Human Serum from Patients with Septic Shock Activates Transcription Factors STAT1, IRF1, and NF-κB and Induces Apoptosis in Human Cardiac Myocytes*

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Proinflammatory cytokines have been linked to depression of myocardial contractility in vivo in patients with acute septic shock and in vitro models employing isolated myocytes exposed to serum from such patients. The key pathways involved in mediating this septic organ dysfunction (cell adhesion molecule expression, inducible nitric-oxide synthase induction, and apoptosis) are known to be regulated by transcription factors STAT1, IRF1, and NF-κB. Utilizing a model that mimics human disease, we have demonstrated activation of the transcription factors STAT1, IRF1, and NF-κB in human fetal myocytes exposed to human septic serum. Both reporter and electrophoretic mobility shift assays demonstrated a 5–19-fold increase in activation of transcription factors STAT1, IRF1, and NF-κB in response to incubation with human septic serum. The addition of human septic serum to human fetal myocytes induced apoptosis in human fetal myocytes and activation of the mitogen-activated protein kinase c-Jun NH2-terminal kinase and caspase 1 as measured by Western blot. These data suggest that transcription factor activation and early myocyte apoptosis play a mechanistic role in septic myocardial depression and sepsis-induced organ dysfunction.

Septic shock is a serious disorder with significant morbidity and mortality despite appropriate antibiotic and supportive therapy. Ultimately, patients with fatal septic shock succumb to either refractory hypotension associated with acute cardiovascular failure or multiple organ failure. In either case, cardiac dysfunction is typical (1, 2). Myocardial depression in human septic shock has been linked to circulating myocardial depressant substances (3, 4). Although several potential mechanisms have been implicated in septic myocardial depression, including NO generation (5), adhesion molecule expression (6, 7), and apoptosis (8–10), the specific intracellular signaling mechanisms underlying the process have yet to be elucidated.

In this study, we have characterized molecular signaling pathways that may occur in the heart during sepsis using a variation of a well-defined tissue culture model that mimics the human disease process. Specifically, we have determined the activation status of the mitogen-activated protein kinase family member c-Jun NH2-terminal kinase (11) (JNK)2 and transcription factors signal transducers and activators of transcription (12) (STAT), interferon regulatory factor (13) (IRF), and NF-κB (14) family members with the addition of serum harvested from patients with septic shock (human septic serum; HSS) to human fetal myocyte (HFM) cells. These transcription factors have been selected because they represent major downstream regulatory signaling mechanisms of the proinflammatory cytokines that regulate genes known to drive septic organ dysfunction (15). In addition, these transcription factors have central roles in the induction of apoptosis (8, 16–23), which has, in recent years, been shown to be important in sepsis-related myocardial dysfunction (9, 10, 24) and septic mortality (25–28) in animal models. Since JNKs play a critical role in the balance of cytokine-mediated cell survival and cell death (11), we also examined the status of JNKs in our model of sepsis. To further elucidate the potential role of cytokine-mediated apoptotic signaling in septic organ dysfunction, the effect of human septic serum on caspase 1 activation and cell apoptosis was examined.

MATERIALS AND METHODS

Study Patients from Whom HSS Was Obtained—Serum was derived from 10 patients who were in the acute phase of septic shock as defined by modified American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference criteria (29). Patients were required to have all (rather than a minimum of two) of the following four criteria for systemic inflammatory response syndrome: 1) a body temperature greater than 38 °C or less than 36 °C; 2) a heart rate greater than 90 beats/min; 3) tachypnea, manifested by a respiratory rate greater than 20 breaths/min or hyperventilation, as indicated by a PaCO2 of less than 32 mm Hg; 4) an alteration in the white blood cell count (i.e. white blood cells greater than 12,000/mm3, less than 4000/mm3, or the presence of more than 10% immature neutrophils). In addition, all patients whose serum samples were utilized exhibited positive blood cultures with a defined focus of infection (e.g. peritonitis or pneumonia) and required substantial pressor therapy (greater than 0.5 μg/kg/min norepinephrine) to maintain mean arterial pressure greater than 65 mm Hg. Serum samples were obtained within 24 h of presentation with septic

References

1 The abbreviations used are: JNK, c-Jun NH2-terminal kinase; STAT, signal transducers and activators of transcription; IRF, interferon regulatory factor; iNOS, inducible nitric-oxide synthase; HSS, human septic serum; HFM, human fetal myocyte; DMEM, Dulbecco’s modified Eagle’s medium; IFN, interferon; FMK, fluoromethyl ketone; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; IL, interleukin; EMSA, electrophoretic mobility shift assay; Z, benzoyloxycarbonyl; TPCK, tosylphenylalanyl chloromethyl ketone.

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Human septic serum was obtained after informed consent under an approved Institutional Review Board (Rush University) approved protocol. As part of that protocol, left ventricular contractility was assessed in each patient at presentation by portable radionuclide angiography as previously described (1). Subjects contributing HSS for this study were not known to have preexisting structural heart disease. Immediately prior to cineangiography, 10 cm3 of blood was drawn from the patient and centrifuged for 10 min at 1700 × g. The supernatant representing the serum was aliquoted and stored at −70 °C. Before use in experiments, the serum was heated-inactivated at 56 °C for 45 min. Key characteristics, radionuclide-determined ejection fraction and cytokine profiles of serum donors with septic shock is shown in TABLE ONE. Normal human serum was obtained from laboratory personnel and commercial sources (Sigma).

Cells—The HFM cells used in the preliminary studies are primary cells obtained from an aborted fetus at 20 weeks (Clonetics Laboratories, Inc.). Myocytes were isolated by Ficol gradient, and viable myocytes was confirmed by staining for myosin IgG, sarcomeric actin IgM, and α SMC actin.

Enzyme-linked Immunosorbent Assays—Concentrations of TNFα and IL-1β in human septic serum was determined by an enzyme-linked immunosorbent assay using a sandwich immunoassay (purchased from Roche Applied Science). The assay is based on an enzyme-linked immunosorbent assay with anti-secondary antibody conjugated to horseradish peroxidase. Tetramethylbenzidine is the substrate.

Construction of STAT, IRF, and NF-kB Reporter Constructs—We used the pLuc luciferase vector for the STAT and NF-kB luciferase reporters. The pLuc vectors contain an enhancer restriction enzyme multicloning site 5’ to a functional TATATA RNA polymerase initiation binding site, which itself is 5’ to the luciferase gene. We cloned the STAT regulatory element 5’-TTCCGGGAA-3’ blunt using Smal into the pLuc vector multicloning site and selected for two forward copies of this STAT regulatory element and used this construct in the experiments. The NF-kB pLuc luciferase reporter construct is driven by the NF-kB regulatory element multimerized 5’-(TGGGAGCTTCCGCGC)5-3’ and was purchased from Stratagene. The Gbp-2 promoter (−550/+1) was cloned upstream of the pGL2 vector and contains the IRF binding sequence 5’-AAGTTGA-3’. Transfection of these reporter constructs into HFM cells was performed as previously described (30). HFM cells (2 × 106 cells/10-cm dish) were incubated separately with no serum, 10% normal serum, 10% HSS, 106 HFM cells (10-cm plate) treated with no serum, 10% normal serum, or 10% HSS in DMEM as indicated. The JNK inhibitor SP600125 (Sigma; stock solution prepared in Me2SO at a concentration of 10 mM and used at final tissue culture concentration of 10 μM); the NF-kB inhibitor TPCK (Sigma; stock solution prepared in Me2SO at a stock concentration of 10 mM and used at a final tissue culture concentration of 10 μM); the caspase 1, 4, and 5 inhibitor Ac-YVAD-FMK; the caspase 1 pseudoinhibitor Z-FA-FMK; the caspase 2 inhibitor Z-VD-VAD-FMK; the caspase 3, 6, 7, 8, and 10 inhibitor AC-DEVD-CHO; and the pancaspase inhibitor Z-VAD-FMK (Calbiochem; caspase inhibitors were prepared in Me2SO at a stock concentration of 10 mM and used at a concentration of 10 μM in tissue culture) were added individually at the same time as the 10% normal serum or 10% septic serum. After 18 h, the cells were trypsinized, and viable cells were counted using a trypsin blue exclusion assay.

HSS-induced Genomic DNA Laddering in HFM Cells—2 × 106 HFM cells (10-cm plate) were treated with no serum, 10% normal serum, or 10% HSS in DMEM for 18 h. The cells were washed with PBS, harvested by scraping in PBS, and microcentrifuged. The cell pellets were treated for 10 s with lysis buffer (0.5% Nonidet P-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). The resuspended pellet was centrifuged, and supernatants that contained the genomic DNA were brought to 1% SDS and treated for 2 h with RNase A (final concentration 5 mg/ml) and subsequently with proteinase K for 4 h. After the addition of one-half volume of 10 mM ammonium acetate, the DNA was precipitated with 2.5 volumes of ethanol, dissolved in gel loading buffer. The genomic DNA was fractionated by 1% agarose electrophoresis.

Western Blot Analysis—2 × 106 HFM cells (10-cm plate) were treated with 10% normal human serum or 10% HSS for 12 h. The cells cultured in DMEM were washed with PBS and scraped in 1 ml of PBS, transferred to microcentrifuge tubes, and microcentrifuged. The supernatant was removed, and the cell pellets were resuspended in 100 μl of radiomun precipitation lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and incubated on ice for 20 min. The whole cell extracts were microcentrifuged, and 100 μg of the supernatant representing the whole cell extract was analyzed on a 10% SDS-polyacrylamide gel and subsequently wet electrotransferred to polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was blocked with 5% blotto. Polyclonal caspase 1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added at a 1:200 dilution, polyclonal phospho-JNK antibody (Cell Signaling; Thr183/Tyr185) was added at a 1:2000 dilution, and JNK monoclonal antibody (Santa Cruz Biotechnology) was added at 1:200 dilution in the TBST (0.1% Tween) binding buffer. The plots were washed with 1 × TBST (0.1% Tween). The appropriate secondary antibodies were incubated with the blots at a 1:5000 dilution. The blots were washed with 1 × TBST (0.1% Tween). The blots were subjected to ECL using Pierce reagents, and imaging and densitometry were performed using the Bio-Rad ChemiDoc XRS system.

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Immediate prior to cineangiography, 10 cm3 of blood was drawn from the patient and centrifuged for 10 min at 1700 × g. The supernatant representing the serum was aliquoted and stored at −70 °C. Before use in experiments, the serum was heated-inactivated at 56 °C for 45 min. Key characteristics, radionuclide-determined ejection fraction and cytokine profiles of serum donors with septic shock is shown in TABLE ONE. Normal human serum was obtained from laboratory personnel and commercial sources (Sigma).

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TABLE ONE

Infecting organism and serum cytokine profile of patients with septic shock

| Patient no. | Clinical infection       | Infecting organism          | Ejection fraction | Serum TNFα concentration | Serum IL-1β concentration |
|-------------|-------------------------|-----------------------------|-------------------|--------------------------|--------------------------|
|             |                         |                             | %                 | pg/ml                    | pg/ml                    |
| 1           | Pyelonephritis           | Escherichia coli            | 21                | 111                      | 264                      |
| 2           | Pneumonia                | Escherichia coli            | 18                | 26                       | 78                       |
| 3           | Pneumonia                | Streptococcus pneumoniae    | 17                | 294                      | 108                      |
| 4           | Peritonitis              | Multiple enteric organisms  | 38                | 141                      | 30                       |
| 5           | Pneumonia                | Staphylococcus aureus       | 33                | 512                      | 78                       |
| 6           | Cholangitis              | Escherichia coli            | 23                | 903                      | 158                      |
| 7           | Neutropenic sepsis       | Klebsiella pneumoniae       | 25                | 319                      | 394                      |
| 8           | Pyelonephritis           | Escherichia coli            | 40                | 447                      | 2                        |
| 9           | Necrotizing fasciitis    | Group A streptococci       | 26                | 133                      | 30                       |
| 10          | Intra-abdominal abscess  | Pseudomonas aeruginosa      | 37                | 466                      | 17                       |
|             |                         |                             | Mean              | 28 ± 3                   | 335 ± 82                 | 146 ± 47                 |

*E. coli, Enterococcus faecalis, Candida albicans.*

**Statistical Analysis**—Data are expressed as means ± S.E. of the mean. All group comparisons were performed by two-tailed Student’s t test. A Bonferroni correction for multiple comparisons of transcription factor activation (TABLE TWO) mandated an α of 0.0133 for significance.

**RESULTS**

**Serum and Serum Donor Characterization**—Human septic serum donors had a mean age of 56 ± 3 years, with six males and four females. Eight of the 10 eventually succumbed to septic shock or sepsis-related organ failure. HSS donors, in addition to demonstrating evidence of myocardial depression in vivo (as manifested by decreased left ventricular ejection fraction), also had, in aggregate, significantly elevated levels of TNF-α and IL-1β, as indicated in TABLE ONE. TNF-α and IL-1β levels in serum derived from healthy volunteers were not measurable at the detection limit of the assay, 0.5 pg/ml. All 10 HSS samples had previously been shown to exert substantial myocardial depressant effect in previous series of experiments, whereas such activity was absent in serum from normal volunteers (3–5, 31).

**Kinase Assay**—To determine whether HSS treatment of HFM cells could activate JNK, immunoblot experiments were performed using an antibody that recognized the phosphorylated active form of JNK. HSS treatment of HFM cells (lanes 5–8) activated JNK by 4.8-fold on average as compared with normal serum treatment (lanes 1–4) and normalized to JNK full-length expression (Fig. 1).

**Transcription Factor Assays**—To determine whether HSS treatment could activate transcription factors STAT, NF-κB, and IRF family members in HFM cells, reporter assays using luciferase as the reporter gene were performed. Transfection efficiency was determined by co-transfection with a thymidine kinase (tk- Renilla) promoter-driving Renilla construct. This promoter was not activated in response to any of the inducers. The STAT-, NF-κB, and IRF luciferase-dependent activities were normalized to tk-Renilla expression levels (TABLE TWO).

The STAT GAS regulatory element 5′- (TTCGGGAA) 3′ (12, 32–35) used to drive this pLuc luciferase construct has been shown to be regulated by STAT1, STAT3, and STAT4. The addition of 10% HSS to HFM cells transfected with the STAT-luciferase construct induced the reporter by an average of 10.1-fold (range 4.6–18.4 fold) over base line. Induction was increased 6.6-fold compared with the response to 10% normal serum controls (mean 1.53; range 0.9–2.5) (TABLE TWO). As a positive control, these cells were treated with IFN-γ, which is known to activate members of the STAT family, as indicated in TABLE TWO; this treatment induced the STAT-dependent reporter construct to a similar degree as HSS. The IRF hexamer regulatory element 5′- AAGTGA-3′, located in the Gbp2 promoter (36), was used to drive the pGL2 luciferase construct and has been shown to be regulated by IRF1 and IRF2. The addition of 10% HSS to HFM cells transfected with the IRF-luciferase construct induced the reporter by a mean of 8.1-fold (range 4.7–14.2) over base line. Compared with 10% normal serum controls (mean 1.5-fold increase; range 0.9–2.0), induction was increased 5.3-fold (TABLE TWO). As a positive control, IFN-γ treatment induced the IRF-dependent reporter construct to an extent comparable with the effect generated by HSS (TABLE TWO). The NF-κB regulatory element 5′- (TGGGGACTTTCCGC) 3′ (14, 37–41) was used to drive this pLuc luciferase construct and has been shown to be regulated by p50, p52, RelA, RelB, and c-Rel. The addition of 10% HSS to HFM cells transfected with the NF-κB-luciferase construct induced the reporter by a mean of 9.8-fold (range 6.2–19.3) over base line. Normal serum (10%) controls generated a mean 1.8-fold (range 0.9–3.1) increase for a relative 5.5-fold increase (TABLE TWO). Again, as a positive control, TNF-α treatment induced the NF-κB-dependent construct to an extent similar to that generated by HSS (TABLE TWO).

These transfection experiments indicate that STAT, IRF, and NF-κB transcription family members are activated in response to HSS in HFM cells. Since the regulatory elements used to drive the reporter constructs are not specific for a particular member of a transcription factor family (i.e. the STAT-luciferase construct could have been induced by STAT1, STAT3, or STAT4), this set of experiments cannot distinguish the STAT family member that induced the reporter. The same rationale applies for the IRF and NF-κB transfection assays.

To determine the activation status and subunit composition of HSS-activated STAT, IRF, and NF-κB family members, we have performed EMSA experiments. HFM cells were treated separately with no serum,
10% HSS, 10% normal serum, IFN-γ, or TNF-α for times as indicated, and nuclear protein extract was harvested. EMSAs were performed using radiolabeled DNA regulatory elements specific for STAT, IRF, and NF-κB family members. Fig. 2, A–C, represents typical results of a single sample of HSS and normal serum. The STAT GAS regulatory element 5′-TTCCGGGAA-3′ was used as the radiolabeled probe and has been shown to bind to STAT1, STAT3, and STAT4. In Fig. 2A, we have performed a time course activation of transcription factor STAT1

### Table Two

Mean transcription factor induction in human fetal myocytes by human septic serum (mean increase over baseline ± S.E.)

| Transcription factor | Serum-free media | Normal human serum (10%) | Human septic serum (10%) | Cytokine |
|----------------------|------------------|--------------------------|-------------------------|---------|
| STAT1                | 0.58 ± 0.04      | 1.53 ± 0.19              | 10.05 ± 1.26<sup>b</sup> | 10.62 ± 0.39 |
| IRF-1                | 0.75 ± 0.05      | 1.52 ± 0.14              | 8.08 ± 0.88<sup>b</sup>  | 6.81 ± 1.18 |
| NF-κB                | 0.49 ± 0.04      | 1.77 ± 0.29              | 9.76 ± 1.29<sup>b</sup>  | 9.97 ± 0.28 |

<sup>a</sup> IFN-γ (100 units/ml) for STAT1 and IRF and TNFα (20 ng/ml) for NF-κB.

<sup>b</sup> p < 0.0001 versus normal human serum.
with HSS treatment of HFM cells. Transcription factor STAT1 is activated in HFM cells treated with 10% HSS at the 30 min, 2 h, 4 h, and 8 h time points (lanes 3, 8, 13, and 18, respectively). As a positive control, IFN-γ treatment at 30 min, 2 h, 4 h, and 8 h (lanes 4, 9, 14, and 19, respectively) activates transcription factor STAT1. Normal serum treatment of the HFM cells does not activate STAT1 at these time points (lanes 2, 7, 12, and 17). This experiment indicated that HSS contains unique factors that can activate STAT1. Since a negative control TNF-α treatment did not activate STAT1 (lanes 5, 10, 15, and 20). The HSS complex binding to the GAS probe was identified as minimally containing STAT1, since the addition of STAT1 antibody to the EMSA reaction supershifted the complex (lane 21). As a positive control, the IFN-γ-activated complex was also supershifted by STAT1 antibody (lane 22).

Fig. 2B EMSA was performed as in Fig. 2A except that the IRF hexamer DNA regulatory element 5′-(AAGTGA)₄-3′ was used as the radiolabeled probe that has been shown to bind to IRF1 and IRF2 (position −49 to −54 human Gbp2 promoter, multimerized four times). HFM cells were treated with 10% HSS for the time points indicated, nuclear cell extract was harvested, and EMSA was performed. Transcription factor IRF1 is activated in HFM cells treated with 10% HSS at the 2-h time point (lane 8). As a positive control, IFN-γ treatment at 30 min and 2 h activated transcription factor IRF1 (lanes 4 and 9). Normal serum treatment of the HFM cells did not activate IRF1 (lanes 2 and 7) at these time points. TNF-α treatment did not activate IRF1 (lanes 5 and 10). HSS treatment of these cells activated two complexes to the IRF DNA element with similar mobilities as observed with IFN-γ treatment. The complexes, as indicated in Fig. 2B, were identified as IRF1, since preincubation of septic serum-treated cell extract with IRF1 antibody reduced binding to the IRF probe. Fig. 2C EMSA was performed as in Fig. 2A except that a consensus NF-κB element 5′-TGAGGGACTTTCCGC-3′ was used as the radiolabeled probe and has been shown to bind to p50, p52, RelA, RelB, and c-Rel. In Fig. 2C, treatment of HFM cells for 30 min with 10% HSS activated transcription factor NF-κB (lane 2) as compared with normal serum treatment (lane 1). The HSS-activated complex was identified as NF-κB minimally containing the p65 subunit, since p65 antibody incubated in the reaction buffer ablated the complex (lane 3).

**Apoptosis-related Assays**—Incubation of HFM cells separately with 10 normal human sera for 18 h resulted in 98% cell viability (2.0 ± 0.2% apoptosis). In contrast, treatment of HFM cells separately with 10 human septic sera for 18 h yielded an average 81% cell viability (19.0 ± 1.0% apoptosis) (p < 0.0001) (TABLE THREE). As indicated in Fig. 3, treatment of HFM cells with HSS (lane 1) for 18 h induced genomic DNA laddering as compared with normal serum (lane 2) or no serum (lane 3) treatment.

To determine whether caspase 1 was activated by HSS treatment of HFM cells, Western blot experiments were performed. Procaspase 1 is present in nonapoptotic cells as a latent protein of 45 kDa, containing 404 amino acids. Apoptotic stimuli such as synergistic combinations of cytokines, DNA-damaging agents, and γ-ray radiation led to the activation of caspase 1 through cleavage at caspase recognition sites within the procaspase precursor (42). The 45-kDa caspase 1 precursor is cleaved at amino acids 103 and 297 to form caspase 1 subunits of p20 and p10 kDa. The p20 and p10 subunits heterodimerize to form active caspase 1 enzyme. Fig. 4 demonstrates Western blot data from HFM cells treated with 10% HSS from four patients with septic shock. A caspase 1 antibody that recognizes the 45-kDa precursor form of caspase 1 but not the p10 or p20 subunits was utilized for this assay. HSS treatment of these cells for 12 h induced the formation of a caspase 1 intermediate (lanes 3–7), suggesting that HSS activates caspase 1.

**TABLE THREE**

**Percentage of human fetal myocyte apoptosis following incubation with human serum**

| Inhibitor | Factor inhibited | Normal serum | Septic serum* |
|-----------|------------------|--------------|---------------|
| No inhibitor | | 2 ± 0.2 | 19 ± 1.0 |
| SP600125 | JNK | 1 ± 0.1 | 11 ± 0.7 |
| TPCk | NF-κB | 2 ± 0.2 | 22 ± 1.1 |
| Ac-YVAD-FMK | Caspase 1, 4, and 5 | 1 ± 0.2 | 11 ± 0.9 |
| Z-VDVAD-FMK | Caspase 2 | 2 ± 0.3 | 14 ± 1.2 |
| Ac-DEVD-CHO | Caspase 3, 6, 7, 8, and 10 | 2 ± 0.3 | 7 ± 0.7 |
| Z-VAD-FMK | Pancaspase | 2 ± 0.2 | 5 ± 0.5 |
| Z-FA-FMK | Pseudocaspase | 1 ± 0.2 | 18 ± 0.9 |

* p < 0.0001 versus normal human serum.

**FIGURE 3.** HSS induces genomic DNA laddering in HFM cells. HFM cells were treated with 10% HSS for 18 h, and genomic DNA was harvested and fractionated by 1% agarose gel electrophoresis. ss, septic serum; ns, normal serum; nt, no treatment; M, 100-base pair marker.

**FIGURE 4.** HSS treatment of HFM cells induces the formation of a caspase 1 intermediate as determined by immunoblot assay. HFM cells were treated with 10% normal serum (lanes 1–4) or 10% HSS (lanes 5–8) as indicated for 12 h, and Western blot was performed using polyclonal caspase 1 antibody. ss, septic serum; ns, normal serum.
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concurrently with the 10 septic sera. The JNK inhibitor SP600125 reduced HSS induced apoptosis to 11%. The NF-κB inhibitor increased HSS induced apoptosis to 22%, the caspase 1, 4, and 5 inhibitor reduced apoptosis to 12%; the caspase 2 inhibitor reduced apoptosis to 14%; the caspase 3, 6, 7, 8, and 10 inhibitor reduced apoptosis to 7%; the pan-caspase inhibitor reduced apoptosis to 5%; and the negative control caspase 1 pseudoinhibitor did not have an effect on HSS-induced apoptosis, 18% of cells undergoing apoptosis.

DISCUSSION

Among the organs affected during septic shock and sepsis-associated multiple organ failure, the heart is crucial. The mechanism by which the heart becomes dysfunctional in septic shock and sepsis-associated organ failure has been the subject of recent investigations. Studies have implicated a circulating substance generated during human septic shock (3, 4, 31). Serum from patients with septic shock directly induces depression of maximum extent and peak velocity of contraction in beating rat ventricular myocytes in culture. The degree of depression is related to the degree of myocardial depression found in the donor patient as measured by depression of the radionuclide cineangiogram-derived ejection fraction. This substance has also been linked to evidence of organ failure (increased lactate) (1, 3) and risk of death during sepsis (3–5, 31). Several lines of evidence suggest that this substance represents a synergistic combination of several cytokines, including TNFα and IL-1β (4).

Despite the implication of proinflammatory cytokines in the pathogenesis of septic organ dysfunction, strategies designed to reduce the mortality of sepsis and septic shock by modulation of single proinflammatory gene products (e.g. TNFα, IL-1β, iNOS, etc.) or nonpeptide mediators (e.g. PAF, prostaglandin, or leukotriene inhibitors) have been unsuccessful to date. One reason for this may be the redundant and overlapping intramolecular signaling pathways associated with these factors. For example, both TNF-α and IL-1β signaling pathways culminate in the activation of transcription factor NF-κB. Similarly, the Toll-like receptors that have been shown to mediate cell signaling by endotoxin, Gram-positive antigens, and bacterial nucleic acids have an intracellular domain identical to that of the IL-1β receptor, a receptor known to have a key role in the pathogenesis and pathophysiology of sepsis (43). Due to these redundancies, it may not be feasible to modulate the broad ranging processes that have been implicated in sepsis-dependent illness by targeting a single cytokine. Supporting this notion, both TNF-α and IL-1β levels varied extensively in the septic serum utilized in this study (TNF-α levels varied by 35-fold, and IL-1β levels varied by 197-fold) (TABLE ONE). In contrast, transcription factor levels varied only 8–10-fold compared with controls. Transcription factors may represent a more uniformly elevated and highly conserved set of downstream targets of therapy for sepsis and septic shock (comparing transcription factor activation levels within the 10-HSS treatment set) (TABLE TWO).

The addition of each of the 10 septic sera to HFM cells activated transcription factors STAT1, IRF1, and NF-κB as determined by reporter assays and EMSA (TABLE TWO and Fig. 2, A, B, and C). This finding is consistent with the observation that TNF-α and IL-1β levels were elevated in the serum of patients with acute, catecholamine-dependent septic shock compared with normal volunteers in this study (TABLE ONE). Previously published data using these same serum samples demonstrate that these same serum samples exert substantial myocardial depressant activity in an in vitro assay of myocardial contractility (3–5, 31). In addition, the septic serum donors demonstrated substantial in vivo myocardial depression (as measured by depressed echocardiographic and radionuclide cineangiogram-derived left ventricular ejection fraction) at the time the serum samples were obtained (3, 44).

Each of the transcription factors examined in this study potentially has a significant role in the pathophysiology of sepsis. Septic shock survival in humans has been predicted by increased binding activity of NF-κB in nuclear extracts of circulating polymorphonuclear leukocytes and mononuclear cells (45, 46). NF-κB regulates production and cellular effects of a variety of proinflammatory cytokines including TNF-α and IL-1β (14, 15, 33, 47). Downstream processes regulated include processes key to sepsis pathophysiology, including adhesion molecule and iNOS expression (48). For example, in vivo inhibition of NF-κB activation prevents iNOS expression and arterial hypotension in a rat model of septic shock (49). With respect to the heart, NF-κB regulates cytokine-induced nitric oxide production in cardiac myocytes (50), and iκB, an inhibitor of NF-κB, confers resistance to endotoxin-induced contractile depression to cardiac tissues (51).

Compared with NF-κB, much fewer data exist regarding the role of STATs and IRFs in the context of sepsis. STAT4 and STAT6 appear to have a crucial role in the innate immune response to sepsis. STAT4- and STAT6-deficient mice (−/−) appear to be resistant to septic peritonitis with improved mortality and increased peritoneal proinflammatory cytokine levels (52). Further, IL-4–induced activation of STAT6 is associated with increased mortality in a cecal ligation and perforation model of murine septic shock (53). A STAT-like factor has also been implicated in lipopolysaccharide, IL-1, and IL-6 signaling (54). Data on the potential role of IRF in sepsis are even more limited. IRF1 and IRF2 regulate IFN-dependent COX-2 expression, and IRF1 is necessary for iNOS expression in murine peritoneal macrophages (23, 55). Cyclooxygenase products and iNOS have been shown to have crucial roles in the pathogenesis of sepsis pathophysiology (56, 57).

In addition to their role in other elements of the inflammatory response, the STAT, IRF, and NF-κB families of transcription factors have been implicated as both positive and negative regulators of apoptosis. NF-κB is well known to have a central role in control of apoptosis. For example, NF-κB inhibits TNF-α–induced cardiac myocyte apoptosis in vitro (8) and is associated with increased hepatic apoptosis in a murine burn injury model (16). Similarly, mice that overexpress heart-specific TNFα demonstrate increased activity of both pro- and antiapoptotic pathways in that organ (17). Our data indicate that treatment of HFM cells with HSS and the NF-κB inhibitor TPCK induced a modest 16% increase in apoptosis. STAT factors also play central roles in cytokine-mediated apoptosis and particularly myocardial apoptosis during injury. A mutant fibroblast cell line that lacks STAT1 was defective in TNF-α–induced apoptosis as a consequence of constitutively low levels of caspases 1, 2, and 3 (18) and IFN-γ-dependent induction of caspase 1 gene expression required STAT1 (58). Several studies have demonstrated that the JAK/STAT pathway is central to myocardial apoptosis associated with ischemia and reperfusion (19, 20). The association of myocardial apoptosis is less direct but still powerful. Several lines of evidence suggest that inducible nitric-oxide synthase has a prominent role in cytokine-induced apoptosis in heart and other tissues (21, 22). Other data suggest that IRF1 activation is required for iNOS gene induction (23) and γ-radiation-dependent induction of caspase 1 (59).

Our data indicate that the addition of HSS to HFM cells induced apoptosis as determined by trypan blue exclusion assays (TABLE THREE) and chromosomal cleavage agarose gel electrophoresis experiments (Fig. 3). To determine the mechanism of HSS induced apoptosis, we examined the status of caspase 1. The addition of HSS to HFM cells induced the formation of a caspase 1 intermediate as compared with
normal serum controls (Fig. 4). We performed inhibitor studies to determine the role of caspases in HSS-induced HFM cell apoptosis. Ac-YVAD-FMK (inhibitor of caspases 1, 4, and 5) treatment reduced HSS-induced apoptosis by 42%; Ac-DEVD-CHO (inhibitor of caspase 2) treatment reduced HSS-induced apoptosis by 26%; Ac-DEV-D-CHO (inhibitor of caspases 3, 6, 7, 8, and 10) treatment reduced HSS-induced apoptosis by 63%; Z-VAD-FMK (pan-caspase inhibitor) treatment reduced HSS-induced apoptosis by 74%; and Z-FA-FMK (negative control pseudocaspase 1 inhibitor) treatment did not affect HSS-induced apoptosis. These caspase inhibitor studies indicate that multiple caspases mediate HSS-induced HFM cell apoptosis.

The JNK family is composed of three members (JNK1, JNK2, and JNK3) and transduces signals from a broad range of extracellular stimuli including cytokines, viruses, and environmental stress and mediates downstream cellular processes, which include cell growth, cell cycle control, and apoptosis (11). In particular, JNKs have been shown to act as positive and negative regulators of cytokine-induced apoptosis (11, control, and apoptosis (11). In particular, JNKs have been shown to act downstream of cytokines (11). Multiple pathways have been shown to transduce the cytokine-induced activation of JNKs to transcription factors, which include cell growth, cell cycle control, and apoptosis. The JNK family is composed of three members (JNK1, JNK2, and JNK3) and transduces signals from a broad range of extracellular stimuli including cytokines, viruses, and environmental stress and mediates downstream cellular processes, which include cell growth, cell cycle control, and apoptosis (11). In particular, JNKs have been shown to act as positive and negative regulators of cytokine-induced apoptosis (11, control, and apoptosis (11). In particular, JNKs have been shown to act downstream of cytokines (11).

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