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Selective Proinflammatory Activation of Astrocytes by High-Mobility Group Box 1 Protein Signaling

Marco Pedrazzi,‡* Mauro Patrone,† Mario Passalacqua,* Elia Ranzato, ‡ Diego Colamassaro,* Bianca Sparatore,* Sandro Pontremoli,* and Edon Melloni*

Extracellular high-mobility group box 1 protein (HMGB1) triggers inflammatory events in the brain. We demonstrate that astrocytes, the main glial cells in the brain, acquire a specific reactive phenotype when exposed to HMGB1. This cell activation, which involves the receptor for advanced glycation end-products and the MAPK/ERK1/2 cascade, results in the transcriptional/translational induction of a restricted number of inflammatory mediators, including cyclooxygenase-2, matrix metalloproteinase-9, and several chemokines of the CC and CXC families. The mixture of factors released by HMGB1-reactive astrocytes displays a potent chemotactic activity on human mononcytoid cells. This study is the first to suggest that HMGB1/astrocyte interaction plays a specific functional role in the progression of inflammatory processes in the CNS by facilitating local leukocyte infiltration. The Journal of Immunology, 2007, 179: 8525–8532.

High-mobility group box 1 protein (HMGB1) is a widely distributed DNA-binding protein in mammalian tissues (1). Maximal expression of HMGB1 is detectable during embryonic life and in several proliferating normal or transformed phenotypes (2–4). Once exported, HMGB1 behaves as a potent cytokine at a local or systemic level (5). Although extracellular HMGB1 can contribute to tissue development and regeneration, it is also able to sustain pathological conditions by enhancing inflammatory responses, tumor cell invasiveness and lethal endotoxemia (6). Recently, high levels of HMGB1 have been detected in specific areas of the adult brain, including the extracellular matrix (7). The local extracellular accumulation of this cytokine in the brain, as a result of its intracerebroventricular administration or its release from dying neurons during cerebral ischemia, elicits local inflammatory events in mice (8–10). Moreover, HMGB1 also promotes neuroinflammation in postischemic rat brain (9) and in the neurodegenerative processes associated to Alzheimer’s disease (11, 12). Hence, new information on the role of single phenotypes of excitatory amino acids from subcellular re-sealed particles of astrocyte origin (17). Thus, it is conceivable that astrocytes may be a target of extracellular HMGB1.

The local concentration of extracellular HMGB1 can rapidly increase in the CNS through active exportation of this leaderless cytokine or as a consequence of passive cell leakage. It has previously been shown that several cells of the CNS can actively export HMGB1 when exposed to appropriate stimuli (9, 16, 18) and a massive accumulation of HMGB1 outside necrotic cells occurs immediately after cerebral ischemic injury (9). Once released, HMGB1 can interact with several components of the extracellular matrix such as the proteoglycans neurocan and phosphacan, which have been identified as high-affinity ligands for HMGB1 in the CNS (19, 20). The receptor for advanced glycation end products (RAGE), TLR2 and TLR4 are further transmembrane receptors, which can be engaged and activated by HMGB1 in brain cells (21, 22). Hence, HMGB1 can trigger neuronal and glial cell responses through RAGE-dependent and -independent mechanisms (16, 23–25).

To determine whether HMGB1 promotes a specific proinflammatory program on primary astrocytes, we analyzed the effect of this cytokine on the expression of inflammatory gene markers related to cell stress and death, immunity, signaling, adhesion, metabolism, and proteolysis. Our findings indicate that astrocytes exposed to HMGB1 acquire a reactive profile that is characterized by the release of potent mediators involved in the local progression of physiopathological processes with an inflammatory component.

Materials and Methods

Animals

Rats of the Milan strain (26) were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light-dark schedule.
Astrocyte primary cultures

Cultures were prepared from brains of 1- to 2-day-old rats, essentially as described previously (27). In brief, cerebral cortices were triturated, filtered on a 70-μm cell strainer (BD Italia) and placed in 25-cm² cell culture flasks containing DMEM (Sigma-Aldrich), 10% FBS (Euroclone), 100 U/ml penicillin (Sigma-Aldrich), 100 μg/ml streptomycin (Sigma-Aldrich) and glutaMAX (Invitrogen Life Technologies). Flasks were kept in an atmosphere of 5% CO₂ inside a humidified incubator for 2–4 wk. Microglial cells were then detached by shaking the flasks at 37°C for 1 h. Astrocytes were collected by trypsinization and replated at 40,000 cells/cm². All experiments were performed on day 3 or 4 after replating. Immunostaining for glial fibrillary acidic protein (GFAP, astrocyte marker) and CD11b (microglia marker) revealed that microglial contamination of astrocyte cultures was below 1%. The potential contribution of residual contaminating microglia to the inflammatory responses was assessed by measuring NO and TNF-α production following 72 h cell exposure to 10 μg/ml LPS (28). Only LPS-unresponsive astrocyte cultures were used in additional experiments. Cells were placed in fresh medium containing 2% FBS 24 h before treatments.

Astrocyte stimulation

Cells were stimulated with purified recombinant HMGB1 or with a complete cytokine mixture (CCM) previously characterized as an artificial approximation to a full inflammatory condition (28). Alternatively, astrocytes were incubated in the presence of LPS (Sigma-Aldrich) at concentrations corresponding to the possible contamination present in HMGB1 preparations. Stimulated cells were used for RNA extraction and for two-dimensional liquid chromatography analysis, whereas the conditioned medium was used for zymography, multiplex sandwich ELISA (SearchLight rat chemokine 7-Plex Array; Pierce Endogen) and chemotaxis assay. Where indicated, 40 μg/ml blocking Ab mouse anti-RAGE (Chemicon International) or 50 μM PD098059 (MEK inhibitor) (Sigma-Aldrich) was added 15 min before cell treatments.

Chemotaxis Assay

Mono Mac 6 cells (provided by Dr. Stifanese, Department of Experimental Medicine, Biochemistry Section, University of Genoa, Genoa, Italy), cultured as previously reported (29), were seeded at 2 × 10⁶/ml in the upper compartment of Transwell chambers (8 μm pore size; Corning Costar). HMGB1, dissolved in DMEM containing 2% FBS, and the conditioned medium from stimulated astrocytes were placed in the lower compartment. Cells were allowed to migrate for 1.5 h at 37°C in a 5% CO₂ atmosphere. Medium from stimulated astrocytes were placed in the lower compartment. HMGB1, dissolved in DMEM containing 2% FBS, and the conditioned medium from stimulated astrocytes were placed in the lower compartment. Cells were allowed to migrate for 1.5 h at 37°C in a 5% CO₂ atmosphere. Cells on the lower surface were scraped from the upper surface of the filter. Cells on the lower surface were fixed with methanol and stained with 0.1% (w/v) toluidine blue for 15 min at 4°C. Five random fields were counted for each triplicate sample.

Semi-quantitative reverse transcriptase-PCR

Astrocyte total RNA was extracted by using RNeasy columns with a DNase digestion step included (Qiagen). cDNA was prepared by using 1 μg of RNA, random hexamer primers and ThermoScript RT-PCR system (Invitrogen Life Technologies) according to the manufacturer’s instructions. One microliter of cDNA was amplified by means of 0.05 U/μl recombinant TaqDNA polymerase (Invitrogen Life Technologies) and the following primers for the targets indicated.

PCR products were resolved on a 1.7% agarose gel containing 0.5 μg/ml ethidium bromide (Sigma-Aldrich) and quantified densitometrically by means of Chemi Doc XRS (Bio-Rad) at cycle numbers between 10 and 40 to determine the appropriate number of cycles at which exponential amplification of products occurred and to select the number of cycles at which discrimination was sufficient to accurately quantify increases or decreases in gene expression. The reaction mixtures were subjected to 30 cycles (25 cycles for GAPDH) of PCR in the following conditions: 94°C for 20 s, 58°C for 30 s, and 72°C for 30 s.

Purification of HMGB1

Eukaryotic recombinant HMGB1 protein was produced by using the baculovirus system, and was purified as described (30), with some modifications. Briefly, 72 h infected SF9 cells were lysed by sonication in 50 mM sodium borate buffer (pH 8.5) containing 0.15 M NaCl, 0.2% (v/v) Triton X-100, 10 μg/ml aprotinin, 100 μg/ml leupeptin and 2 mM Pefabloc SC (Fluka Sigma-Aldrich). The lysate was cleared by centrifugation at 100,000 × g for 10 min at 4°C and the supernatant was applied to a 1 ml HiTrap heparin column (GE Healthcare). The column was eluted with a linear gradient of 0.15–1.5 M NaCl in 50 mM sodium borate buffer (pH 8.5). Fractions containing recombinant HMGB1 were pooled and subjected to anion exchange chromatography using a MonoQ 5/50 GL column (GE Healthcare) under the same conditions. Purified HMGB1 was applied to an AffinityPak Detoxi-Gel endotoxin-removing column (Pierce) according to the manufacturer’s instructions. The amount of LPS in HMGB1 preparations was assayed by the LAL test (International pbi) and found to be <0.002 EU/μg HMGB1, corresponding to 0.3 pg/μg HMGB1.

Immunoblotting

Proteins were separated by SDS-10%PAGE and transferred onto nitrocellulose membranes. Membranes were blocked in 20 mM sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl, 5% (w/v) nonfat dry milk, 0.1% (v/v) Tween 20 (Sigma-Aldrich) and incubated with one of the following primary Abs (16 h at 4°C): rabbit anti-phospho-ERK1/2, 1:1000; rabbit anti-ERK1/2, 1:1000 (Cell Signaling Technology, Celbio S.p.A, Mi, Italy); mouse anti-inducible NO synthase (iNOS), 1:2500 (BD Italia); goat anti-cyclooxygenase-2 (COX-2) 1:2000 (Santa Cruz Biotechnology); mouse anti-actin 1:5000 (Sigma-Aldrich). Peroxidase-conjugated secondary Abs (1 h at 20°C) were: anti-rabbit, 1:3000; anti-mouse, 1:4000 (Cell Signaling Technology); anti-actin 1:6000 (Chemicon International). Blots were washed with 0.15% (v/v) Tween 20 (Sigma-Aldrich) and incubated with one of the following Abs: mouse anti-phospho-p44/42 MAPK (1:1000; Cell Signaling Technology); anti-actin 1:6000 (Chemicon International). Immunoreactive signals were developed by means of an ECL Plus Western blotting detection system (GE Healthcare) and quantified by using Chemid Doc XRS, as specified above.

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<th>Target</th>
<th>Forward Primer (5′)</th>
<th>Reverse Primer (3′)</th>
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<tr>
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<td>5′-TCTCTACCACCTCTTCTG-3′</td>
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<tr>
<td>IL-6</td>
<td>5′-AGGAGTACCACCCACACA-3′</td>
<td>5′-TTTGGGACATCCACATTT-3′</td>
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<td>5′-GCAACAAATTGATGTTGCTCCA-3′</td>
<td>5′-TCCACCGATGACCTGATATT-3′</td>
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Table I. Major proteins up-regulated by HMGB1 in primary astrocytes

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<th>Protein</th>
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<th>Score</th>
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<tr>
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<td>67</td>
</tr>
<tr>
<td>Ubiquitin and ribosomal protein L40 precursor (gi 13928952)</td>
<td>ND</td>
<td>73</td>
</tr>
<tr>
<td>Histone H2B1 (gi 12025236)</td>
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<td>73</td>
</tr>
<tr>
<td>Alpha-enolase (gi 56757324)</td>
<td>2.5</td>
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</tr>
<tr>
<td>GFAP (gi 5030428)</td>
<td>3.1</td>
<td>161</td>
</tr>
<tr>
<td>Golgi phosphoprotein 3 (gi 62461580)</td>
<td>3.6</td>
<td>1.58d</td>
</tr>
<tr>
<td>Similar to lipase A precursor (gi 109460058)</td>
<td>8.1</td>
<td>88</td>
</tr>
<tr>
<td>Vimentin (gi 14389299)</td>
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<td>119</td>
</tr>
<tr>
<td>Vimentin (gi 14389299)</td>
<td>3.4</td>
<td>73</td>
</tr>
</tbody>
</table>

a Protein scores greater than 59 are significant (p < 0.05) using MASCOT search engine.

b ND. Not detectable in unstimulated cells.

c Phosphorylated protein forms.

d This protein was recognized (p < 0.05) with ALDENTE search engine (www.exspasy.org).

Zymography

MMP (matrix metalloproteinase)-2 and MMP-9 were assayed by zymography (31). Briefly, 30 μl of astrocyte-conditioned medium were subjected to electrophoresis, without boiling or reduction, through a 10% polyacrylamide gel copolymerized with 1 mg/ml gelatin at 4°C. The gel was then incubated for 1 h at 25°C in 2.5% (v/v) Triton X-100 (Sigma-Aldrich), washed twice with water (20 min each time), and then maintained overnight at 37°C in 0.05 M Tris (pH 7.4) containing 10 mM CaCl₂, 0.15 M NaCl and 0.05% BRIJ35 (Calbiochem, VWR International). As a control, duplicate samples were loaded onto gels that were incubated in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.05% EDTA, to inhibit MMP proteolytic activity. The gels were fixed with 40% methanol-H₂O, 0.1% TFA in acetonitrile. The relative levels of MMP activity were calculated by computer-assisted planimetry of the lytic area using Chemi Doc XRS.

Chemokine assay

The amounts of CCL3 (MIP1α), CCL5 (RANTES), CXCL1 (GROα), CXCL2 (MIP2α) (detection limit, 1.56 pg/ml), CCL2 (MCP1), CCL20 (MIP3), and CX3CL1 (Fractalkine) (detection limit, 3.13 pg/ml) were measured in the astrocyte-conditioned medium by multiplex sandwich ELISA (Pierce Endogen) according to the manufacturer’s instructions.

Proteomic analysis

Astrocytes were lysed in 62.5 mM Tris (pH 7.8 – 8.2) containing 7.5 M urea, 2.5 M thiourea, 12.5% (v/v) glycerol, 2.5% (w/v) octylglucoside, 6.25 mM Tris(2-carboxy-ethyl)phosphine hydrochloride, 10 μg/ml aprotinin, 100 μg/ml leupeptin, and 2 mM Pefabloc SC (Fluka Sigma-Aldrich). The lysates were cleared by centrifugation at 20,000 × g for 1 h at 18°C, and the buffer was then exchanged with the PF2D start buffer (pH 8.5 ± 0.1; Beckman Coulter) by using a PD10 column (GE Healthcare). Proteins were collected and quantified by means of a MicroBCA protein assay kit (Fierce), and 0.7 mg of each sample was injected into a chromatofocusing column.

Proteomic analysis was conducted by using a ProteomeLab PF2D system (Beckman Coulter, Inc.) which separates proteins according to pI by means of a chromatofocusing column in the first dimension, and by means of nonporous reversed-phase HPLC in the second dimension.

The first-dimension method was started at a flow rate of 0.2 ml/min by washing the column with 100% start buffer for 45 min 1 ml fractions were collected at 5 min intervals. The linear pH gradient was generated by running 100% eluent buffer (pH 4.5 ± 0.1; Beckman Coulter) for 1 h; fractions were collected every 0.3 pH units. When the eluent reached pH 4.5, the column was washed with 10 volumes of 1 M NaCl to remove residual proteins.

The fractions generated in the first dimension were sequentially injected (250 μl) onto the second-dimension reversed-phase HPLC column. The second-dimension method consisted of a 30 min linear gradient of 5–100% B/A at 0.75 ml/min, where A is 0.10% trifluoroacetic acid (TFA) in water and B is 0.08% TFA in acetonitrile. The column was kept at 50°C, and the eluted proteins were detected at 214 nm. Data were collected and analyzed by means of ProteoVue/DeltaVue software (Beckman Coulter).

 Peaks of interest were collected and digestion was performed overnight at 37°C in 20 μl of 25 ng/μl sequencing-grade trypsin (Roche) reconstituted in 50 mM NH₄HCO₃. Each sample was then purified by means of a reversed-phase ZipTip (Millipore) as recommended by the manufacturer. The eluted tryptic digest (1 μl) was mixed with 1 μl of matrix solution (2.5-dihydroxybenzoic acid (Fluka Sigma-Aldrich) in 50% acetonitrile/ H₂O, 0.1% TFA) and spotted on a MALDI sample plate. All mass spectrometry data were collected by means of a MALDI-TOF Voyager-DE Pro.

FIGURE 1. Levels of COX-2 and iNOS in astrocytes stimulated with HMGB1. 20 μg of total astrocyte proteins was subjected to Western blotting. A) astrocytes were left untreated (ctrl), or treated for 16 h with the indicated additions; B) astrocytes were left untreated, or pretreated for 15 min with the indicated additions and then stimulated with HMGB1 for 9 h. Blots are representative of three separate experiments.

FIGURE 2. Effect of HMGB1 on phosphorylation of ERK2. 20 μg of total astrocyte proteins was subjected to Western blotting. A) time-course analysis of phospho-ERK1/2 (p-ERK1/2) in astrocytes stimulated with 40 nM HMGB1. The total amounts of ERK1/2 are also shown. B) quantification of p-ERK1/2 in astrocytes untreated (ctrl), or treated for 10 min with HMGB1. Anti-RAGE was added 15 min before HMGB1 stimulation. Numbers under the p-ERK2 immunoreactive bands refer to their relative amounts. The total levels of ERK1/2 are also shown. Blots are representative of three separate experiments.
Biospectrometry Workstation (Applied Biosystems) and acquired in the positive ion-reflector delayed-extraction mode. Each sample was internally calibrated on the trypsin autolysis peaks (at \( m/z \) 1153.5741, 2163.0570 and 2273.1600). Data were processed by means of Data Explorer software 4.0.0.0 (Applied Biosystems). Proteins were identified using a MASCOT search engine (www.matrixscience.com). One missed cleavage per peptide was allowed, and an initial mass tolerance of 50 ppm was adopted. If the proteins were not significantly identified, the mass tolerance was extended to 100 ppm.

**Statistical analysis**

Data were analyzed by means of the Prism 4.0 software package (GraphPad). Results are expressed as the mean ± SE and were compared by \( t \) test.

**Results**

**Proteomic analysis of astrocytes stimulated with HMGB1**

To evaluate whether HMGB1 modulates major proteins expressed by primary astrocytes, cells were cultured in the absence or presence of this cytokine, and cell proteins were then separated by two-dimensional liquid chromatography. Protein peaks that increased >2-fold in samples from HMGB1-stimulated astrocytes were selected for further mass spectrometry analysis. Nine of the up-regulated proteins were identified by means of a peptide mass fingerprint procedure. As shown in Table I, HMGB1 induced a 3-fold increase in the levels of phosphorylated GFAP and vimentin, two glial intermediate filament protein markers of inflamed astrocytes (32, 33). HMGB1 also promoted a 2.5-fold up-regulation of \( \alpha \) enolase, one of the major proteins previously found to be over-expressed in astrocytes stimulated with a CCM (14). Among the other major proteins found to be up-regulated in astrocytes exposed to HMGB1, only Hsp10 has previously been related to inflammatory processes (34). This proteomic evidence indicates that HMGB1 induces a proinflammatory-like response in primary astrocytes and that its signaling activity results in the modulation of several major gene products in these cells.

**Molecular mechanisms involved in HMGB1 signaling**

Because the enzymes COX-2 and iNOS are relevant to the inflammatory response, we analyzed whether HMGB1 induced their expression in primary astrocytes. As reported in Fig. 1A, although resting astrocytes did not express the inflammation-specific proteins COX-2 and iNOS, the addition of CCM to the cell cultures induced both gene products. In contrast, HMGB1 only induced a dose-dependent up-regulation of COX-2, suggesting that this cytokine displays a selective proinflammatory activation of astrocytes. Starting from a previous observation that astrocytes express RAGE (17), we analyzed the involvement of this receptor as an upstream mediator of the HMGB1-stimulated production of COX-2. As shown in Fig. 1B, an anti-RAGE Ab able to block RAGE activation prevented the HMGB1-promoted induction of COX-2, indicating that RAGE plays a crucial role in the inflammatory response of astrocytes to HMGB1. Because induction of rat cortical astrocyte COX-2 mainly requires the activation of the MAPK/ERK1/2 pathway (35), we tested the possible role of ERK1/2 as a mediator of HMGB1-induced expression of this enzyme. It was found (Fig. 1B) that the COX-2 immunoreactive signal disappeared when cells were stimulated with HMGB1 in the presence of the MEK inhibitor PD098059.

A time-course analysis of ERK1/2 activation was conducted by using an Ab that recognizes rat phospho-42 ERK (p-ERK2) only increased 5-fold in astrocytes exposed to HMGB1.
when phosphorylated at Thr183 and Tyr185. HMGB1 stimulated the rapid phosphorylation of this kinase, the maximum level of p-ERK2 being 2.5-fold higher than in unstimulated cells after 10 min (Fig. 2A). This effect subsided almost completely within 2 h. Conversely, astrocytes stimulated with HMGB1, even at a concentration of the cytokine up to 400 nM, did not show activation of p38 MAPK or NF-κB, nor degradation of IkBα, events which are often related to the generation of inflammatory mediators (data not shown). The level of phosphorylated ERK2 after 10 min cell stimulation with HMGB1 was markedly reduced in the presence of the anti-RAGE Ab (Fig. 2B).

These results provide clear evidence of the involvement of the MAPK/ERK1/2 cascade as a down-stream transducer of HMGB1/RAGE signaling in primary astrocytes.

**Pattern of inflammatory genes induced by HMGB1 on primary astrocytes**

The profile of astrocytes activated by HMGB1 was further defined by the semiquantitative RT-PCR analysis of several genes which are up-regulated in the fully inflammatory transcriptome of these cells (14). As shown in Fig. 3, HMGB1 induced a significant increase in the levels of CCL5 and the ELR-chemokines CXCL1 and CXCL2. However, the level of the monocyte chemoattractant protein CCL7 transcript was not affected, suggesting that HMGB1 is a specific, rather than a general, activator of CC chemokine transcription in primary astrocytes. This specificity is further supported by the fact that HMGB1 induced a 2-fold increase in the level of the astrocyte MMP-9 transcript, leaving MMP-2, MMP-3 and the tissue inhibitor of the matrix metalloproteinase (TIMP)2 unaffected. However, as expected, HMGB1 also produced a 2-fold increase in the level of the COX-2 transcript. In contrast, several inflammatory genes related to immunity (caspase 4, IFN-induced protein with tetratricopeptide repeat (IFIT)3 and IL6), signaling (IFN regulatory factor-1 (IRF1) and TNF-α) and cell adhesion (VCAM1), did not show any HMGB1-dependent modulation.

Moreover, LPS (0.3 pg/ml, corresponding to the possible contamination present in 40 nM HMGB1) did not induce expression of the chemokines genes up-regulated by HMGB1. We only observed an increase in MMP-9 gene expression significantly lower than that obtained with HMGB1 (Fig. 3).

**FIGURE 5.** Quantification of CXC chemokines released by astrocytes stimulated with HMGB1. The experimental conditions were as specified in the legend to Fig. 5. *, p < 0.05; #, p < 0.01 vs cells stimulated 24 h with 40 nM HMGB1.

**FIGURE 6.** Quantification of MMP-2 and 9 in astrocyte-conditioned medium. A and B) the conditioned medium (30 μl) of astrocytes cultured for 24 h in the presence of the indicated concentrations of HMGB1 were subjected to zymography and analyzed by means of the Quantity One Software. Numbers above each lytic band indicate the amounts relative to the unstimulated cell condition. Where indicated, the HMGB1-stimulation mixture also contained 50 μM PD098059. Experiments were performed three times and one representative outcome is shown.

**FIGURE 7.** Chemotactic properties of conditioned medium from HMGB1-stimulated astrocytes. The conditioned medium (CM, 600 μl) of astrocytes cultured for 24 h in the presence of the indicated concentrations of HMGB1 or CCM or LPS and a 40 nM HMGB1 solution were placed in the bottom of a 24-well plate. Mono Mac 6 cells (100 μl) were seeded in the upper compartment of Transwell® chambers (8 μm pore size) at a density of 2 × 10^5/ml. After 1.5 h, cells on the filter were counted; data are expressed as percentage of cells that migrated in the absence of a chemotactic stimulus. Five random fields were counted for each duplicate sample.
Hence, in primary astrocytes HMGB1 activates the transcription of a restricted number of inflammatory genes, which include chemoattractive molecules for specific subsets of leukocytes and a proteolytic enzyme that facilitates tissue infiltration by inflammatory cells.

### Analysis of chemokines and metalloproteinases released by HMGB1-stimulated astrocytes

To determine whether the HMGB1-dependent induction of specific chemokines corresponded to their enhanced exportation from primary astrocytes, we used ELISA. As reported in Fig. 4, the release of CCL5 was highly up-regulated by HMGB1, and the extracellular amounts of this chemokine depended on the time of stimulation and on the concentration of HMGB1 added. Furthermore, HMGB1 stimulated the cell production of CCL2, CCL20, and CCL3. To assess the role of the MAPK/ERK1/2 cascade in this process, astrocytes were also stimulated with 40 nM HMGB1 for 24 h in the presence of the MEK inhibitor PD098059. In this condition, the release of the various CC chemokines was markedly decreased. The CXC chemokines CXCL1 and CXCL2, both transcriptionally induced by HMGB1 (Fig. 3), were also actively released by astrocytes upon stimulation with this cytokine in a time- and concentration-dependent manner (Fig. 5). Moreover, we detected an HMGB1-dependent cell release of the CX3CL1 chemokine, a known activator of microglia and a positive modulator of leukocyte infiltration in rat brain (36). The exportation of CXC and CX3C chemokines was markedly reduced by PD098059, thus providing further evidence that the ERK1/2 MAPK cascade is a fundamental mediator in the inflammatory activation of astrocytes by HMGB1.

Zymography analysis (Fig. 6A) revealed that the conditioned medium of astrocytes subjected to stimulation with HMGB1 for 24 h contained increased amounts of MMP-9. The proteinase was released in an HMGB1-dose-dependent manner; again, this cell response to HMGB1 was prevented by PD098059 (Fig. 6B). In contrast, MMP-2 was not modulated by HMGB1, and the MEK inhibitor PD098059 did not affect its release.

Taken together, these results corroborate the transcriptional profile and further extend the number of chemokines that are actively released when astrocytes are exposed to nanomolar amounts of HMGB1.

### Functional properties of HMGB1-activated astrocytes

To establish the functional properties of HMGB1-activated astrocytes, the conditioned medium was tested for its capacity to induce chemotaxis on the monocytic cell line Mono Mac 6.

These cell targets were selected because it had previously been shown that nanomolar concentrations of extracellular HMGB1 did not affect monocyte chemotaxis (37). As shown in Fig. 7, we confirmed that Mono Mac 6 were also insensitive to 40 nM HMGB1 and demonstrated that the conditioned medium from astrocytes stimulated with 4 and 40 nM HMGB1 displayed a 3- and 6-fold increase, respectively, in chemotactic activity in comparison with the medium of unstimulated cells. Moreover, the conditioned medium of astrocytes exposed to 40 nM HMGB1 displayed a level of chemotactic activity similar to that obtained by their exposure to the CCM. The conditioned medium of astrocytes exposed to 0.3 pg/ml LPS did not display chemotactic activity on the monocytic cell line Mono Mac 6 (Fig. 7). In conclusion, although the complex mixture of bioactive molecules released by HMGB1-stimulated astrocytes has not so far been fully characterized, these results indicate that it displays potent chemotactic properties.

### Discussion

Our results demonstrate that HMGB1 displays a proinflammatory activity on primary astrocytes and that engagement of the transmembrane receptor RAGE and the subsequent activation of the MAPK/ERK1/2 cascade are involved in mediating the inflammatory response to this cytokine. They also show that the reactive phenotype of astrocytes, obtained in the presence of nanomolar concentrations of extracellular HMGB1, expresses a significant increase in the levels of several major proteins, such as the phosphorylated forms of intermediate filament proteins GFAP and vimentin, which are classical markers of astrogliosis in the CNS (38). Among the numerous transcripts and gene products involved in the astrocyte inflammatory process, we observed that the most prominent cluster of genes regulated by HMGB1 includes several chemokines and MMP-9 all of which are known to be actively involved in chemotaxis and to facilitate the invasion of inflamed tissues (39). The present study demonstrates that nanomolar concentrations of HMGB1 are sufficient to induce a 5–6-fold up-regulation of astrocyte CXCL1 and CXCL2 chemokine transcripts, followed by a marked increase in the release of these gene products. In accordance with the role of these chemokines in the CNS (40), our findings suggest that HMGB1 causes astrocytes to acquire a reactive profile that is able to stimulate cell migration and immune surveillance in the CNS. Furthermore, our results indicate that, among the ELR-negative chemokines, HMGB1 does not affect the expression levels of astrocyte CCL7, but that it induces a sizeable up-regulation of CCL5 and CCL2, which are inducers of monocyte/macrophage and microglia migration (41). Because astrocytes are abundant in the CNS and are localized in proximity to the blood-brain barrier, their up-regulation of CCL5 and CCL2 could directly contribute to rapid proinflammatory responses within the CNS by attracting both resident microglia and circulating monocytes. Moreover, owing to the role of CCL5 as an inducer of multiple chemokines and cytokines in astrocytes (42), we speculate that CCL5, once released by HMGB1-stimulated astrocytes, could amplify inflammatory events in the brain. In contrast, we found that HMGB1 also induced the release of CX3CL1. This chemokine is a modulator of neuron-microglia interaction and counteracts the neurotoxicity triggered by activated microglia in proinflammatory conditions (43), thus indicating that astrocytes exposed to HMGB1 release a mixture of chemokines characterized not only by specific leukocyte-attracting activities but also by neuromodulatory functions.

Kim and coworkers (9) have recently reported that HMGB1 induces several proinflammatory markers in microglial cultures. However, these authors found a discrepancy between the relatively low release of NO induced by HMGB1 in microglia and the severity of neuroinflammation triggered by HMGB1 in the postischemic brain. Our results suggest that the inflammatory profile expressed by astrocytes in the presence of extracellular HMGB1 released by dying neurons could be responsible for the amplified inflammatory responses measured in vivo following cerebral ischemic injury.

In this study, we observed that HMGB1 also induces astrocyte COX-2. This enzyme is a marker of oxidative stress and plays a major role in the inflammatory reactions involved in neurodegenerative processes, also by activating the expression of MMP-9 (44, 45). Because inhibitors of COX-2 prevent neuroinflammatory disorders, the enzyme is a promising target in the management of CNS inflammation triggered by extracellular HMGB1. Accordingly, the increased expression of MMP-9 by astrocytes exposed to
HMG1 may be the result of a direct cell stimulation followed by a secondary activation promoted by prostaglandin E2. Furthermore, because the ERK signaling pathway plays a pivotal role in the production and release of MMP-9 (46), the induction and release of the proteinase is favored by the rapid activation of ERK1/2 detected in HMG1-stimulated astrocytes. These cells are a major source of MMP-9 in the inflamed brain (47), where the proteinase is involved in the degradation of specific components of the CNS tissue matrix and blood-brain barrier (48). The close proximity of astrocytes and endothelial cells in the brain suggests that an enhanced production of MMP-9 by astrocytes could modulate neuroinflammatory processes associated with blood-brain barrier dysfunction.

In conclusion, we report that a complex mixture of signal molecules is released into the extracellular milieu of HMG1-stimulated astrocytes and that it is functionally involved in the stimulation of monocyte chemotaxis. Accordingly, it can be proposed that nanomolar concentrations of extracellular HMG1 are sufficient to induce a reactive astrocyte phenotype that promotes astrocyte activation and thus the production of a potent mixture of bioactive protein factors involved in inflammatory/immune responses in the brain.

Disclosures
The authors have no financial conflict of interest.

References
3. Daston, M.M., and N. Ratner. 1994. Amphoterin (P30, HMG-1) and RIP are functional properties of other sets of leukocytes. Taken together, the results reported in this study demonstrate that HMG1 behaves as a typical immunoregulatory cytokine by promoting astrocyte activation and thus the production of a potent mixture of bioactive protein factors involved in inflammatoryimmune responses in the brain.

Regulation of monocyte migration by amphoterin (HMGB1). Blood 104: 1174–1182.


