine nucleotides, creatine, phosphocreatine and lactate in canine heart.

Metabolite	Non-ischemic zone (A)	Ischemic zone (B)	(B:A) x 100%
ATP	28.4 ± 1.55	8.72 ± 2.56*	30.7
ADP	4.1 ± 0.6	2.45 ± 0.26	59.8
AMP	0.81 ± 0.1	0.32 ± 0.03*	39.5
ΣAN	33.32 ± 0.91	10.49 ± 2.7*	31.5
PCr	33.89 ± 2.21	4.87 ± 1.36*	14.4
Cr	66.6 ± 1.76	79.47 ± 4.56*	119.3
ΣCr	100.48 ± 3.97	84.33 ± 5.66	83.9
Lactate	1.58 ± 0.57	34.28 ± 5.97*	2169.6

Values are expressed in μmol/g dry weight.
* - $P < 0.05$ versus non-ischemic region.

CD95 has been also described in neurons after ischemia and reperfusion *in vivo* [19]. These data indicate the possible involvement of CD95 receptor in reperfusion-induced death of cardiomyocytes and other cell types. However, it is not clear which form of CD95 protein is synthesized in control cardiomyocytes and during ischemia and reperfusion. Existence of multiple forms of this molecule as a result of alternative splicing has been shown in different systems [3, 4]. Some of these CD95 receptors are exposed on cell surface and can bind CD95-ligand but they can not initiate the apoptotic cascade because of deletion of the death domain [3]. Other CD95 molecules do not have transmembrane or both transmembrane and cytoplasmic domains and can be secreted as soluble proteins capable to compete with CD95-bearing cells for the binding of CD95 ligand [4].

Taken together the data presented in combination with results published in literature suggest the following model of cell injury during ischemia and reperfusion. Damages induced during ischemia are capable of initiating apoptosis but if ischemia is long and the amount of accumulated damages is too high, cells die by necrosis. Another reason for mainly necrotic cell death during prolonged ischemia is the lack of energy production and, possibly, of some other metabolic processes for the apoptotic pathway initiated by ischemia to proceed. Thus, during reperfusion cells die by apoptosis because reperfusion creates conditions for the apoptotic pathway which has been initiated during ischemia. Without reperfusion these cells die by necrosis.

Of course the scheme proposed is a simplification of real events. Reperfusion *in vivo* leads to the appearance of additional factors which can induce cell death, e.g. complement activation, infiltration with neutrophils etc. (see rev. [6]). Induction of CD95 gene shows one more possible way for initiation of cardiomyocyte death during ischemia. Regardless of the nature of the triggering signals, the involvement of apoptosis in ischemia/reperfusion-induced heart injury indicates the possibility of new ways for the treatment of acute myocardial infarction based on the modification of the apoptotic pathway.

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