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Prevention of rat neonatal cardiomyocyte apoptosis induced by simulated *in vitro* ischemia and reperfusion

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Abstract

Apoptosis, or programmed cell death, is an active metabolic response to physiological signals or exposure to cytotoxic agents. Recent evidence has shown that the cell death response can be modified by agents presumed to be unrelated to the initial signal, but capable of interfering with the molecular mechanisms of the apoptotic pathway progression. Here we show the results of investigations on the use of a phospholipid-based pharmaceutical preparation for suppression of myocardial damage. First, we show that serum or serum/glucose deprivation, *in vitro* ischemia with subsequent simulated reperfusion, inhibition of protein synthesis, and treatment with ceramide, staurosporine, adriamycin, cis-platinum and menadione induce apoptotic death in a primary culture of rat neonatal cardiomyocytes. Then we demonstrate that a mixture of specific phospholipids, which has been originally purified from soy flour on the basis of its anti-apoptotic activity, prevents cardiomyocyte death induced by serum or serum/glucose deprivation, by ischemia with subsequent simulated reperfusion, and by ceramide, but not by other cytotoxic treatments. This suggests that ceramide, a lipid secondary messenger which triggers apoptosis induced by some cytotoxic agents, may be involved in the process of signaling ischemia/reperfusion induced apoptotic death of cardiomyocytes. These results further demonstrate that an active pharmaceutical preparation for the suppression of cardiomyocyte death can be formulated based upon a novel strategy of apoptosis modification.

Keywords: apoptosis; cardiomyocytes; phospholipids; ceramide; ischemia; reperfusion

Abbreviations: PA, phosphatidic acid; PI, phosphatidylinositol; LPA, lysophosphatidic acid; LPI, lysophosphatidylinositol; LPC, lysophosphatidylcholine; SLF, a lipid fraction purified from soy flour; ROM, reconstituted optimized mixture of phospholipids; DAG, diacylglycerol

Introduction

The most important consequence of acute myocardial ischemia is the death of individual cells which leads to organ dysfunction. Early reperfusion decreases heart damage but at the same time the massive death of cells that remain viable by the end of ischemia occurs with the restoration of blood flow (Karmazin, 1991; Fox, 1992).

Two forms of cell death, necrosis and apoptosis, have been described and are now intensively and widely investigated (Kerr *et al*, 1972; Umansky, 1996; Vaux and Strasser, 1996). Necrosis is generally considered to be a result of severe irreversible cell damage. It is characterized by early swelling of the cell and its cytoplasmic organelles with subsequent rupture of the outer membrane. Apoptosis is a much more widespread phenomenon and is an important component of normal development, tissue homeostasis, as well as the pathogenesis of severe diseases. It is usually initiated by specific signals, but also can be induced by mild, non-catastrophic cell injury. Apoptosis is characterized by morphological changes marked by reduction of cell volume and shrinkage of organelles, early chromatin condensation and margination, and membrane blebbing. Specific internucleosomal DNA fragmentation is a hallmark for many, but notably not all, instances of apoptotic death. Several genes and gene families involved in signal transduction and modulation of the apoptotic pathway are already described (Umansky, 1996; Vaux and Strasser, 1996). By definition, necrosis can be prevented only by decreasing cell injury. Whereas, apoptosis is active cell response to a physiological or damaging signal, that can be inhibited by interfering with metabolic processes involved in the apoptotic pathway, without any requirement to prevent cell damage or promote its repair. Prevention of apoptosis by upregulation of bcl-2 and bcl-x expression or by inhibitors of ICE-like proteases are typical examples of this approach to modification of apoptotic cell death (Umansky, 1996; Vaux and Strasser, 1996; Nunez *et al*, 1994; Whyte, 1996).

Recent data indicate that apoptosis plays a significant role in heart injury induced by ischemia and subsequent reperfusion (Gottlieb *et al*, 1994; Ito *et al*, 1995; Umansky *et al*, 1995, 1996). Severe cell damage during prolonged ischemia appears to result in the necrotic death of myocardial cells. However, if ischemia is relatively limited in extent and duration, the apoptotic pathway is initiated and the restoration of blood flow creates conditions for ongoing apoptosis. IGF and calpain inhibitors, which prevent apoptosis in different systems, also inhibited apoptosis in cardiomyocytes following ischemia and reperfusion both *in vivo* and *in vitro* (Umansky *et al*, 1995; Buerke *et al*, 1995).

Recently, we have purified from soy flour a lipid fraction (SLF) which is capable of preventing apoptotic death

induced in C3H-10T1/2 mouse embryonic cells by serum deprivation (Tomei *et al*, 1993). SFL is a mixture of phospholipids, comprised of phosphatidic acid (PA), phosphatidylinositol (PI), lysophosphatidic acid (LPA), lysophosphatidylinositol (LPI), and lysophosphatidylcholine (LPC). A series of synthetic mixtures of these phospholipids was analyzed in the above mentioned cell system. The highest anti-apoptotic activity was observed with the mixture of PA : PI : LPA : LPI : LPC with the ratio of 10 : 10 : 8 : 2 : 4, respectively, and referred to as ROM (*r*econstituted *o*ptimized *m*ixture).

Experiments were designed to determine the ability of SFL and ROM to inhibit apoptosis in primary cultures of rat neonatal cardiomyocytes induced by different treatments. The results demonstrated that various treatments, including serum and serum/glucose deprivation, simulated ischemia/reperfusion, as well as exposure to adriamycin, staurosporine, cis-platinum, and ceramide induce the apoptotic

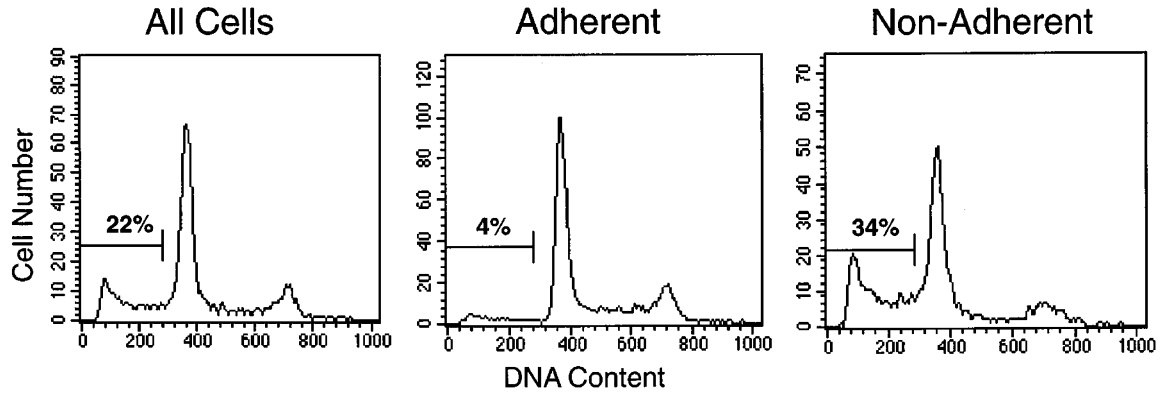

Figure 1 DNA histograms of rat neonatal cardiomyocytes after ischemia and reperfusion. Numbers indicate per cent of <2C DNA cells.

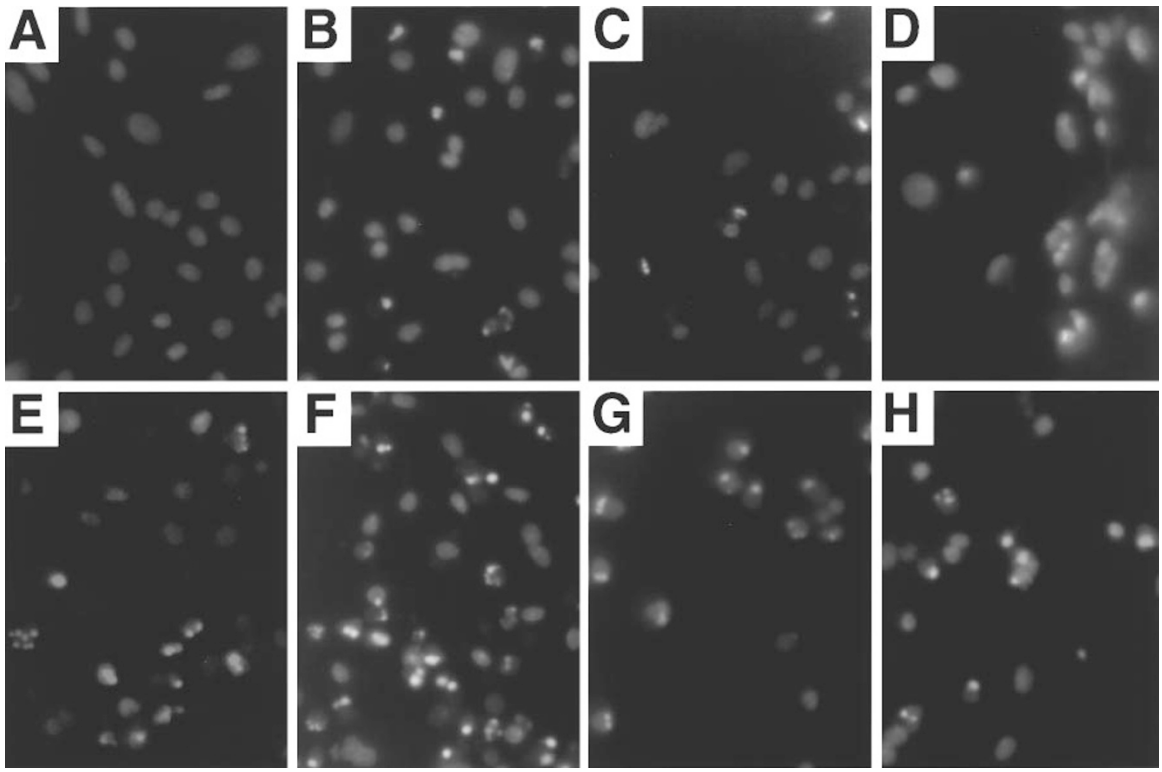

Figure 2 Luminescent microscopy of rat neonatal cardiomyocytes after different treatments. (**A**) control; (**B–H**) cells were subjected for 24 h to serum deprivation (**B**) or treated with 30 ng/ml of staurosporine (**C**), 15 μM menadione (**D**), 75 μmol cisplatin (**E**), 10 μg/ml of cycloheximide (**F**), 3 μg/ml of puromycin (**G**) or 12.5 μmol C2-ceramide (**H**).

death of cardiomyocytes. Both SFL and ROM inhibit apoptosis induced by serum and serum/glucose deprivation, simulated ischemia/reperfusion and ceramide but have no effect on apoptotic cell death initiated by either adriamycin, staurosporine, cis-platinum.

Results

Cardiomyocyte death induced by ischemia/reperfusion

Earlier, using morphological criteria, flow cytometry and DNA electrophoresis, we have shown that ischemia (oxygen, serum and glucose deprivation) with subsequent simulated reperfusion (readdition of oxygen, serum and glucose) of rat neonatal cardiomyocytes induces both necrotic and apoptotic cell death (Umansky *et al*, 1995). DNA degradation is a widely used hallmark of apoptosis and, thus, apoptotic cells can be measured by flow cytometry by determining the proportion of cells that contain less than 2C DNA (Afanasyev *et al*, 1986, 1993). Figure 1 shows that about 34% of dead non-adherent cells contain less than 2C DNA and are presumed to be apoptotic based on this criterion. To model ischemia in these experiments we subjected cells to simultaneous oxygen, serum and glucose deprivation and then restored all these components to simulate reperfusion. Apoptotic cell death was observed predominately during reperfusion (Umansky *et al*, 1995), which is in accord with data obtained in intact myocardium *in vivo* (Gottlieb *et al*, 1994; Umansky *et al*, 1996). Following 24 h serum deprivation, approximately 20% cell death was observed, whereas, 30–40% cell death was observed in combined serum and glucose deprivation (data not shown). Nuclear morphology (Figure 2B) and internucleosomal DNA fragmentation (Figure 3, lane 1) revealed that cell death was primarily apoptotic. Ischemia with the subsequent reoxygenation but in the continued absence of serum and glucose, resulted in slightly more dead cells than in the presence of a normal glucose level (data not shown). However, the number of apoptotic cells, containing less than 2C DNA, was substantially lower after reoxygenation (Figure 4), suggesting that necrotic cell death was encountered at significantly higher rate in the absence of restored energy production. These results are in a good agreement with *in vivo* data which indicate that the energy sources, in particular creatine phosphate, are necessary for the apoptotic cell death (Umansky *et al*, 1996).

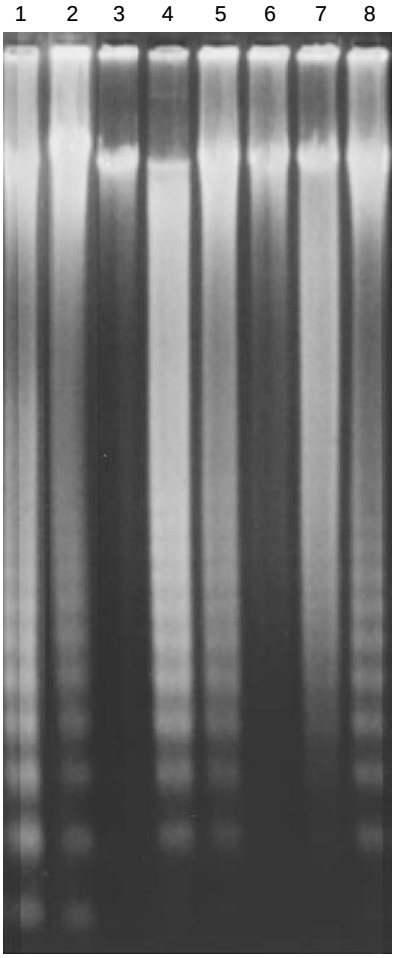

Figure 3 Agarose gel electrophoresis of DNA isolated from rat neonatal cardiomyocytes after 24 h incubation in different conditions. (**1**) serum and glucose deprivation; (**2**) 10 μmol C2-ceramide; (**3**) control; (**4**) 3 μg/ml of puromycin; (**5**) 10 μg/ml of cycloheximide; (**6**) 15 μmol menadione; (**7**) 5 μg/ml of adriamycin; (**8**) 100 ng/ml of staurospine.

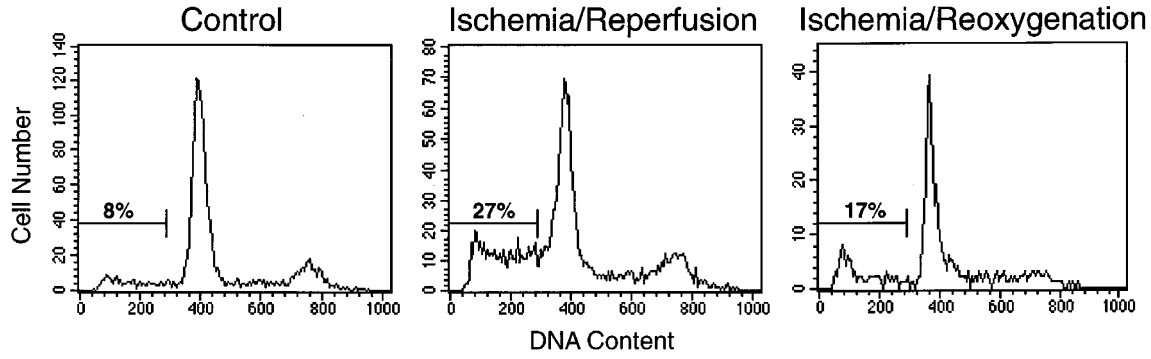

Figure 4 DNA degradation in rat neonatal cardiomyocytes after ischemia with the subsequent reperfusion or reoxygenation. Numbers on DNA histograms indicate per cent of $<$2C DNA cells.

Cardiomyocyte death induced by divers pro-apoptotic agents

Myocardial cell apoptosis has become a subject of study only recently. Little or no data has been published regarding the mechanism of cardiomyocyte death induced by divers cytotoxic stimuli. Using rat neonatal cardiomyocytes, we analyzed the cytotoxic effects of agents shown to have different mechanisms of action (Figure 5). Agents studied were: adriamycin, a chemotherapeutic drug thought to involve topoisomerase II inhibition and to generate reactive oxygen species (Olson and Mushlin, 1990; Duran *et al*, 1966), staurosporine, a nonspecific protein kinase inhibitor capable of inducing apoptotic cell death in many systems (Tamaoki *et al*, 1986); cis-platinum, believed to produce DNA–DNA and DNA-protein crosslinking (Zamble and Lippard, 1995); puromycin and cycloheximide, protein synthesis inhibitors, and menadione which leads to generation of reactive oxygen species (Thor *et al*, 1982). Adriamycin has been of special interest because it is known to produce severe cardiotoxicity in humans (Olson and Mushlin, 1990). All these compounds have been found to be toxic for cardiomyocytes (Figure 5) in dose ranges similar to those in other cell systems, and to induce the morphological changes of nuclei characteristic of apoptosis (Figure 2C–G). With the exception of menadione, all agents tested also induce internucleosomal DNA fragmentation (Figure 3, lanes 4–8). It is interesting that in the same cell type some agents induce DNA fragmentation and chromatin condensation and menadione induces only the latter. That means that, as in some other systems (Tomei *et al*, 1993; Cohen *et al*, 1992), in cardiomyocytes chromatin condensation is not dependent on the internucleosomal DNA cleavage.

Effect of SFL and ROM on cardiomyocyte death induced by different agents

The ability to protect C3H 10T1/2 cells from serum deprivation induced apoptosis has been used as a criterion for the purification of SFL. Therefore, we started the investigation of the effect of SFL and ROM on cardiomyocyte death by using a serum deprivation assay. Both SFL and ROM inhibited the death of rat neonatal cardiomyocytes induced by serum and serum/glucose deprivation (Figure 6A and B). Protection has been evaluated by measurement of the amount of adherent cells (Figure 6), by analysis of cell morphology (Figure 7) and by inhibition of internucleosomal DNA fragmentation (Figure 8, lanes 2 and 3). Because of the similar effect of SFL and

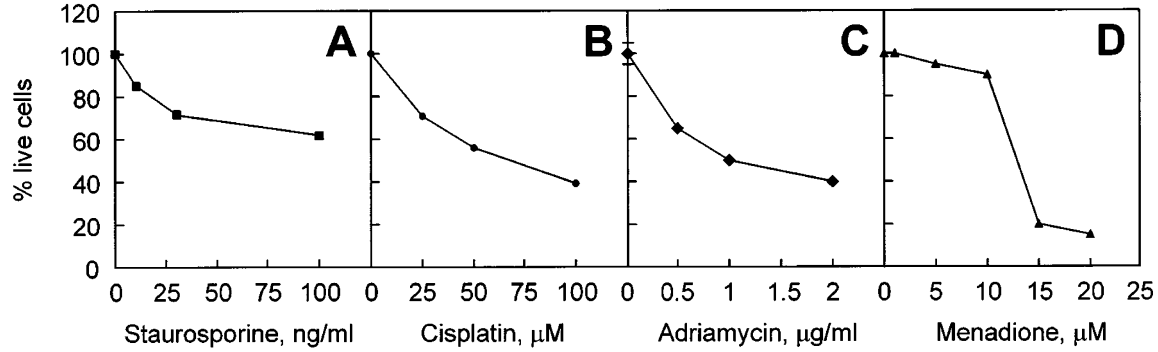

Figure 5 Cardiomyocyte death induced by different cytotoxic agents. Cardiomyocytes were treated with different agents at concentrations indicated, and 24 h later cell death was measured by the decrease in the amount of adherent cells as described in Materials and methods.

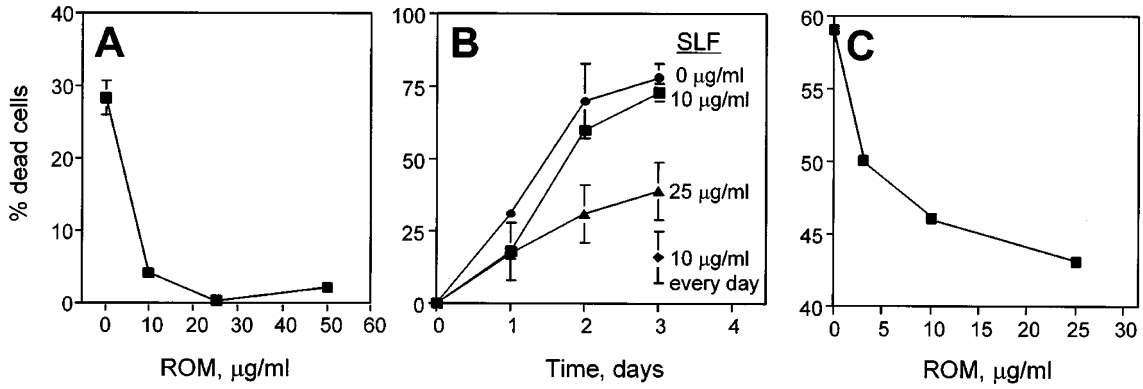

Figure 6 Prevention of cardiomyocyte death by SLF or ROM. (**A**) 24 h serum deprivation in the absence or presence of ROM; (**B**) kinetics of cell death in a serum and glucose free medium. SLF was added once at the beginning of experiments (0, 10 or 25 μg/ml) or each 24 h (10 μg/ml). (**C**) after 8 h ischemia glucose and ROM were added and the cultures were returned to a normal oxygen gas overlay for 16 h.

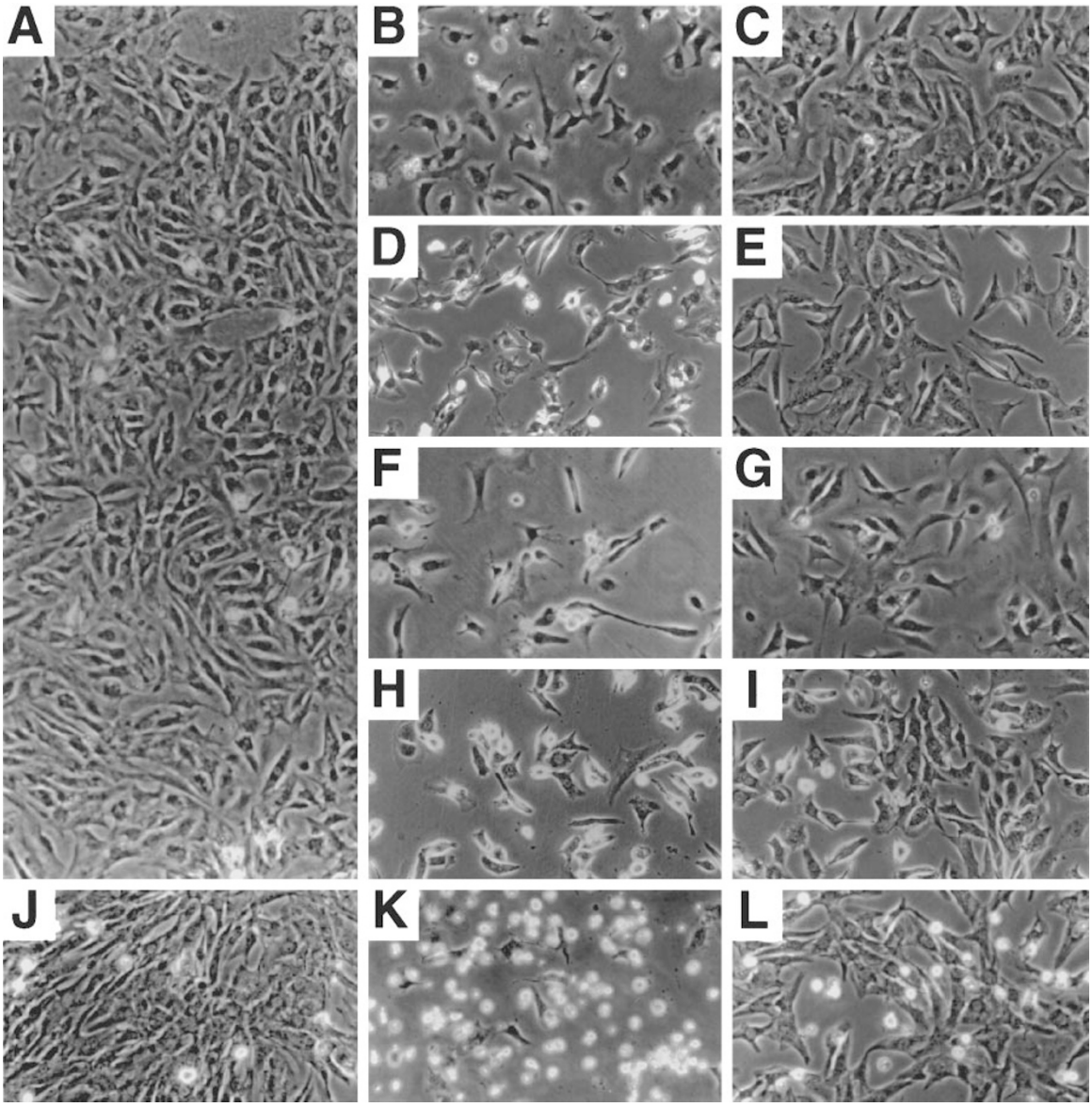

Figure 7 Light microscopy of rat neonatal cardiomyocytes after different treatments in the absence or presence of ROM or SLF. (**A–I**) and (**J–L**) 24 and 72 h incubation respectively. (**A**) and (**J**) controls; (**B**) and (**C**) serum deprivation; (**D,E,K**) and (**L**) serum and glucose deprivation; (**F**) and (**G**) ischemia and reperfusion; (**H**) and (**I**) cells were treated with 10 μmol C2-ceramide. (**B,D,F,H**) and (**K**) no additions; (**C,E,G**) and (**I**) 10 μg/ml of ROM were added at the beginning of the experiment; (**L**) 10 μg/ml SLF were added each day.

ROM in this and other systems, we continued to use ROM mainly in following studies.

As will be explained below, experiments with ROM required simulated reperfusion to be provided in the presence of oxygen and glucose, but in the absence of serum. Figure 6C and 7F, G shows the dose dependent protection of cardiomyocytes by ROM added after ischemia. 25 μg ROM per ml causes about 25% reduction in the amount of dead cells. Taking into account the permeability data (Umansky *et al*, 1995) that shows that about 20–30% of cells die during 8 h ischemia, ROM prevents the death of 40–50% of the cardiomyocytes which die during simulated reperfusion. We found that by varying the time of ischemia, we could modify the amount of cardiomyocytes that die by the end of reperfusion and the proportion of cells which die during ischemia and reperfusion. As ischemia was shortened, the amount of cells dying by the beginning of simulated reperfusion was lessened and the portion of cells which die during reperfusion became greater. If, in the absence of ROM, the amount of dead cells was 60%, 49% and 38%, the amount of dead cells decreased by 26.7%, 40.8% and 50%, respectively, with the addition of 5 μg ROM. Thus, in agreement with our predictions, protection with ROM added after ischemia was more pronounced when the total amount of dead cells without ROM was lower. Addition of ROM before ischemia is not more protective than ROM treatment during simulated reperfusion only. Thus, ROM does not decrease damage induced by ischemia and does not prevent the

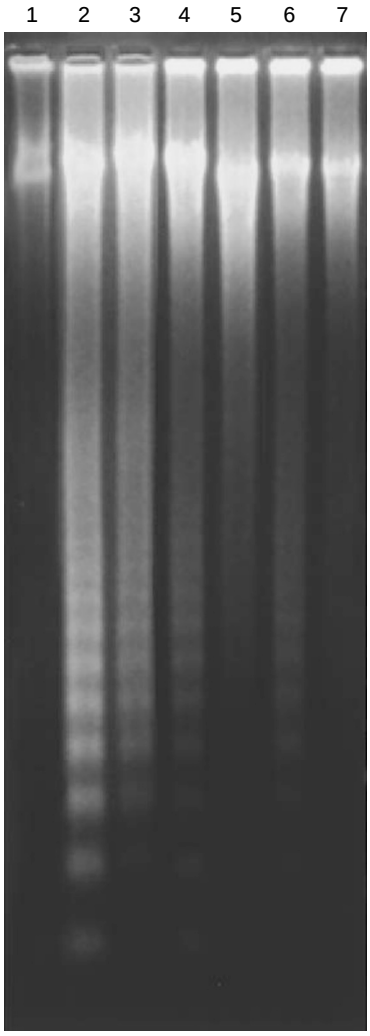

Figure 8 Agarose gel electrophoresis of DNA isolated from rat neonatal cardiomyocytes after different treatments in the absence or presence of 10 μg/ml of ROM. (**1**) control; (**2** and **3**) serum and glucose deprivation in the absence and presence of ROM, respectively; (**4** and **5**) 10 μmol C2-ceramide in the absence and presence of ROM, respectively; (**6** and **7**) 7.5 μmol C2-ceramide in the absence and presence of ROM, respectively.

necrotic cell death that occurs before the beginning of reperfusion. Taken together, these data demonstrate the ability of SLF and ROM to prevent the apoptotic death of cardiomyocytes induced by serum or serum/glucose deprivation and by simulated ischemia/reperfusion.

To investigate whether ROM possesses anti-apoptotic activity against other cytotoxic treatments it was added to cardiomyocytes together with cycloheximide, puromycin, adriamycin, staurosporine, cis-platinum, or menadione. In all these cases we could not find a significant decrease in the amount of dead cells (data not shown).

ROM blocks the apoptotic pathway initiated by ceramide

Despite of the similarity of the final stages of apoptosis induced by different agents initial signaling mechanisms depend upon the nature of physiological or damaging cytotoxic stimuli and are not well understood. During the last few years, the involvement of ceramide, a lipid secondary messenger generated from sphingomyelin by acidic and neutral sphingomyelinases, in triggering apoptosis has been intensively investigated (Hannun and Obeid, 1995; Kolesnick and Fuks, 1995). Ceramide is involved as a secondary messenger in the transduction of pro-apoptotic signal from the TNF-receptor and CD95 (Fas/APO-1) (Dbaibo *et al*, 1993; Cifone *et al*, 1993; Kolesnick and Golde, 1994; Tepper *et al*, 1995), as well as in the induction of apoptosis by γ-irradiation (Haimovitz-Friedman *et al*, 1994), serum deprivation (Esteve *et al*, 1995; Jayadev *et al*, 1995) and antibiotic daunorubicin (Bose *et al*, 1995). Soluble synthetic ceramides are also capable of inducing apoptotic death in different cell types (Obeid *et al*, 1993).

Figure 9A shows that synthetic C2-ceramide kills rat neonatal cardiomyocytes in a dose dependent manner. Cell morphology (Figure 2H), accumulation of cells containing less than 2C DNA (data not shown) and internucleosomal DNA fragmentation (Figure 3, lane 2) demonstrate that the cells are dying by apoptosis.

ROM effectively prevents ceramide-induced cardiomyocyte death (Figure 7H,I, 11B) and internucleosomal DNA

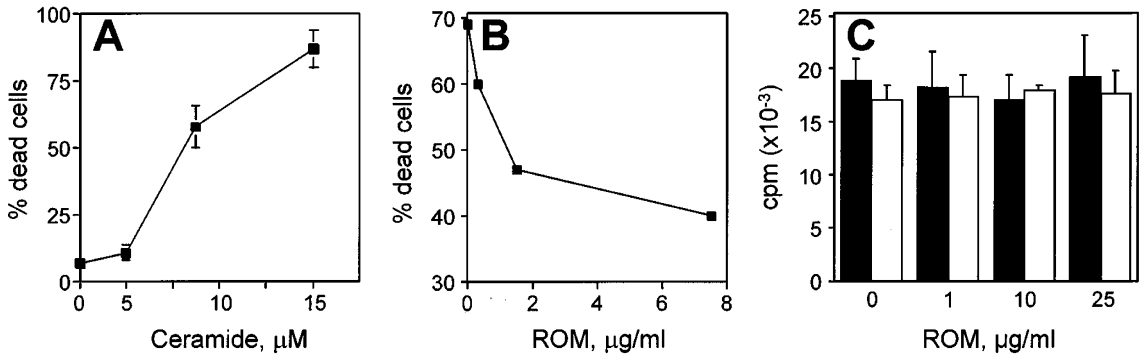

Figure 9 Ceramide-induced death of cardiomyocytes and its prevention by ROM. (**A**) dose dependence of C2-ceramide-induced cardiomyocyte death; (**B**) cells were treated with 15 μmol ceramide in the presence of the indicated amounts of ROM. (**C**) 10 μmol [^{3}H]C2-ceramide (a kind gift of Dr Alicja Bielawska) and different amounts of ROM were added to cardiomyocytes in RPMI. After 1 h (black bars) or 3 h (white bars) incubation cells were washed five times with RPMI, trypsinized, collected onto filters and their radioactivity was measured.

degradation (Figure 8, lanes 4–7). Since ROM is a mixture of phospholipids and forms micelles in water solutions, one can suggest that ceramide is sequestered in these complexes, thus preventing the interaction of ceramide with cardiomyocytes. To check this possibility, we incubated cardiomyocytes with ^{3}H-labeled ceramide in the presence of increasing concentrations of ROM and measured the amount of ceramide bound to cells after 1 and 3 h (Figure 9C). In the absence of ROM, cadiomyocytes bound about 25–30% of added ceramide. ROM had no effect on ceramide binding even at doses much higher than ones which protect cardiomyoctes from ceramide toxicity.

Discussion

Now it is well established that ischemia and reperfusion cause necrotic and apoptotic death of cardiomyocytes both *in vitro* and *in vivo* (Gottlieb *et al*, 1994; Ito *et al*, 1995; Umansky *et al*, 1995, 1996). Data presented show that other cytotoxic agents with different mechanisms of action also induce apoptosis in myocardial cells. Particularly, this result is important to interpret the effect of adriamycin on cardiac cells since its use as a chemotherapeutic agent is limited by its cardiotoxicity. The understanding of the mechanisms of cardiomyocyte death is not a purely theoretical problem. As we have discussed above, the approaches for the modification of necrotic and apoptotic cell death are different. To inhibit necrotic death we can only prevent the cell injury and early reperfusion and the use of antioxidants are the part of this therapeutic strategy. However, apoptosis can be also prevented by the interfering with signaling mechanisms and the apoptotic pathway.

In this study we employed this approach to prevent cardiomyocyte death by using a new anti-apoptotic compound originally purified from soy flour on the basis of its anti-apoptotic activity. Both the preparation obtained from flour and the reconstructed mixture with optimized ratio of five phospholipids were used and quite similar results have been obtained. SLF and ROM inhibit cardiomyocyte death induced by serum and serum/glucose deprivation, and partially prevent simulated ischemia/reperfusion-induced death of cardiomyocytes but they have no effect on apoptosis induced by inhibitors of protein synthesis, adriamycin, menadione, cis-platinum, and staurosporine.

Apoptosis can be triggered by different mechanism, and the initial stages of apoptosis can vary depending on the nature of the pro-apoptotic signal. Recently, ceramide was found to be a secondary messenger in apoptosis induced by signaling through the TNF-receptor (Dbaibo *et al*, 1993; Kolesnick and Golde, 1994) and CD95 (Fas/APO-1) (Cifone *et al*, 1993; Tepper *et al*, 1995), by irradiation (Haimovitz-Friedman *et al*, 1994), serum deprivation (Esteve *et al*, 1995; Jayadev *et al*, 1995) and some other treatments. Accumulation of ceramide is usually a result of sphingomyelinase activation which leads to hydrolysis of sphingomyelin into ceramide and phosphocholine but the induction of ceramide synthesis *de novo* has also been observed (Bose *et al*, 1995). Triggering of apoptosis by ceramide occurs through activation of protein kinase cascade including a stress-activated protein kinase (Westwick *et al*, 1995; Verheji *et al*, 1996). Some data indicate that ceramide-mediated and p53-mediated apoptosis are distinct and independent.

Data presented above show that the addition of ceramide induces apoptotic death in rat neonatal cardiomyocytes and, thus, all components necessary for ceramide-mediated apoptosis are present in cardiomyocytes. Ceramide measurement is necessary to find out which pro-apoptotic stimuli induce cardiomyocyte death through this ceramide-mediated pathway. However, the data on prevention of ceramide-induced apoptosis by ROM suggest that ceramide may also be involved in triggering cardiomyocyte death induced by serum or serum/glucose deprivation and by ischemia/reperfusion which also is inhibited by ROM. The possible mechanisms of ROM interference with ceramide-mediated apoptosis are not clear. One of the active components of ROM is LPA. LPA alone can partially protect cardiomyocyte from apoptosis induced by serum deprivation (data not shown). Unfortunately, the solubility of LPA in water solutions is very low and LPA is easily adsorbed to glass, plastic and other surfaces, and rapidly disappears from solution. The combination of several phospholipids in ROM leads to formation of stable micelles and keeps LPA in the medium. It is known that during blood coagulation LPA is liberated from platelets and binds to albumin (Moolenar, 1995). Thus, any serum contains LPA adsorbed to albumin (Tigyi and Miledi, 1992). Therefore all experiments with ROM were performed in the absence of serum. LPA effects on cell growth and differentiation have been described (Moolenar, 1995) and our data indicate the possible involvement of LPA in the regulation of apoptosis. It is known that LPA can transduce a signal from the cell surface through a LPA-receptor and the putative receptor has been purified recently (Guo *et al*, 1996). Among other intracellular events induced by LPA is the increase of DAG concentration (Moolenar, 1995) and DAG has been shown to be an inhibitor of ceramide-induced apoptosis (Jarvis *et al*, 1994). Of course any suggestion as to the mechanisms of ROM or LPA anti-apoptotic activity is now speculative but it indicates the potential approaches for future studies.

Thus, the data presented here demonstrate the important role of apoptosis in myocardial cell injury induced by different agents. A mixture of phospholipids possessing the anti-apoptotic activity in different systems is also effective in the prevention of cardiomyocyte apoptosis induced by serum deprivation, simulated *in vitro* ischemia/reperfusion and ceramide. The latter finding indicates a possible involvement of ceramide in triggering ischemia/reperfusion induced apoptosis of cardiomyocytes. Recently we have shown that ischemia/reperfusion induced an increase of the ceramide levels both *in vitro* and *in vivo* (Bielawska *et al*, manuscript in preparation).

The data presented in this paper demonstrate the efficacy of a new approach in the treatment of the consequences of ischemia, based on interference with the apoptotic pathway.

Materials and methods

Isolation of rat neonatal cardiomyocytes

Cardiomyocytes were prepared from hearts of day-old Sprague Dawley rats by trypsinization and mechanical disaggregation (Simpson, 1985). The cells were resuspended in MEM, 1 × MEM vitamins (Gibco), 5% fetal bovine serum and 50 U/ml penicillin-G and pre-plated for 30 min to reduce contamination of non-myocytes. The non-adherent cardiac myocytes were separated and seeded in 2 ml in 35 mm dishes at a density of 3.5×10^5 viable cells per ml. The cells were allowed to adhere for 16–24 h in a 37°C/5% CO_2 humidified incubator.

Cardiomyocyte treatment

Following the initial incubation period, each culture plate was washed with fresh medium prior to addition of RPMI/10% fetal bovine serum containing either adriamycin, menadione, staurosporine or cis-platinum. The cultures were then incubated for 24 h.

For serum deprivation, the medium was replaced with fresh serum-free RPMI, whereas serum/glucose deprivation was performed using glucose-free RPMI. The induction of cell death by C_2-ceramide was accomplished by the addition of the agent prepared in serum-free RPMI. As a model of ischemia, cultures in serum and glucose free RPMI were placed in an airtight chamber and the latter was continuously perfused with oxygen-free gas overlay of 95% N_2/5% CO_2 for 8 h at 37°C. To model reperfusion of the ischemic cells, 10% fetal bovine serum, 2 g/L of glucose were added and the cultures were returned to a normal oxygen gas overlay (37°C/5% CO_2) in a humidified incubator for 16 h.

Purification of SFL

Purification of the anti-apoptotic factor from soy flour was performed using the 10T1/2 apoptotic cell assay to monitor the anti-apoptotic activity. (Tomei *et al*, 1993). Soy flour, type 1 (Sigma, St Louis, MO) was defatted with 70% acetone and extracted with 50% ethanol. The extract was concentrated 100 times by ultrafiltration through 10 kDa membrane (Filtron, Boston, MA) and lyophilized. The powder was extracted with the mixture water : methanol : chloroform (3 : 8 : 4). The soluble fraction containing the majority of the anti-apoptotic activity was reclaimed by rotary evaporation and lyophilized.

For these experiments, SFL or ROM were prepared by sonication at the concentration 10 mg/ml in 105 mmol NaCl/50 mmol ammonium bicarbonate, pH 8.0 and added to cells in serum free medium at the concentrations indicated.

Measurement of cell death

Since cardiomyocytes are differentiated non-dividing cells, viability was determined by measurement of the decrease in the relative number of adherent cells. The measurement of non-adherent cells was found to be less reproducible because of their rapid lysis following release from adhesion substrate. Adherent cardiomyocytes were collected from culture dishes using 0.25% Trypsin/0.05% EDTA and counted on Coulter Counter ZM and Coulter Channelyzer 256.

Analysis of DNA degradation

For flow cytometry analysis, adherent and non-adherent cells were combined. Cells were fixed in 50% ethanol, treated 30 min with 100 μg/ml RNase, stained with 40 μg/ml propidium iodide and the amount of cells containing <2C DNA was registered by FACScan. DNA was isolated from combined adherent and non-aherent cells and analyzed by agarose gel electrophoresis. 10 μg DNA were loaded on each lane.

Each experiment was repeated 6–8 times and typical data are presented.

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