Neurobiology of Disease

The HMGB1 Receptor RAGE Mediates Ischemic Brain Damage

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In ischemic stroke, the necrotic core is surrounded by a zone of inflammation, in which delayed cell death aggravates the initial insult. Here, we provide evidence that the receptor for advanced glycation end products (RAGE) functions as a sensor of necrotic cell death and contributes to inflammation and ischemic brain damage. The RAGE ligand high mobility group box 1 (HMGB1) was elevated in serum of stroke patients and was released from ischemic brain tissue in a mouse model of cerebral ischemia. A neutralizing anti-HMGB1 antibody and HMGB1 box A, an antagonist of HMGB1 at the receptor RAGE, ameliorated ischemic brain damage. Interestingly, genetic RAGE deficiency and the decoy receptor soluble RAGE reduced the infarct size. *In vitro*, expression of RAGE in (micro)glial cells mediated the toxic effect of HMGB1. Addition of macrophages to neural cultures further enhanced the toxic effect of HMGB1. To test whether immigrant macrophages in the ischemic brain mediate the RAGE effect, we generated chimeric mice by transplanting RAGE $^{-/-}$ bone marrow to wild-type mice. RAGE deficiency in bone marrow-derived cells significantly reduced the infarct size. Thus, HMGB1–RAGE signaling links necrosis with macrophage activation and may provide a target for anti-inflammatory therapy in stroke.

Key words: RAGE; HMGB1; microglia; cerebral ischemia; stroke; macrophage

Introduction

Stroke is the second most common cause of death worldwide. Despite this, the only treatment of ischemic stroke available is recanalization of the occluded vessel by thrombolysis. Thrombolysis is safe and effective only within 3 h of the onset of symptoms. This short time window results in a low treatment rate and warrants safer treatment regimes. In stroke, energy depletion leads to necrotic cell death in the most severely affected area of ischemia. However, pathology may spread to the so-called penumbra. In the penumbra, ischemia itself is not sufficiently severe to cause cell death. Toxic factors from the core of the lesion may pose an additional risk for cells in the penumbra. One such factor is spreading depression, which has been shown to promote the enlargement of ischemic lesions (Takano et al., 2007). In addition, soluble mediators from the necrotic core area may diffuse to the adjacent penumbra and elicit a delayed inflammatory response that contributes to neuronal cell death. Here, we provide evidence that the receptor for advanced glycation end products (RAGE) and its ligand high mobility group box 1 (HMGB1) qualify for a role as mediators of ischemic inflammation and neurodegeneration.

HMGB1 is a ubiquitous and abundant nuclear protein that is released from necrotic and inflammatory cells and induces an inflammatory response (Lotze and Tracey, 2005). It binds to RAGE and apparently also to toll-like receptor (TLR)2 and TLR4. HMGB1 contains two DNA binding domains (box A and box B) and an acidic C-terminal tail (see Fig. 2*D*). Box A acts as a specific antagonist for the interaction between HMGB1 and RAGE (Bianchi and Manfredi, 2007). RAGE is a member of the Ig superfamily of cell surface receptors (Bierhaus et al., 2005) that is activated by several ligands including HMGB1 but also by advanced glycation end products (AGEs), S100 proteins, and amyloid β-peptide (Aβ). In the brain, RAGE is present on neurons, glia, and endothelial cells (Yan et al., 1996; Deane et al., 2003; Arancio et al., 2004; Bierhaus et al., 2004). However, the role of RAGE in stroke has not been studied in detail.

Materials and Methods

Subjects. Stroke patients and control subjects represent a randomly selected subgroup of a previous study (Inta et al., 2008). Control subjects were age- and sex-matched to stroke patients. All subjects gave their informed consent. This study was approved by the local ethics committee.

ELISA and real-time RT-PCR. To determine HMGB1 concentrations, we used an ELISA kit (Shino-test). The detection threshold of this assay is <1 ng/ml. The between-assay coefficient of variation is $<8\%$. Serum samples were stored at $-80°C$ before measurement.

We purified RNA from whole cortex of the ischemic hemisphere and from whole blood using RNAPure (Peqlab) and Mouse RiboPure kit

Received May 30, 2008; revised Sept. 8, 2008; accepted Sept. 29, 2008.

This work was supported by a grant from the Landesstiftung (Network Aging Research) Baden-Württemberg to A.B. and M.S., the Deutsche Forschungsgemeinschaft (SFB 405), and the Hopp Stiftung to P.P.N. K.J.T. is consultant to Critical Therapeutics, which has sponsored research and license agreements with The Feinstein Institute for Medical Research for patents related to inhibiting HMGB1. Grazisa Rossetti is employed by HMGBiotech, a company providing HMGB1-related reagents.

The authors declare competing financial interests.

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DOI:10.1523/JNEUROSCI.2435-08.2008

 0270-6474/08/2812023-09$15.00/0

(Ambion), respectively. After reverse transcription using the High Capacity cDNA Archive kit (Applied Biosystems), we performed real-time PCR with the following Taqman assays on demand: HMGB1, Mn00849805_gH; glucuronidase, Mm00446953_m1; hypoxanthine phosphoribosyl-transferase 1, Mm00446968_m1; and TATA box binding protein, Mm00446973_m1. Quantified results for HMGB1 cDNA were normalized to a mean value of the three house-keeping genes. For measurement of RAGE cDNA, the Absolute Blue QPCR SYBR Green Mix (Thermo Scientific) and the following primers were used: RAGE forward, 5′-ATT CAG CTG TTG GTT GAG CCT-3′, RAGE reverse, 5′-CCA TCC TTT ATC CAG TGG ACC T-3′ (amplicon length, 113 bp); cyclophilin forward, 5′-AGG TCC TGG CAT CTT GTC CAT-3′, cyclophilin reverse, 5′-GAA CCG TTT GTG TTT GGT CCA-3′ (amplicon length, 51 bp). Quantified results of RAGE cDNA were normalized to cyclophilin. The purity of the amplified products was checked by the dissociation curve.

Immunohistochemistry and TUNEL staining. For immunohistochemistry, sections or cells were fixed in 4% paraformaldehyde (PFA) for 30 min. After blocking in 5% normal horse serum, 5% normal goat serum, or 1% bovine serum albumin, the following primary antibodies were applied: goat anti-RAGE antibody (1:200, AGE 001; Biologo), rabbit anti-Iba1 antibody (0.5 μg/ml, 019-19741; Wako), rat anti-CD11b (1:50, MCA 74GA; Serotec), mouse anti-NeuN (1:50, MAB377; Millipore), mouse anti-GFAP (1:50, s.c.-33673; Santa Cruz Biochemicals), rabbit anti-HMGB1 (1:100) (Yang et al., 2004). Then, the cells or sections were washed with PBS before the secondary antibodies were applied: Cy3-conjugated donkey anti-goat antibody (1:200, 705-165-147; Jackson ImmunoResearch Laboratories), alexa 488-conjugated donkey anti-rabbit (1:100, A21206; Invitrogen), alexa 488-conjugated donkey anti-rat (1:100, A21208; Invitrogen), TRITC-conjugated goat anti-mouse (1:150, 115-025-003; Jackson ImmunoResearch Laboratories), alexa 488-conjugated donkey anti-mouse (1:100, A21202; Invitrogen), Cy3-conjugated goat anti-rabbit (1:100, 111-165-144; Jackson ImmunoResearch Laboratories). Finally, sections or cells were mounted with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) (Vectashield; Vector Laboratories) containing medium.

For staining of rabbit anti-HMGB1 antibody, sections were fixed in acetone, treated with 1.5% hydrogen peroxide in methanol and incubated for 1 h with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody (1:100, ECL, NA934V; Amersham Pharmacia). 3, 3′-diaminobenzidine was used as substrate.

For terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick end labeling (TUNEL), coronal cryosections (20 μm thick, 400 μm caudal to the anterior commissure) were fixed in 4% PFA at room temperature, stained and mounted with medium containing DAPI as described previously (Zhang et al., 2005). Slides were scanned completely using laser scanning cytometry (LSC) (Harnett, 2007) with a 20× objective (CompuCyte) as described previously (Herrmann et al., 2005). Cells positive for DAPI and TUNEL or CD11b were visualized in an *x*- versus *y*-position scattergram on a template of brain contours corresponding to the brain area analyzed.

Fluorescence-activated cell sorting analysis. Fluorescence-activated cell sorting (FACS) analysis of brain cells was performed as described previously [method 2 in Campanella et al. (2002)] using PerCP-labeled anti-CD45 antibody (#557235; BD PharMingen), PerCP-labeled IgG_{2b},κ isotype control (#552991; BD PharMingen), PE-labeled anti-CD11b antibody (#557397; BD PharMingen) and PE-labeled IgG_{2b},κ isotype control (#553989; BD PharMingen).

Preparation of soluble RAGE. For preparation of recombinant soluble RAGE (sRAGE), the coding sequence of the mouse extracellular domain of RAGE (1030 bp) was cloned into pET-DEST42 (Invitrogen). The recombinant plasmid was transformed into the *E. coli* strain BL21. Protein expression was induced by isopropyl D-thiogalactopyranoside. sRAGE was purified by using Protino Ni-TED 2000 columns (Macherey-Nagel), and purity was estimated to be >90% by Coomassie stained SDS-PAGE. Endotoxin content was determined by the E-Toxate Kit (Sigma-Aldrich) and subsequent endotoxin contamination removed by affinity chromatography EndoTrap blue 5/1 (Profos AG) Total protein concentration was assessed by the Lowry method.

Table 1. Characteristics of stroke patients and control subjects on admission

	Stroke patients	Control subjects
Women, *n*	3	3
Men, *n*	5	5
Age, mean ± SEM	66.3 ± 5.9	65.1 ± 5.6
Risk factors, *n*		
No risk factor	0	2
One risk factor	1	3
Two risk factors	6	1
Three or more risk factors	1	2
Stroke classification, *n*		
Large-vessel disease	3	
Cardioembolic disease	3	
Others	2	
Rankin scale, median (range)	3 (2–6)	
NIHSS, median (range)	4 (1–13)	

Stroke subtype was classified according to the TOAST (Trial of Org 10172 in Acute Stroke Treatment) criteria (Adams et al., 1993). Risk factors were atrial fibrillation, hypertension, hypercholesterinemia, and smoking. NIHSS, National Institutes of Health Stroke Scale.

Cell culture. Mixed glial cells were prepared from the brains of neonatal (postnatal day 2) wild-type or $RAGE^{-/-}$ mice as has been described (Marriot et al., 1995). Cells were cultured in DMEM (PAA) containing L-glutamine (0.5 mM), 10% FCS (PAA), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Primary cortical neurons were prepared from brains of embryonic (E16) Naval Medical Research Institute mice (Potrovita et al., 2004). Neurons (200,000 per well) were either incubated in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), L-glutamine (0.5 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) on poly-D-lysine (50 μg/ml) coated 24-well plates or in DMEM containing all supplements on top of confluent glial cells. Mouse peritoneal macrophages were prepared as described previously (Dory, 1989). Cells (400,000 per well) were plated on mixed neural cultures 24 h before stimulation with HMGB1. Mixed cultures were used 6 d after plating the neurons, whereas pure cortical neurons were used on day 10 *in vitro*. Recombinant HMGB1 (HMGBiotech) was added in Neurobasal medium without B27 supplement 24 or 48 h before activity of lactate dehydrogenase (LDH) was measured using the Cytotoxicity Detection Kit (Roche).

Models of cerebral ischemia. $RAGE^{-/-}$ mice (Constien et al., 2001) were backcrossed for 14 generations on a C57BL/6 background. Therefore, we used age-matched C57BL/6 mice as controls. All mice were male and were anesthetized at the age of 3–4 months by intraperitoneal injection of 150 μl 2.5% tribromoethanol per 10 g of body weight. We used a model of distal permanent middle cerebral artery occlusion (MCAO). In this model, neuroprotective effects are less readily observed than in models of transient cerebral ischemia (Chan et al., 1993; Endres et al., 1998). It is afflicted by a rather low mortality (10–12% within 2 d, 20% within 14 d in this study). A skin incision was made between the ear and the orbit on the left side. The temporal muscle was removed by electrical coagulation. The stem of the MCA was exposed through a burr hole and occluded by microbipolar coagulation (Erbe). Surgery was performed under a microscope (Hund). A body temperature of 37°C was maintained in the mice by using a heating pad. After the time indicated, mice were deeply reanesthetized with tribromoethanol and perfused intracardially with Ringer's solution. Coronal cryosections of the brains (20 μm in thickness) were cut every 400 μm and stained with a silver technique. Infarct volumes were corrected for brain edema as has been described before (Potrovita et al., 2004; Herrmann et al., 2005; Zhang et al., 2005). Surgery was performed and ischemic damage was measured without knowledge of the treatment group or the genotype. In a separate cohort of animals, the femoral artery was cannulated to measure arterial blood gases and mean arterial blood pressure. Arterial blood gases, glucose, and hemoglobin were measured 10 min before and 10 min into MCAO in a blood sample of 100 μl. For laser Doppler measurements, the probe (P415-205; Perimed) was placed 3 mm lateral and 6 mm posterior to the

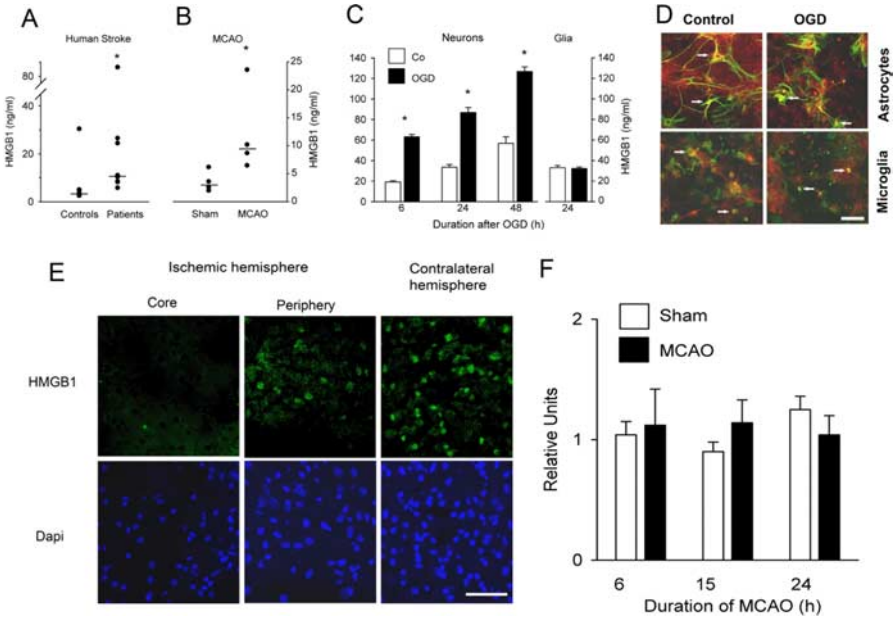

Figure 1. Release of HMGB1 from neural cells during ischemia. ***A***, Stroke patients had significantly higher HMGB1 serum concentrations than controls. Patients were included into the study within 24 h after onset of symptoms. The time interval between symptom onset and blood sampling was 8.8 ± 2.3 h. HMGB1 levels were determined by ELISA. $*p < 0.05$ ($n = 8$, Mann–Whitney test). ***B***, Elevated HMGB1 serum levels in mice 4 h after MCAO compared with sham-operated controls. $*p < 0.05$ ($n = 4$, Mann–Whitney test). ***C***, OGD for 4.5 h stimulated the release of HMGB1 from cortical neurons but not from glia. Values are means ± SEM ($n = 8$). $*p < 0.001$ compared with control group (Co) (*t* test with Bonferroni correction). ***D***, In glial cultures, immunocytochemistry of GFAP or Iba1 [FITC (fluorescein isothiocyanate), green] and RAGE (Cy3, red) showed that GFAP-positive astrocytes and Iba1-positive microglia retained HMGB1 after OGD. Scale bar, 50 μM. ***E***, HMGB1 staining disappeared in the core of the ischemic lesion and was reduced the periphery 4 h after onset of MCAO. Scale bar, 50 μm. ***F***, At the mRNA level, HMGB1 expression was not affected by cerebral ischemia. mRNA was measured by real-time RT-PCR. Values are means ± SEM ($n = 4$–9).

Results

Release of HMGB1 in cerebral ischemia

Clinical characteristics of our stroke patients and control subjects are detailed in Table 1. In patients with ischemic stroke, HMGB1 serum concentrations measured by ELISA were higher than in age- and sex-matched control patients admitted for eye surgery (Fig. 1*A*). In an experimental stroke model in mice, we already noted increased serum concentrations of HMGB1 4 h after ischemia (Fig. 1*B*).

OGD mimics ischemia *in vitro* and led to neuronal cell death (LDH release from primary cortical neurons 1.00 ± 0.07 relative units, $n = 8$; release during 24 h after OGD 1.66 ± 0.10 relative units, $n = 8$, $p < 0.05$). In parallel, OGD induced HMGB1 release from neurons (Fig. 1*C*). The mild OGD for 4.5 h only induced a slight increase in LDH release from glial cultures (LDH release 1.00 ± 0.07 relative units, $n = 8$; release during 24 h after OGD 1.28 ± 0.09 relative units, $n = 8$, $p < 0.05$) that was not accompanied by a release of HMGB1 from glial cells (Fig. 1*C*). Indeed, HMGB1 could be detected by immunocytochemistry in GFAP-positive astrocytes and Iba1-positive microglia after OGD (Fig. 1*D*). Within 4 h after onset of cerebral ischemia, immunostaining of nuclear HMGB1 in the cerebral cortex disappeared in the core of the infarct and was markedly reduced in the periphery (Fig. 1*E*) in accordance with a release into the extracellular space. The well known leakiness of the blood–brain barrier in cerebral ischemia (see Fig. 3*C*) may favor the evasion of HMGB1 from brain. These data suggest that the increase in serum levels of HMGB1 is probably attributable to a spill-over from necrotic neural cells. In contrast, mRNA levels of HMGB1 did not change after MCAO (Fig. 1*F*). Notably, under the same conditions, other genes were markedly upregulated at the mRNA level (Herrmann et al., 2005).

HMGB1 contributes to ischemic brain injury

To test the functional significance of HMGB1 release in our mouse stroke model, we used a neutralizing antibody against HMGB1. The rabbit polyclonal anti-HMGB1 antibody was injected at a dose of 600 μg 15 min before MCAO. Controls received the same amount of a nonspecific rabbit IgG. Treatment with anti-HMGB1 significantly reduced the infarct volume (Fig. 2*A*). To determine the number of dead cells, TUNEL-positive cells were counted on a coronal section (400 μm caudal to the anterior commissure) by LSC. The number of TUNEL-positive cells in the periphery of the ischemic area was significantly decreased by neutralizing HMGB1 reflecting the protective effect of anti-HMGB1 (Fig. 2*B*). To evaluate the penetration of the rabbit anti-HMGB1 antibody into brain, we stained parallel sections with a HRP-labeled secondary antibody. Twelve and 48 h after administration of anti-HMGB1 and MCAO, we detected anti-HMGB1 antibody in the ischemic territory. In contrast, no signal was found if mice were not treated with antibody (Fig. 2*C*).

The N-terminal part of HMGB1, box A, has been shown to antagonize binding of HMGB1 to its receptor RAGE (Bianchi

bregma. Relative perfusion units were determined (Periflux 4001; Perimed).

The neutralizing anti-HMGB1 antibody (600 μg per mouse in 200 μl) (Yang et al., 2004) and sRAGE (50 μg per mouse in 200 μl) were administered by intraperitoneal injection 15 min before MCAO (anti-HMGB1, sRAGE) plus 90 min after MCAO (sRAGE). HMGB1 box A (600 μg per mouse in 360 μl; HMGBiotech) was administered by intraperitoneal injection 15 min before MCAO. To investigate blood–brain barrier leakage, mice were reanesthetized with tribromoethanol 48 h after MCAO. Sodium fluorescein (1.2 mg in 200 μl PBS; Sigma) was injected into the femoral vein. Thirty minutes later, mice were perfused and the left and right cortices dissected. After tissue extraction (Schoch et al., 2002), we measured fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Animal experiments were approved by local authorities.

Oxygen glucose deprivation (OGD) was used as an *in vitro* model of ischemia. One hour before OGD start, 2-deoxy-D-glucose (5 mM) was added in Neurobasal medium without B27 supplement. After 45 min, cells were placed in an anaerobic chamber flushed with 5% CO_2 in 95% N_2 for 15 min before the chamber was sealed and incubated for 4.5 h at 37°C. Then, the medium was replaced with fresh Neurobasal medium without B27 supplement, and the cells were allowed to recover for 24 h under normal conditions.

Bone marrow transplantation. Bone marrow was obtained aseptically from femurs and tibias of wild-type, ACTB–EGFP (Okabe et al., 1997), or $RAGE^{-/-}$ mice after euthanizing animals by cervical dislocation. Unfractionated bone marrow cells were resuspended in 0.25 ml sterile PBS and injected retro-orbitally into 10- to 13-week-old C57BL/6 mice that had been lethally irradiated (10 Gy) 1 d before. Six weeks after bone marrow reconstitution, we confirmed successful engraftment by RT-PCR of RAGE mRNA in whole blood or FACS analysis of $EGFP^{+}$ cells and subjected mice to MCAO. Eleven percent of mice that were subjected to bone marrow transplantation died during the procedure.

and Manfredi, 2007). HMGB1 box A has been used as a pharmacological tool to inhibit the effects of HMGB1 that are mediated through RAGE (Yang et al., 2004; Sitia et al., 2007). Intraperitoneal treatment of mice 15 min before MCAO with 600 μg HMGB1 box A significantly reduced the infarct volume (Fig. 2*D*).

RAGE contributes to ischemic brain injury

To investigate the role of RAGE in cerebral ischemia, we subjected RAGE-deficient mice to MCAO. Two days after the onset of ischemia, RAGE$^{-/-}$ mice had significantly smaller infarcts than controls (Fig. 3*A*). Treatment of RAGE$^{-/-}$ mice with the neutralizing anti-HMGB1 antibody did not further decrease the infarct volume (Fig. 3*A*) in accordance with the notion that the effect of HMGB1 is mediated by RAGE. In the experiment depicted in Figure 3*A* wild-type and RAGE$^{-/-}$ control mice received nonspecific IgG to control for anti-HMGB1 treatment. We observed a similar difference in infarct volumes between wild-type and RAGE$^{-/-}$ mice if animals were not treated with IgG (data not shown). The difference between RAGE$^{-/-}$ and wild-type mice persisted 2 weeks after MCAO, although the absolute infarct size was smaller at this time (Fig. 3*B*), showing that RAGE deficiency does not just delay ischemic neurodegeneration but actually prevents it.

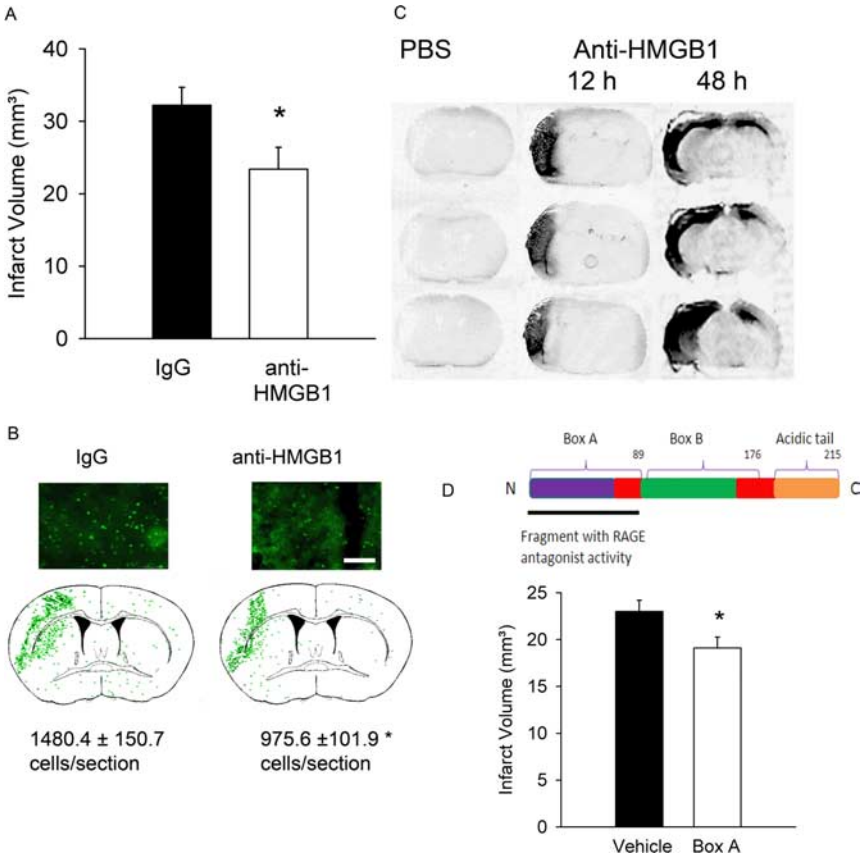

Figure 2. Inhibition of HMGB1 is neuroprotective in cerebral ischemia. ***A***, Intraperitoneal injection of an anti-HMGB1 antibody (600 μg/mouse) reduced the infarct size 48 h after onset of ischemia. IgG, Control antibody. Values are means ± SEM ($n = 8$). *$p < 0.05$ (*t* test). ***B***, Anti-HMGB1 treatment reduced the number of TUNEL-positive cells 48 h after MCAO. TUNEL-labeled cells were detected by LSC on a coronal section 400 μm caudal to the anterior commissure. The distribution of TUNEL-labeled cells in an individual brain section from each group is overlaid on a brain scheme. *$p < 0.05$ (*t* test, $n = 5$ mice). Scale bar, 50 μm. ***C***, Immunostaining of rabbit Ig demonstrating presence of the anti-HMGB1 antibody into the ischemic territory 12 and 48 h after MCAO (right side). For comparison, staining of sections from mice that did not receive antibody is shown (left side). ***D***, The N-terminal part of HMGB1, box A, antagonizes activation of RAGE by HMGB1. Intraperitoneal injection of HMGB1 box A (600 μg/mouse) reduced the infarct size compared with controls that received equal volumes of vehicle. Infarct size was measured 48 h after MCAO. Values are means ± SEM, $n = 18$. *$p < 0.05$ (*t* test).

Because RAGE is expressed by endothelial cells and is reported to play an important role in the blood–brain barrier (Deane et al., 2003), we further evaluated the possibility that RAGE deficiency indirectly affects infarct size by influencing the permeability of the blood–brain barrier. Blood–brain barrier function was investigated by intravenous injection of sodium fluorescein, which is largely retained if the barrier is intact. Two days after MCAO there was considerable evasion of sodium fluorescein into the ischemic brain, showing that the blood–brain barrier had been disrupted in the ischemic hemisphere (Fig. 3*C*). The sodium fluorescein evasion after MCAO was not significantly different between RAGE$^{-/-}$ and control mice, demonstrating that leakage through the blood–brain barrier remained unchanged. In addition, several physiological parameters that may influence the infarct size did not differ between controls and RAGE$^{-/-}$ mice (Table 2).

A soluble form of RAGE functions as a decoy receptor that prevents ligands from interacting with the full-length membrane receptor (Schmidt et al., 1994). sRAGE provides a pharmacological tool to translate results obtained in RAGE$^{-/-}$ mice into a more clinical setting. Administration of sRAGE (i.p., 50 μg 15 min before MCAO plus 50 μg 90 min after MCAO) reduced the infarct size significantly (Fig. 3*D*). To evaluate the effect of sRAGE on the inflammatory response, we performed immunohistochemistry for the microglia/macrophage marker CD11b and quantified CD11b$^+$ cells by LSC. CD11b$^+$ cells were distributed beyond the infarct area over the whole hemisphere and even on the contralateral side (Fig. 3*E*). sRAGE significantly decreased the number of CD11b$^+$ cells in parallel to the reduced infarct size (Fig. 3*E*). In addition, sRAGE was neuroprotective in mixed neural cultures and cortical neurons exposed to OGD (data not shown).

Brain macrophages mediate the HMGB1–RAGE induced injury

RAGE is expressed by glia, neurons, and endothelial cells (Deane et al., 2003; Bierhaus et al., 2004; Bianchi et al., 2007). To elucidate in which cell type RAGE exerts its detrimental effect in cerebral ischemia, we turned to neural cell culture and evaluated cell death by measuring LDH release. Surprisingly, treatment with recombinant HMGB1 for 24 h did not induce cell death in either primary cortical neurons, glial cultures (Fig. 4*A*), pure astrocytes or microglia (data not shown). A coculture of glial cells and neurons (later referred to as mixed neural cultures) was required for HMGB1 to exert a toxic effect on cells (Fig. 4*A*). Prolonged treatment of mixed neural cultures for 48 h further elevated LDH release (Fig. 4*B*). HMGB1 treatment decreased the number of NeuN-positive neurons (Fig. 4*C*) indicating that neurons are dying if cultures are treated with HMGB1. In these mixed neural

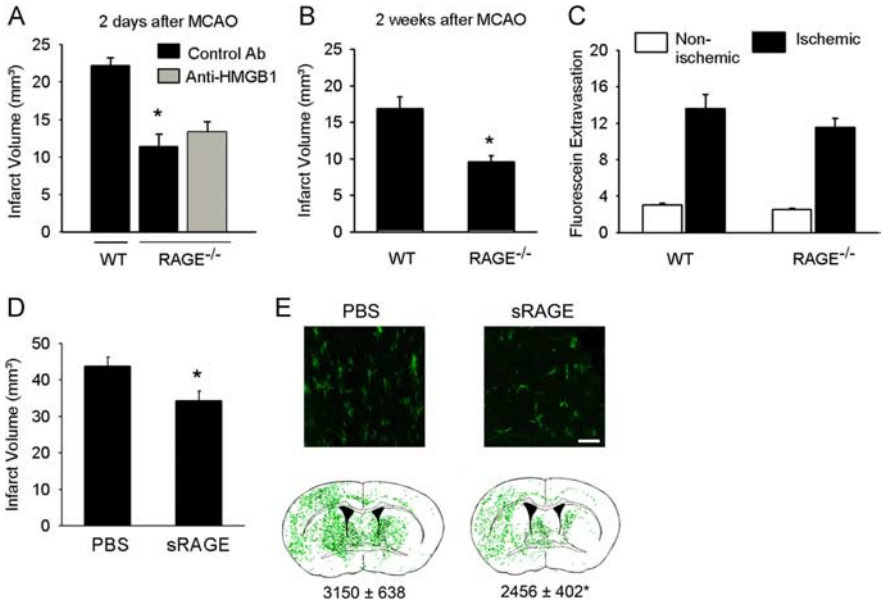

Figure 3. The HMGB1 receptor RAGE contributes to ischemic brain damage. ***A***, RAGE $^{-/-}$ mice had reduced infarcts compared with wild-type mice (WT) 2 d after MCAO. Treatment with anti-HMGB1 (600 μg/mouse, i.p.) did not show additional protection. IgG, Control antibody. Univariate ANOVA, $F_{(2,20)} = 17.917$, $p < 0.001$. *$p < 0.001$ compared with WT (Tukey–honestly significant differences *post hoc* test). Values are means ± SEM. ***B***, RAGE $^{-/-}$ mice had smaller infarcts than WT 2 weeks after MCAO. *$p < 0.05$ (*t* test, $n = 8$). ***C***, Fluorescein extravasation reflecting leakage through the blood–brain barrier was higher in the ischemic than in the nonischemic hemisphere 48 h after MCAO. However, there was no difference in fluorescein extravasation into the ischemic hemisphere between WT and RAGE $^{-/-}$ mice. Values are means ± SEM ($n = 5$). ***D***, sRAGE (twice 50 μg) compared with solvent (PBS) reduced the infarct volume 48 h after MCAO. *$p < 0.05$ (*t* test, $n = 8$). ***E***, sRAGE treatment also decreased the number of CD11b-expressing microglial cells. Typical immunostainings in the two groups are shown on top. CD11b-positive cells were quantified by LSC. The distribution of positive cells in individual brains from the two groups is overlaid on a brain scheme. *$p < 0.05$ (*t* test, $n = 8$). Scale bar, 50 μm.

Table 2. Physiological parameters of control and RAGE$^{-/-}$ mice 10 min before and 10 min after MCAO

Parameter	RAGE$^{+/+}$		RAGE$^{-/-}$	
	Before MCAO ($n = 6$)	After MCAO ($n = 6$)	Before MCAO ($n = 6$)	After MCAO ($n = 6$)
MABP (mm Hg)	62.0 ± 2.4	56.8 ± 5.6	60.0 ± 7.3	56.2 ± 5.3
Heart rate (per min)	274.7 ± 34.1	275.6 ± 19.6	276.0 ± 30.6	266.0 ± 25.6
Glucose (mg/dl)	229.3 ± 128.6	266.6 ± 168.4	220.7 ± 42.8	227.7 ± 69.2
Arterial pH	7.2 ± 0.09	7.13 ± 0.06	7.3 ± 0.06	7.21 ± 0.06
Arterial pO_2 (mm Hg)	107.7 ± 11.7	93.9 ± 5.7	99.7 ± 12.6	86.6 ± 16.9
Arterial pCO_2 (mm Hg)	41.1 ± 11.2	47.2 ± 4.2	42.7 ± 5.1	43.6 ± 6.5
Base excess	−10.7 ± 2.5	−12.2 ± 4.5	−7.3 ± 4.3	−10.3 ± 2.6
Hb (g/l)	15.1 ± 1.8	13.2 ± 1.6	16.6 ± 0.9	15.0 ± 1.8
Laser doppler (% before MCAO)		15.7 ± 4.7		12.3 ± 4.2
Body weight	31.5 ± 3.0		28.0 ± 2.6	

There was no significant difference between the genotypes (*t* test). MABP, Mean arterial blood pressure.

cultures, Iba1-positive microglial cells expressed RAGE (Fig. 4*F*). The morphology of the double-stained cells and an increased expression of RAGE on activation of the cells by OGD or LPS treatment suggest that RAGE is expressed by activated microglia. However, neurons and astrocytes were also stained by the anti-RAGE antibody (data not shown). To distinguish the function of glial and neuronal RAGE in HMGB1-induced cell death, we cocultured glia and neurons from different genotypes. The toxic effect of HMGB1 was lost if glial cells were prepared from RAGE $^{-/-}$ and not from wild-type mice (Fig. 4*D*). Thus, glial RAGE is required for the detrimental effect of HMGB1 *in vitro*.

In stroke, reactive microglia are recruited to a considerable extent from bone marrow-derived cells of myeloid origin (Priller et al., 2001; Lalancette-Hebert et al., 2007). To evaluate the potential role of immigrant macrophages we added peritoneal macrophages to our mixed neural cultures. The presence of macrophages potentiated the HMGB1-induced cell death (Fig. 4*E*).

To investigate the role of immigrant macrophages *in vivo*, we generated chimeric mice by bone marrow transplantation. We characterized bone marrow-derived cells in brain by transplanting bone marrow from ACTB-EGFP mice (Okabe et al., 1997) to wild-type mice. FACS analysis showed that in blood of chimeric mice 87 ± 2% ($n = 5$) of CD45$^+$ leukocytes were GFP$^+$ versus 93 ± 2% ($n = 5$) in ACTB–EGFP, confirming a high bone marrow transplantation efficiency. In the nonischemic hemisphere, a small fraction of cells was EGFP$^+$ indicating that they were derived from bone marrow (CD45$^+$EGFP$^+$, 2.7 ± 0.4%). Further analysis showed that 1.1 ± 0.1% of cells were EGFP$^+$ and expressed the microglial markers CD45^{lo}/CD11$^+$ (Sedgwick et al., 1991). The low turnover rate of microglia from bone marrow in the nonischemic hemisphere of our chimeric mouse model is in accordance with previous reports and may be attributable to brain irradiation (McMahon et al., 2002; Hess et al., 2004; Mildner et al., 2007). Interestingly, in the ischemic hemisphere, there was a marked increase in bone marrow-derived cells (CD45$^+$EGFP$^+$, 18.2 ± 3.2% of all cells). Two days after MCAO, most of the bone-marrow derived EGFP$^+$ cells in the ischemic hemisphere were CD45^{hi}/CD11b$^+$ (15.5 ± 2.8% of all cells), suggesting that they are immigrant macrophages (Fig. 5*A*). Immunohistochemistry confirmed that most EGFP$^+$ cells expressed the macrophage marker Iba1 (Fig. 5*B*).

We used the chimeric mouse model to investigate whether immigrant macrophages mediate the effect of RAGE in cerebral ischemia. Wild-type mice that received RAGE$^{-/-}$ bone marrow (RAGE$^{-/-}$>WT) had a 93.8 ± 2.3% reduction of RAGE mRNA levels in whole blood compared with the WT>WT group ($n = 8$–10, $p < 0.0001$) (Fig. 5*C*), demonstrating the efficiency of bone-marrow transplantation. Double staining of RAGE and the macrophage marker Iba1 confirmed that RAGE expression in brain macrophages was reduced in the RAGE$^{-/-}$>WT group (Fig. 5*D*). Interestingly, wild-type mice who received RAGE$^{-/-}$ bone marrow transplants had significantly smaller infarcts than animals that received wild-type bone marrow (Fig. 5*E*,*F*). Together with the *in vitro* data, this strongly indicates that RAGE expressed by immigrant macrophages is required for the development of ischemic brain damage, probably by binding HMGB1 released from necrotic cells in the core of the ischemia.

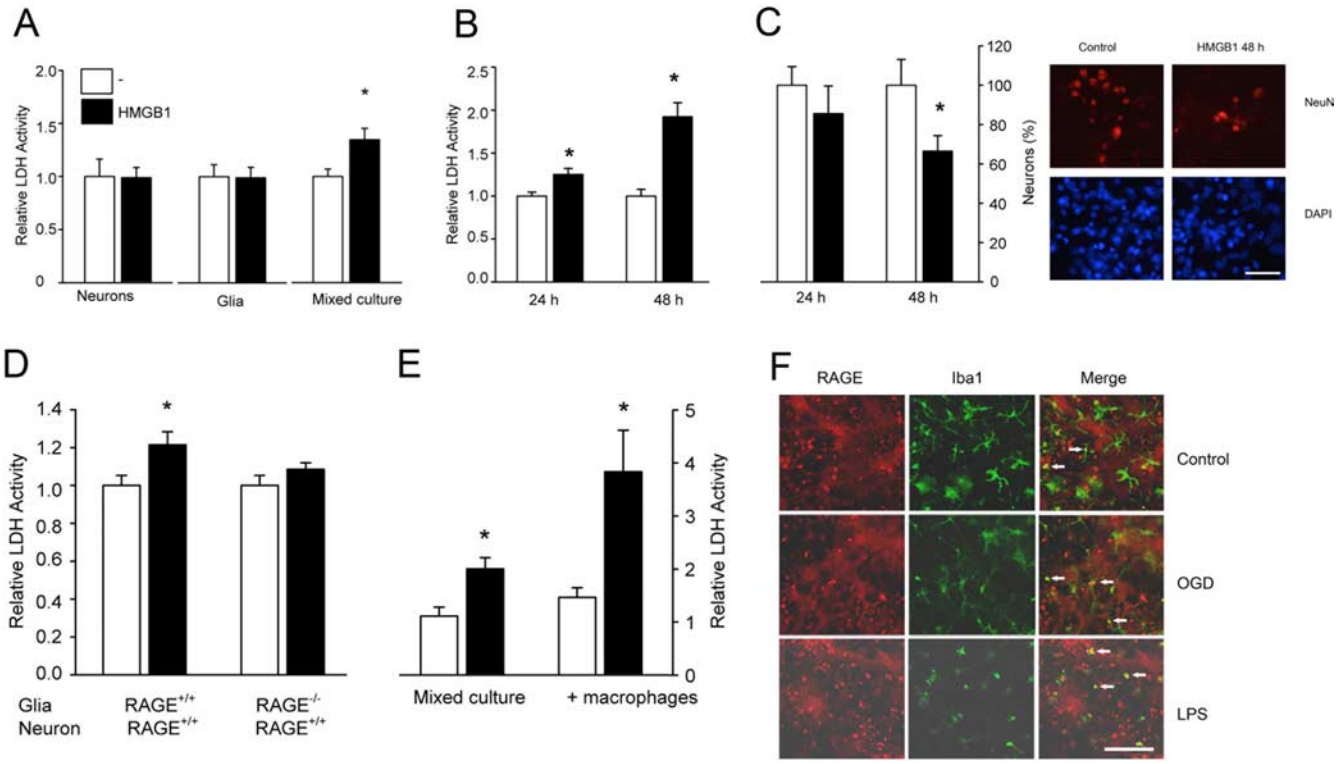

Figure 4. Glial RAGE mediates the effect of HMGB1 *in vitro*. ***A***, HMGB1 (500 ng/ml) stimulated LDH release in mixed neural cultures but not in pure cortical neurons or glial cells. Cells were exposed to HMGB1 for 24 h. $*p < 0.05$ (*t* test, 3 experiments with $n = 6$ each). ***B***, Time-dependent effect of HMGB1 (500 ng/ml) on LDH release from mixed neural cultures. $*p < 0.05$ (*t* test, 3 experiments with $n = 6–9$ each). ***C***, Treatment of mixed neural cultures with HMGB1 (500 ng/ml) reduced the number of NeuN positive cells indicating death of neurons. $*p < 0.05$ (*t* test, 3 experiments with $n = 6–9$ each). Representative NeuN immunostainings after 48 h of HMGB1 are shown. Scale bar, 50 μm. ***D***, Stimulation of LDH release by HMGB1 was abolished in the absence of glial RAGE. Mixed neural cell cultures were prepared by plating RAGE $^{+/+}$ neurons on RAGE $^{+/+}$ or RAGE $^{-/-}$ glial cells. $*p < 0.05$ (*t* test, $n = 6$). ***E***, Addition of peritoneal macrophages to mixed neural cultures increased the HMGB1-induced LDH release after 48 h of treatment. $*p < 0.05$ (*t* test, compared with HMGB1-stimulated cultures without macrophages, $n = 6–9$). ***F***, In glial cell cultures, immunohistochemistry showed that Iba1-positive microglia were partly stained by anti-RAGE antibody. After OGD or LPS treatment the number of Iba1- and RAGE-positive cells increased. Scale bar, 50 μm.

Discussion

This study shows that RAGE mediates ischemic brain injury. Previous work has indicated that RAGE may be involved in the pathophysiology of liver and cardiac ischemia (Zeng et al., 2004; Bucciarelli et al., 2006; Andrassy et al., 2008). Combining genetic RAGE deficiency and an *in vivo* model of cerebral ischemia, our data demonstrate that RAGE plays a key role in the pathophysiology of acute ischemia.

RAGE is a multiligand membrane receptor. Several of the established ligands may activate RAGE in cerebral ischemia. Hyperglycemia, a common finding in stroke patients (Scott et al., 1999), enhances the production of AGEs that have been shown to contribute to neurotoxicity during ischemic stroke (Zimmerman et al., 1995). In addition, there is evidence that Aβ is involved in the pathogenesis of ischemic brain injury (Koistinaho and Koistinaho, 2005) and that S100 proteins are released during ischemia and modulate its outcome (Kogel et al., 2004). However, the most obvious RAGE activator in stroke seems to be HMGB1 that is known to be released from necrotic cells (Degryse et al., 2001; Scaffidi et al., 2002). Indeed, cortical neurons released HMGB1 if cell death was induced by OGD, an *in vitro* model of cerebral ischemia. *In vivo*, release from necrotic cells is reflected by a reduction of nuclear HMGB1 staining in the ischemic area (Fig. 1*E*). Qiu et al. (2008) demonstrated that nuclear HMGB1 staining predominantly disappeared in neurons during cerebral ischemia. However, prolonged OGD and other noxious stimuli are able to trigger release of HMGB1 also from astrocytes (Faraco et al., 2007; Qiu et al., 2008). This may explain why HMGB1 staining was completely lost in the core of the ischemic lesion (Fig. 1*E*). Thus, release from necrotic neural cells is the likely source of elevated serum concentrations of HMGB1, which we found in stroke patients as well as mice subjected to focal cerebral ischemia. A recent report of elevated serum concentrations of HMGB1 in stroke patients confirms our finding (Goldstein et al., 2006). Moreover, interference with HMGB1 by a neutralizing antibody (Fig. 2*A*) (Liu et al., 2007), HMGB1 box A (Fig. 2*D*), or a short hairpin RNA (Kim et al., 2006) ameliorated brain damage.

RAGE is localized on all types of brain cells. Activation of RAGE on neurons has been shown to stimulate the production of reactive oxygen species and to lead to the death of neuron-like cell lines (Yan et al., 1996; Vincent et al., 2006). However, it has been reported that activation of RAGE induces neurite outgrowth and that induction of neuronal RAGE by hypoxia-inducible factor-1α protects against hypoxia (Pichiule et al., 2007). The relevance of the latter finding for stroke is questionable because we found no induction of RAGE mRNA (data not shown) and, in contrast to that report, interference with RAGE signaling reproducibly improved brain damage in our stroke model. Furthermore, the effect of RAGE we observed was unequivocally mediated by bone marrow-derived (non-neuronal) cells. RAGE is involved in the recruitment of neutrophils by HMGB1 (Orlova et al., 2007), and neutrophil recruitment contributes to ischemic brain injury, at least in transient cerebral ischemia (Zhang et al., 1995), suggesting that the cells mediating the RAGE effect could

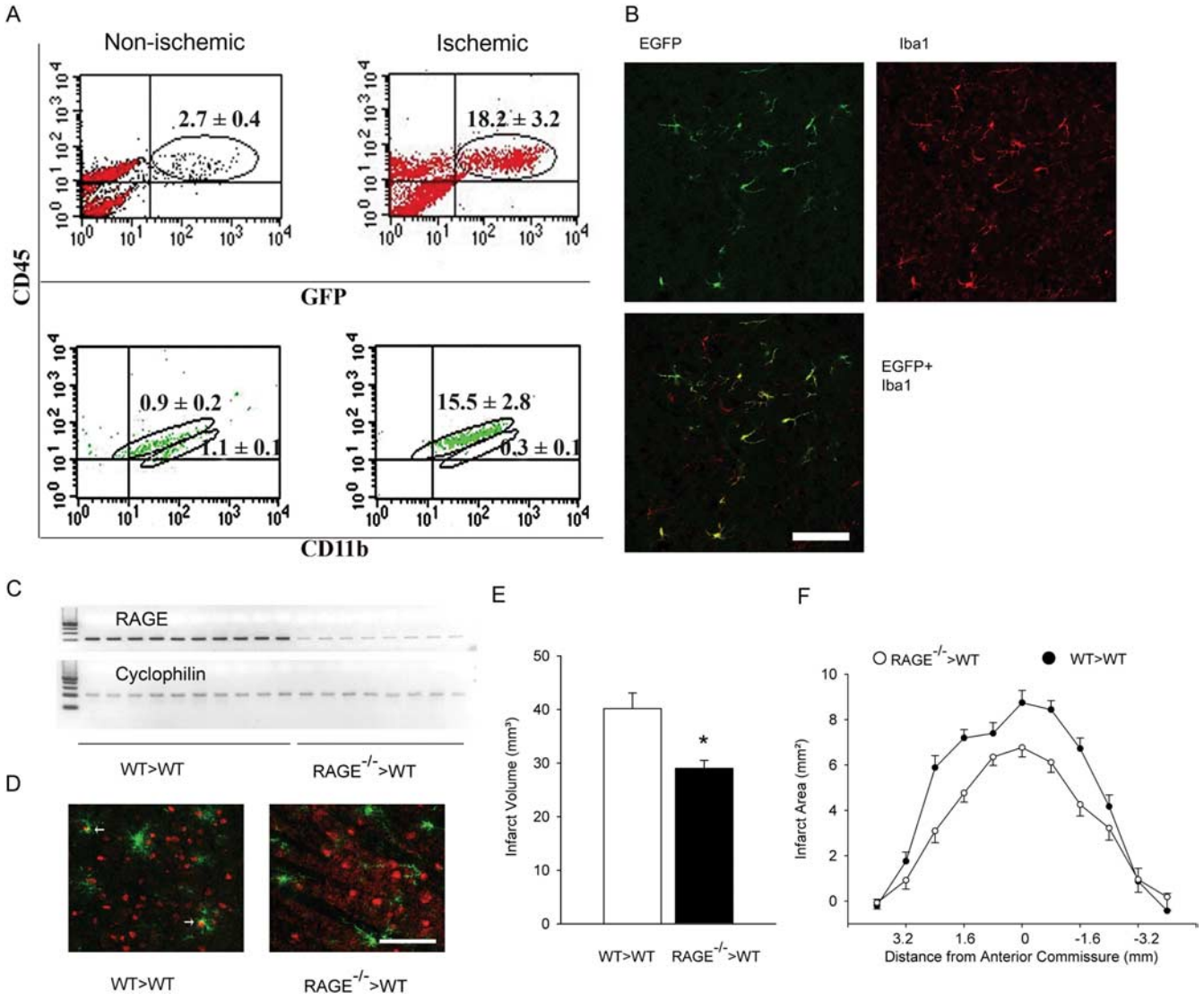

Figure 5. Immigrant macrophages mediate the detrimental effect of RAGE on the infarct size. ***A***, Bone marrow of ACTB–EGFP mice was transplanted to wild-type mice. Six weeks after transplantation, MCAO was performed. Two days later, FACS analysis showed that GFP$^+$/CD45$^+$ cells increased in the ischemic hemisphere compared with the nonischemic contralateral hemisphere (top row). GFP$^+$ cells (bottom row) were mainly CD45^{hi}/CD11$^+$ in the ischemic hemisphere. ***B,*** Immunohistochemistry of Iba1 showed colocalization of EGFP and Iba1. Scale bar, 50 μm. ***C***, Detection of RAGE and cyclophilin mRNA in blood by real-time RT-PCR confirmed the efficiency of bone-marrow transplantation. After PCR, products were resolved on an agarose gel next to a DNA marker (top, 100 bp marker; bottom, 25 bp marker). For quantification, see Materials and Methods. WT>WT, Transplantation of wild-type bone marrow to wild-type mice. RAGE$^{-/-}$>WT, Transplantation of RAGE$^{-/-}$ bone marrow to wild-type mice. ***D***, Immunohistochemical double-staining of RAGE (Cy3, red) and the microglial marker Iba1 [FITC (fluorescein isothiocyanate), green] in WT>WT and RAGE$^{-/-}$>WT brains. Arrows, Double-labeled cells. Scale bar, 50 μm. ***E***, Infarct volume in WT>WT was larger than in RAGE$^{-/-}$>WT mice 48 h after MCAO. *$p < 0.05$ ($n =$ 8–10, *t* test). ***F***, Distribution of infarcts on coronal sections.

be neutrophils. However, our *in vitro* results argue for a role of brain macrophages. In cerebral ischemia, brain macrophages consist partly of activated microglia and partly of immigrant macrophages. Up to now, transplantation of labeled bone marrow is the only definite way to distinguish between the two populations. Two recent studies have indicated that the differentiation of bone marrow-derived cells into microglia (accounting for <5% of microglia) may be an artifact attributable to brain irradiation and the mobilization of bone marrow cells (Ajami et al., 2007; Mildner et al., 2007). These studies investigated brain pathologies, which were not associated with overt blood–brain barrier disruption. In contrast, the disrupted blood brain barrier in stroke allows a massive invasion of mononuclear cells independent of previous irradiation (Dirnagl et al., 1999). Thus, bone marrow transplantation appears as an efficient way to target blood-derived macrophages selectively and to spare most resident microglia. Our data in chimeric mice indicate that RAGE expressed by immigrant macrophages mediates ischemic brain injury. In addition, resident microglia may contribute to this effect. In accordance with this notion, sRAGE reduced the number of CD11b$^+$ brain macrophages *in vivo*, mice with RAGE$^{-/-}$ bone-marrow had smaller infarcts, and macrophages enhanced the toxic effect of HMGB1 on the viability of mixed neural cultures. Thus, we propose that RAGE activated by HMGB1 functions as a sensor of necrotic cell death at the core of the ischemia and mediates the activation of brain macrophages, mainly immigrant macrophages. The scavenger receptor CD36 seems to have a similar function in cerebral ischemia (Cho et al., 2005; Kunz et al., 2008).

Macrophages are a well known contributor to brain injury in

several diseases (Block et al., 2007). Nitric oxide (NO) production is stimulated by HMGB1 treatment of primary microglia (Kim et al., 2006). Another potential effector of HMGB1-stimulated brain macrophages may be prostaglandin E2 (PgE2). PgE2 and NO are well known toxic mediators in stroke (Iadecola et al., 1997; Kawano et al., 2006).

The effect of HMGB1 box A, a functional antagonist of extracellular HMGB1 activity and HMGB1 interaction with RAGE, provides evidence that the HMGB1 effect is mediated by RAGE. This conclusion is supported by the observation that genetic RAGE deficiency mimics the effect of HMGB1 antagonists (Fig. 3) and *in vitro* data showing that the HMGB1 effect depended on the expression of RAGE (Fig. 4*D*). However, we cannot exclude HMGB1 signaling through other membrane receptors beside RAGE in cerebral ischemia. TLR2 and TLR4 are other membrane receptors that bind HMGB1 in addition to RAGE (Park et al., 2004, 2006), although a recent study has questioned this finding (Tian et al., 2007).

The current findings, however, clearly indicate that sRAGE, a neutralizing anti-HMGB1 antibody, or the HMGB1 antagonist box A may be specific tools to interfere with both inflammation and delayed cell death in stroke. These agents have high molecular weights that prevent their access to the intact brain. However, in stroke they can cross the leaky blood–brain barrier (Fig. 3*C*), suggesting that translation of the present findings in the clinic may be feasible. In this study, we used a rabbit anti-HMGB1 antibody in mice. Experience with enlimomab have taught us that cross species antibody administration may be detrimental (Furuya et al., 2001). Thus, a fully humanized anti-HMGB1 antibody would be required to move to the clinic.

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