Multiple Effects of High Mobility Group Box Protein 1 in Skeletal Muscle Regeneration

Roberta De Mori, Stefania Straino, Anna Di Carlo, Antonella Mangoni, Giulio Pompilio, Roberta Palumbo, Marco E. Bianchi, Maurizio C. Capogrossi, Antonia Germani

Objective—High mobility group box 1 protein (HMGB1) is a cytokine released by necrotic and inflammatory cells in response to injury. We examined the role of HMGB1 in skeletal muscle regeneration after hindlimb ischemia.

Methods and Results—Unilateral hindlimb ischemia was induced in mice by femoral artery dissection. HMGB1 levels increased in regenerating skeletal muscle and the blockade of endogenous HMGB1 by the administration of its truncated form, the BoxA, resulted in the reduction of vessel density. In contrast, intramuscular administration of HMGB1 enhanced perfusion and increased the number of regenerating fibers. To separately study the myogenic and the angiogenic effects of HMGB1, in vitro experiments were performed with isolated myoblasts and endothelial cells. Myoblasts were found to express the HMGB1 receptor RAGE and TLR4 which were downregulated during in vitro myogenic differentiation. HMGB1 was extracellularly released by differentiated myoblasts and exerted a chemotactic activity on myogenic cells. This effect was partially dependent on RAGE and was inhibited by BoxA treatment. Finally, HMGB1 stimulated tubular-like structure formation by endothelial cells through the activation of extracellular signal-regulated kinase (ERK) and JNK signal transduction pathways.

Conclusions—HMGB1 plays a role in skeletal muscle regeneration modulating, in an autocrine-paracrine manner, myoblast and endothelial cell functions. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: hindlimb ischemia ■ HMGB1 ■ cytokines ■ regeneration

Skeletal muscle regeneration, which occurs after tissue damage, is a complex multistep process leading to the formation of myotubes and to the restoration of the vascular network.1 Muscle injury triggers signals important for neutrophil and macrophage chemotraction in the damaged tissue. Further, inflammatory cells remove cellular debris and produce growth factors and cytokines which orchestrate the regenerative process activating muscle satellite cells to proliferate and differentiate into myotubes.2

High Mobility group box 1 protein (HMGB1) is a multifunctional ubiquitous protein of 215 amino acids mostly located in the nucleus where it binds DNA regulating physical interaction between transcription factors and chromatin.3 In addition to its nuclear role, HMGB1 is an extracellular mediator of the immune response to infection and injury. HMGB1 reaches the external milieu either by passive release from necrotic cells or active secretion by immune cells stimulated with cytokines and bacterial endotoxins (eg, Lipopolysaccharide [LPS]).4-5 The extracellular protein signals tissue injury and evokes inflammatory response inducing the release of a variety of proinflammatory cytokines by monocytes, neutrophils, and dendritic cells.8 Moreover, HMGB1 exhibits a chemotactic effect on smooth muscle cells,9 stem cells of vascular origin10,11 and endothelial precursor cells.12-14 Further, it induces endothelial cell sprouting in vitro12-14 and myogenic differentiation of rat L6 myoblasts.15 We recently demonstrated that HMGB1 is a potent regenerative cytokine: in fact, its delivery in infarcted mouse hearts induces cardiac stem cell activation and differentiation into myocytes.16 HMGB1 effects are mediated trough its interaction with the Receptor for Advanced Glycation End products (RAGE)17 and members of the Toll like receptors (TLR) family.18-20

In the present study we provide evidences of the involvement of HMGB1 in skeletal muscle regeneration. Specifically we show that either endogenous HMGB1 blockade or HMGB1 administration, in a mouse model of hindlimb ischemia, modulate myoblast function and neovascularization.

Materials and Methods

Please refer to the online data supplement at http://atvb.ahajournals.org for details.

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18546/H18546
Animals and Hindlimb Ischemia Model
Hindlimb ischemia was performed in C57Bl6J mice as previously described.21

HMGB1 Immunoassay
HMGB1 levels were measured using an ELISA assay (Shino Test Corporation) according to manufacturer’s instructions, as shown in the online supplements.

Chemotaxis Assays
Chemotaxis was performed in 48-microwell chemotaxis chambers (Neuroprobe) using 8-µm pore-size polycarbonate filters (Costar Scientific Corporation) coated with murine collagen type IV (Becton-Dickinson) as described.22

Results
Please see supplemental Figures I through V, online at http://atvb.ahajournals.org.

HMGB1 Expression in Skeletal Muscle
HMGB1 expression was investigated during skeletal muscle regeneration, after hindlimb ischemia induced by femoral artery dissection. In the absence of tissue damage, HMGB1 localized in the nucleus of myotubes as well as in the nucleus of satellite cells, that are cells closely associated to skeletal muscle fibers expressing the adhesion molecule M-cadherin (Figure 1A and supplemental Figure I). HMGB1 was also detected in vascular structures (supplemental Figure I). At day 3 after femoral artery dissection, HMGB1 staining was strongly evidenced in inflammatory cells, which occupied most of the damaged area (supplemental Figure I). After 1 week, regenerating skeletal muscle fibers, distinguishable from normal fibers because of their small size and central nuclei, exhibited diffuse cytoplasmic HMGB1 labeling (Figure 1B and supplemental Figure I). In mature fibers, as well as in regenerated muscle at 21 days after ischemia, HMGB1 expression returned to the basal level in fiber nuclei (Figure 1C and supplemental Figure I). Western blot analysis revealed that HMGB1 levels slightly increased at day 3 after surgery, peaked at day 7 and returned to basal levels at day 21 (Figure 2A). F4/80, a macrophage expressed protein,23 was expressed at day 3 after ischemia but not at later time points, suggesting that macrophages may contribute to HMGB1 expression only at day 3 (Figure 2A).

HMGB1 expression was also investigated in 2 animal models of skeletal muscle tissue damage, ie, dystrophin deficient mice (mdx) and myotubularin (MTM1) knockout mice. The lack of dystrophin in mdx mice results in the formation of immature fibers that undergo cycles of degeneration/regeneration.24 MTM1 is a ubiquitously expressed phosphatase, that, when mutated, leads to muscular disorder characterized by generalized hypotonia and muscle weakness.25 Inflammatory infiltrates and fibrosis, key components of myopathy associated to dystrophin deficiency, were undetectable in MTM1−/− mice.25 Western blot analysis revealed that HMGB1 levels were higher in skeletal muscle obtained from both mdx and MTM1−/− mice compared with their wt counterparts (Figure 2B).

The reduced number of inflammatory cells in MTM1−/− skeletal tissue, as well as the presence of high HMGB1 levels at day 7 after hindlimb ischemia, when the inflammatory process had declined, supported the involvement of muscle cells in maintaining elevated HMGB1 levels during regeneration.

HMGB1 Enhances Skeletal Muscle Regeneration
To evaluate whether HMGB1 plays a role on skeletal muscle regeneration, a single dose of HMGB1 (200 ng) was admin-
istered in the skeletal adductor muscle immediately after the induction of ischemia. Laser Doppler Perfusion Imaging (LDPI) was used to document changes in hindlimb blood flow in untreated and HMGB1-treated mice at the indicated time points. In both strains, blood flow was drastically reduced immediately after femoral artery dissection and progressive recovery was detected between day 7 and day 21 (Figure 3A). HMGB1 delivery to ischemic hindlimbs increased blood flow in comparison to untreated mice: at day 7 after surgery the Doppler flow ratio was significantly higher in HMGB1-treated mice (0.51 ± 0.06 versus 0.33 ± 0.07), and this difference was still detected at day 14 after surgery (0.81 ± 0.05 versus 0.65 ± 0.04; Figure 3A). Tissue sections from adductor muscle were examined histologically at day 7 after surgery, when blood flow analyzed by LDPI exhibited the most significant difference between the 2 groups. Although capillary density was similar in both untreated and HMGB1-treated mice (supplemental Figure II), length density of arterioles, 4 to 41 μm in diameter, was significantly increased in HMGB1-treated mice (22.1 ± 1.5 mm/mm^3 versus 12.8 ± 3 mm/mm^3; Figure 3B and supplemental Figure II).

To evaluate whether HMGB1 treatment affects myogenic differentiation in vivo, the number of regenerating fibers were counted on adductor muscle sections at day 7 after ischemia and HMGB1 delivery. At this time point, both neovascularization and the number of regenerating fibers were enhanced in the HMGB1-treated group when compared with untreated animals (88.6 ± 21/mm^3 versus 29 ± 13 mm^3; Figure 3C and supplemental Figure II).

To further confirm the involvement of HMGB1 in skeletal muscle regeneration and neovascularization, endogenous HMGB1 was inhibited in the ischemic skeletal muscle by the administration of the HMGB1-containing the DNA binding domain A (BoxA), a truncated form of the protein which acts as a competitive antagonist, inhibiting HMGB1 binding to its receptor RAGE.26,27 BoxA delivery every day did not affect LDPI (Figure 3A) but markedly decreased, at day 7 after ischemia, the length density of both capillary (579 ± 62/mm^3 versus 249 ± 9/mm^3) and arterioles 4 to 11 μm (7 ± 1.8 versus 3.8 ± 1.1 mm/mm^3 densities), whereas it induced a trend in the reduction of length density of arterioles 11 to 21 and 21 to 41 μm in diameter (supplemental Figure II). This effect resulted in the significant reduction of arteriole density 4 to 41 in diameter (13 ± 2.9 versus 9 ± 0.7 mm/mm^3; Figure 3B and supplemental Figure II). The evaluation of fiber number

Figure 2. HMGB1 accumulates in regenerating skeletal muscle. A, Western blot analysis of total extracts from adductor muscles of C57Bl6/J mice prior to and at 7, 14, 21, and 28 days after hindlimb ischemia. F4/80 is used to quantify the levels of macrophages in the adductor muscle. To normalize loading, filters are probed with anti-α-tubulin mAb. Lower panel: average results of densitometric analysis of Western blots. B, Western blot analysis to detect HMGB1 in adductor muscle of mdx, MTM1−/− mice and their wt counterparts.

Figure 3. HMGB1 enhances vascularization and myofiber formation. A, Graphs show ratio of ischemic to nonischemic perfusion in plantar region of mice assessed by LDPI (n=10, *P<0.04). Perfusion ratio before ischemia was 1. B, Arteriole density and (C) number of fibers, 7 days after ischemia in untreated (Saline), HMGB1 and BoxA-treated mice (n=7; *P<0.001, †P<0.01, and **P<0.01 vs Saline).
did not reveal differences between untreated and BoxA-treated animals (Figure 3C and supplemental Figure II).

**HMGB1 Is an Angiogenic Molecule**

The following experiments were aimed at determining whether the improved neovascularization in HMGB1-treated ischemic skeletal muscle resulted from a direct angiogenic effect of HMGB1.

Matrigel supplemented either with HMGB1 or saline solution were injected subcutaneously into the midlower abdominal region of C57 mice. In this model, host endothelial cells and smooth muscle cells migrated and formed vascular network in the Matrigel implants. Quantitative analysis of the Matrigel plugs 8 days after implantation revealed a higher number of blood vessels in the Matrigel plugs containing HMGB1 versus control (Figure 4A and supplemental Figure III). Further, HMGB1 stimulated in vitro the formation of tubular structures in Matrigel-cultured human umbilical vein endothelial cells (HUVECs). This effect was similar to that obtained with vascular endothelial growth factor (VEGF) and complete medium (EGM; Figure 4B). To investigate the mechanisms underlying HMGB1-induced HUVEC differentiation, we cultured cells in the presence of HMGB1, and we showed increased phosphorylation of extracellular-regulated kinases (ERKs) and of c-Jun N-terminal kinase (JNK), peaking at 15 minutes (Figure 4C). The activation of these pathways is known to be involved in VEGF-induced tubular structure formation.28 Accordingly, either the addition of the ERK inhibitor PD038059 or the JNK inhibitor, SP600125, to the Matrigel-cultured HUVECs, prevented both VEGF- and HMGB1-induced tubular structure formation (Figure 4B). On the contrary, both inhibitors had no effect on tubular structure formation induced by complete medium (EGM; Figure 4B), even in presence of increased ERK and JNK phosphorylation (supplemental Figure III). The activation of alternative pathways by serum- and growth factor–containing EGM may account for the lack of PD038059 and SP 600125-mediated inhibitory effects in this culture condition.

**HMGB1 Expression and Distribution on Skeletal Myoblast In Vitro**

To study the effect of HMGB1 on skeletal muscle cell function, a model of myogenic differentiation was reproduced in vitro using either primary myoblasts (satellite cells) or a cell line derived from murine satellite cells, ie, C2C12 myoblasts. Satellite cells and C2C12 cells proliferate when cultured in growth medium (GM) and, after 48 to 72 hours in differentiation medium (DM), cells fuse to form multinucleated myotubes. We first assessed the expression of HMGB1 and its receptors RAGE and TLR4, in GM- and DM-cultured C2C12 myoblasts. Western blot analysis of C2C12 lysates showed that both HMGB1 and RAGE decreased over a 5-day period in DM when compared with GM-cultured cells (Figure 5A). The less characterized HMGB1 receptor TLR4 was expressed in GM-cultured C2C12 cells but was undetectable from day 1 to 5 of culture in DM. Changes in the expression of HMGB1 and its receptors paralleled a progressive increase in myosin heavy chain expression (MyHC), a marker of C2C12 differentiation (Figure 5A), used as a control.

Immunofluorescence studies showed that HMGB1 was located in the nucleus of GM-cultured primary myoblasts and C2C12 cells (Figure 5B). By day 3 of culture in DM, HMGB1 staining increased in the cytoplasm of myotubes.
derived from both primary myoblasts and C2C12 cells (Figure 5B). In accordance to Western blot analysis, RAGE and TLR4 receptors were present in proliferating myoblasts but only RAGE was still detected in 3 days differentiated myotubes (Figure 5C).

To investigate whether the cytoplasmic localization of HMGB1 in DM-cultured C2C12 cells may reflect HMGB1 secretion during differentiation, DM was collected after 1 and 3 days of culture and assayed for the presence of HMGB1 by Western blotting and ELISA assay. HMGB1 levels increased in DM during differentiation (Figure 5D and supplemental Figure IV). The amount of HMGB1 in DM-conditioned media at 1 and 3 days was 90±27 ng/mL and 91±1 ng/mL, respectively whereas it was very low in GM cultured cells (9±6 ng/mL; supplemental Figure IV). LDH levels were similar between GM and 1 day DM-cultured cells demonstrating that necrosis did not account for extracellular HMGB1 at this time point. In contrast, necrotic cells might contribute to HMGB1 release at day 3 of culture (supplemental Figure IV).

Role of HMGB1 on In Vitro Cultured Skeletal Myoblasts

In this first set of experiments it was examined whether HMGB1 and its receptor RAGE may modulate C2C12 cell invasion. In this assay, cells in the upper chamber migrate through an extracellular matrix protein (ECM)-coated nucleopore filter to a lower chamber which contains the chemotactic agent. Under the experimental conditions of the present study, HMGB1 exhibited a chemotactic activity on C2C12 myoblasts that, at the concentration of 100 ng/mL, was higher to that induced by GM (Figure 6A). The HMGB1-mediated migratory effect was abolished in presence of BoxA (Figure 6A).

Figure 5. Expression and localization of HMGB1 during myogenic differentiation in vitro. A, HMGB1, RAGE, and TLR4 expression decreases during C2C12 differentiation. HMGB1 (B) and RAGE and TLR4 (C) localization in myogenic cells cultured for 3 days either in GM or in DM. D, ELISA determination of HMGB1 in supernatants from C2C12 cells cultured in GM and in DM for 1 and 3 days. Value are expressed as Optical Density (OD).

Figure 6. HMGB1 induces myoblast migration. A, HMGB1 induces C2C12 migration and BoxA inhibits HMGB1-chemotactic activity (*P<0.01 vs −, †P<0.05 vs + and HMGB1). B, dnRAGE partially inhibits HMGB1-induced chemotaxis of C2C12 cells. (n=4, *P<0.05 vs HMGB1-induced migration of GFP/pCDNA3 transfected cells; †P<0.01 vs GFP/pCDNA3 transfected cells with 0.1% BSA; ††P<0.001 vs Controls).
To assess whether the HMGB1-induced myoblast migration was mediated by its interaction with RAGE, C2C12 cells were cotransfected with plasmids expressing dominant negative (dn) RAGE, a mutant form lacking the cytoplasmic domain, and green fluorescence protein (GFP), to identify transfected cells (Figure 6B). In our experimental conditions dnRAGE-transfected cells were partially impaired in their ability to migrate in response to HMGB1. In contrast, dnRAGE did not affect the migratory response induced by GM (supplemental Figure V).

Then it was evaluated the effect of HMGB1 treatments on myogenic differentiation. The myogenic program is marked by Muscle Regulatory Factors (MRFs) activation, including MyoD and myogenin which controls the expression of specific muscle gene such as MyHC. To assess whether HMGB1 may induce myoblast differentiation, C2C12 cells were cultured in DM in the presence or absence of HMGB1 for the indicated time points and then Western blot analysis was performed on total cellular extracts to determine MyoD, Myogenin and MHC expression. Although it has been previously reported that HMGB1 induced myogenic differentiation of rat myoblast cell line L6E9, this effect was not detected on C2C12 cells in our culture conditions (supplemental Figure V). Accordingly, the administration of BoxA to DM-cultured C2C12 cells did not affect the myogenic program (data not shown).

**Discussion**

Several studies have associated local expression of HMGB1 with tissue damage and inflammation. This HMGB1 released by necrotic cells activates neutrophils and macrophages which, in turn, induce and sustain local inflammation producing inflammatory cytokines including HMGB1 as well. However, HMGB1 also acts as a regenerative cytokine: we recently demonstrated that its delivery in a mouse model of myocardial infarction induces myocardial regeneration activating “resident” cardiac stem cells. Notably, HMGB1 attracts intraarterial delivered mesoangioblasts in uninjured skeletal muscle and promotes endothelial precursor cells (EPC) homing to ischemic tissue.

In the present study it was examined the role of endogenous HMGB1 in skeletal muscle regeneration and in myogenic cell functions. In vivo, HMGB1 was widely expressed by skeletal muscle; it localized in mature fiber nuclei, in the cytoplasm of regenerating fibers, in satellite cells and in vascular structures. In agreement with its well-established role in the inflammatory response, HMGB1 levels increased both in α-sarcoglycan and dystrophin-null muscles which are characterized by extensive degeneration/regeneration processes. The high amount of HMGB1 in regenerating muscles was due both to muscle damage and to the presence of inflammatory cells. Indeed, HMGB1 accumulated in skeletal muscle of MTM mice which are characterized by extensive regeneration and reduced inflammatory response. In the mouse model of hindlimb ischemia, HMGB1 levels increased in skeletal muscle at day 3 after ischemia and remained above control until day 14. It is noteworthy that in our experimental model inflammatory cells may contribute to HMGB1 accumulation in ischemic skeletal muscle at day 3. However, at later time points, inflammation declined and satellite cells as well as regenerating fibers could account for the persistence of high HMGB1 levels in the regenerating skeletal muscle. Our data support the hypothesis that, through this mechanism, fibers sustain the regenerative process modulating satellite cell functions and vessel formation.

According to in vivo studies, HMGB1 accumulated in the cytoplasm of both C2C12 skeletal myoblast cell line and primary myoblasts (satellite cells) when they differentiated into myotubes. This event is followed by the intracellular decrease of HMGB1 and an increase of its levels in the culture medium. To date, signals involved in extracellular release of HMGB1 are not understood. In monocytes, HMGB1 accumulates in secretory lysosomes that undergo exocytosis, and acetylation represents a signal required for its localization into these organelles. Whether a similar mechanism occurs in myoblasts is unknown.

The involvement of extracellular HMGB1 in skeletal muscle regeneration was further supported by the evidence that its administration, immediately after the induction of hindlimb ischemia, enhanced the number of regenerating fibers as well as blood vessel formation. Therefore, HMGB1 could positively affect the differentiation potential of myogenic cells. Although Sorci et al demonstrated that HMGB1 accelerated myogenic differentiation a rat cell line L6E9, we were not able to reproduce this result probably because our experimental approach/cells were different.

We found that HMGB1 is a powerful chemoattractant in vitro for skeletal myoblasts suggesting that its persistence in regenerating tissue may be required to recruit satellite cells into the injury site. The migratory effect of HMGB1 was inhibited by BoxA and partially involved the receptor RAGE suggesting that other receptors could account for the migratory response of myoblasts.

Importantly, HMGB1 improved vascularization. By LDPI we found an enhanced recovery of perfusion at day 3, 5, and 7 after ischemia and HMGB1 delivery. Accordingly, arteriole length density was significantly higher in HMGB1-treated muscle.

In vitro experiments demonstrated that HMGB1 induced tubular structure formation by HUVECs, which expressed RAGE, through a mechanism involving the activation of ERK and JNK signaling. Moreover, its delivery in a Matrigel plug subcutaneously in mice enhanced vessel formation. These data are in agreement with 2 recent published reports in which HMGB1 was shown to induce endothelial cell migration and sprouting as well as neovascularization in vivo in the chick embryo chorioallantoic membrane assay. We further demonstrated that HMGB1 activates the arteriogenic response in vivo. Notably, arteriogenesis is a process which requires vascular smooth muscle cell (VSMC) migration, and proliferation and HMGB1 has such effects on VSMC.

Regeneration induced by HMGB1 in vivo could be partially prevented by the administration of the BoxA. BoxA-treated ischemic muscle showed a significant reduction of 4- to 11-μm arteriole length density) whereas only a trend was observed in the density of 11- to 41-μm larger arterioles). This effect could account for the lack of modification in
blood flow measured by LDPI and in the number of regenerating fibers when animals were treated with BoxA.

In conclusion, we have extended our previous findings demonstrating that HMGB1 is a cytokine with muscle regeneration potential in vivo. The ability of HMGB1 to induce skeletal muscle regeneration is attributable both to a direct effect on skeletal myoblasts, enhancing their recruitment in the site of injury, as well as to its arteriogenic action. Therefore, preventing the pathologic actions of HMGB1 and promoting its repair responses may represent a therapeutic strategy for the treatment of diseases in which regeneration is impaired.

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Disclosures
The authors declare no direct financial interest. However, M.E. Bianchi is founder and part owner of HMGBiotech, a company that provides goods and services related to HMGB proteins.

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ON LINE SUPPLEMENTAL DATA

DETAILED FIGURE LEGENDS

Figure 4. HMGB1 is an angiogenic molecule in vivo and in vitro

(A,B) HMGB1 enhances vessel number in Matrigel plugs. (A) Bar graph of the total number of vessels over the whole area of Matrigel plugs supplemented with HMGB1 or saline solution (n=6; * p<0.006). (B) Representative photomicrographs of sections analyzed in (A) (Trichrome-Masson staining, x 40 magnification). (C) HMGB1 induces tubular-like structure formation in vitro.

HUVEC were cultured on a substrate of Matrigel without growth factors in presence either of HMGB1 (200ng/ml). Both Endothelial Growth Medium (EGM) and VEGF (50ng/ml) were used as positive controls. The ERK inhibitor, PD038059 and the JNK inhibitor, SP600125, blocked HMGB1 and VEGF -induced tubular structure formation, while they had no effect in EGM cultured cells. In the lower panel is shown the bar graph of the total number of branching points over 5 representative fields for each well of culture (n=5;* p<0.01, vs respective treatments). (D) HMGB1 stimulates ERK and JNK phosphorylation. HUVEC were treated for the indicated time points with HMGB1 (200 ng/ml) and cell extracts analyzed by western blot with anti-phosphorylated ERK and JNK antibodies. As a loading control blots were stripped and reprobed with antibodies for total ERK and JNK and the average results of densitometric analysis were shown in the lower panel.

Figure 5. Expression and localization of HMGB1 during myogenic differentiation in vitro.

(A) HMGB1, RAGE and TLR4 expression decreases during C2C12 differentiation. Total cellular extracts obtained from growth medium (GM)- and 1, 3, 5 days differentiation medium (DM)-cultured C2C12 cells, were analyzed by Western blot with the indicated antibodies. Myosin heavy chain (MyHC) expression was shown to monitor myogenic differentiation. The same filter was probed with anti α-tubulin mAb to show equal protein concentration. (B) HMGB1, (C) RAGE and TLR4 localization in myogenic cells cultured for 3 days either in GM or in DM. (D) ELISA
determination of HMGB1 in supernatants from C2C12 cells cultured in GM and in DM for 1 and 3 days. Value are expressed as Optical Density (OD).

Figure 6. Effect of HMGB1 on myoblast functions in vitro

(A) HMGB1 induces C2C12 migration and BoxA inhibits HMGB1-chemotactic activity. C2C12 cells were placed in the upper compartment of the modified Boyden chamber and HMGB1 (100 ng/ml) added to the lower compartment in absence and in presence of the BoxA (2 μg/ml). DMEM containing 0.1% BSA and 10% FCS were used as negative (-) and positive (+) controls, respectively. Data are expressed as the number of migrated cells and represent the average ± SD of four experiments performed in duplicate. (*p<0.01 vs -, †p<0.05 vs + and HMGB1).(B) HMGB1-mediated C2C12 migration is partially dependent on RAGE. C2C12 cells were transfected with the dominant negative form of RAGE (dnRAGE) or with an empty plasmid as control (pcDNA3), together with pGFP. Cells were induced to migrate in presence of HMGB1 (100 ng/ml). dnRAGE partially inhibited HMGB1 induced chemotaxis of C2C12 cells. Data are expressed as the fold increase in the number of migrated GFP expressing cells relative to the number of GFP migrated cells in the absence of factor (migration index) and are the means ± SD of at least 4 independent experiments performed in duplicate. (* p<0.05 vs HMGB1-induced migration of pCDNA3 transfected cells).
MATERIALS AND METHODS

In vivo studies

Immediately after surgery, HMGB1 (200 ng) or BoxA (1 μg), resuspended in 30 μL PBS, were delivered at three levels (proximal, medial, distal, 10 μL each) of the adductor muscle, along the femoral artery site after its removal.

Laser Doppler Perfusion Imaging (LDPI-Lisca) was used to record serial blood flow measurements over the course of 21 days postoperatively as previously described 22.

All experimental procedures complied with the Guidelines of the Italian National Institutes of Health, with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee.

Histology, Immunohistochemistry and Morphometric Analysis

Anesthetized mice were perfused with phosphate buffer (0.2 mol/L, pH 7.4) containing 5000 U/mL heparin (Roche Molecular Biochemical, Monza, MI, Italy) followed by 10% buffered formalin for 10 min via the abdominal aorta at 100 mm Hg. Adductor muscles were removed, fixed in formalin for 48 hours and embedded in paraffin (Bio-plast special; melting point 52–54°C). Sections from each sample were cut at a thickness of 3 μm and both capillary number and arteriole length density analysed as described 1. Sections were incubated with anti HMGB1 (1 μg/ml Abcam, Cambridge, UK), anti m-Cadherin (1μg/ml St.Cruz Biotechnology, USA) and anti Laminin (1μg/ml SIGMA Milan, Italy) followed by incubation with rabbit anti-mouse antibody coupled to TRITC (1:40) for HMGB1 and rabbit anti-mouse antibody coupled to FITC for m-Cadherin and Laminin. Sections were counterstained with Hoechst to identify nuclei. In other experiments HMGB1 was detected using biotinylated secondary antibodies (7.5 μg/ml, Vector Laboratories, Peterborough, UK) and
then with avidin-biotinylated peroxidase complex (ABC Elite Kit, Vector Laboratories). Sections were counterstained with hematoxylin to identify nuclei. Control reactions included the omission of the primary antibody, which was substituted by non-immune rabbit serum.

**Immunofluorescence**

C2C12 and satellite cells, cultured in GM and DM, were fixed in PBS with 4% paraformaldehyde and permeabilized in PBS with 0.1% Triton X-100. Coverslips were rinsed and blocked in PBS with 0.2% BSA prior to incubation with antibodies. Fixed cells were incubated with the following antibodies: HMGB1 rabbit polyclonal antibody (pAb, 0.5 μg/ml Abcam), anti RAGE pAb (0.4 μg/ml Santa Cruz Biotechnology, St. Cruz CA); TLR4 pAb (0.5 μg/ml St. Cruz ) for 1 h followed by incubation with rabbit anti-mouse antibody coupled to FITC (1:40) or TRITC (1:40). The coverglasses were mounted and analyzed with a Zeiss microscope equipped for epifluorescence. Cells were lysed in RIPA buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 1% deoxycolic acid, 0.1% sodium dodecyl sulfate (SDS), 10% glycerol and protease inhibitors. Equal amounts of total cellular proteins (100 ng/lane) were resolved by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK).

**Cell culture, treatments and western blot analysis**

The murine myoblast C2C12 cell line (a kind gift of Dr. F. Martelli) was cultured in Dulbecco's modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum (FCS, Euroclone Inc., Milan, Italy), 20 mM Glutamine, 100 U/ml Penicillin and 100 mg/ml Streptomycin (Growth medium-GM, Gibco BRL, Paisley, UK). To induce differentiation, cells at 70-80% confluence were shifted to DMEM supplemented with 2% horse serum (Differentiation Medium-DM). Primary satellite cells were isolated from mouse hindlimb skeletal muscle as previously described and grown in DMEM supplemented with 10% horse serum (Sigma) and 3% chick extract (ICN-
Biomedicals). Transfection experiments were performed with plasmids pdn RAGE (1μg/μl), pCDNA3 and pGFP using the LipofectAMINE Plus reagent as recommended by the supplier (Invitrogen, Carlsbad CA).

Human umbilical vein endothelial cells (HUVEC) (Cambrex, Bergamo, Italy) were cultured in EGM-2 which contained growth factors including hEGF, VEGF, bFGF, IGF1 (Cambrex). Cells were used between passage 4 and 6. For starvation experiment EBM-2 was used (Cambrex).

HUVEC were treated with the JNK inhibitor, SP 600125 (Sigma), in a concentration of 10 μM and with the ERK inhibitor PD038059 (Sigma), in a concentration of 30 μM. Then cells were collected for cell extract, western blot analysis or Matrigel assay.

For Western Blot analysis, membranes were probed with HMGB1 rabbit polyclonal antibody (pAb), (0.5 μg/ml Abcam); RAGE pAb (0.4 μg/ml Santa Cruz Biotechnology, St. Cruz CA); TLR4 pAb (0.5 μg/ml St. Cruz ); α -tubulin monoclonal antibody (mAb) 0.1 μg/ml , Oncogene Science Inc., Cambridge, MA); MyoD rabbit pAb (0.4 μg/ml BD Pharmingen, San Diego, CA), Myogenin mAb(0.4 μg/ml, Santa Cruz Biotechnology); Myosin heavy chain mAb (MyHC) (1:40, MF20); F4/80 rat antibody (0.5 μg/ml Serotec, Oxford, UK), followed by horseradish peroxidase-coupled secondary antibodies and developed by a chemiluminescence-based detection system (ECL, Amersham, Milan, Italy).

**In vivo and in vitro angiogenesis assays**

Growth factor-free reconstituted basement membrane proteins Matrigel (Becton Dickinson) was mixed either with HMGB1 and PBS and injected subcutaneously in mice. Each mouse received 400 μl of matrigel containing or not 400 ng of HMGB1. After 8 days from Matrigel injection, plugs were removed and processed for histologic analysis. Sections (5 μm thickness) were stained with Trichrome-Masson (Bio-Optica, Milan, Italy). The vessels within the plugs were recognized by both morphology and presence of red blood cells. Angiogenesis was evaluated blindly, by
considering at least three different sections per Matrigel plug; each section was 100 µm from the next. The total number of neovessels over the whole area of Matrigel was measured and expressed as number of vessels/mm² as described ³. Immunoistochemical analysis with anti-α actin mAb was performed in different sections in order to quantify arteriole length density. In vitro angiogenic assay was performed as described ³.

HMGB1 immunoassay

C2C12 plated in 60 mm dishes (10⁵ cells/dish) were cultured either in GM for 1 day or in DM for 1 and 3 days. Conditioned medium (CM) was collected at the indicated time points and concentrated 10 times with centricon (Viviscience Sartotius, Firenze, Italy). Values were assayed in triplicate and corrected for the LDH protein amounts measured using Cytotox96 assay (Promega, Milan, Italy) according to manufacturer’s instructions.
REFERENCES


SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I.
Expression of HMGB1 in skeletal muscle in vivo. HMGB1 expression in normoperfused skeletal muscle (A), in vessels (C) and in ischemic skeletal muscle (D-E). HMGB1 and m-cadherin expression in serial sections of normoperfused skeletal muscle to evidence satellite cells (arrows) (A,B). HMGB1 was also expressed in nuclei of mature fibers (arrowheads). At day 3 after ischemia HMGB1 was detectable in satellite cells and in the inflammatory infiltrate (D). At day 7 after ischemia HMGB1 was present in the cytoplasm of regenerating fibers (E). At day 21 after ischemia HMGB1 expression was back to control condition (F). HMGB1 and m-cadherin were detected using biotinylated secondary antibodies and then with avidin-biotinylated peroxidase complex. Sections were counterstained with hematoxylin to identify nuclei.

Supplemental Figure II.
(A) Capillary number and (B-D) arteriole density, 7 days after ischemia in untreated (Saline), HMGB1 and BoxA treated mice. (n=7 *p<0.001; † n=6, p<0.04). (E) Representative pictures of sections stained with smooth muscle actin (SMA) to detect arterioles. (F) Representative hematoxylin and eosin to evidence regenerating fibers.

Supplemental Figure III.
(A) HMGB1 enhances vessel number in Matrigel plugs in vivo. Representative photomicrographs of Matrigel plug sections supplemented with HMGB1 or saline solution (Trichrome-Masson staining, × 40 magnification). (B) EGM stimulated ERK and JNK phosphorylation. HUVEC were cultured in EGM for the indicated time points and cell extracts analyzed by western blot with anti-phosphorylated ERK and JNK antibodies. As a loading control blots were stripped and reprobed with antibodies for total ERK and JNK (n=2).
Figure IV
HMGB1 is released by differentiated myoblasts. (A) Western blot analysis of supernatants obtained from C2C12 cells cultured in GM and in DM for 1 and 3 days. The same filter was probed with anti-lactate dehydrogenase (LDH) mAb (lower panel). (B, C) ELISA determination of HMGB1 and lactate dehydrogenase (LDH) in supernatants analyzed in (A). Values are expressed as ng of HMGB1 normalized for mg of protein in cell extracts and optical density (OD), respectively (n=3, *p<0.05 vs. GM).

Figure V
(A) dnRAGE does not affect growth medium (GM)-induced chemotaxis of C2C12 cells. C2C12 were transfected either with dnRAGE or the empty plasmid pCDNA3 together with pGFP. Cells were induced to migrate in response to 0.1% BSA (-) or GM (+). Data are expressed as the fold change in the number of migrated GFP/pCDNA3 and GFP/dnRAGE cells relative to the number of GFP/pCDNA3 migrated cells with 0.1% BSA (migration index) and are the means ± SD of at least 4 independent experiments performed in duplicate (* p<0.01 vs GFP/pCDNA3 migrated cells with 0.1% BSA).

(B) HMGB1 does not affect myoblast differentiation. Western blotting analysis of total extract from C2C12 cells cultured in DM for 1 and 3 days either in absence (Control) or in presence of 100 ng/ml HMGB1 (HMGB1). The expression of muscle regulatory factors MyoD, Myogenin and MyHC were unchanged between untreated and HMGB1- treated cells.
Figure I
Figure II

A

B

C

D

E

F
Figure III

(A) Saline

(B) Time (min): 0 5 15 30

- p-ERK
- T-ERK
- p-JNK
- T-JNK

Fold increase

Time (min): 0 5 15 30
Figure IV
Figure V

A

Migration index

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- MyHC
- MyoD
- Myogenin
- α-tubulin
Authors Conflict of Interest Disclosure Questionnaire

Journal: ATVB
Manuscript number: 153429
Author's name: ROBERTA DE NORI

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Date: 29.8.07
Authors Conflict of Interest Disclosure Questionnaire

Journal: ATUB
Manuscript number: 153478
Author’s name: HARCO E. BLANCHI
Page 1 of 2

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Manuscript number: $\text{M34 29}$
Author's name: $\text{MARCO E. BIANCHI}$
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