Published in final edited form as:

Biochem Biophys Res Commun. 2008 June 27; 371(2): 298–303. doi:10.1016/j.bbrc.2008.04.070.

Macrophage migration inhibitory factor induces cardiomyocyte apoptosis

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Abstract

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that causes cardiac contractile dysfunction, whereas inactivation of MIF improves cardiac function in experimental animal models of sepsis. We used cultured cardiomyocytes to determine whether MIF-induced contractile dysfunction was mediated in part by myocyte apoptosis and to identify MIF-activated intracellular signaling pathways in this process. MIF treatment significantly increased myocyte apoptosis in a dose-dependent manner to $15.5 \pm 3.9\%$ and $26.0 \pm 7.1\%$ TUNEL positive nuclei (20 and 30 ng/ml MIF for 24 h) vs control ($3.7 \pm 0.9\%$). This effect was attenuated by inactivation of MIF with the chemical inhibitor, ISO-1. MIF-induced cleavage of caspase 3 and reduction of Bcl-xL/Bax were similarly attenuated by ISO-1 pre-treatment. MIF stimulated the rapid, transient phosphorylation of stress kinases, p38MAPK and JNK. Thus, MIF induces cardiomyocyte apoptosis by activating stress kinases and mitochondria-associated apoptotic mechanisms, whereas inactivation of MIF pro-inflammatory activity improves cardiomyocyte survival.

Keywords

MIF; ISO-1; Cytokines; Apoptosis; Cardiomyocyte; Macrophage migration inhibitory factor; JNK; p38MAPK; ERK; Caspase 3; Bcl-xL/Bax

Macrophage migration inhibitory factor (MIF) has been recognized as a potent pro-inflammatory mediator in the progression of systemic inflammation [1,2], and it has been shown to accumulate in the plasma of humans during severe gram-negative and gram-positive sepsis with high levels correlating with mortality [3,4]. Animal studies have shown that administration of neutralizing anti-MIF antibodies or deletion of the MIF gene, protected mice from lethality induced by endotoxemia or sepsis [4,5]. Our recent reports showed that inhibition of the pro-inflammatory activity of MIF using a small molecule inhibitor (ISO-1), protected against lethal endotoxemia when administered up to 24 h after the induction of sepsis [6,7]. Furthermore, cardiac contractile dysfunction associated with

polymicrobial sepsis was significantly improved with inhibition of MIF activity [8]. We showed that MIF was produced in the lung and released into the circulation during experimental sepsis, and this release was temporally related to activation of the MAPK stress signaling pathways in the myocardium [9].

MIF is produced by a variety of cell types but unlike most other pro-inflammatory cytokines, most notably TNF α , IL-1 β , and IL-6, whose synthesis is induced in the myocardium response to injury [10], MIF is preformed in the cardiomyocyte [11] and has been shown to be secreted in response to oxidative stress [12]. Circulating levels of MIF have been reported to be increased following acute myocardial infarction in humans [13,14], and after cardiopulmonary bypass surgery in adults and children [15–17].

Pro-inflammatory cytokines have been shown to induce production of superoxide anions and peroxynitrite in the heart that desensitize the myofilament to calcium, leading to contractile dysfunction [18], and to the development of cardiac hypertrophy [19]. Chronically, cytokines are involved in the remodeling process by altering extracellular matrix by activating matrix metalloproteinases [20], changing integrin expression [21], and stimulating vascular and myocyte regeneration [reviewed in 22]. To determine whether MIF has a direct effect on cardiac function, Garner et al. [11] treated isolated perfused normal mouse hearts with recombinant MIF at concentrations approximating that in serum of patients with septic shock, and showed that rMIF led to significant decreases in LV pressure, $\pm dP/dt_{\max}$, and rate of LVP rise, indicating significant systolic and diastolic dysfunction. The mechanisms by which MIF induces these effects remain unknown.

Although the immunoregulatory and inflammatory properties of MIF have been well studied [reviewed in 23], the present studies were undertaken to identify potential molecular mechanisms by which MIF induces cardiac dysfunction. We have made use of a small molecule inhibitor of the pro-inflammatory activity of MIF (ISO-1) to interrogate potential mechanisms of action of MIF on cultured primary cardiomyocytes.

Methods

Isolation of neonatal rat ventricular myocytes and treatment protocols

Animals were treated in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHHS Publication No. 85-23). Ventricular myocytes were isolated from hearts of 2-day-old rats by collagenase digestion as previously described [24]. Myocytes were maintained in serum-free medium containing transferrin (5 mg/L) and selenium (5 μ g/L) for 24 h prior to treatment with recombinant mouse MIF (R&D Systems; Minneapolis, MN) or ISO-1 [6,7] or vehicle (5% DMSO). For ISO-1 studies, MIF was pre-incubated with ISO-1 (3 μ M) for 30 min in culture medium at 37 °C before treatment. ISO-1 [(*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester] was synthesized as previously described [6].

Protein extraction and Western blot analysis

Cells were homogenized in buffer containing 20 mM Hepes, pH 7.9, 10 mM KCl, 1 mM EDTA, 10% glycerol, 0.2% NP-40 plus protease and phosphatase inhibitors, and fractionated by centrifugation at 12,000g for 1 min at 4 °C. Proteins were resolved by polyacrylamide gel electrophoresis as previously published [24]. Antibodies used for immunoblot analysis were directed against phospho-CREB (Ser133) (Upstate Cell Signaling Solutions, Lake Placid, NY), and phospho-c-jun (Ser63), phospho-JNK (Thr183/Tyr185), JNK, phospho-Akt (Thr308), Akt, phospho-p44/42 MAPK (Thr202/Tyr204), p42 MAPK, phospho-p38MAPK (Thr180/Tyr182), phospho-p90RSK (Ser380), cleaved caspase 3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly,

MA), and p38MAPK, Bax, and Bcl-xL (Santa Cruz Technology, Santa Cruz, CA). Secondary antibodies used were either goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxidase. Protein bands were detected using chemiluminescence reagent (PerkinElmer, Boston, MA), visualized by exposure to X-ray film, and quantified by laser scanning densitometry (GS-800 densitometer; Bio-Rad).

Apoptosis analysis by TUNEL and annexin V staining

Cardiomyocytes were cultured in 2-well chamber slides (Nalge Nunc; Rochester, NY) and treated with MIF (20 or 30 ng/ml), ISO-1 (2.5 μ M) or with MIF plus ISO-1 for 24 h. Cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100. The number of apoptotic cells was determined by nuclear DNA fragmentation using the deoxynucleotidyltransferase-mediated dUTP nick-end labeling assay (DeadEnd™ Fluorometric TUNEL System; Promega, Madison, WI) according to the manufacturer's recommendations. Using fluorescence microscopy (Olympus IX70 Fluoview), the number of TUNEL positive nuclei (green fluorescence) and total nuclei (propidium iodide stained) were counted in nine microscopic fields at 20 $\times$ power, thus providing the number of TUNEL positive nuclei within the total number of nuclei per field. The percent TUNEL positive nuclei were obtained by averaging from nine fields per treatment condition. Early stages of apoptosis were assessed by annexin V-FITC fluorescence microscopy using a commercially available kit (BD Biosciences/Pharmingen), with modifications appropriate for adherent cells as published by Casciola-Rosen et al. [25]. Cardiomyocytes cultured on chamber slides were washed twice with ice-cold PBS prior to incubation with annexin V-FITC in binding buffer (15 min at room temperature). Cells were then washed with binding buffer, fixed with 2% paraformaldehyde followed by permeabilization with 0.5% Triton X-100. Nuclei were then stained with propidium iodide (5 μ g/ml). The number of annexin V positive cells and total propidium iodide stained nuclei were then viewed by confocal fluorescence microscopy. As described above, the percent annexin V positive cells were determined by counting cells in nine fields (20 $\times$ power) per slide per treatment and this was repeated in three separate cell experiments providing 27 observations per treatment.

Data analysis

Data are presented as means $\pm$ SE derived from a minimum of two separate cell preparations. One-way analysis of variance or unpaired Student's *t*-test was used for the statistical analysis of mean values between experimental groups. Data were analyzed using SigmaStat (Systat Software, Inc., Richmond, CA).

Results

ISO-1 attenuates MIF-induced cardiomyocyte apoptosis

Treatment of cultured cardiomyocytes with MIF (30 ng/ml) for 24 h resulted in significant cell apoptosis above basal levels as evidenced by positive annexin V staining, $21.5 \pm 2.4\%$ vs $9.3 \pm 1.5\%$ of total cell number, respectively (Fig. 1A). TUNEL analysis that measures DNA fragmentation occurring at later stages of apoptosis, showed that MIF treatment increased cardiomyocyte apoptosis above basal levels in a dose-dependent manner with $15.5 \pm 3.9\%$ and $26.0 \pm 7.1\%$ TUNEL positive cells with 20 and 30 ng/ml MIF, respectively (Fig. 1B). The pro-apoptotic action of MIF was attenuated by ISO-1, resulting in $4.1 \pm 0.6\%$ TUNEL positive cells, which was not significantly different from basal levels ($3.7 \pm 0.9\%$). ISO-1 treatment alone was without effect on cardiomyocyte apoptosis ($3.0 \pm 0.7\%$ TUNEL positive) (Fig. 1B). To further substantiate the pro-apoptotic effects of MIF, we measured cleaved caspase 3, a key player in the apoptotic process. Immunoblot analysis showed a significant increase in activated caspase 3 in cardiomyocytes treated with MIF, and this effect was blocked by ISO-1 pre-treatment (Fig. 2). To determine which death pathway is

activated by MIF, mitochondria-associated pro-survival Bcl-xL and pro-apoptotic Bax were measured as representative intrinsic signaling proteins. The Bcl-xL/Bax ratio was reduced in MIF-treated cardiomyocytes, whereas inhibition of MIF with ISO-1 resulted in a significant increase in Bcl-xL/Bax (Fig. 2).

MIF activates intracellular stress signaling pathways

Based on these data, we sought to determine the molecular pathways by which MIF-induced myocyte apoptosis. Included among the prominent signaling molecules activated by a variety of cellular stress signals are the mitogen-activated protein kinases (MAPK) including the JNK (Jun N-terminal kinase) and p38MAPK families [27]. Treatment of cardiomyocytes with MIF resulted in a rapid but transient phosphorylation of JNK1/2 occurring within 7 min, followed by dephosphorylation to pre-stimulation levels by 30 min (Fig. 3A). The temporal response of JNK phosphorylation to MIF was quantified by densitometric analysis of the immunoblots and is plotted as the ratio of pJNK to total JNK protein and compared to untreated cells (0 min) (Fig. 3A). This effect of MIF was blocked completely by pre-treatment of the cardiomyocytes with ISO-1, as shown by Western blot analysis of pJNK in cells harvested 7 min after treatment (Fig. 3B). ISO-1 alone had no effect. Activated pJNK can translocate to the nucleus where it can phosphorylate one of its substrates, c-Jun, thereby regulating transcription. This is supported by the transient phosphorylation of c-Jun occurring after phosphorylation of JNK (Fig. 3A).

Similarly, phosphorylation of p38MAPK by MIF treatment occurred rapidly, but the effect was transient with dephosphorylation of p38MAPK occurring by 10 min (Fig. 3C).

MIF-activated ERK signaling can be blocked by ISO-1

In contrast to the stress-activated kinases JNKs or p38MAPKs, activation of the MEK/ERK (extracellular signal regulated kinases) signaling pathway has been associated with cell survival and cardiac hypertrophy [27,28]. Studies have shown that the ERK signaling pathway is activated by MIF in embryonic fibroblasts [29] and cardiomyocytes [30]. Therefore, we examined whether MIF activated the ERK signaling pathway and whether this effect could be inhibited by ISO-1 pre-treatment. Western blot analysis using phospho-specific antibodies showed that phosphorylation of ERK1/2 increased rapidly in response to MIF treatment, and this effect was transient (Fig. 4A). When MIF was incubated with ISO-1 prior to treatment of the myocyte cultures, phosphorylation of ERK1/2 at 7 min was prevented (Fig. 4B). Treatment with ISO-1 alone was without effect.

We examined whether activation of ERK1/2 resulted in phosphorylation of a known substrate, p90RSK (p90 ribosomal S6 kinase) [31], and further downstream substrates, such as CREB which is phosphorylated by MSK1/2 (mitogen- and stress-activated protein kinases) that are in turn activated by p38MAPK and ERK1/2 [32]. Addition of MIF to the cultured cardiomyocytes resulted in rapid, transient phosphorylation of both CREB and p90RSK (Fig. 4A).

Discussion

We have previously reported that polymicrobial sepsis induced in experimental animals resulted in elevated circulating levels of MIF produced by the lungs, and that inactivation of MIF using ISO-1 significantly improved cardiac function 30 h after induction of sepsis [8,9]. These data suggested that MIF pro-inflammatory activity may be responsible, at least in part, for the cardiac dysfunction observed in late stages of sepsis. The goal of the present study was to determine the potential mechanisms by which MIF acts on the cardiomyocyte. The salient finding of this study is that MIF can induce cardiomyocyte apoptosis, and this

effect can be attenuated by inactivation of MIF using the small molecule inhibitor, ISO-1. Although several previous reports have implicated a cardio-depressive role for MIF in the heart [11], the mechanism by which this occurs is unclear. In one study, the use of neutralizing anti-MIF antibodies in mice 24 h after LPS challenge reduced cardiac myocyte apoptosis as measured by caspase 3 activation; however, myocardial production of IL-6, IL-1 α β , and TNF α was also reduced suggesting that the effect of MIF on myocyte apoptosis might be indirect [33]. Ha et al. [34] treated septic mice with glucan phosphate and showed that cardiac function was preserved and MIF expression was attenuated, while the number of apoptotic cardiac myocytes and cleaved caspase 3 were reduced. These published reports are supportive of our present results. In contrast to the potential pro-apoptotic effects of MIF on the cardiomyocyte, a recent report proposes that MIF plays a role in AMP-activated protein kinase (AMPK) activation and may protect the heart in ischemic injury [35]. Under hypoxic conditions when AMPK is maximally activated, it remains to be ascertained whether MIF plays a significant role. The effect of MIF on AMPK activity in the myocardium in sepsis and autoimmune diseases remains unknown, and will require further investigation.

The molecular mechanisms of action of MIF on the cardiomyocyte have not been rigorously investigated. We therefore, undertook studies to examine potential signal transduction pathways activated by MIF treatment of purified ventricular myocytes. As has been previously reported, constitutively expressed MIF is released from cardiac tissue in response to hypoxic conditions or LPS challenge, and can act as an autocrine/paracrine factor [11,12,30]. Extracellular MIF signal transduction is initiated by binding to the CD74/CD44 complex in various cell types including macrophages and fibroblasts [36], and our unpublished data show that CD74 and CD44 are present in the ventricular myocyte. Our present data in cardiomyocytes show that MIF activates stress signaling pathways, JNK and p38MAPK, and although JNK has been shown to be a direct activator of mitochondrial death machinery in cardiomyocytes [37], the pro-apoptotic role of the p38MAPK pathway remains controversial [reviewed in 26]. These data support a pro-apoptotic role of MIF initiated by activation of the JNK and p38MAPK signaling cascades preferentially involving the mitochondrial death pathway. Several studies in non-cardiac cells have shown that MIF transiently activates the ERK1/2 signaling pathway [29]. In cardiomyocytes, oxidative stress, hypoxia and agonist-induced activation of ERK1/2 have generally been associated with protection from apoptosis and induction of hypertrophy [26–28]. Our observation that MIF treatment of cardiomyocytes transiently activates the ERK1/2 pathway resulting in phosphorylation of the p90 ribosomal S6 kinases (RSK), that in turn have been shown to promote cell viability, will require further investigation of the complex interactions of intracellular signaling pathways.

In summary, the present data support a role of MIF in promoting cardiomyocyte apoptosis that may underlie the cardiac dysfunction that we have observed in late stages of sepsis. Thus, these results support the potential therapeutic benefit of targeting the pro-inflammatory activity of MIF in medical conditions expressing elevated levels of MIF.

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Fig. 1.

MIF-induced cardiomyocyte apoptosis was attenuated by ISO-1 treatment. (A) Cardiomyocytes were treated with MIF (30 ng/ml) for 24 h, then incubated with annexin V-FITC (green). Nuclei were stained with propidium iodide (PI) (red). The % annexin V positive cells were quantified in nine fields per slide per experiment and an average value obtained from three separate experiments as represented by the bar graph showing means $\pm$ SE. * $p < 0.001$ MIF vs control. (B) DNA fragmentation was assessed by fluorometric TUNEL assay. % TUNEL positive nuclei (green fluorescence) are expressed as percent of total nuclei identified by PI staining. Cardiomyocytes treated with MIF showed a dose-dependent increase in TUNEL positive nuclei (* $p < 0.001$; vs all other groups). Pre-treatment with ISO-1, reduced % TUNEL positive nuclei in MIF-treated (20 ng/ml) cardiomyocytes. Bar graph shows means $\pm$ SE. ** $p < 0.001$, MIF vs MIF+ISO-1 and MIF vs ISO-1. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Fig. 2.

MIF activates pro-apoptotic pathways with decreased Bcl-xL/Bax ratio and caspase 3 activation. Representative Western blot analyses showing cleaved caspase 3 (17 kDa), Bcl-xL (30 kDa), Bax (21 kDa) and GAPDH in cytosolic fractions (50 μ g protein per lane) of cardiomyocytes treated with MIF (30 ng/ml), MIF plus ISO-1, ISO-1 alone or vehicle (CTL). Bar graph represents the densitometric quantitation (arbitrary units, a.u.) of Bcl-xL to Bax protein ratio relative to CTL, and cleaved caspase 3. Data (means $\pm$ SE; $n = 4-6$) are derived from two separate cell experiments. ** $p < 0.01$ vs all treatment groups; * $p < 0.01$ vs MIF + ISO-1.

Fig. 3.

MIF activation of the JNK and p38MAPK stress signaling pathways. (A) Temporal effects of MIF treatment of cardiomyocytes on JNK phosphorylation analyzed by Western blotting of whole cell lysates. Quantitation of phospho-JNK normalized to total JNK protein by densitometric analysis is shown in the bar graph. * $p < 0.01$ vs 0 min; $n = 4-6$ from three separate cell experiments. The same Western blots were stripped and reprobed with anti-phospho-c-Jun antibody. (B) Representative immunoblot analysis of pJNK1/2 in total cell lysates derived from cardiomyocytes treated for 7 min with MIF (20 ng/ml), MIF plus ISO-1, ISO-1 alone and vehicle (CTL). (C) Effect of MIF on phosphorylation of p38MAPK over a time course of 60 min is shown by a representative Western blot. Reprobing with anti-p38MAPK antibody shows similar amount of total p38MAPK protein per time point.

Fig. 4.

MIF activation of the ERK1/2 pathway is blocked by ISO-1. (A) Representative Western blots of cardiomyocyte lysates showing rapid phosphorylation of ERK1/2 (p-p42/44 ERK) in response to MIF treatment. The blots were reprobed with anti-ERK antibody. Quantitation of phospho-p42/44 ERK by densitometric analysis and normalized to total ERK is shown in the bar graph (means $\pm$ SE). * $p < 0.01$ vs all other time points. Cell lysates harvested at similar time points were analyzed for phosphorylated CREB and p90RSK and representative Western blots are shown. The immunoblots of the time course of CREB phosphorylation were quantified by densitometry and the data shown in the bar graph. * $p < 0.01$ vs all other time points; $n = 6$ from two separate experiments. (B) Representative Western blot showing phospho-ERK1/2 in cardiomyocytes treated with MIF (20 ng/ml), MIF plus ISO-1, ISO-1 alone and vehicle (CTL) for 7 min.