Tumor necrosis factor-α in mechanic trauma plasma mediates cardiomyocyte apoptosis

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Li S, Jiao X, Tao L, Liu H, Cao Y, Lopez BL, Christopher TA, Ma XL. Tumor necrosis factor-α in mechanic trauma plasma mediates cardiomyocyte apoptosis. Am J Physiol Heart Circ Physiol 293: H1847–H1852, 2007. First published July 6, 2007; doi:10.1152/ajpheart.00578.2007.—Mechanical traumatic injury causes cardiomyocyte apoptosis and cardiac dysfunction. However, the signaling mechanisms leading to posttraumatic cardiomyocyte apoptosis remains unclear. The present study attempted to identify the molecular mechanisms responsible for cardiomyocyte apoptosis induced by trauma. Normal cardiomyocytes (NC) or traumatic cardiomyocytes (TC, isolated immediately after trauma) were cultured with normal plasma (NP) or traumatic plasma (TP; isolated 1.5 h after trauma) for 12 h, and apoptosis was determined by caspase-3 activation. Exposure of TC to NP failed to induce significant cardiomyocyte apoptosis. In contrast, exposure of NC to TP resulted in a greater than twofold increase in caspase-3 activation (P < 0.01). Incubation of cardiomyocytes with cytomix (a mixture of TNF-α, IL-1β, and IFN-γ) or TNF-α alone, but not with IL-1β or IFN-γ alone, caused significant caspase-3 activation (P < 0.01). TP-induced caspase-3 activation was virtually abolished by an anti-TNF-α antibody, and TP isolated from TNF-α−/− mice failed to induce caspase-3 activation. Moreover, incubation of cardiomyocytes with TP upregulated inducible nitric oxide (NO) synthase (iNOS)/NADPH oxidase expression, increased NO/superoxide production, and increased cardiomyocyte protein nitration (measured by nitrotyrosine content). These oxidative/nitrative stresses and the resultant cardiomyocyte caspase-3 activation can be blocked by neutralization of TNF-α (anti-TNF-α antibody), inhibition of iNOS (1400W), or NADPH oxidase (apocynin) and scavenging of peroxynitrite (FP15) (P < 0.01). Taken together, our study demonstrated that there exists a TNF-α/iNOS/NADPH oxidase-dependent, peroxynitrite-mediated signaling pathway that contributes to posttraumatic myocardial apoptosis. Therapeutic interventions that block this signaling cascade may attenuate posttraumatic cardiac injury and reduce the incidence of secondary organ dysfunction after trauma.

apoptosis; signal transduction; heart; cytokines

IT IS WELL RECOGNIZED that blunt chest trauma causes direct heart damage (i.e., cardiac contusion and vascular lacerations) from mechanical force. However, several groups have reported that blunt chest trauma causes myocardial infarction (MI) even in the absence of coronary artery dissection or direct mechanical cardiomyocyte injury (10, 23, 24). Moreover, a recent multi-hospital clinical study (10) demonstrated that although blunt chest trauma creates the greatest risk (8-fold) for MI, abdominal or pelvic trauma also dramatically increases the risk for MI (6-fold). These results strongly suggest that besides the primary injury to the heart that occurs immediately after trauma, trauma may cause secondary heart injury through yet to be identified mechanisms.

In a recent study, we demonstrated that significant cardiomyocyte apoptosis, a cell death pathway that contributes to MI, occurs in mice subjected to a nonlethal mechanical trauma. Administration of a nonselective caspase inhibitor immediately after trauma not only blocked cardiomyocyte apoptosis but also attenuated posttraumatic cardiac dysfunction (22). These results demonstrated that traumatic injury causes cardiomyocyte apoptosis, which contributes to posttraumatic organ dysfunction. Since mechanical trauma may cause cardiomyocyte apoptosis by initiating a pathological signal locally within the heart or as a result of systemic injury, it is important to further investigate whether mechanical trauma causes cardiomyocyte apoptosis by cardiomyocyte-localized factors or by systemic circulatory mediators.

Peroxynitrite is the biradical reaction product of nitric oxide (NO) and superoxide and is a highly toxic species that can oxidize or nitrate a variety of molecules and alter their function (3, 19). We (22) have previously demonstrated that in the traumatic heart, NO and superoxide production were both significantly increased and peroxynitrite was formed. However, a causative link between peroxynitrite overproduction and posttraumatic cardiomyocyte apoptosis has not been established. Moreover, the molecular sources that are responsible for increased NO and superoxide production in the traumatized heart remain unknown. Finally, signaling mechanisms that cause upregulation of NO/superoxide producing enzymes have not been identified.

Therefore, the aims of the present study were to 1) determine the origin [i.e., traumatic cardiomyocytes (TC) vs. traumatic plasma (TP)] of the pathological mediators that cause cardiomyocyte apoptosis after trauma; 2) identify specific factor(s) responsible for initiating cardiomyocyte apoptosis after trauma; and 3) delineate the intracellular signaling pathway by which cardiomyocyte apoptosis is initiated following traumatic injury.

MATERIALS AND METHODS

Induction of sham traumatic or traumatic injury in adult male mice. Nonlethal mechanical trauma was induced as previously described (22). In brief, male adult Swiss Webster mice were anesthetized with pentobarbital sodium (40 mg/kg). Mice were placed in a Noble-Collip drum, a 12-in.-diameter plastic wheel with internal shelves on which a mouse is traumatized as the wheel is rotated (200 revolutions at a rate of 40 rpm). Sham traumatic mice were subjected to the same procedure but were not rotated.
revolutions, but animals were taped to the inner wall of drum, thus avoiding traumatic injury. After completion of the procedure, mice were either immediately killed (for cardiomyocyte culture as described below) or allowed to recover in a warmed chamber and killed at the time points specified in RESULTS (for plasma collection). Experiments were performed in adherence with National Institutes of Health guidelines on the use of laboratory animals and were approved by the Thomas Jefferson University Committee on Animal Care.

**Cardiomyocyte culture and treatments.** Hearts from sham traumatized or traumatized mice were removed and perfused at 37°C for ~3 min with a Ca²⁺-free bicarbonate-based buffer. The enzymatic digestion was initiated by adding collagenase type B/D and protease type XIV to the perfusion solution. When the hearts became swollen and hard after ~3 min of digestion, 50 μM Ca²⁺ was added to the enzyme solution. Approximately 7 min later, the left ventricle was removed, cut into several chunks, and further digested in a shaker for 10 min at 37°C in the same enzyme solution. The supernatant containing the dispersed myocytes was filtered into a sterilized tube and centrifuged at 800 g for 1 min. The cell pellet was then resuspended in bicarbonate-based buffer containing 125 μM Ca²⁺. After the myocytes had been pelleted by gravity for ~10 min, the supernatant was aspirated, and myocytes were resuspended in bicarbonate-based buffer containing 250 μM Ca²⁺. Myocytes were plated at 0.5–1 × 10⁶ cells/cm² in culture dishes precoated with mouse laminin. After 1 h of culture in a 5% CO₂ incubator at 37°C, the medium was changed to FBS-free MEM.

To determine the origin of the pathological mediators that cause cardiomyocyte apoptosis after trauma, cardiomyocytes were isolated from either sham traumatic mice [normal cardiomyocytes (NC)] or traumatic mice (TC) immediately after the trauma procedure and cultured in vitro for 12 h with medium containing 10% plasma (4) obtained from sham trauma [normal plasma (NP)] or trauma animals (TP; isolated from a different group of animals 1.5 h after trauma). To identify the specific factor(s) responsible for initiating cardiomyocyte apoptosis after trauma, cardiomyocytes isolated from traumatized mice were incubated with one of the following factors for 12 h: 1) NP; 2) TP; 3) Cytomix (a mixture of 1,000 U/ml IFN-γ, 1 ng/ml IL-1β, and 10 ng/ml TNF-α) (18); 4) individual components of Cytomix at one to three times the concentration used in the mixture; or 5) TP plus anti-TNF-α antibody (1 μg/ml) (11). To delineate the role of oxidative/nitrite stress in cardiomyocyte apoptosis initiated by traumatic injury, cardiomyocytes isolated from traumatized mice were subjected to one of the following treatments: 1) NP; 2) TP; 3) TP plus 1400W [a highly selective inducible NO synthase (iNOS) inhibitor, 400 μM (9)]; 4) TP plus apocynin [a NADPH oxidase inhibitor, 100 μM (11)]; or 5) TP plus FP15 [a peroxynitrite decomposition catalyst, 100 μM (20)]. Each experimental condition listed above was examined in triplicate using cardiomyocytes isolated from the same animal. Results from the same animal were averaged and counted as one sample. At least seven to nine animals were used in each group. At the end of the experiments, cardiomyocytes were lysed in lysis buffer, and apoptosis was determined by caspase-3 activity assay as described in our previous study (21).

**Determination of total NOx content in cardiomyocyte culture medium.** At the end of in vitro experiments, cardiomyocyte culture medium was collected. NO and its metabolic products (NO₂⁻ and NO₃⁻), collectively known as NOx, were determined in culture medium using a chemiluminescence NO Detector (Siever 280 NO Analyzer) as described in our previous study (22).

**Determination of myocardial superoxide generation and nitrotyrosine content.** Myocardial superoxide content was determined by lucigenin-enhanced luminescence, and cardiac nitrotyrosine content (a footprint of in vivo peroxynitrite formation) was determined by an ELISA method as described in our recent study (22).

**Immunoblot analysis.** Proteins from cardiomyocytes were separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and Western blotted with monoclonal antibodies against gp91phox or iNOS (Santa Cruz Biotechnology, Santa Cruz, CA) followed by secondary antibody incubation. The blot was developed with Super Signal Femto Reagent (Pierce Biotechnology, Rockford, IL) and visualized with a Kodak Image Station 400. Blot densities were analyzed with Kodak 1D software.

**RESULTS**

**Proapoptotic mediators present in TP.** Noble-Collip drum exposure is a well-accepted traumatic model that results in a whole body nonpenetrative mechanical trauma (6). Proapoptotic mediators can thus be produced either within TCs themselves or by remote organs and circulate to the heart. In a recent study, Carlson et al. (4) reported that the addition of 10% plasma harvested from rats subjected to burn trauma to culture medium caused significant cardiomyocyte apoptosis, suggesting that plasma from burn trauma animals contains proapoptotic factors. However, whether mechanical trauma may also result in the accumulation of proapoptotic factors in the plasma, thus stimulating posttrauma cardiomyocyte apoptosis, remains unknown. Moreover, whether traumatic injury may activate apoptotic machinery within cardiomyocytes themselves has never been previously investigated. As shown in Fig. 1, the addition of TP to TCs caused a 2.2-fold increase in caspase-3 activity. No significant caspase-3 activation was observed when TCs were cultured with NP. However, significant caspase-3 activation (1.9-fold) was observed when TP was added to NCs. These results provide clear evidence that proapoptotic mediators are present in the plasma of traumatic animals, not within TCs themselves.

**Time course of cytokine production in traumatic animals.** Substantial evidence exists that cytokines, including TNF-α, IFN-γ, and IL-1β, are proapoptotic factors that are responsible for cardiomyocyte apoptosis under many pathological conditions (2, 5, 15). Having demonstrated that proapoptotic mediators in traumatic animals are present in plasma, we wanted to determine whether plasma levels of cytokines are elevated in traumatic animals. Animals were killed at different time points after trauma, blood samples were obtained, and plasma IFN-γ, IL-1β, and TNF-α concentrations were determined using ELISA assay kits. As shown in Fig. 2, plasma IL-1β, IFN-γ, and TNF-α levels were all significantly elevated in traumatic animals, although individual cytokines exhibited different time courses after trauma. Noticeably, all three cytokines peaked at or before 1.5 h after traumatic injury, a time point that is ~2 h ahead of significant cardiomyocyte apoptosis occurring after trauma (22).

**Identification of a specific cytokine responsible for the induction of cardiomyocyte apoptosis.** Since levels of TNF-α, IFN-γ, and IL-1β were all significantly increased in traumatic animals (Fig. 2) and a mixture of these three cytokines, termed Cytomix, has been previously shown to stimulate apoptotic cell death in fibroblasts (17) and in β-cells (16), we then determined whether these three cytokines (Cytomix) may cause significant apoptosis in TCs and, if so, to further identify the...
specific cytokine(s) responsible for trauma-induced cardiomyocyte apoptosis. As shown in Fig. 3, incubation of TCs with NP supplemented with Cytomix (a mixture of 1,000 U/ml IFN-γ, 1 ng/ml IL-1β, and 10 ng/ml TNF-α) (18) for 12 h resulted in significant caspase-3 activation that was comparable with that seen in cardiomyocytes treated with TP, suggesting that cytokines are likely to be responsible for TP-induced cardiomyocyte apoptosis. The addition of IFN-γ or IL-1β alone (at the same concentration used in the mixture, as shown in Fig. 3, or up to 3 times of the concentrations used in the mixture, data not shown) had no significant effect on caspase-3 activity. However, the addition of TNF-α alone at the concentra-

To obtain more evidence to support a causative role of TNF-α in TP-induced cardiomyocyte apoptosis, two additional experiments were performed. In the first series of experiments, an anti-TNF-α antibody was added to the TP at 1 μg/ml, a concentration that blocks TNF-α-induced cardiomyocyte apoptosis (11). As shown in Fig. 4, neutralization of TNF-α almost completely blocked TP-induced cardiomyocyte caspase-3 activation. In the second series of experiments, TNF-α−/− mice were subjected to traumatic injury as described above, and their plasma was isolated 1.5 h after trauma and added to cardiomyocytes isolated from wild-type mice subjected to traumatic injury. As shown in Fig. 4, the addition
of TP from TNF-α−/− mice failed to induce significant caspase-3 activation. These results provide firm evidence that the increased TNF-α concentration in traumatic animals is the primary cause for the cardiomyocyte apoptosis observed in traumatic animals.

Downstream signaling pathway linking TNF-α overproduction and cardiomyocyte apoptosis. In a recent study, we (22) demonstrated that NO and superoxide production were markedly increased in traumatic hearts. Having demonstrated that TNF-α is the primary cytokine responsible for posttrauma cardiomyocyte apoptosis, we further tested the hypothesis that TNF-α may initiate cardiomyocyte apoptosis by upregulating iNOS/NADPH oxidase expression with resultant oxidative/nitrative stress. As shown in Figs. 5–7, iNOS and gp91phox (a major component of NADPH oxidase) protein expression were upregulated, NO and superoxide production were increased, and the production of peroxynitrite, a proapoptotic/cytotoxic molecule, was significantly increased in cardiomyocytes exposed to TP. More importantly, neutralization of TNF-α with anti-TNF-α antibody almost completely blocked TP-induced oxidative and nitrative stress (Figs. 4–6). These results provide clear evidence that TNF-α present in the TP is the primary factor that causes significant oxidative/nitrative stress in TCs.

To further dissect the cause-effect relationship between TP-initiated oxidative/nitrative stress and TP-induced cardiomyocyte apoptosis, cardiomyocytes were treated with 1400W (a selective iNOS inhibitor), apocynin (a NADPH oxidase inhibitor), or FP15 (a peroxynitrite decomposition catalyst) immediately after the addition of TP. As shown in Fig. 8, treatment with 1400W significantly reduced NO production (A) without affecting superoxide production (B). In contrast, treatment with apocynin significantly reduced superoxide production without affecting NO production (Fig. 8B). Treatment with FP15 affected neither NO nor superoxide production (Fig. 8, A and B). However, all three treatments significantly reduced myocardial nitrotyrosine content (Fig. 8C) and reduced TP-induced caspase-3 activation (Fig. 8D). Taken together, our data show that blockade of iNOS/NADPH oxidase activity with 1400W/apocynin or increasing peroxynitrite decomposition with FP15 markedly reduced posttraumatic cardiomyocyte apoptosis. These results demonstrated that TNF-α-initiated
oxidative/nitrative stress plays a causative role in TP-induced cardiomyocyte apoptosis.

**DISCUSSION**

We have made several novel observations in the present study. First, we demonstrated, for the first time, that proapoptotic mediators that trigger cardiomyocyte apoptosis in traumatic animals are present in the TP rather than the cardiomyocytes themselves. Second, we identified that TNF-α is the most critical factor that contributes to posttraumatic cardiomyocyte apoptosis. This result is consistent with a recent study (12) demonstrating that TNF-α plays a critical role in myocardial dysfunction and apoptosis during hindlimb ischemia and reperfusion. However, our result is different from that reported by Carlson et al. (4) in a burn trauma model in which endotoxin, not TNF-α, was shown to be responsible for cardiomyocyte apoptosis, indicating that proapoptotic factors differ in different traumatic models. Finally, we provided clear evidence that peroxynitrite, the biradical reaction product between NO and superoxide, is the downstream molecule that mediates TC apoptosis. Taken together, our present study demonstrates that there exists a TNF-α-initiated, cardiomyocyte iNOS/NADPH oxidase-dependent, and peroxynitrite-mediated signaling pathway that contributes to posttraumatic myocardial apoptosis.

In a recent study, we (22) demonstrated that the mild trauma model used in our study causes cardiomyocyte death by apoptosis, not by necrosis. Our present study further demonstrated that pathological factors that mediate cardiomyocyte apoptosis are not formed by cardiomyocytes themselves. The proapoptotic effect of TNF-α has been extensively investigated in a variety of in vitro and in vivo models (7, 13). TNF-α causes apoptotic cell death by two different yet interrelated mechanisms (14). First, by binding to its cell surface receptors, TNF-α triggers apoptosis signaling through the activation of caspase-8 and subsequent caspase-3 activation (extrinsic pathway). Second, TNF-α is a powerful proinflammatory factor that upregulates the expression of many inflammatory genes, including genes encoding iNOS and NADPH oxidase (14). Therefore, TNF-α can also stimulate apoptosis through the activation of caspase-9 (which, in turn, activates caspase-3) as a result of overproduction of reactive oxygen/nitrogen species and mitochondrial injury (intrinsic pathway). Our present results demonstrated that although multiple cytokines are significantly increased in traumatic animals and the addition of Cytomix induced significant cardiomyocyte apoptosis, only TNF-α, not IFN-γ or IL-1β, induced significant cardiomyocyte apoptosis when added alone. Moreover, TP-induced oxidative/nitrative stress and cardiomyocyte apoptosis were significantly reduced when TNF-α was neutralized by an anti-TNF-α antibody.

Fig. 7. TP-induced peroxynitrite formation (as measured by nitrotyrosine content) and its inhibition by anti-TNF-α antibody. n = 7–9 mice/group. **P < 0.01 vs. the NP group; ##P < 0.01 vs. the TP group.

In addition, TP isolated from TNF-α−/− mice failed to induce significant caspase-3 activation. These results clearly demonstrated that TNF-α is the most important proapoptotic molecule that causes posttraumatic cardiomyocyte apoptosis.

Fig. 8. Effect of iNOS inhibition (1400W), NADPH oxidase inhibition (apocynin), and peroxynitrite scavenging (FP15) on TP-induced NO production (A), superoxide generation (B), peroxynitrite formation (C), and caspase-3 activation (D). n = 7–9 mice/group. **P < 0.01 vs. the NP group; ##P < 0.01 vs. the TP group.
It is well recognized that TNF-α is the primary cytokine responsible for the upregulation of iNOS and NADPH oxidase gene expression under inflammatory conditions (8). Moreover, considerable evidence exists that increased NO and superoxide production results in the formation of peroxynitrite, a highly cytotoxic molecule that causes significant cardiomyocyte apoptosis (2). Our present study demonstrated that treatment with an iNOS inhibitor, a NADPH oxidase inhibitor, or a peroxynitrite scavenger dramatically attenuated posttraumatic cardiomyocyte apoptosis. These results strongly suggest that the TNF-α-induced overproduction of reactive oxygen/nitrogen species is the primary mechanism by which TNF-α results in cardiomyocyte apoptosis in this traumatic injury model.

In conclusion, our study demonstrated that cardiomyocyte apoptosis caused by mechanical trauma is initiated by TNF-α produced by injured peripheral tissues and mediated by overproduction of cytotoxic reactive oxygen/nitrogen species within cardiomyocytes. As posttraumatic multiorgan failure is becoming a critical factor determining the overall mortality and quality of life after trauma, those therapeutic interventions that are capable of blocking this signaling cascade may attenuate posttraumatic cardiac injury and reduce the incidence of secondary organ dysfunction after trauma.

REFERENCES


