Burn plasma mediates cardiac myocyte apoptosis via endotoxin

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Carlson, Deborah L., Ellis Lightfoot, Jr., Debora D. Bryant, Sandra B. Haudek, David Maass, Jureta Horton, and Brett P. Giroir. Burn plasma mediates cardiac myocyte apoptosis via endotoxin. Am J Physiol Heart Circ Physiol 282: H1907–H1914, 2002; 10.1152/ajpheart.00393.2001.—Thermal trauma is associated with cardiac myocyte apoptosis in vivo. To determine whether cardiac myocyte apoptosis could be secondary to burn-induced cytokines or inflammatory mediators, we investigated the effects of tumor necrosis factor-α (TNF-α) and burn plasma on a murine cardiac myocyte cell line and primary culture myocytes. HL-1 cells were exposed to plasma isolated from burned or sham rats. Burn, but not sham plasma, induced significant increases in caspase-3 activity and DNA fragmentation. Similar results were obtained in primary culture rat myocytes. A dose-dependent increase in caspase-3 activity was observed when HL-1 cells were incubated with increasing concentrations of TNF-α. Even though TNF-α increased apoptosis, enzyme-linked immunosorbent assay detected no TNF-α in burn plasma. Burn plasma also failed to induce TNF-α mRNA, eliminating an autocrine mechanism of TNF-α secretion and binding. Also, treatment of burn plasma containing rhuTNFR:Fc failed to inhibit apoptosis. To examine the possibility that endotoxin within burn plasma might account for the apoptotic effect, burn plasma was preincubated with rBPI21. Caspase-3 activity was reduced to control levels. These data indicate that burn plasma induces apoptosis in cardiac myocytes via an endotoxin-dependent mechanism and suggest that systemic inhibition of endotoxin may provide a therapeutic approach for treatment of burn-associated cardiac dysfunction.

Although apoptosis is often evolutionarily adaptive, it may also be associated with the progression of human diseases. Cardiac myocyte apoptosis has been implicated in the pathogenesis of heart failure of diverse etiologies, including myocarditis (20), ischemia-reperfusion injury (13), chronic pressure overload (2, 3), congestive heart failure (28), and sepsis (27). Recently, Horton (14) demonstrated that myocyte apoptosis occurred in the ventricular myocardium of rats undergoing severe thermal trauma and was temporally correlated with the development of cardiac depression.

There are several potential explanations for the development of myocyte apoptosis following thermal trauma. It is possible that a decrement in coronary perfusion pressure, despite volume resuscitation, may result in ischemic myocardial damage. Ischemia could be worsened by transient hypoxemia from acute lung injury and increased lung permeability following burn trauma (19, 31). However, it is also possible that apoptosis is secondary to cytokines present in the circulation following injury. A prime candidate is tumor necrosis factor-α (TNF-α), which may be increased after thermal trauma and which has been associated with the disruption of endothelial integrity and the loss of cardiac function (6, 11, 12). Presumably, TNF-α might cause apoptosis via activation of the intracellular death domain of TNFR1 with subsequent triggering of the caspase cascade (18).

The purpose of this study was to determine whether plasma obtained from thermally injured rats could induce apoptosis, and furthermore to identify which factor(s) present in plasma accounts for myocyte apoptosis in this model. The identification of a proapoptotic factor could provide a therapeutic target potentially capable of ameliorating cardiac failure following burn injury.

METHODS

Animals. Pathogen-free adult male Sprague-Dawley rats (325–350 g) were purchased from Harlan Laboratories, (Houston, TX). All animals were acclimated to their sur-
roundings for 5 days before experimentation. The research protocol was conducted in accordance with the guidelines of the Institutional Review Board for Animal Research at The University of Texas Southwestern Medical Center and within the guidelines of the American Physiological Society and The National Institutes of Health.

**Burn procedure and plasma isolation.** Rats were deeply anesthetized with methoxyflurane and secured in a constructed template device. They were subjected to full-thickness dermal burns comprising 43 ± 1% of the total body surface area, as previously described (17). Sham-burned rats were subjected to an identical procedure except they were immersed in room temperature water. After immersion, the rats were immediately dried and placed in individual cages to recover from anesthesia. Burned rats did not display discomfort or pain, and all rats consumed food and water within 45 min of the burn procedure.

Plasma was harvested from either burned or sham-burned rats 4 h postinjury. The rats were deeply anesthetized with methoxyflurane, and cardiac puncture was used to collect blood into two 1 ml separator tubes containing heparin (Microtainer). The tubes were then spun at 200 g for 10 min. The plasma was aliquotted and stored at −80°C.

**Cardiomyocyte isolation.** To examine several aspects of burn-mediated cardiomyocyte dysfunction, hearts were collected at several times postburn; time-matched sham burns were included to provide appropriate controls. All animals collected at several times postburn; time-matched sham burns were included to provide appropriate controls. All animals were treated with increasing increments of calcium (100, 200, 500 μM). After the final pelleting step, the supernatant was removed, and the pellet was resuspended in minimum essential medium (MEM) [which was prepared by adding 10.8 g MEM (Sigma M-1018), 1 g NaHCO3, 2.3 g HEPES, and 10 ml penicillin-streptomycin (100×, Gibico-BRL 1540-122) with 950 mM water; total volume is then adjusted to 1 liter. At the time of MEM preparation, the medium was bubbled with 95% O2-5% CO2 for 15 min, the pH was adjusted to 7.1 with 1 M NaOH, and the solution was then filter sterilized and stored at 4°C until use]. At the final concentration of calcium, the cardiomyocyte cell number was calculated per milliliter, and viability was determined.

**Cell culture.** The HL-1 cell line was obtained from Dr. William C. Claycomb, Louisiana State University Medical Center. HL-1 cells are a cardiac muscle cell line derived from the AT-1 subcutaneous mouse atrial cardiomyocyte tumor lineage, which maintain the morphological, biochemical, and electrophysiological phenotype of adult myocytes in culture (5). HL-1 cells can be passaged serially and cultured as adherent cells in T-25 flasks as described (5). We estimated bioactivity of TNF-α by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) analysis that ~8% of confluent HL-1 cells undergo apoptosis without stimulation. The cells are grown in Ex-Cell 320 medium part A and B (JRH Biosciences; Lenexa, KS), 10% fetal bovine plasma, 10 μg/ml insulin, 50 μg/ml endothelial cell growth supplement, 1 μM retinoic acid, 10 μM norepinephrine, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 pg/ml amphotericin, and an additional 1× nonessential amino acids. Cells were stimulated with 1–50 ng/ml recombinant mouse TNF-α. Cells were stimulated after reaching confluency for 24 h. Confluent HL-1 cells were treated with 50 ng/ml rhuTNFR:Fc (Immunex), a soluble TNF-α receptor, which binds and neutralizes the bioactivity of TNF-α. Confluent HL-1 cells were also treated with plasma isolated from either burned or sham-burned rats, which had been incubated with 2 μg/ml of a recombinant NH2-terminal fragment of bacterial permeability-increasing protein (rBPI21, XOMA, Berkeley, CA). Incubation with rBPI21 occurred for 30 min before exposure to HL-1 cells to neutralize any endotoxin present in the plasma (25). All cells were removed from the flasks by gentle scraping and agitation for analysis.

**TUNEL assay.** Twenty microliters (1 × 106 cells/ml) of HL-1 cells were harvested from six-well macrotiter plates by gentle agitation, transferred to a ProbeonPlus slide (Fisher Scientific; Houston, TX), and allowed to adhere for 1 h at room temperature. The slides were then fixed in 10% formalin for 10 min at room temperature, washed in phosphate-buffered saline (PBS; pH 7.4) twice for 5 min at room temperature, postfixed in ethanol-acetic acid (2:1 vol/vol) for 5 min at −20°C, and washed twice in PBS (pH 7.4) for 5 min at room temperature. Cells were stained using in situ apoptosis detection reagents (Intergen; Purchase, NY). To accomplish this, cells were preincubated in equilibration buffer (134 mM NaCl, 5.5 mM KCl, 1.0 mM KH2PO4, 20 mM NaHCO3, 10 mM KHCO3, 20 mM glucose; and 0.5× basal medium Eagle amino acids (40×, Gibico-BRL 11130-051); 10 mM HEPES; 30 mM taurine; 2.0 mM carnitine; and 2.0 mM creatine, which was bubbled with 95% O2-5% CO2. The cells were then cannulated via the aorta and perfused with heart medium at the rate of 12 ml/min for a total of 5 min in a nonrecirculating mode. Enzymatic digestion was initiated by perfusing the heart with a digestion solution; 60 ml of digestion solution was prepared by adding 50 mg of collagenase II (Worthington 4177, Lot No. MOB8771), 50 mg of bovine serum albumin (BSA), fraction V (Gibico-BRL 11018-025), 0.5 ml trypsin (2.5%, 10×, Gibco-BRL 15090-046), 7.5 μl CaCl2 (100 mM) to 34.5 ml of heart medium prepared as described above, and 15 ml 2,3-butanedionemonoxide (BDM) stock (40 mM). Enzymatic digestion was accomplished by recirculating this solution through the heart at a flow rate of 12 ml/min for 20 min. The temperature of the heat exchange perfusion apparatus was maintained such that all solutions perfusing the heart were constant at 37°C. At the end of the enzymatic digestion, the ventricles were removed and mechanically dissociated in 6 ml of enzymatic digestion solution plus 6 ml of 2× BDM/BSA solution [prepared by adding 5 g BSA, fraction V (Gibico-BRL 11018-025) to 150 ml of BDM stock (40 mM)]. After mechanical dissociation by mincing with fine scissors, the tissue homogenate was filtered through a mesh filter into a conical tube; cells adhering to the filter were collected by washing with an additional 10-ml aliquot of 1× BDM-BSA solution that was prepared by combining 100 ml of BDM stock, 40 mM; 100 ml of heart medium prepared as described above, and 2 g of BSA, fraction V (Gibico-BRL 11018-025). Cells were then allowed to pellet in the conical tube for 10 min. The supernatant was removed, and the pellet was resuspended in 10 ml of 1× BDM-BSA. The cells were then washed and pelleted further in BDM-BSA buffer with increasing increments of calcium (100, 200, 500 μM, and a final concentration of 1,000 μM). After the final pelleting step, the supernatant was removed, and the pellet was resuspended in minimum essential medium (MEM) (which was prepared by adding 10.8 g MEM (Sigma M-1018), 1 g NaHCO3, 2.3 g HEPES, and 10 ml penicillin-streptomycin (100×, Gibico-BRL 1540-122) with 950 ml water; total volume is then adjusted to 1 liter. At the time of MEM preparation, the medium was bubbled with 95% O2-5% CO2 for 15 min, the pH was adjusted to 7.1 with 1 M NaOH, and the solution was then filter sterilized and stored at 4°C until use]. At the final concentration of calcium, the cardiomyocyte cell number was calculated per milliliter, and viability was determined.

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for 30 min at room temperature in a dark, moist chamber (13 μL/cm²) and washed three times in PBS (pH 7.4) for 5 min at room temperature. The slides were counterstained with propidium iodide (3 μL/cm²) (Ventana Medical Systems; Tucson, AZ) at room temperature in the dark and sealed with a glass coverslip mount. Slides were stored at −20°C until examination.

Cells were examined with a Nikon Optiphot-2 fluorescent microscope at ×430 magnification. The number of apoptotic cells was counted in a total of 1,000 myocytes over several random fields, and the percent TUNEL-positive cells was calculated. The examiner was blinded to the experimental conditions.

Caspase-3 activity assay. Caspase-3 activity was quantified by measuring a relative synthetic peptide composed of aspartic acid, glutamic acid, arginine, and aspartic acid (DEVDase), or caspase, cleavage activity. The assay was carried out using the ApoAlert Caspase-3 Assay Kit (Clontech). HL-1 cells were incubated in six-well plates to confluence and were stimulated with TNF-α or plasma as described in RESULTS. Negative control cells were not stimulated. Caspase activity was measured according to the manufacturer’s instruction. All results were calculated against a standard pNa calibration curve. To confirm the correlation between protease activity and signal detection, we also performed the control reaction of incubating a TNF-α-induced sample with caspase-3 inhibitor (DEVD-fmk) before adding substrate.

DNA-based ELISA. Mono- and oligonucleosomes produced by endogenous endonucleases were detected using mouse monoclonal antibodies. HL-1 cells were grown in 48-well plates until confluent and were stimulated with varying concentrations of TNF-α and/or 10% burn or sham-burned plasma for 24 h. The Cell Death Detection ELISA PLUS kit (Roche Diagnostics) was used to assess apoptosis, and the manufacturer’s directions were followed for the assay with the exception that after lysis, 5 μl instead of 20 μl from the supernatant were transferred into the streptavidin-coated microtiter plate. All samples were done in duplicate, and the absorbance values were averaged. Results were calculated after subtracting the background value of the immunoassay from the average of the absorbance values.

Mouse TNF-α immunoassay. After a 24-h exposure to plasma isolated from either burned or sham-burned rats, HL-1 cells were collected into a 1.5-ml Eppendorf tube and were resuspended in 10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 20 μg/ml leupeptin. After the cells were lysed with a 25-g needle, they were then microcentrifuged for 20 min at 14,000 g. Fifty microliters of each resulting supernatant were used for the enzyme-linked immunosorbent assay (ELISA) assay to measure TNF-α. The assay was performed as per the manufacturer’s instructions (Quantikine Murine, R&D Systems).

RT-PCR. All reagents and primers were purchased from Gibco-BRL. RNA (1.5 μg) was reverse transcribed with the use of six units of SuperScript RTIII, 12.5 ng/ml of oligo (dT)12–18, and 100 μM of each dNTP in a solution containing 1 unit Rnasin solution, 5 μM 1,4-dithiothreitol, and 1× buffer (50 mM Tris·HCl, pH 8.3; 75 mM KCl, and 3 mM MgCl₂) in a total volume of 20 μl. The reaction was carried out at 37°C for 60 min, followed by 95°C for 5 min to heat inactivate the reverse transcriptase. The primers for ‘TNF-α’ were 5’ GCC GGT ACC CTC AGA TCA TCT TCT CAA AAT 3’ and 5’ TTC TCC AGC TGG AAG ACT CC 3’.

Reverse-transcribed cDNA (~0.5 μg in 2 μl) was then mixed with 1× PCR buffer (20 mM Tris·HCl, pH 8.4; 50 mM KCl); 1 mM MgCl₂; 20 μM of each dNTP; 20 ng/μl of each primer set, and 1 unit platinum Taq in a 20-μl reaction. The

Fig. 1. Burn plasma causes apoptosis in HL-1 cardiac myocytes. A: caspase-3 activity as measured by caspase (DEVDase) activity. +, Addition of burn plasma, control plasma, or tumor necrosis factor-α (TNF-α). Burn or control plasma was added to HL-1 cells to 10% vol/vol for 24 h. Control plasma, plasma isolated from sham-burned rats; burn plasma, plasma isolated from rats 4 h postburn injury. TNF-α was added to a concentration of 20 ng/ml for 24 h. B: DNA-based enzyme-linked immunosorbent assay (ELISA). ELISA assay measuring the absorbance the enrichment of mono- and oligonucleosomes in treated and control HL-1 cells.

Order is identical to A. Values are expressed as means ± SE. *P < 0.05 compared with control.

Fig. 2. Burn plasma causes apoptosis in primary culture cardiac myocytes. Caspase-3 activity as measured by caspase (DEVDase) activity. +, Denotes the addition of burn or control plasma. Burn or control plasma was added to isolated rat myocytes to 10% vol/vol for 24 h. Control plasma, plasma isolated from sham-burned rats; burn plasma, plasma isolated from rats 4 h postburn injury. *P < 0.05 compared with control.

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following conditions were used for TNF-α amplification: one cycle of 94°C for 2 min, followed by 27 cycles of (94°C for 45 s, 63°C for 45 s, 72°C for 45 s) and then one cycle of 72°C for 5 min.

RESULTS

Plasma isolated from burned rats stimulates apoptosis in HL-1 and primary culture cardiac myocytes. Confluent HL-1 cardiac myocytes were exposed to medium containing 10% plasma harvested from either burned or sham-burned rats for a 24-h period. Plasma was harvested 4 h after burn wound based on caspase-3 activity assays and a DNA-based ELISA, which measured the number of nucleosomes containing single- or double-stranded DNA. Maximum HL-1 cell apoptosis was observed using plasma harvested 4 h postburn compared with 2, 8, 18, and 24 h after burn trauma (data not shown).

A significant 3.6-fold increase in caspase-3 activity was observed in HL-1 cells treated with plasma harvested from burned versus sham-burned animals (Fig. 1A). This increase was comparable to the apoptotic rise observed in confluent HL-1 cells treated with 20 ng/ml TNF-α for 24 h. Consistent with the caspase-3 data, a 3.4-fold increase in apoptosis was also detected using the DNA-based ELISA (Fig. 1B).

To confirm that the results observed in HL-1 cells were representative of cardiac myocytes, primary culture myocytes were harvested from adult male Sprague-Dawley rats. The myocytes were stimulated for 24 h with 10% plasma harvested from either burned or sham-burned rats. No significant difference in caspase-3 activity was detected between control cells and those exposed to sham-burned plasma, but a two-fold increase in caspase-3 activity was observed when...
primary culture myocytes were exposed to plasma harvested from burned animals (Fig. 2). These results were confirmed with the TUNEL assay (data not shown).

TNF-α concentrations were measured by ELISA in the primary culture myocytes both at the time of harvest and 24 h after plasma stimulation. Less than 1 pg/ml of TNF-α was detected at any time point in either the control, sham, or burn-stimulated cells.

In an independent experiment, the effect of burn plasma on HL-1 cells was also examined using TUNEL. The results are shown in Fig. 3. As can be seen in Fig. 3, B and D, when HL-1 cells are exposed to either TNF-α as a positive control or 10% burn plasma, apoptotic cells appear as green bodies as a result of the TUNEL-FITC staining. In contrast, in Fig. 3, A and C, when HL-1 cells are either untreated as a control or exposed to 10% plasma isolated from sham-burned rats, the cells appear red from the propidium iodide staining. These results are consistent with the findings presented in Fig. 1. We conclude that plasma harvested from burned, but not sham-burned, animals, is capable of causing apoptosis in HL-1 cells.

**Burn plasma-induced apoptosis is TNF-independent.** It has previously been demonstrated that TNF-α causes apoptosis in a variety of cell types, including myocytes (27). We confirmed that TNF-α also induced a dose-related increase in apoptosis in HL-1 cardiac myocytes. A sixfold increase in caspase-3 activity was observed between cells treated with 1 ng/ml TNF-α and 50 ng/ml TNF-α (Fig. 4A). As an independent confirmation of apoptosis, we also observed a sevenfold increase in the enrichment of mono- and oligosomes (Fig. 4B), confirming the dose-dependent increase in apoptosis using the ELISA assay. A fourfold increase in TUNEL-positive cells was observed (data not shown) between control HL-1 cells and those incubated with 50 ng/ml TNF-α. Taken together, these results confirm that exposure of HL-1 cardiac myocytes to TNF-α results in apoptosis.

Because burn trauma is associated with an increase in both local and systemic levels of TNF-α, we hypothesized that the apoptotic response observed was due to TNF-α. However, this hypothesis was refuted by three independent groups of experiments. First, an ELISA assay specific for mouse TNF-α detected no measurable TNF-α in either burn or sham burn plasma (data not shown). To investigate the possibility that TNF-α was synthesized by HL-1 cells in response to burn plasma, the TNF-α ELISA was also performed on the culture medium after the 24-h coincubation of burn plasma with HL-1 cells. Again, no TNF-α was detected in culture medium (data not shown). Despite the lack of TNF-α in the medium, it was theoretically possible that TNF-α might be secreted in small quantities by HL-1 cells and act in an autocrine fashion such that TNF-α might not be detected in the culture medium. To investigate this possibility, we performed RT-PCR to sensitively detect TNF-α mRNA in the stimulated HL-1 cells. No TNF-α mRNA was detected by RT-PCR (Fig. 5).

To conclusively demonstrate that TNF-α was not involved in HL-1 cell apoptosis, we pretreated plasma with 50 ng/ml of rhuTNFR:Fc before incubation with HL-1 cells. Despite blockade of TNF-α bioactivity, no decrease in apoptosis was observed in samples treated with rhuTNFR:Fc, as measured by both the caspase-3 assay and the DNA-based ELISA. These experiments indicated that TNF-α is not responsible for cardiac myocyte apoptosis induced by burn plasma (Fig. 6, A and B).
Burn plasma-induced apoptosis is endotoxin dependent. Because apoptosis was not caused by TNF-α, we next hypothesized that plasma endotoxin might be responsible for inducing apoptosis. To determine whether endotoxin was capable of inducing apoptosis in HL-1 cardiac myocytes, we incubated cells with lipopolysaccharide (LPS, 10 ng/ml to 1 μg/ml) for 24 h. A significant increase in caspase activation was detected at LPS concentrations higher than 100 ng/ml (Fig. 7A). The LPS-stimulated increase in caspase activity could be alleviated by the addition of 2 μg/ml of a recombinant NH₂-terminal bactericidal permeability-increasing protein (rBPI21) to the LPS containing medium for 30 min before exposure to HL-1 cells (Fig. 7A). To determine whether the LPS-induced apoptosis was secondary to increased TNF-α secretion, we also examined caspase activity in HL-1 cells treated with 50 ng/ml of rhuTNFR:Fc. No significant decrease in caspase-3 activity was observed (data not shown).

To determine whether endotoxin contained within the burn plasma was associated with HL-1 apoptosis, we neutralized any endotoxin activity by preincubation of the burn plasma with 2 μg/ml of rBPI21. Pretreatment with rBPI21 resulted in a 2.7-fold decrease in caspase activity as compared with non-rBPI-treated samples, indicating that endotoxin within the plasma collected from burned rats was causally associated with the observed apoptosis (Fig. 7, B and C). Higher concentrations of rBPI21 (2, 5, 10, and 20 μg/ml) were also tested to determine whether apoptosis could be completely blocked. No significant difference in inhibition was observed (data not shown). Lower concentrations of rBPI21 resulted in a dose-dependent response. To confirm the specificity of the rBPI21, we repeated the experiment, preincubating the burn plasma with 2.5, 5, 10, and 20 μg/ml of BSA, respectively, before burn plasma. The addition of BSA to culture had no effect on burn plasma-induced apoptosis.

**DISCUSSION**

Myocyte apoptosis occurs in several cardiac diseases, including myocarditis (20), ischemia-reperfusion injury (13), chronic pressure overload (2, 3), congestive heart failure (28), and sepsis (27). Recently, we demonstrated that myocyte apoptosis occurred in the ventricular myocardium of rats following severe thermal trauma and was temporally correlated with the development of cardiac dysfunction (14). Here, we demonstrate by multiple methods, that myocyte apoptosis following thermal injury is mediated, at least in part, by factors in burn plasma. Apoptosis was neither associated with the presence of TNF-α in burn plasma, nor the induction of TNF-α in either HL-1 cells or primary culture myocytes exposed to burn plasma. However, apoptosis was significantly reduced by inhibition of endotoxin activity in burn plasma by rBPI21, a recombinant NH₂-terminal fragment of the bactericidal permeability-increasing protein.

The ability of burn plasma to induce apoptosis was examined in HL-1 cells, a cardiac muscle cell line that maintains an ultrastructure similar to primary cardiac myocytes and maintains the ability to spontaneously contract while remaining in a mitotic state typical of normal in vivo immature mitotic cardiomyocytes (5).
HL-1 cells represent a particularly useful model, because it has been recently shown that human cardiac myocytes are capable of entering mitosis and undergoing division after injuries such as myocardial infarction (1). We show that in HL-1 cells, caspase-3 activity was induced by burn plasma to levels at least three times greater than control levels. Caspase-3 activation confirms that at least one apoptotic signal transduction pathway has been activated. In addition to caspase signaling, we also demonstrated the presence of late-stage apoptotic cells via TUNEL staining and a DNA-based ELISA measuring the enrichment of mono- and oligosomes from the systematic breakdown of the nucleosomal DNA.

Whereas HL-1 cells represent a useful and relevant myocyte model, we further demonstrated that burn plasma was capable of stimulating apoptosis in primary culture rodent myocytes. Using the caspase-3 assay, a twofold increase was observed in myocytes that were exposed to plasma isolated from burned animals, confirming the HL-1 cells as a relevant model system.

Because TNF-α is a potent inducer of cardiac myocyte apoptosis (22) and because TNF-α is frequently induced by severe systemic insults, we fully expected that TNF-α would account for the apoptotic activity of burn plasma. This hypothesis proved completely false. No TNF-α was detected in burn plasma, and no TNF-α or TNF-α mRNA was produced by HL-1 cells. This lack of TNF-α in burn plasma is not entirely unexpected, because elevation of plasma TNF-α was uncommon in multiple studies of severe burn injury in humans (9, 10, 29).

Because TNF-α was not directly involved in the pathogenesis of apoptosis in this model, we investigated additional possibilities. Burn trauma results in a multitude of cellular changes that may trigger the apoptotic cascade, including increases in intracellular calcium concentration, a rise in reactive oxygen metabolites, and an increase in both local and systemic levels of interleukin-1 (16, 21, 30). In addition, burn injury can also lead to transient intestinal ischemia, contributing to a disruption of the normal gut barrier function and translocation of indigenous bacteria or bacterial products (7, 8, 15). Because endotoxin infusion was recently associated with cardiac myocyte apoptosis in a rat model (26), we hypothesized that endotoxin present in the plasma of burned animals might also account for cardiac apoptosis in the burn model.

rBPI21, a recombinant form of the neutrophil-derived protein binds with high affinity to the lipid A portion of LPS and inhibits all LPS bioactivity, including its ability to promote apoptosis (4). Bactericidal permeability-increasing protein is known to neutralize LPS activities in vitro and in vivo (24). When burn plasma was preincubated with rBPI21, the caspase-3 activity dropped 2.7 times compared with the activity observed in using untreated burn plasma. These results indicate that a significant portion of the observed apoptotic activity is due to endotoxin contained within the plasma collected from the burned animals. The observation that pretreatment of the cells with rBPI21 does not result in the abolition of all apoptosis, suggests the presence of other unidentified proapoptotic factors within the burn plasma. The source of endotoxin could be from the gut via translocation or from colonization of the burn wound itself. Whichever the specific origin, these data suggest that the systemic inhibition of endotoxin may provide a therapeutic approach for the treatment of burn-associated cardiac dysfunction.

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