Cell Death in Cultured Adult Rat Cardiomyocytes: Use of the Comet Assay to Distinguish Apoptosis from Necrosis

M. Trevor Page[(1)], Penelope J.E. Quintana[(2)], Joseph A. Ligutti[(1)], and Roger A. Sabbadini[(1)]

(1) Department of Biology and (2) Graduate School of Public Health, San Diego State University

Abstract

Apoptosis is a form of programmed cell death that has only recently been implicated in the pathogenesis of cardiac diseases, including ischemia and reperfusion damage and in response to cytokines and other apoptotic triggers. Methods to study apoptosis range from measures of early changes in morphology to late events such as DNA laddering. In studying apoptosis in the heart, the application of some of these techniques is limited due to low sensitivity of detection and difficulties in quantification, especially when working with adherent cell cultures of cardiomyocytes. We present here results on the modification of the single cell gel electrophoresis or comet assay in order to assess apoptosis of adult cardiomyocytes in primary cell cultures. The comet assay quantifies DNA fragmentation in individual cell nuclei embedded in agarose and then electrophoresed. We present data on TNFα and sphingosine-induced apoptosis in adult rat cardiomyocytes as assessed by the comet assay. By using a combination of the alkaline, neutral and subcellular versions of the comet assay, we were able to accurately differentiate apoptosis from necrosis in cardiomyocytes. We conclude that the comet assay is a sensitive and useful method for the detection of apoptosis in cardiomyocytes.

Key words: apoptosis, cardiomyocytes, comet assay, necrosis, sphingosine, TNFα.

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Apoptosis is a form of programmed cell death whereby a cell undergoes a controlled form of cellular suicide [18]. Apoptosis can be distinguished from another form of cell death known as necrosis. Necrosis is defined as an uncontrolled process whereby a cell undergoes rapid cytoplasmic and organelle swelling when subjected to severe physical injury or an extreme variance in the cellular environment. Ultimately, the cell membrane becomes permeabilized which culminates in cell lysis. The disintegration of the cell causes the release of harmful cytoplasmic contents, such as lysosomal enzymes, which can damage healthy cells adjacent to necrotic cells [37]. Often, necrosis produces a substantial inflammatory response that also cause damage to cells surrounding the initial site of insult. On the other hand, apoptosis is a pre-lytic process, whereby all apoptotic events occur before there is a loss in plasma membrane integrity [3]. Early in the apoptotic process is the activation of a caspase cascade either by the release of cytochrome C from the mitochondria or through activation of death receptors such as Fas or TNFR1 [32].

On a morphological level, apoptosis is distinguished by unique features, including cell shrinkage, the formation of pyknotic nuclei, and double-stranded DNA fragmentation. Double-stranded DNA fragmentation is one of the most widely accepted criteria used to characterize apoptotic cell death because it is discernible from the random single-stranded DNA breaks produced by necrosis [9, 38]. DNA "laddering" is the most common assay for visualizing the double-stranded oligonucleosomal DNA fragments on agarose gels; however, this technique is difficult to quantify [6] and not particularly sensitive as an extensive number of cells (greater than 10^6 cells/sample) must be caught in synchronous fragmentation to produce the oligonucleosomal ladders [11]. The TUNEL assay also suffers from lack of specificity and cannot distinguish apoptosis from necrosis unless a second marker is used [4].

A technique known as the "comet assay" has been developed to assess DNA fragmentation patterns capable of distinguishing apoptosis from necrosis in a single assay ([8, 30, 33] also see reviews by [5, 7]). This assay

involves single cell microgel electrophoresis of individual nuclei to visualize characteristic DNA fragmentation patterns that are produced after the electrophoresis of cells damaged by apoptosis [28, 30, 33]. The undamaged nuclei of healthy cells typically appear as tight, concentric, spheroids after electrophoresis. The electrophoresis of nuclei that exhibit DNA damage will produce a characteristic DNA fragmentation pattern that resembles a celestial comet. The comet produced can be characterized by both the amount of DNA that remains in the nucleus or "head" and the amount and pattern of DNA that has migrated away from the nucleus forming the tail. Furthermore, software programs that employ computer imaging can analyze comet data for calculation of what is known as the comet tail moment [7]. The comet assay requires very few cells, and is a very rapid and sensitive technique that can measure the DNA fragmentation during the early stages of apoptosis [7, 10, 12, 19, 27]. Additionally, the comet is a relatively simple technique that allows one to determine not only the percentage of apoptotic nuclei, but also the extent of genotoxic damage induced by an apoptotic trigger.

Apoptosis in the heart was first linked to fetal cardiac organogenesis and postnatal cardiac maturation [13, 16]; however, new research suggests that apoptosis, rather than necrosis, may be responsible for the cell death observed in human cardiac pathologies [1, 2, 15, 39]. Specific triggers known to induce apoptosis in non-cardiac cells are the subject of intense investigation as potential apoptotic stimuli in the heart and should provide the most insight into the mechanism(s) underlying cardiac apoptosis.

The purpose of the current study was to apply the comet assay to adherent primary cultures of cardiomyocytes in an attempt to distinguish apoptosis from necrosis and direct genotoxic damage caused by various apoptotic triggers, including TNFα and sphingosine. The comet assay has previously been applied to non-muscle cells from many tissue types to assess apoptotic DNA damage [26, 33]. However, prior to our previous report [20] and the current study, the comet assay had neither been applied to adherent cells in culture nor had the comet assay been used to examine apoptosis in cultured cardiomyocytes.

Methods

Materials

Type A Collagenase (lot # 83229823) and the *in situ* cell death detection (TUNEL) kit, fluorescein was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. Heparin and sphingosine were purchased from CalBiochem, La Jolla, CA. Collaborative Biomedical Products in Bedford, MA, were the providers of human fibronectin (lot # 905446) and mouse laminin (lot # 903836 and lot # 908155). Corning cover glass (24x60 mm) and premium fully frosted microscope slides were purchased from Fisher, Tustin, CA. Comet-slides were purchased from Trevigen, Inc., Gaithersburg, MD. Rat recombinant TNFα (lot # B7091 and lot # B6724) was purchased from Genzyme Diagnostics, Cambridge, MA. Dulbecco's Modified Eagles Medium/F12 and Fetal Calf Serum were obtained from Life Technologies, Grand Island, NY. Molecular Probes of Eugene, OR provided YOYO-1. The Buffer Puffer horizontal minigel electrophoresis system (12 cm W x 14 cm L) was purchased from Owl Scientific, Inc., Woburn, MA. Low melting agarose was obtained from SeaKem Gold, FMC Bioproducts, Rockland, ME. Ampicillin, amphotericin B, Hoechst bisbenzimide (#33342), Type I Collagenase (lot # 27H0626), kanamycin, propidium iodide, pyruvate, and sodium pentobarbitol were purchased from Sigma, St. Louis, MO.

Cardiomyocyte isolation

Adult ventricular myocytes were isolated from Sprague-Dawley male rats (200-350 g) and cultured as previously described [22], with the following modifications: the rats were first injected intraperitoneally (IP) with 0.5 mL of 0.0128 g/mL heparin dissolved in Ca^{2+}-free Tyrode's (140 mM NaCl, 5.4 mM KCl, .5 mM $MgCl_2$, 10 mM HEPES, 0.25 mM NaH_2PO_4, pH 7.3) 15-20 minutes before the procedure. The rats were then anesthetized with an IP injection of 0.4 mL of a 50 mg/mL sodium pentobarbital solution in Ca^{2+}-free Tyrode's. Once the anesthetized rats exhibited a loss of reflexes, yet maintained a normal rate and rhythm of respiration, the thoracic cavity was opened via an anterior vertical incision along the midline at the costal cartilages and a transverse incision just inferior to the length of the diaphragm. The aorta was than clamped, the heart was subsequently removed, and the aorta was then immediately affixed to a cannula attached to 20 cc syringe. The heart was initially perfused with 3-5 cc of ice cold Tyrode's (140 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 10 mM HEPES, 0.25 mM NaH_2PO_4, 1 mM $CaCl_2$, pH 7.3) at a rate not exceeding 2-3 drops/second to clear the coronary arteries. The cannulated heart was removed from the syringe and immediately placed on a Langendorff apparatus, pre-set to a constant drip rate of 1-2 drops/second, and perfused for 2-2.5 minutes with warm oxygenated Tyrode's maintained at 37°C using a jacketed H_2O bath. The heart was then perfused with Ca^{2+}-free Tyrode's for 5-5.5 minutes by diverting the flow of perfusion solutions via a three-way luer-lock stopcock. Subsequently, the heart was perfused with digesting solution (45 mg Type A Collagenase in 2.5 mL of Tyrode's and 50 mL of Ca^{2+}-free Tyrode's, 37°C) for 8-8.5 minutes or until the texture of the atria reached a gummy consistency. The ventricles were then excised and placed in 15-20 mL of digesting solution and oxygenated for 1 minute while the tissue was first diced and

then carefully teased apart. The tissue suspension was then incubated at 37°C in 15-20 mL of digesting solution for 30 minutes. Every 10 minutes throughout the course of the 30 minute digestion the cells were removed from the incubator, oxygenated for 1 minute, agitated, and then observed under a microscope. Subsequently, the cell suspension was filtered through four layers of cheesecloth, dampened with Ca^{2+}-free Tyrode's, to remove any cellular debris. The isolated cardiomyocytes in solution were settled for 10 minutes under oxygenated conditions. The clear cell free supernatant was removed and the concentrated cells were resuspended in 10-20 mL of Ca^{2+}-free Tyrode's depending on the concentration of viable cells. The freshly dissociated ventricular cardiomyocytes were plated directly onto laminin-coated (50 µg/mL) frosted microscope slides or Trevigen Cometslides which were placed individually within 100 x 15 mm Petri dishes. The cardiomyocytes applied to each slide were incubated without media and within an oxygen chamber filled with 95% O_2/5% CO_2 for 10 minutes to allow for the maximum number of viable cells to bind to the laminin-coated slides. Following the media-free incubation, the cells were removed from the oxygen chamber and each Petri dish was carefully filled, as measure to reduce the number of cardiomyocytes displaced off each slide and coverslip, with approximately 10 mL of Dulbecco's Modified Eagles Medium (DMEM)-F12/10% fetal calf serum (FCS) (supplemented with 0.05 mg/mL ampicillin, 0.04 mg/mL fungizone, 0.6 mg/mL glutamine, 0.1 mg/mL kanamycin, and 0.25 mg/mL pyruvate). The cells were then incubated for 1 hour at 37°C prior to washing 3 times with 1:1 DMEM (supplemented with 0.05 mg/mL ampicillin and 0.1 mg/mL kanamycin) to remove any non-viable or injured cardiomyocytes that do not bind to laminin. Following completion of the wash, the cells were incubated in 10 mL of DMEM-F12/10% FCS at 37°C with or without a test agent present for various time periods.

Single-cell microgel electrophoresis (comet assay)

Cultured cardiomyocytes were treated with or without a test agent (e.g. SPH, TNFα) for a specific period of time and were subjected to three variations of single-cell microgel electrophoresis (comet assay) as described below. The alkaline comet assay was performed as previously described [34] with the following modifications: agarose was directly applied to adherent cells on a coverslip, and the lysis, unwinding and electrophoresis times were shortened to times detailed below. The cultured cells plated on the clear glass coverslips were also examined for viability (controls) and to determine if the density of cardiomyocytes (approximately 30-40 cells per field viewed by light microscopy under 10X magnification) was sufficient to initiate the comet assay. The microscope slides were carefully removed from each Petri dish. Subsequently, 135 µL of a 0.5% agarose in 1X PBS (pH 7.4), maintained at a temperature not exceeding 43°C, was applied to each individual microscope slide plated with cardiomyocytes. Immediately, a 24 x 60 mm coverslip was applied to each agarose-coated slide in such a manner that the agarose layer was evenly spread over the length of each slide. The slides were quickly cooled on an ice-chilled glass plate so that the agarose solidified to embed the cells *in situ*. After 1 minute of chilling, the slides were allowed to thaw before each coverslip was carefully removed, leaving an even layer of agarose affixed each slide. For use of the Trevigen Cometslides as a substitute for full-length frosted slides, the cells were plated in the circular wells of the Cometslides that had been pretreated with laminin. Agarose could then be applied directly over the cells and allowed to solidify without using a coverslip.

The adherent cells were then placed in a Coplin staining jar filled with 40-50 mL of freshly made refrigerated lysis buffer (0.1 M EDTA, 2.5 M NaCl, 1% Na-lauryl sarcosinate, 10 mM Tris base, 1% peroxide and carbonyl free Triton X-100, pH 10) for 15 minutes at 4°C. Following cell lysis, the slides were immersed for 20 minutes in refrigerated alkaline electrophoresis buffer (10 mM EDTA, 2% dimethyl sulfoxide, 0.1% 8-hydroxyquinoline, 300 mM NaOH) allowing DNA within the nuclei to unwind. Unwinding was followed by electrophoresis for 5 minutes at 2 V/cm using a Buffer Puffer re-circulating electrophoresis assembly. After electrophoresis, the buffer was allowed to cool for approximately 1 minute before each slide was carefully removed and placed on an ice-chilled glass plate to re-solidify the agarose. Subsequently, the slides were then placed in a Coplin jar containing 0.4 M Tris base (pH 7.4) and allowed to incubate for 1 hour as a step to remove all salts and neutralize the pH of the agarose embedding the cells. Finally, each slide was stained with of 75 µL YOYO-1 (3 µmol/L dissolved in 2X PBS, pH 7.4), covered with a 24x60 mm coverslip, and stored in a dark humid chamber prior to visualization using an Nikon Labophot-2 fluorescence microscope equipped with an Olympus FITC filter cube.

The neutral comet assay was performed as described for the alkaline comet assay with the following modifications: two layers of 0.5% agarose were applied over the cultured cells instead of one. The second layer gives the embedding gel added strength to withstand the slightly more strenuous procedure of the neutral comet assay. Following removal of each coverslip, the slides were placed within a Coplin jar containing the alkaline lysis solution (pH 10.0), as described above, for 15 minutes at 4°C. The slides were then removed and allowed to unwind for 30 minutes in room temperature neutral electrophoresis buffer (1X TBE, 0.1% hydroxyquinoline, 0.02% DMSO, pH 8.0). The pH was adjusted

the following manner: the pH was first increased to 11.0 using NaOH pellets, allowing the chemicals to enter solution, and was then decreased to the working neutral pH of 8.0. The neutral electrophoresis buffer was maintained at room temperature to prevent loss of solubility. Following unwinding, the slides were electrophoresed for 5 minutes at 2.5 V/cm using the Buffer Puffer assembly. Upon completion of electrophoresis, the buffer was first drawn from the assembly to a level just below that of the platform supporting the slides and then each slide was carefully removed and placed on an ice bath for 1 minute. The pH of the slides was then neutralized in 0.4 M Tris for 1 hour followed by staining with 75 µL of YOYO-1 (3 µmol/liter) and visualization with fluorescence microscope fitted with a FITC cube.

The subcellular comet assay was performed essentially as described [17] with the following modifications: untreated adherent cells were layered with 0.5% agarose (43°C) and placed on an ice bath to solidify the gel after which each coverslip was removed. A second layer of 0.5% agarose was applied by repeating the same steps as above. The slides were then placed in the same alkaline lysis solution (pH 10.0) for 15 minutes at 4 C and then washed twice in 0.4 M Tris-HCl buffer (pH 7.5) for 5 minutes. Following cell lysis each slide was placed individually in 100x15 mm Petri dishes each filled with 10 mL of 0.4 M Tris-HCl buffer with or without a test agent. The embedded nuclei were then incubated with or without the test agent for 1 hour at 37°C. This treatment step allows the test agent to come into direct contact with the DNA of each cell. The slides were then removed and again washed twice in 0.4 M Tris-HCl buffer (pH 7.5) for 5 minutes. Subsequently, the slides were subjected to the same unwinding and electrophoresis steps as described in the alkaline comet assay. The cells were neutralized in 0.4 M Tris-HCl, pH 7.5, for 1 hour and were finally stained with 75 µL of YOYO-1 (3 µmol/liter) for visualization.

Hoechst/propidium iodide staining

Cardiomyocytes were cultured in DMEM-F12/10% FCS either on the Trevigen Cometslides or on circular coverslips placed in the same 100x15 mm Petri dishes that contained the cells cultured on frosted slides. The coverslips were removed and subjected to Hoechst/propidium iodide (H/PI) staining [36], while the frosted microscope slides which had been cultured simultaneously were subjected to the alkaline comet assay (see Methods). Alternately, the Trevigen Cometslides proved more useful for combining H/PI staining with comet analysis of the same cells. At the conclusion of each incubation with a particular test agent, the cells that were cultured on the Cometslides were treated with H/PI to evaluate the percent red-staining cells as an indication of necrosis. The cells were then subsequently processed for the comet assay as described above. For the H/PI staining, Hoechst bisbenzimide (0.1 µg/mL) was added directly to the cells and allowed to incubate for 5-10 minutes at room temperature in the dark. Subsequently, propidium iodide (0.5 µg/mL) was added directly (no wash necessary) to the media in which each coverslip or Cometslide that had been cultured. The media was then incubated for 5 minutes, after which they were viewed using a fluorescence microscope equipped with an UV filter cube, using excitation wavelength of 350 nm.

TUNEL assay

Terminal deoxynucleotidyl transfer-(TDT)-mediated dUTP nick end labeling of fragmented nuclei (TUNEL assay) was performed on cardiomyocytes that had been plated on laminin-coated coverslips and cultured in DMEM-F12/10% FCS in the presence or absence of a test agent. The in situ TUNEL assay was performed as described by the manufacturers protocol (see Materials) with the following modifications: cardiomyocytes adherent to laminin-coated (50 µg/mL) circular coverslips were placed in 6-well plates (1 coverslip/well) and fixed with 1mL/well of 4% paraformaldehyde for 30 minutes at room temperature. The fixed cells were washed 3 times with 500 µL/well with refrigerated PBS (pH 7.4) and then placed in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 30 minutes on ice. The permeabilization solution was then drawn from each well and the coverslips in each well were washed 3 times with cold PBS. The coverslips were then removed and the excess was liquid blotted from the bottom prior to placement within a humid chamber. The label and enzyme were mixed according to manufacturer's protocol to make the labeling solution, of which 100 µL was added to each coverslip and followed by a 1 hour incubation at 37°C. The coverslips were then placed back into the 6-well plates and 500 µL of cold final wash solution (PBS, 0.1% Triton-X 100, and 1% BSA, pH 7.4) was paced in each well of the plate, which was then placed on a shaker for 5 minutes. The final wash step was repeated 3 times. Finally, 1 drop of Vectashield was added to each coverslip which were then mounted by inverting each slip so that the adherent cells came into direct contact with the clear microscope slide that each slip was placed upon. The cells were then visualized using a fluorescence microscope using an UV filter cube.

Results & Discussion

We initially used the second messenger, sphingosine (SPH), as an apoptotic trigger to investigate the effectiveness of the comet assay in evaluating cardiomyocyte apoptosis, since SPH and other sphingolipids have been shown to induce apoptosis in a variety of other cell types [14, 24, 25] and because our laboratory has shown that sphingolipids are important signaling molecules in cardiomyocytes [20, 23, 29]. Experiments

were designed to assess the ability of the comet assay to accurately detect and quantify apoptosis in experiments involving a large number of individual cells exposed to an apoptotic trigger. The alkaline comet assay was applied to cardiomyocytes treated for 18 hours with or without SPH (10 µmol/L) (Figure 1A, B). As evident by the large number of undamaged, type A nuclei, fragmentation was not observed in untreated (control) cells because the nuclear DNA of each cell received minimal damage produced by single or double-stranded breaks. Treatment with SPH induced DNA fragmentation in a large percentage of cardiomyocytes as demonstrated by the many comets that were produced. The majority of the DNA fragmentation patterns produced by SPH were apoptotic type C comets; however, a few type B comets were also evident.

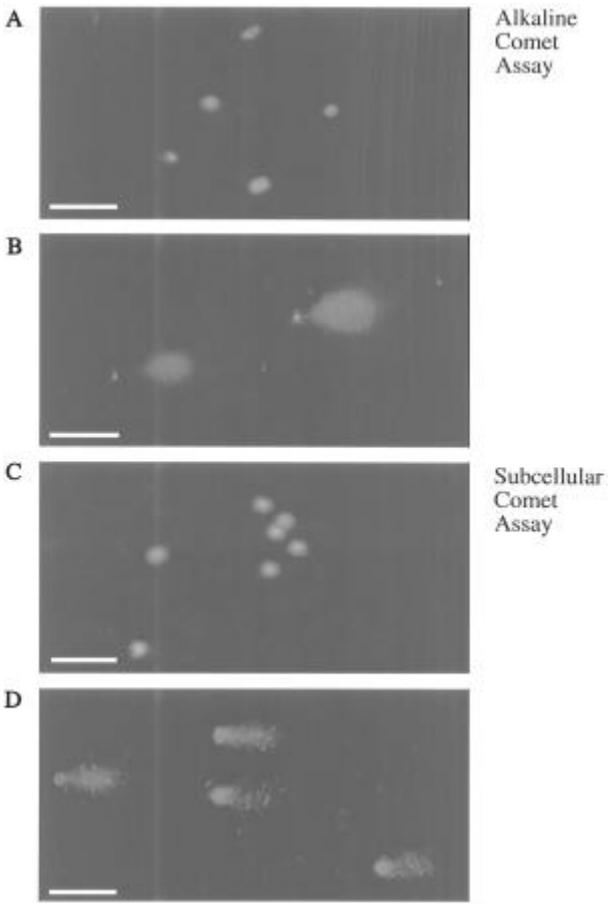

Figure 1. Comparison of the alkaline and subcellular comet assays. Cultured adult rat cardiomyocytes were incubated for 18 hr in the absence (A) or presence (B) of 10 mmol/L sphingosine (SPH). Following treatment, the cardiomyocytes were then subjected to the alkaline comet assay (A-B). Untreated cells were also subjected to the subcellular comet assay (C-D), whereby the cells were lysed following an 18 hr incubation and then treated with or without either 10 mmol/L SPH (C) or 100 mmol/L H_2O_2 (D) for 1 hr before the unwinding and electrophoresis steps of the comet assay were performed (See Methods). The cells were then visualized by YOYO-1 staining under fluorescence microscopy. Bar represents 50 mm.

In order to determine if the SPH induced DNA fragmentation resulted from the activation of an apoptotic death program and not from direct genotoxic damage, we applied the subcellular comet assay [30] to the cultured cardiomyocytes (Figure 2C, D). In this figure, adult rat cardiomyocytes taken from the same preparation of cells isolated as shown in Figure 1A and B were plated and cultured (see Methods). However, for the subcellular comet assay, the cells were embedded in agarose and lysed before treatment with the test agent, which in this case was SPH. Lysing the agarose-embedded cells before treatment allowed the DNA to be directly exposed to the test agent. If the agent is genotoxic, it will directly damage the nuclear DNA and subsequent electrophoresis will produce a comet fragmentation pattern reminiscent of type B comets (Figure 1D). As expected, the subcellular comet assay did not reveal any DNA fragmentation within cardiomyocytes whose DNA was directly exposed to SPH (10 µmol/L) for 1 hour (Figure 1C). This is in stark contrast to the prominent SPH-induced DNA fragmentation patterns produced when intact cells were treated with SPH before the alkaline comet procedure was initiated (Figure 1B). Consequently, the ability of SPH to induce DNA damage must be dependent upon the active processes of an intact cell.

To demonstrate that DNA fragmentation produced by TNFα requires an intact cell and was not the result of direct genotoxic damage, as demonstrated for SPH, we employed the subcellular comet assay to TNFα-treated cells. We have previously shown that TNFα is a potent trigger of apoptosis in adult cardiomyocytes [20], and others have shown similar results in neonatal rat cardiomyocytes [35]. We compared the results of cells that were incubated with or without TNFα (3 nmol/L) or SPH (10 µmol/L) for 18 hours and subjected to either the alkaline comet assay or to the subcellular comet assay (Figure 2). The control cells (untreated cells) were undamaged and displayed 3.1±0.5% type C comets for the alkaline comet assay and 2.6±0.5% for the subcellular assay. As expected, the cells treated with TNFα and SPH (positive control) and subjected to the alkaline comet assay produced an increase in apoptotic nuclei; 50.9±1.0% and 98.2±0.5% type C comets respectively. In contrast, the overwhelming majority of cells exposed to TNFα or SPH and processed in the subcellular comet assay remained undamaged as demonstrated by less than 5% type B and type C comets present for either treatment. The subcellular comet data confirmed that TNFα, like SPH, required the cellular machinery to be

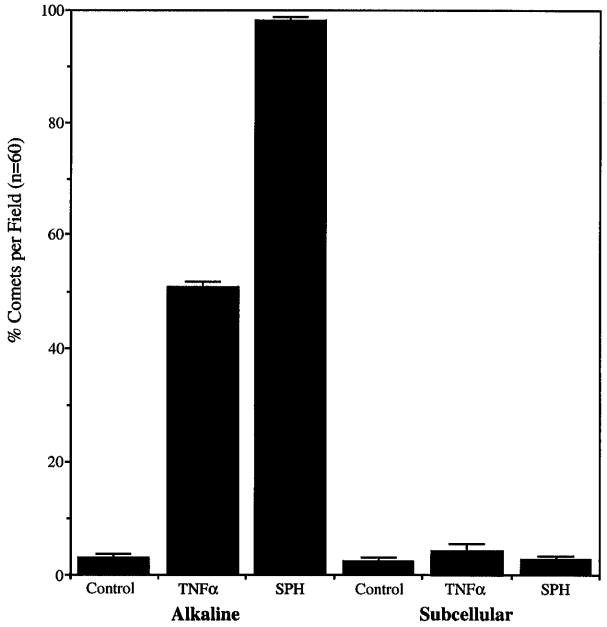

Figure 2. Comparison of alkaline and subcellular comet assays to reveal potentially direct genotoxic damage. Adult rat cardiomyocytes were incubated in the absence (control) or presence of either TNFα (3 nmol/L) or SPH (10 mmol/L) for 18 hr. The cells for each treatment were then subjected to the alkaline comet assay (see Methods). Untreated cells were also subjected to the subcellular comet assay, whereby the cells were lysed following an 18 hr incubation and then treated with or without either TNFα (3 nmol/L) or SPH (10 mmol/L) for 1 hr before the unwinding and electrophoresis steps of the comet assay were performed. All the cells were then stained with YOYO-1 and quantified by visual analysis. The values in the bar graph represent the average percentage of nuclei per field that exhibited type C fragmentation patterns for each treatment. Two replicate experiments were performed for both the alkaline and the subcellular comet assay in which a total of 63 fields (±SEM) were quantified per treatment for both comet assays. Between 450-550 nuclei were scored for each treatment point per individual experiment, adding to a total of roughly 6,000 nuclei that were quantified.

intact to elicit an apoptotic response and did not act directly on the cellular DNA in a genotoxic manner.

It is not known if the partial DNA fragmentation manifested as type B comets represented single-stranded DNA cleavage associated with necrosis or, alternately, represented 'transitional' comets on their way to becoming full-blown type C comets. It is assumed that type C comets can only result when two double-strand breaks occur on the same piece of DNA. Otherwise, one could not visualize the characteristic separation of the comet tail from the remnant nucleus (e.g., Figure 1B). Alternately, the type B comets can be explained by limited endonuclease-dependent DNA cleavage, producing only one double-stranded DNA break before the death program was interrupted by termination of the experiment.

In order to determine the origin of type B comets, the time-dependency of type B and C comet production was assessed in response to SPH (Figure 3). Cardiomyocytes were treated with SPH (10 µmol/L) for 4 and 18 hours and subjected to the alkaline comet assay (Figure 3). As expected, the untreated cells for each time course remained undamaged, where as the SPH treated cells that were incubated for 4 hours displayed 42.2±1.0% type B comets and 34.5±0.8% type C comets (Figure 3). However, after 18 hours of SPH treatment, only 3.3±0.9% of the cardiomyocytes were type B comets, while 94.3±1.4% of these cells were type C comets. The decrease in the number of type B comets coincided with a marked increase of type C comets as the time of treatment with an apoptotic agent increased. Furthermore the type C comets produced by the 18 hour SPH treatment displayed the characteristic gap between the "pin" head

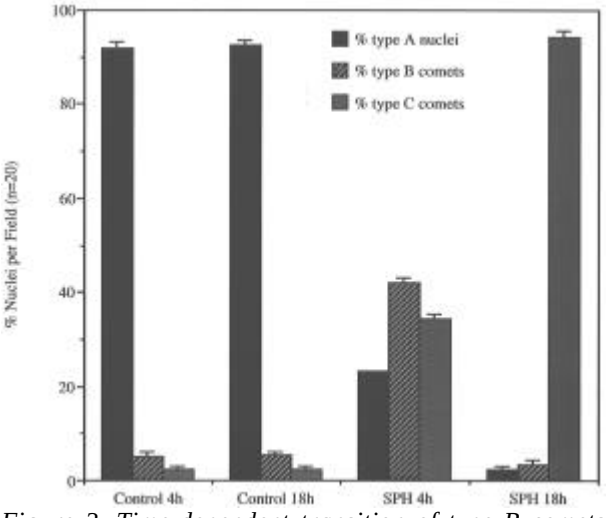

Figure 3. Time-dependent transition of type B comets into type C comets. Cultured adult rat cardiomyocytes were incubated with or without (control) 10 mmol/L SPH for 4 and 18 hr and were then subjected to the alkaline comet assay and visualized by YOYO-1 staining under fluorescence microscopy. Nuclei were categorized as either type A nuclei, type B or type C comets and quantified per field using visual analysis (see Methods). The bar graph represents the average percentage of nuclei per field that exhibit type A, B, or C fragmentation patterns. Approximately 1,500 cells were scored, with 350-450 nuclei per treatment point, following the quantification of 20 fields (±SEM) for each treatment performed.

and the "puffy" tail of each comet which indicates double-strand DNA breaks. This shift of type B comets to type C comets as a component of time lends support to the contention that type B comets induced by an apoptotic agent could indeed be transitional.

Additional experiments were designed to provide further evidence that the alkaline comet assay can distinguish apoptotic nuclei with double-stranded DNA breaks from nuclei with single strand or random DNA double strand breaks. The DNA fragmentation patterns produced by SPH were compared to the effects of a known DNA single-strand breaking agent, hydrogen peroxide (H_2O_2) (Figure 4). We compared the DNA fragmentation patterns produced by both alkaline (pH 12+) and neutral (pH 8) conditions during unwinding and electrophoresis of the comet assay.

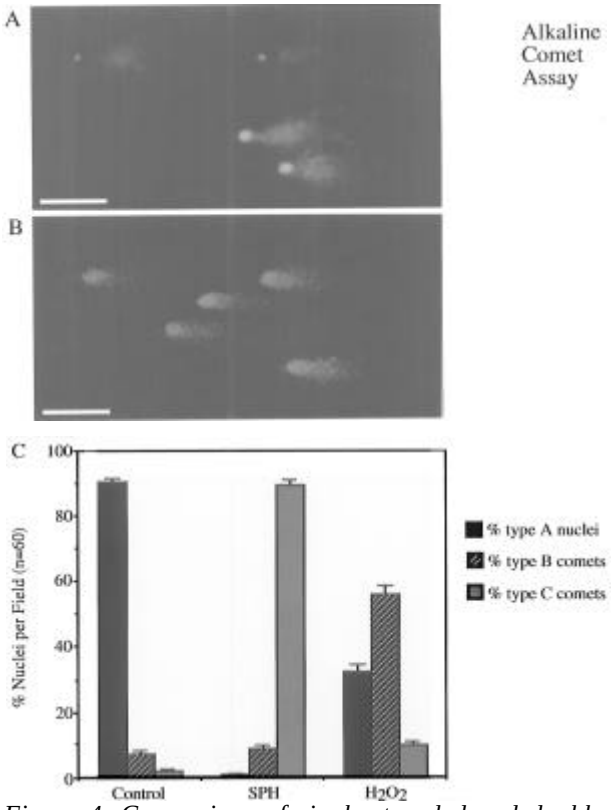

Figure 4. Comparison of single-stranded and double-stranded DNA breaks as revealed by the alkaline comet assay. Adult rat cardiomyocytes were incubated for 18 hr in the absence (control) or presence of SPH (10 mmol/L). Cells were also treated with H_2O_2 (100 mmol/L) for 30 min following a 18 hr incubation without treatment. The cardiomyocytes were then subjected to the alkaline comet assay, visualized by staining with YOYO-1 under fluorescence microscopy, and quantified by visual analysis. (A-B) Panels display the characteristic DNA fragmentation patterns produced following electrophoresis of nuclei from cells that have been treated with SPH (A) and H_2O_2 (B), respectively. The values of the bar graph (C) represent the average percentage of nuclei per field that display type A, B, or C fragmentation patterns. Between 350-450 nuclei were scored for each treatment performed for three experiments, contributing to a total of approximately 4,500 cells that were quantified. A total of 60 fields were scored (±SEM). Bar represents 50 mm.

Cells that were treated with SPH (10 µmol/liter) displayed many type C comets characterized by a "pin head" separated by a space followed by a "puffy" tail (Figure 4A). However, when cells from the same heart preparation were treated for 30 minutes with H_2O_2 (100 µmol/L), followed by 18 hours in culture, very few type C comets resulted. On the other hand, the majority (56.3±2.2%) of nuclei from cells exposed to H_2O_2 resembled type B comets, characterized by a large amount of DNA in the "head" followed by a tail in which most of the DNA fragments were still attached to the nuclear core (Figure 4B). Therefore, the cells treated with H_2O_2 produced DNA fragmentation patterns indicative of single-stranded DNA breaks and were quite distinct from the type C comets characteristic of the double-stranded DNA breaks induced by the apoptotic trigger SPH.

To further distinguish apoptotic DNA fragmentation patterns from those of genotoxicity or necrosis, we performed the neutral comet assay [19, 30] on cells exposed to either SPH or H_2O_2 (Figure 5). Unlike the alkaline comet assay, which denatures and separates the two strands of the DNA double helix, the neutral pH condition of the neutral comet assay prevents denaturing of the DNA during unwinding and electrophoresis [19]. Thus, single-stranded DNA breaks will not produce a comet tail because each damaged strand is still attached to the undamaged strand, which in turn is still connected to the bulk of nuclear DNA. Therefore, any significant fragmentation patterns observed would only be the result of double-strand DNA breaks.

As anticipated, SPH (10 µmol/L) produced 93.0±0.8% type C comets under neutral conditions (Figure 5A, C), adding more support to the hypothesis that SPH produces the characteristic double-strand breaks of apoptosis. In contrast to the type C comets induced by SPH with alkaline comet assay, the "heads" were slightly larger and the tails were long, thin, and more linear (Figure 5A) than after electrophoresis under neutral conditions. On the other hand, most (84.1±1.2%) of the cells that were treated with H_2O_2 (100 µmol/L) and subjected to the neutral comet assay displayed no DNA fragmentation. Interestingly, 15.3±1.2% of the H_2O_2-treated cells displayed a small degree of fragmentation (Figure 5C). The fragmentation patterns produced by H_2O_2 resembled large type A nuclei that appeared as a if they were just on the verge of transforming into type B comets with very small

tails (Figure 5B). Theoretically, it is possible but not likely that four or more single-strand breaks between histones could be cleaved to produce a piece of DNA that will separate entirely from the nucleus. It is most likely that, occasionally, two single-strand breaks could occur in the same internucleosomal position. This could have resulted in a thread of damaged DNA that would migrate away from the nucleus but still remained attached, accounting for the rare observation of H_2O_2-damaged DNA fragments that were seen extending a small distance from the nucleus of a cell.

Alkaline comet conditions allowed most of the single-stranded DNA breaks induced by H_2O_2 to be manifested by type B comets (Figure 4B, C). However, a great majority of the cells treated with H_2O_2, and then unwound and electrophoresed under neutral conditions, as expected, did not show DNA fragmentation (Figure 5B, C). The lack of DNA fragmentation produced by the neutral comet confirms that few double-stranded DNA breaks are induced by H_2O_2 and the comets produced by H_2O_2 under

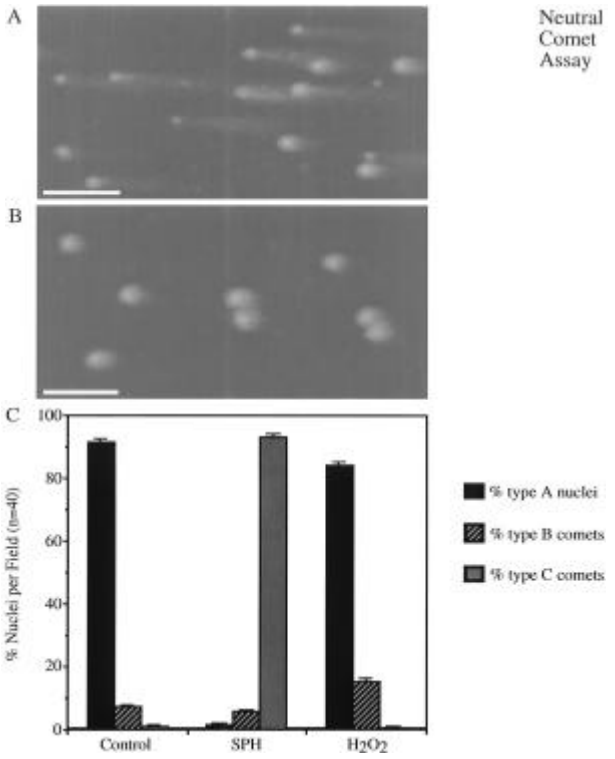

Figure 5. Double-stranded DNA breaks as revealed by the neutral comet assay. Adult rat cardiomyocytes isolated from the same heart prep as Figure 4 were incubated for 18 hr in the absence (control) or presence of SPH (10 mmol/L). Cells were also treated with H_2O_2 (100 mmol/L) for 30 min following a 18 hr incubation without treatment. The cardiomyocytes were then subjected to the neutral comet assay (see Methods), visualized by staining with YOYO-1 under fluorescence microscopy, and quantified by visual analysis. The characteristic apoptotic double-stranded DNA fragmentation patterns produced by SPH (A) and lack of fragmentation patterns produced by H_2O_2 (B) are displayed in the upper panels, respectively. The values of the of the bar graph (C) represent the average percentage of nuclei per field that exhibit type A, B, or C fragmentation patterns. Between 350-450 nuclei were scored for each treatment performed for two experiments, contributing to a total of approximately 2,700 cells that were quantified. A total of 40 fields were scored (±SEM). Bar represents 50 mm.

Figure 6. Comparison of apoptosis and necrosis by Hoechst bisbenzimide (H) and propidium iodide (PI) staining. Adult rat cardiomyocytes were incubated in the absence (A) or presence of 10 mmol/L SPH (B). Cells were also treated with 100 mmol/L H_2O_2 (C) for 30 min following an 18 hr incubation without treatment. The cardiomyocytes were then stained with a combination of Hoescht (0.1 mg/mL) and propidium iodide (10 mg/mL) (see Methods) and analyzed using a fluorescence microscope with an UV filter cube with a maximum excitation wavelength of 350 nm. The nuclei of intact cells appear blue following Hoescht staining, while PI will only stain nuclei, appearing red/violet, of cells that have been permeabilized. The inset (B) is a higher magnification (100X) image of a cell, treated with SPH and stained with H/PI, which displays characteristic apoptotic cellular (plasmalemmal blebbing and cell shinkage with conserved membrane integrity) and nuclear morphology (pyknosis). Bar represents 50 mm.

alkaline conditions were the result of single-stranded DNA breaks. Therefore, we can conclude that the vast majority of comets produced by SPH under alkaline and neutral conditions were the result of double-strand DNA breaks. Thus, it is possible that the neutral comet assay can be used to differentiate between apoptotic double-stranded DNA damage and the single-strand DNA damage often seen in necrosis or genotoxicity.

Plasma membrane integrity is a hallmark that is often used to distinguish apoptosis from necrosis. Therefore,

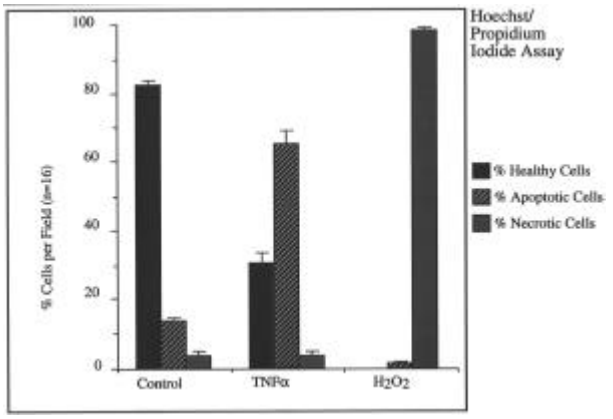

*Figure 7. TNFa-induced apoptosis as revealed by the Hoechst/propidium iodide assay. Adult rat cardiomyocytes were incubated in the absence (A) or presence of 3 nmol/L TNF**a** (B). Cells were also treated with 100 mmol/L H_2O_2 (C) for 30 min following a 18 hr incubation without treatment. The cardiomyocytes were then stained with a combination of Hoescht (0.1 mg/mL) and propidium iodide (10 mg/mL) (see Methods) and analyzed using a fluorescence microscope with an UV filter cube having a maximum excitation wavelength of 350 nm. The nuclei of intact cells appear blue following Hoescht staining, where as PI will only stain the nuclei of cells that have been permeabilized a red/violet color. (B) The inset is higher magnification (100X) of a cell, treated with TNF**a** and stained with H/PI, which displays characteristic apoptotic cellular (plasmalemmal blebbing and cell shinkage with conserved membrane integrity) and nuclear morphology (pyknosis). Bar represents 50 mm. The values in the bar graph (D) represent the average percentage of cells per field that exhibited H/PI staining characteristic of healthy, apoptotic, and necrotic cells. Healthy cells were characterized as blue cells that displayed the typical rod shape, crisp myofibril striations, and blue elongated nuclei. Apoptotic cells were classified by morphology as described above for the inset in (B) and necrotic cells were characterized by the presence of red/violet nuclei. Sixteen fields (±SEM) were quantified for each treatment performed on a total of 1,700 cells.*

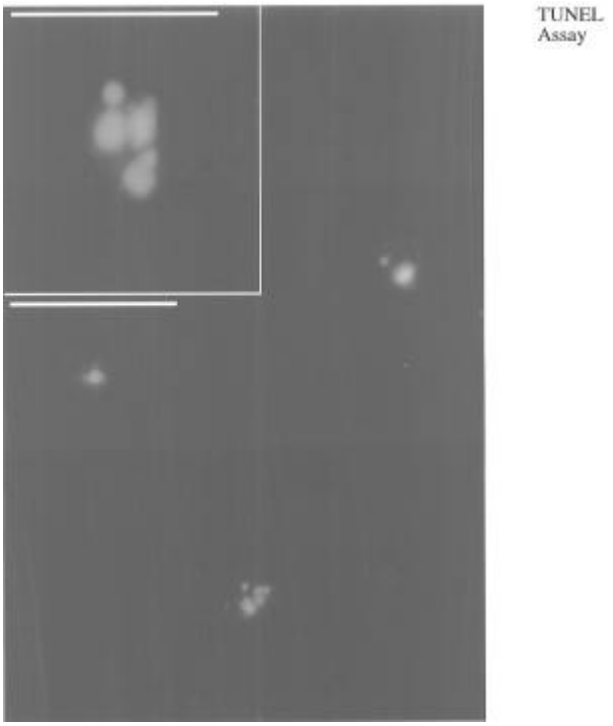

*Figure 8. In situ morphological assessment of TNF**a**-induced apoptosis in adult cardiomyocytes. Cultured adult rat cardiomyocytes were treated with 3 nmol/L TNF**a** for 18 hr followed by staining for DNA damage by in situ terminal deoxynucleotidyl transfer-mediated end labeling of fragmented nuclei (TUNEL assay). Myocytes were visualized under fluorescence to illuminate nuclei damaged by DNA fragmentation. Bar represents 50 mm. The pyknotic nucleus of an apoptotic cell is displayed at a higher magnification (100X) in the inset (bar represents 25 mm).*

experiments were devised to test the ability of cardiomyocytes to maintain plasma membrane integrity after exposure to the apoptotic triggers, SPH, TNFα and the necrotic agent, H_2O_2. In Figure 6, cells were treated with either SPH or H_2O_2 and then stained with H/PI. Figure 6A demonstrates that the vast majority of the untreated cells had intact plasma membranes, as determined by the absence of red/violet nuclei following exposure to H/PI. The characteristic rod shaped morphology with crisp striations (Figure 6A) indicated that cells also remained viable and were not permeable to calcium, which can enter the cell if the plasma membrane integrity is compromised and cause supercontracture of the cardiomyocyte. The alkaline comet assay was performed concurrently and determined that 93.2±1.4% of the untreated cells had not suffered DNA damage (i.e., exhibited type A nuclei). As expected, the cells treated for 18 hours with SPH induced substantial apoptosis as judged by characteristic morphological changes, such as cell shrinkage, plasmalemmal blebbing and nuclear pyknosis (Figure 6B).

However, the pyknotic nuclei of apoptotic cells still fluoresced blue, a clear indication that the plasma membrane had not been compromised (Figure 6B). The alkaline comet assay revealed that 74.2±1.55% of the same cardiomyocytes treated with SPH for 18 hours suffered apoptotic type C DNA fragmentation. In contrast to the cells treated with SPH, the cells incubated with H_2O_2 displayed a loss of plasma membrane integrity as evidenced by the magnitude of red-stained nuclei after exposure to Hoescht/PI (Figure 6C).

In parallel experiments, the necrosis-producing effects of H_2O_2 were compared to apoptotic trigger, TNFα, and quantified by the H/PI assay (Figure 7). Cardiomyocytes were treated with or without TNFα (3 nmol/liter) for 18 hours prior to H/PI staining. A positive control for permeabilization consisted of exposing cells with H_2O_2 (100 µmol/liter) for 30 minutes following an 18 hour incubation without treatment. As expected, 82.5±1.3% of untreated cells demonstrated the maintenance of plasma membrane integrity appeared healthy as determined by blue stained rod shaped cells with elongated nuclei. However, 98.7±0.5% of cells treated with H_2O_2, a positive control for necrosis and direct genotoxic damage, stained red/violet. The majority of nuclei (65.5±3.7%) from cells treated with TNFα appeared apoptotic with blue pyknotic nuclei, cell shrinkage, and plasmalemmal blebbing) and were stained positive for the TUNEL assay (Figure 8). The ability of TNFα to exhibit apoptotic features in the H/PI and TUNEL assays is consistent with the data in Figure 2 showing by the alkaline comet assay, that TNFα treatment triggers apoptosis in adult rat cardiomyocytes in culture.

Conclusions

Taken together, these data establish that the alkaline comet assay in combination with H/PI staining can serve as an effective method to differentiate apoptosis from necrosis or genotoxic damage. Additional value comes from comparing the results obtained from alkaline, the neutral, and the subcellular comet assays, particularly to investigate if a test agent is genotoxic through direct cleavage of the DNA or through cellular pathways. Importantly, the ability of the alkaline comet to detect SPH-triggered apoptosis within 4 hours of treatment suggested that the comet assay might serve as a very sensitive method to detect early apoptotic events.

Importantly, the comet assay has demonstrated that SPH and TNFα are potent inducers of apoptosis in primary cultures of adult rat cardiomyocytes. TNFα receptors are expressed by rat cardiomyocytes [21] and this pro-inflammatory cytokine has been implicated in the activation of the sphingomyelin signal transduction cascade in cardiomyocytes [20, 29]. Moreover, TNFα production from the ischemic heart has been suggested as part of the pathophysiology associated with acute ischemic events [23].

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Address correspondence to:

Roger A. Sabbadini, Ph.D., Department of Biology, San Diego State University, 5500 Campanile Dr., San Diego, CA 92182 4614, U.S.A., tel. (619) 594 6272, fax (619) 594 0626, Email rsabba@sunstroke.sdsu.edu.

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