The Amphoterin (HMGB1)/Receptor for Advanced Glycation End Products (RAGE) Pair Modulates Myoblast Proliferation, Apoptosis, Adhesiveness, Migration, and Invasiveness

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We reported that RAGE (receptor for advanced glycation end products), a multiligand receptor of the immunoglobulin superfamily expressed in myoblasts, when activated by its ligand amphoterin (HMGB1), stimulates rat L6 myoblast differentiation via a Cdc42-Rac-MKK6-p38 mitogen-activated protein kinase pathway, and that RAGE expression in skeletal muscle tissue is developmentally regulated. We show here that inhibition of RAGE function via overexpression of a signaling deficient RAGE mutant (RAGEΔcyto) results in increased myoblast proliferation, migration, and invasiveness, and decreased apoptosis and adhesiveness, whereas myoblasts overexpressing RAGE behave the opposite, compared with mock-transfected myoblasts. These effects are accompanied by a decreased induction of the proliferation inhibitor, p21

We documented that amphoterin (HMGB1), a multifunctional cytokine, is expressed in myoblasts, when activated by its ligand amphoterin, induces cell proliferation and/or modulate myoblast differentiation, tissue formation. These factors will assure an appropriate extent of muscle differentiation and inactivation of proliferation, and deregulation of RAGE expression in myoblasts might contribute to their neoplastic transformation.

Myogenesis is a multistep process in which the precursors of myofibers, the myoblasts, first proliferate and then differentiate into fusion-competent cells that finally fuse with each other to form myotubes (1–3). A similar process occurs in mature skeletal muscles in case of damage; quiescent, mononucleated cells, the satellite cells, that coexist with myofibers, can be activated by a number of extracellular factors to proliferate and then to differentiate as above to repair the damaged myofibers (3). In both cases, proliferation and differentiation are separ-
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Cell Culture Conditions, [3H]Thymidine Incorporation, Transfections, and Apoptosis and Luciferase Assays—Rat L6 myoblasts (clone L6C31) were cultured for 24 h in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin, in a H2O-saturated 5% CO2 atmosphere at 37 °C before decreasing FBS to 2% to induce myoblast differentiation. L6 myoblast clones stably overexpressing RAGE or RAGEΔcyto were selected and characterized as described (15). Experiments were performed using mock-transfected, L6/RAGE and L6/RAGEΔcyto myoblasts in 10 or 2% FBS as indicated in the legends to figures. The anti-amphoterin antibody (BD Pharmingen) used in some experiments was shown to neutralize culture medium amphoterin (16).

For [3H]thymidine incorporation assay, myoblasts (25 × 103 cells/well) were cultured in 10% FBS for 24 h in 24-multiwell plates, washed with DMEM, serum-starved for 24 h, washed with DMEM, and cultivated in DMEM in the presence of either 10 or 2% FBS for another 24 h in the presence of 1 µCi of [3H]thymidine/ml. Parallel myoblasts treated in the same manner in the absence of [3H]thymidine were incubated with HOECHST 33258 (bisbenzimidazole) as described (25) to normalize incorporated [3H]thymidine to DNA content.

For cell number measurements, myoblasts were cultured in 10% FBS for 24 h in 96-multiwell plates at a density of 4 × 103 cells/well and then in DMEM in the presence of either 10 or 2% FBS for 1–7 days. Cell density was measured by a tetrazolium-based (MTT) colorimetric assay.

To analyze the cell cycle and measure apoptosis, myoblasts were seeded onto 35-mm plastic dishes (18 × 105 cells/dish) in 10% FBS for 24 h, washed with DMEM, and cultivated for 24 or 48 h in DMEM in the presence of either 10 or 2% FBS. Cells were stained with propidium iodide in hypotonic buffer and subjected to fluorescence-activated cell sorting (FACS) analysis as described (26). This procedure allows the determination of the percentage of apoptotic (hypodiploid) nuclei as well as that of normal (diploid) nuclei in the same cell population irrespective of the cell volume. In experiments performed in the presence of the p38 MAPK inhibitor, SB203580 (Calbiochem) (2 µM, final concentration), control cells received an equal volume of vehicle (dimethyl sulfoxide). FACS analysis was also employed to measure the mean cell volume as described (27).

Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Briefly, myoblasts cultured in 10% FBS without antibiotics were transfected with the reporter gene p21190-97-luc, reporter gene cyclin D1-luc, or empty vector. After 6 h, cells were cultivated in 10 or 2% FBS, as indicated in the figure legends. After another 24 h cells were harvested to measure luciferase activity. p21190-97 and cyclin D1 promoter transcriptional activities were normalized for transfection efficiency by cotransfecting cells with a cDNA encoding green fluorescent protein. The percentage of green fluorescent protein-positive cells (20 to 25%) was determined by FACS analysis.

Western Blot Analyses—To detect phosphorylated and total extracellular signal-regulated kinase (ERK) 1/2, phosphorylated and total c-Jun NH2-terminal protein kinase (JNK), phosphorylated and total retinoic acid receptor protein Rb, tubulin, caspase-3, Bcl-2, integrin β1, VCAM, NCAM, and caveolin-3 in myoblast extracts by Western blotting, myoblasts were cultivated as detailed in the legend of pertinent figures, washed twice with PBS, and solubilized with 2.5% SDS, 10 mM Tris-HCl, pH 7.4, 0.1 mM dithiothreitol, 0.1 mM tosylsulfonyl chloromethyl ketone protease inhibitor (Roche). The following antibodies were used: polyclonal antibody specific to phosphorylated (Thr202/Tyr204) ERK1/2 (1:1,000, New England BioLabs), polyclonal anti-ERK1/2 antibody (1:5,000, Sigma), polyclonal anti-phosphorylated (Ser807/Ser811) Rb antibody (1:1,000, Cell Signaling Technology). The following antibodies were used: monoclonal anti-Rb antibody (1:1,000, Cell Signaling Technology), monoclonal anti-c-Jun antibody (1:10,000, Sigma), monoclonal anti-caspase-3 antibody (1:100; Cell Signaling Technology), monoclonal anti-Vinculin antibody (1:100; BD Pharmingen), monoclonal anti-integrin β1 antibody (1:2,000; BD Transduction Laboratories), monoclonal anti-Cyclin D1 antibody (1:100; BD Pharmingen), monoclonal anti-VCAM antibody (1:2,000; BD Pharmingen), monoclonal anti-NCAM antibody (1:2,000; Sigma), and monoclonal anti-caveolin-3 antibody (1:2,000; BD Transduction Laboratories). The immune reaction was developed by enhanced chemiluminescence (ECL) (SuperSignal West Pico, Pierce).

Adhesion, Migration, and Invasiveness Assays, and F-actin Staining—For adhesion experiments, mock-transfected, L6/RAGE, and L6/RAGEΔcyto myoblasts (50 × 103 cells in 0.1 ml of DMEM containing 10% FBS) were seeded into each well, incubated for 3 h, and further processed as described (28, 29). The supernatant with non-adherent cells was removed by two washes with warme culture medium. Attached cells were fixed with 30% methanol/ethanol for 15 min at room temperature, stained with 0.1% crystal violet (Sigma) in PBS, extensively washed with distilled water, and dried at room temperature. The dye was resuspended with 50 µl of 0.2% Triton X-100/well, and color yield was measured using an enzyme-linked immunosorbent assay reader at 590 nm.
Parallel cells not serum-starved were given 2.5 μg/ml non-immune IgG (that gave similar results to the DM series without additions) is not shown. [3H]Thymidine incorporation, B and C, same as in A except that after culture medium renewal (B) or switch to DM (C), myoblasts were cultivated for the times indicated and processed by MTT assay. D and E, same as in A except that after culture medium renewal (D) or switch to DM (E), myoblasts were cultivated for another 24 or 48 h as indicated and subjected to FACS analysis to measure the fractions of cells in the Go/G1, S, and G2/M phases of the cell cycle. F, same as in E except that at the time of the culture medium switch to DM, myoblasts received either 2.5 μg/ml non-immune IgG or 2.5 μg/ml of an anti-amphoterin antibody for 48 h. Averages of three independent experiments ± S.D. Asterisk, significantly different from control ([3H]thymidine incorporation in L6/mock myoblasts in GM) (p < 0.05) (A); asterisk, significantly different from internal control (individual phases of the cell cycle in L6/mock myoblasts) (p < 0.05) (D–F).

For migration assay, we used Boyden chambers (pore size, 8 μm) (BD Biosciences). Individual myoblast clones (5 × 10^5 cells in 0.5 ml of DMEM) were placed in the upper chamber, and 0.75 ml of DMEM containing 10% FBS was placed in the lower chamber. After 20 h in culture, cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were fixed in methanol for 2 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the underside of the filters were viewed and counted under a microscope. Each clone was plated in triplicate in each experiment. For the invasiveness assay, conditions were as described for migration assay except that bicoid Matrigel invasion chambers (pore size, 8 μm) (BD Biosciences) were used.

For F-actin staining, myoblasts were seeded onto 13-mm glass coverslips in plastic multiwell dishes (7.5 × 10^5 cells/dish) in DMEM containing 10% FBS for 24 h and washed in PBS. Cells were then fixed for 10 min in 3.7% paraformaldehyde in PBS, extensively washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 2 min, washed again, and incubated with fluorescein-labeled phalloidin (Sigma) (1:250 in PBS) for 1 h in a humid chamber at room temperature. After three washes in PBS, the cells were mounted in 80% glycerol, containing 0.02% NaN₃ and p-phenylenediamine (1 mg/ml) in PBS to prevent fluorescence fading and viewed on a Leica DM Rb fluorescence microscope equipped with a digital camera.

Gelatin Zymography—Mock-transfected, L6/RAGE, and L6/RAGEΔcyto myoblasts were cultivated in a 150-cm² flask in DMEM containing 10% FBS, after which the supernatant was collected, centrifuged to remove detached cells, and concentrated. The protein concentration of the supernatant was determined by a BCA Protein Assay Kit (Pierce), 10% of the supernatant was loaded onto a 10% polyacrylamide gel containing 2 mg/ml of gelatin. After electrophoresis, the gel was fixed in 10% trichloroacetic acid. Proteinase activity was quantified by densitometric scanning.

In Vivo Tumor Growth—For tumor growth in vivo, female (NOD/SCID) mice weighing ~20 g were inoculated subcutaneously with 5 × 10^6 L6/wt, L6/RAGEΔcyto, or L6/RAGE myoblasts and monitored for ~3.5 months. The mice were sacrificed by cervical dislocation. Consent was obtained by the Ethics Committee of the University of Perugia.
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Tumor masses were excised and weighed, and tumor volume was calculated by the equation: tumor volume = \( \frac{x^2y}{2} \), where \( x \) and \( y \) correspond to the width and thickness of the tissue, respectively. Tumors were then fixed with 4% paraformaldehyde in PBS (2 days at 4°C), extensively washed in PBS, and paraffin-embedded. Sections were stained with hematoxylin/eosin, and histopathology was performed by an independent pathologist.

Statistical Analysis—The data were subjected to analysis of variance with SNK post-hoc analysis using a statistical software package (GraphPad Prism version 4.00, GraphPad Software, San Diego, CA). Statistical significance was assumed when \( p < 0.05 \).

RESULTS

RAGE Activation in Myoblasts Reduces Proliferation and Stimulates Apoptosis: Role of Amphoterin and p38 MAPK—L6/RAGEΔcytomyoblasts incorporated more \([3H]\)thymidine and L6/RAGEΔcytomyoblasts incorporated less \([3H]\)thymidine than did L6/mock myoblasts in both GM and DM (Fig. 1A). By MTT assay, a larger number of L6/RAGEΔcytomyoblasts and a smaller number of L6/RAGEΔcytomyoblasts were obtained at any day of cultivation in both GM (Fig. 1B) and DM (Fig. 1C) between days 1 and 7, compared with L6/mock myoblasts. Moreover, after 1 and 2 days of cultivation in both GM (Fig. 1D) and DM (Fig. 1E) by FACS analysis a larger fraction of L6/RAGEΔcytomyoblasts and a smaller fraction of L6/RAGEΔcytomyoblasts were in the S and G2/M phases of the cell cycle (with a low percentage of L6/RAGE myoblasts in G2/M phase at 48 h, pointing to the inability of these cells to complete cell division), and a smaller fraction of L6/RAGEΔcytomyoblasts and a larger fraction of L6/mock myoblasts were in the G0/G1 phase, compared with L6/mock myoblasts. Collectively, these data suggested that RAGE might transduce an antiproliferative signal in myoblasts.

Neutralization of culture medium amphoterin with an anti-amphoterin antibody (16) resulted in an increased \([3H]\)thymidine incorporation by L6/RAGE and L6/mock myoblasts compared with their respective controls, and no effects in the case of L6/RAGEΔcytomyoblasts (Fig. 1A). Notably, on administration of anti-amphoterin antibody the levels of \([3H]\)thymidine incorporation were similar in the three L6 clones under study. Neutralization of the culture medium amphoterin with an anti-amphoterin antibody also reduced the fractions of L6/mock and L6/RAGEΔcytomyoblasts in the G0/G1 phase and increased those in S and G2/M phases, whereas without an effect on L6/RAGEΔcytomyoblasts (Fig. 1F). Thus, amphoterin appeared to exert a regulatory role on myoblast proliferation, promoting proliferation arrest via RAGE engagement and stimulation of RAGE transducing activity. This effect of amphoterin would add to the reported promyogenic activity of the protein via activation of a RAGE-Cdc42-Rac-MKK6-p38 MAPK pathway (16).

In addition to reduced proliferation, L6/RAGEΔcytomyoblasts exhibited a larger extent of apoptosis than did L6/mock myoblasts which in turn showed a larger extent of apoptosis than did L6/RAGEΔcytomyoblasts in both GM and DM, with larger percentages in DM than in GM as expected, after 1 day of cultivation (Fig. 2A). Similar results were obtained after 2 days of cultivation (Fig. 2A). Also, neutralization of
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culture medium amphoterin reduced apoptosis in both L6/mock and L6/RAGE myoblasts, whereas without effect in the case of L6/RAGEΔcyto myoblasts (Fig. 2B), suggesting that amphoterin was the RAGE ligand involved in RAGE-dependent stimulation of myoblast apoptosis.

RAGE-dependent activation of p38 MAPK (16) was responsible for RAGE-mediated effects on myoblast proliferation and apoptosis. In fact, treatment with the p38 MAPK inhibitor, SB203580, reduced the fraction of L6/RAGE and L6/mock myoblasts in the S and G2/M phases of the cell cycle and decreased the fraction of these cells in the G0/G1 phase, without affecting L6/RAGEΔcyto myoblasts (Fig. 2C), and decreased apoptosis in L6/RAGE, L6/RAGEΔcyto, and L6/mock myoblasts (Fig. 2D), probably because of lack of inhibition of Raf activity under these conditions (30).

RAGE-dependent regulation of myoblast apoptosis was further investigated by analyzing the levels of activated caspase-3 and the anti-apoptotic factor, Bcl-2. We found that L6/RAGEΔcyto myoblasts in GM and DM exhibited a smaller extent and L6/RAGE myoblasts exhibited a larger extent of caspase-3 activation, compared with L6/mock myoblasts (Fig. 2E). Also, L6/RAGEΔcyto myoblasts exhibited higher levels and L6/RAGE myoblasts exhibited lower levels of Bcl-2, compared with L6/mock myoblasts (Fig. 2E), suggesting that RAGE signaling might negatively regulate Bcl-2 expression in myoblasts. Collectively, these data suggested that, besides promoting myoblast differentiation (16), RAGE activation by amphoterin might cause proliferation arrest and promote apoptosis in myoblasts via stimulation of p38 MAPK.

**RAGE Activation in Myoblasts Increases p21Waf1 Induction and Reduces Cyclin D1 Induction and Rb Phosphorylation**—Because increased levels of the proliferation inhibitor p21Waf1 and decreased levels of cyclin D1 and extents of Rb phosphorylation accompany myoblast proliferation arrest and differentiation (24, 31–33), we next analyzed the role of the amphoterin/RAGE pair in p21Waf1 induction and cyclin D1 induction and extent of Rb phosphorylation. In both GM and DM, L6/RAGEΔcyto myoblasts exhibited a smaller induction of p21Waf1, a larger induction of cyclin D1, and higher levels of phosphorylated Rb, whereas the opposite was observed in L6/RAGE myoblasts, compared with L6/mock myoblasts (Fig. 3, A–C). Neutralization of culture medium amphoterin reduced the levels of p21Waf1 induction in L6/RAGE and L6/mock myoblasts to nearly those detected in L6/RAGEΔcyto myoblasts (Fig. 3A). Similarly, neutralization of culture medium amphoterin resulted in an increase in cyclin D1 induction (Fig. 3B) and the extent of Rb phosphorylation (Fig. 3D) in L6/RAGE and L6/mock myoblasts to the levels observed in L6/RAGEΔcyto myoblasts. These data suggested that induction of p21Waf1 and cyclin D1 and the extent of Rb phosphorylation in myoblasts is under the control of the amphoterin/RAGE pair signaling. Thus, we concluded that the amphoterin/RAGE pair might transduce antiproliferative signals in myoblasts via up-regulation of p21Waf1 induction, down-regulation of cyclin D1 induction, and reduction of Rb phosphorylation.

**RAGE Activation in Myoblasts Results in ERK1/2 and JNK Inactivation**—We have previously shown that the amphoterin/RAGE pair stimulates myogenic differentiation via a Rac1-Cdc42-MKK6-p38 MAPK pathway (16), and data in Figs. 1 and 2 suggest that amphoterin/RAGE-dependent inhibition of myoblast proliferation and stimulation of myoblast apoptosis also might rely on p38 MAPK activation. Because ERK1/2 and JNK are known to exert pro-mitogenic and/or pro-survival effects in myoblasts (15, 34), we next analyzed the extent of their phosphorylation (activation) in the three L6 myoblast clones under study. Higher levels of phosphorylated ERK1/2 (Fig. 4A) and JNK (Fig. 4B) were detected in L6/RAGEΔcyto myoblasts in GM compared with L6/RAGE and L6/mock myoblasts, and low extents of ERK1/2 or JNK phosphorylation were observed in L6/RAGE myoblasts in DM (Fig. 4, A
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**FIGURE 4.** RAGE signaling reduces ERK1/2 and JNK phosphorylation in a p38 MAPK-dependent manner in L6 myoblasts. A and B, L6/mock, L6/RAGEΔcyto, and L6/RAGE myoblasts were cultivated for 24 h in GM (10% FBS) and then in DM for 24 h (A) or 48 h (B), washed and subjected to Western blotting with anti-phosphorylated ERK1/2 or anti-ERK1/2 (A), anti-phosphorylated JNK or anti-tubulin antibody (B). C and D, same as in A and B except that at the time of the switch to DM, myoblasts received 2 μM SB203580 (an inhibitor of p38 MAPK) in Me2SO or an equal volume of Me2SO (control) for 24 h before Western blotting with anti-phosphorylated ERK1/2 or anti-ERK1/2 (C) and anti-phosphorylated JNK or anti-tubulin antibody (D). Mock, Δ, and R stand for L6/mock, L6/RAGEΔcyto, and L6/RAGE myoblasts, respectively. Numbers on top of lanes refer to the relative amount of phosphorylated ERK1/2 or JNK (A-D). One representative experiment of two is shown in A-D.

and B). Moreover, levels of phosphorylated ERK1/2 and JNK were higher in L6/RAGEΔcyto myoblasts compared with L6/mock myoblasts (Fig. 4, A and B), supporting the conclusion that RAGE signaling in L6 myoblasts might depress the activity of these kinases. Inhibition of p38 MAPK with SB203580 resulted in an increase in the extent of ERK1/2 (Fig. 4C) and JNK (Fig. 4D) phosphorylation in L6/RAGE and L6/mock myoblasts to the levels detected in L6/RAGEΔcyto myoblasts. Thus, RAGE engagement caused inactivation of the mitogenic ERK1/2, likely via p38 MAPK-mediated inhibition of the Raf-MEK-ERK1/2 pathway (30), which might explain the antiproliferative and pro-apoptotic activity of RAGE as well as regulatory effects of RAGE signaling on induction of cyclin D1 and Rb phosphorylation, whereas the stimulatory effects of RAGE signaling on p21 

**RAGE Activation in Myoblasts Increases Adhesiveness, Reduces Migration, Increases Cell Volume, and Up-regulates the Expression of Adhesion Molecules**—Changes in adhesiveness and migration occur in myoblasts in coincidence with the initiation of terminal differentiation, and these changes depend on expression of adhesion molecules shown to be important and/or crucial for myogenesis (35–43). Thus, we sought to analyze the three myoblast clones under study for adhesiveness, migration, invasiveness, and expression of adhesion molecules. L6/RAGEΔcyto myoblasts exhibited decreased adhesiveness, increased migration and invasiveness, and decreased cell volume, whereas L6/RAGE myoblasts exhibited the opposite, compared with L6/mock myoblasts. Specifically, in a cell adhesiveness assay, 3 h after plating ~40% less L6/RAGEΔcyto myoblasts and ~2 times more L6/RAGE myoblasts adhered to the substratum compared with L6/mock myoblasts (Fig. 5A). Qualitatively similar results were obtained at 24 h after plating (data not shown). Moreover, in a migration assay using Boyden chambers, a higher percentage of L6/RAGEΔcyto myoblasts and a lower percentage of L6/RAGE myoblasts were recovered in the lower chamber, compared with L6/mock myoblasts (Fig. 5B), suggesting that RAGE signaling might influence myoblast motility. In addition, 1 day after culture in GM, L6/RAGEΔcyto myoblasts exhibited a smaller size and L6/RAGE myoblasts exhibited a larger size, compared with L6/mock myoblasts, as investigated by F-actin decoration with fluorescein-labeled phalloidin (Fig. 5C) and FACS analysis (Fig. 5D). When analyses of cell size were performed on myoblasts in DM, a general increase in mean cell size was registered in individual L6 clones, compared with the respective counterpart in GM, but whereas L6/mock and L6/RAGE myoblasts exhibited a similar size, L6/RAGEΔcyto myoblasts showed a significantly smaller size compared with the two other clones again pointing to different responses of L6/RAGEΔcyto myoblasts from L6/mock and L6/RAGE myoblasts to the switch from GM to DM. Also, the F-actin cytoskeleton appeared well organized in L6/RAGE myoblasts and much less so in L6/RAGEΔcyto myoblasts compared with L6/mock myoblasts (Fig. 5C). These data suggested that the transducing activity of RAGE in myoblasts might not be restricted to stimulation of differentiation (16), inhibition of proliferation, and stimulation of apoptosis (Fig. 1 and 2); RAGE activation also appeared to profoundly modify myoblast morphology and motility, suggesting that these changes might be mechanistically linked to RAGE-dependent stimulation of myoblast differentiation. In particular, functional inactivation of RAGE in L6 myoblasts resulted in a dramatic decrease in cell size along with a less organized F-actin cytoskeleton and increased filopodia formation (Fig. 5), as is typical of proliferating, migrating, and poorly adherent cells, the opposite being observed in myoblasts overexpressing RAGE.

As mentioned above, adhesion molecules (e.g. β1-integrin, NCAM, and VCAM) and caveolin-3 play an important role in myoblast adhesiveness and differentiation (35–43), and RAGE activation has been shown to result in an enhanced expression of VCAM, NCAM, and ICAM in other cell types (17, 18). We found that L6/RAGEΔcyto myoblasts expressed lower levels, and L6/RAGE myoblasts expressed higher levels of β1-integrin, caveolin-3, NCAM, and VCAM, compared with L6/mock myoblasts, as investigated by both Western blotting (Fig. 6A) and immunofluorescence (Fig. 6B), suggesting that RAGE signaling contributed to up-regulate the expression of a set of adhesion molecules playing a fundamental role in myoblast fusion into myotubes. These data were in agreement with the observation that RAGE signaling in myoblasts resulted in enhanced adhesiveness (Fig. 5A) and, incidentally, they provide evidence for the first time that RAGE engagement can induce β1-integrin and caveolin-3 expression.

**RAGE Signaling in Myoblasts Results in Increased Invasiveness and Reduced MMP-1 and MMP-2 Activity**—Given the increased motility of L6/RAGEΔcyto myoblasts and the decreased motility of L6/RAGE myoblasts reported above (Fig. 5B), we sought to determine whether RAGE might be implicated in the regulation of invasive properties of L6 myoblasts.
by an invasiveness assay and determination of MMP activity. In an invasiveness assay using Boyden chambers endowed with a Matrigel barrier, L6/RAGE/H9004 myoblasts migrated to a larger extent through Matrigel, whereas L6/RAGE myoblasts behaved the opposite, compared with L6/mock myoblasts (Fig. 7A). Thus, functional inactivation of RAGE made L6 myoblasts more locally invasive, whereas RAGE activation reduced the invasive properties of L6 myoblasts. Alternatively, the increased volume of L6/RAGE myoblasts (Fig. 5, C and D) might have precluded migration through the 8-μm pores of Boyden chambers.

MMP, i.e. proteases that are liberated into the extracellular space and are implicated in cell migration and invasiveness, have been shown to play a role in myogenesis (44). Specifically, activation of MMP-1 and MMP-2 have been proposed to be important for myoblasts migration and invasiveness both in early phases of skeletal muscle formation during embryogenesis, when proliferating myoblasts have to migrate to the places of muscle formation, and during muscle regeneration, when activated satellite cells have to migrate toward damaged myofibers. Also, the liberation and activity of these two MMPs are remarkably increased in rhabdomyosarcomas (45), which are known to exhibit an exaggerated motility and invasiveness. On the other hand, MMP-9 seems to play a negligible role in myoblast migration and invasiveness normally, its liberation and activity likely being increased in the course of muscle regeneration (44). We analyzed the culture media of individual myoblast clones for MMP-1, MMP-2, and MMP-9 activity by zymography. An increased MMP-1 and MMP-2 activity and remarkably low MMP-9 activity were observed in L6/RAGE/H9004 myoblast culture medium, compared with L6/mock myoblasts (Fig. 7B). By contrast, lower MMP-1 and MMP-2 activities were detected in the culture medium of L6/RAGE myoblasts, compared with L6/mock myoblasts (Fig. 7B). Thus, a close relationship was confirmed between myoblast migration and invasiveness and MMP-1 and -2 activity, and an inverse relationship was established between MMP-1 and -2 activity and the amount of RAGE expressed. Collectively, these data suggested the possibility that RAGE might negatively regulate the release of certain MMPs in myoblasts and that repression of RAGE expression (here exemplified by functional inactivation of RAGE) might confer increased motility and invasiveness upon RAGE Inhibits Myoblast Proliferation and Tumor Formation

FIGURE 5. RAGE signaling increases adhesiveness and reduces motility in L6 myoblasts. A, L6/mock, L6/RAGEcyto, and L6/RAGE myoblasts were seeded at a density of 3 × 10^4 cells/well into a 96-multiwell plate and cultivated for 3 h in GM (10% FBS) after which the medium was aspirated and attached cells were washed twice with culture medium, fixed in methanol/ethanol, and stained with crystal violet. Absorbance at 590 nm was used to calculate the number of adherent cells. B, same as in A except that myoblasts cultivated in GM for 24 h were fixed and stained with fluorescein-labeled phalloidin. Arrows and arrowheads point to stress fibers and filopodia, respectively. C, same as in A except that after 24 h in GM, myoblasts were cultivated in DM for 24 or 48 h and processed for FACS analysis to measure the mean cell volume. Averages of three independent experiments ± S.D. are shown in A. Asterisk, significantly different (p < 0.05) (B–D). One representative experiment of two is shown. Numbers in the legends in the GM and DM panels in D refer to the median distribution of cells. Bars = 250 μm (A) and 20 μm (C).
myoblasts that was also manifested by an increased MMP-1 and -2 activity. As to MMP-9, its low extent of activity in L6/RAGE myoblasts might be in accordance with it not playing a major role in myoblast migration and invasiveness (44).

Inoculation of L6/RAGEΔcyto Myoblasts Results in Tumor Formation in Vivo—Next we inoculated immunocompromised mice with L6/RAGE, L6/RAGEΔcyto, or L6/mock myoblasts, and tumor formation was monitored for ~3.5 months. All five mice injected with L6/RAGEΔcyto myoblasts, three of five mice injected with L6/RAGE myoblasts, and two of five mice injected with L6/mock myoblasts developed a tumor mass (Fig. 8A). However, tumor formation in the case of L6/RAGEΔcyto myoblasts preceded that of L6/RAGE and L6/mock myoblasts by ~4 weeks, and mean volume and weight of tumor masses were ~2.5 times larger in the case of L6/RAGEΔcyto myoblasts compared with L6/RAGE and L6/mock myoblasts (Fig. 8B). Moreover, large areas of necrosis in the central core of the tumor and neovascularization at the tumor periphery were detected in L6/RAGEΔcyto myoblast tumors, whereas L6/RAGE and L6/mock masses were essentially devoid of necrosis (Fig. 8C), and the tumor tissue invaded the neighboring skeletal muscle tissue in the case of L6/RAGEΔcyto myoblasts only (data not shown). Lastly, L6/RAGEΔcyto myoblasts in the tumor mass appeared mostly as densely packed, round cells, whereas L6/RAGE and L6/mock myoblasts appeared elongated and hypertrophic, i.e. similar to myoblasts ready for fusion (Fig. 8C). Indeed, elongated and hypertrophic myoblasts could be detected in L6/mock myoblasts and even more so in L6/RAGE myoblasts (Fig. 8C, arrows). Thus, masses formed in mice inoculated with L6/RAGEΔcyto myoblasts exhibited characteristics of authentic tumors, whereas those formed in mice inoculated with L6/RAGE and L6/mock myoblasts did not, and the higher incidence of tumor mass formation in mice inoculated with L6/RAGE myoblasts compared with those inoculated with L6/mock myoblasts likely depended on the larger volume of L6/RAGE and L6/mock myoblasts rather than on uncontrolled proliferation. In conclusion, overexpression of signaling-deficient RAGE conferred an aggressive potential on L6 myoblasts.

DISCUSSION

RAGE, a multiligand receptor of the immunoglobulin superfamily, has been implicated in the inflammatory response, neuronal trophism,

FIGURE 6. RAGE signaling stimulates integrin β1, VCAM, NCAM, and caveolin-3 expression in L6 myoblasts. A, L6/mock, L6/RAGEΔcyto, and L6/RAGE myoblasts were cultivated for 24 h in GM (10% FBS), switched to DM (2% FBS), and left undisturbed for 6 days under these conditions, washed and subjected to Western blotting with an anti-integrin β1, anti-VCAM, anti-NCAM, or anti-caveolin-3 antibody. A Western blot of tubulin is included to show loading of equal amounts of proteins in individual lanes. Numbers on top of lanes refer to relative amounts of individual adhesion proteins in the three L6 clones after normalization to tubulin. One representative experiment of two is shown. Mock, Δ and R, in E stand for L6/mock, L6/RAGEΔcyto, and L6/RAGE myoblasts, respectively. B, same as in A except that myoblasts were cultivated for 3 days in DM, fixed, and subjected to immunofluorescence with an anti-integrin β1, anti-VCAM, anti-NCAM, or anti-caveolin-3 antibody as indicated. One representative experiment of two is shown. Bars = 20 μm (B).
and neuronal death (depending on the nature of the ligand and the intensity and duration of the stimulus), and tumorigenesis (17). One prominent feature of RAGE is that it is expressed during development, repressed at completion of development, and re-expressed under certain pathological conditions (17). We reported that RAGE is expressed in rat skeletal myofibers during fetal development and up to 11 days after birth, disappearing thereafter (16), suggesting that it might play a regulatory role in skeletal muscle formation. We also found that RAGE is expressed by rat L6 myoblasts and transduces a promyogenic signal via a Cdc42-Rac-MKK6-p38 MAPK pathway upon activation by its ligand, amphoterin (16). Similar results were obtained using the mouse C2C12 myoblast cell line.4 In the present work we show that, additionally, RAGE engagement by amphoterin contributes to myoblast proliferation arrest and apoptosis, two events strictly connected with the activation of the myogenic program (1, 3, 4, 46), and that this activity relies on p38 MAPK activation. We also show that RAGE engagement contributes to the increased adhesiveness and reduced motility of differentiating myoblasts, and, conversely, that reduction of RAGE transducing activity results in a decreased adhesiveness and increased motility and invasiveness of myoblasts. Finally, mice inoculated with L6/RAGE or L6/mock myoblasts, and signs of authentic tumor formation can be observed in masses formed upon inoculation with L6/RAGEcyto myoblasts only.

Several lines of evidence support these conclusions. First, L6/RAGEcyto myoblasts proliferate more and L6/RAGE myoblasts proliferate less than L6/mock myoblasts, as investigated by [3H]thymidine incorporation, MTT assays, and FACS analysis, with consistent changes in the extent of activation of the pro-mitogenic kinases, ERK1/2 and JNK, of induction of the myoblast proliferation inhibitor, p21Waf1, and cyclin D1, and of levels of phosphorylated Rb (Figs. 1 and 4). Second, lower and higher extents of apoptosis and caspase-3 activation were measured in L6/RAGEcyto and L6/RAGE myoblasts, respectively, and higher and lower levels of the anti-apoptotic factor, Bcl-2, were detected in L6/RAGEcyto and L6/RAGE myoblasts, respectively, compared with L6/mock myoblasts (Fig. 2). Third, inhibition of p38 MAPK results in a reduction of RAGE-dependent effects on myoblast proliferation and apoptosis (Fig. 2). Fourth, neutralization of culture medium amphoterin negates the effects of RAGE signaling in L6/mock and L6/RAGE myoblasts with no effects in L6/RAGEcyto myoblasts (Figs. 1–3). Last, a direct relationship is observed between expression of signaling-competent RAGE in myoblasts and myoblast size and adhesiveness, and, conversely, an inverse relationship is observed with respect to migration and invasiveness, L6/RAGEcyto myoblasts exhibiting reduced adhesiveness and enhanced migration and invasiveness compared with

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L6/mock and L6/RAGE myoblasts (Figs. 5 and 7). Consistent with this conclusion, we found that RAGE engagement and signaling positively regulate the expression of adhesion molecules shown to be important for myogenesis and negatively regulate MMP-1 and -2 liberation and activity (Figs. 6 and 7). Last, masses with the histological features of tumors form upon inoculation of immunocompromised mice with L6/RAGE/H9004 cyt, but not L6/mock or L6/RAGE myoblasts (Fig. 8). Most of the experiments described in the present report were performed using three independent L6 stable clones expressing similar amounts of RAGE or RAGE/H9004 cyt, with similar results (data not shown).

Functional inactivation of RAGE signaling results in higher levels of ERK1/2 and JNK phosphorylation compared with L6 mock-transfected myoblasts (Fig. 4), suggesting that signaling competent RAGE might inactivate these mitogenic kinases. Accordingly, levels of ERK1/2 and JNK phosphorylation are very low in myoblasts overexpressing RAGE (Fig. 4). This effect of RAGE signaling depends on activation of p38 MAPK because inhibition of p38 MAPK by use of SB203580 results in higher levels of ERK1/2 and JNK phosphorylation in L6/RAGEΔcyt, L6/mock, and L6/RAGE myoblasts, compared with their respective controls, with similar extents of phosphorylation in the three L6 clones under study (Fig. 4). These findings suggest that RAGE signaling to p38 MAPK might cause inactivation of ERK1/2, likely via inhibition of Raf activity (30), as well as of JNK. Moreover, higher levels of phosphorylated Rb and cyclin D1 and lower levels of the proliferation inhibitor, p21Waf1, are detected in L6/RAGEΔcyt myoblasts, whereas the opposite occurs in L6/RAGE myoblasts, compared with L6/mock myoblasts (Fig. 3). Because proliferation arrest, consequent to ERK1/2 and/or JNK inactivation, is a critical step in the context of myoblast terminal differentiation (at least at early phases of myogenic differentiation) (3, 15, 23, 34), RAGE-dependent inhibition of myoblast proliferation might thus be functional to the subsequent myoblast differentiation operated via p38 MAPK activation. We have previously reported that inhibition of p38 MAPK eliminates the ability of the amphoterin/RAGE pair to stimulate myogenesis (16).

It is known that under differentiation conditions a subpopulation of myoblasts undergoes differentiation and fusion into myotubes, another subpopulation remains undifferentiated and becomes quiescent, and
still another subpopulation undergoes apoptosis (47–51). This latter event might serve to reduce the population of non-fused myoblasts coexisting with myotubes thus keeping the population of quiescent myoblast relatively low. We found that functional inactivation of RAGE signaling results in lower levels of myoblast apoptosis under both proliferation and differentiation conditions via reduction of levels of activated caspase-3 and up-regulation of Bcl-2 expression, compared with mock-transfected myoblasts, whereas the opposite occurs in myoblasts overexpressing RAGE (Fig. 2). Thus, RAGE signaling might contribute to the apoptosis usually taking place during the myoblast differentiation process. The ability of RAGE signaling to inactivate ERK1/2 might be responsible for this effect because inactivation of ERK1/2 by other means (e.g. by use of the ERK1/2 inhibitor, PD98059) also results in increased myoblast apoptosis (52–55).

Amphoterin/RAGE-dependent inactivation of ERK1/2 in myoblasts and the consequent down-regulation of Bcl-2 are at variance with respect to neurons in which RAGE engagement by amphoterin or low doses of S100B, another RAGE ligand, results in protection against apoptosis via stimulation of ERK1/2 and up-regulation of Bcl-2 expression (56). These data, whereas supporting the notion that RAGE can activate various signaling pathways (17), suggest that RAGE engagement might preferentially activate one particular signaling pathway over other ones in the different cell types in which the receptor operates thereby regulating specific functions. In myoblasts, the amphoterin/RAGE pair appears to mainly activate p38 MAPK via Cdc42-Rac1-MKK6 (16) with consequent depression of ERK1/2 and INK activities (Fig. 4) and stimulation of myogenic differentiation (16), inhibition of proliferation, and enhancement of apoptosis (Figs. 1 and 2). These observations suggest that different intermediates acting immediately downstream of RAGE might operate in different cell types to specify the RAGE signaling activity. Additionally (or alternatively), different cell types might express distinct RAGE variants (57) that might account for cell specificity of RAGE effects. Whereas in principle the combination of the inhibitory effect of RAGE signaling on myoblast proliferation and its stimulatory effect on apoptosis might be detrimental during myogenesis and muscle regeneration, leading to a decreased myoblast density, its stimulatory effect on the promyogenic p38 MAPK might instead provide a means for accelerating myoblast terminal differentiation and fusion. Actually, in cultures of 80–90% confluent myoblasts, the overall effect of RAGE signaling is stimulation of myotube formation, which is best evident after the switch from proliferation conditions to differentiation conditions, but also detectable under proliferation conditions (16).

RAGE engagement in myoblasts also result in an increased adhesiveness and a decreased migration and invasiveness (Figs. 5 and 7). Myoblast proliferation arrest and the initiation of terminal differentiation are accompanied by an increased adhesiveness and expression of adhesion molecules and a decreased motility (3). Inhibition of RAGE signaling by overexpression of RAGEΔcyto results in decreased adhesiveness and reduced expression of adhesion molecules (i.e. integrin β1, VCAM, and NCAM) and caveolin-3, and an increased migration and invasiveness, whereas the opposite occurs in myoblasts overexpressing signaling-competent RAGE (Figs. 5–7). Because each one of the molecules listed above plays a fundamental role in myoblast fusion into myotubes (35–43), we conclude that RAGE signaling might reduce myoblast migration and accelerate fusion by contributing to the expression of those adhesion molecules in myoblasts.

Finally, we observe that L6/RAGEΔcyto myoblasts liberate larger amounts of MMP-1 and -2 as inferred by zymography, the opposite occurring in L6/RAGE myoblasts, compared with L6/mock myoblasts (Fig. 5), in accordance with the increased migration and invasiveness of L6/RAGEΔcyto myoblasts and decreased migration and invasiveness of L6/RAGE myoblasts as well as changes in the expression of adhesion molecules mentioned earlier. These findings suggest that RAGE-dependent reduction of MMP-1 and -2 activity might contribute to decrease and/or interrupt myoblast migration thereby contributing to myoblast alignment for subsequent fusion into myotubes and, conversely, repression of RAGE expression might be functional to myoblast migration and/or contribute to the increased motility and invasiveness of, e.g. myoblast neoplastic counterparts. In fact, inoculation of L6/RAGEΔcyto myoblasts into scid mice results in much larger and more aggressive tumor formation, compared with L6/RAGE or L6/mock myoblasts. Also, work in progress suggests that rhabdomyosarcoma cell lines that do not express RAGE undergo proliferation arrest and terminal differentiation, exhibit reduced migration and invasiveness, and grow much smaller tumor masses in vivo upon transfection with signaling-competent RAGE, compared with signaling-incompetent RAGE.5

In conclusion, our data provide evidence that, besides stimulating myoblast differentiation, RAGE engagement by amphoterin contributes to proliferation arrest of differentiating myoblasts, and that all of these effects rely on p38 MAPK activation. We also show that RAGE signaling results in the up-regulation of expression of adhesion molecules (i.e. integrin β1, VCAM, and NCAM) and caveolin-3 that have been shown to play crucial roles in myoblast fusion into myotubes. We speculate that RAGE might be active at precise phases of embryonic myogenesis, i.e. in coincidence with myoblast proliferation arrest, initiation of terminal differentiation, and fusion into myotubes, thereby taking part in these events, and that repression of RAGE expression and/or signaling in myoblasts might contribute to the increased migration and invasiveness occurring in myoblast migration during embryonic myogenesis and/or muscle regeneration, and the increased migration invasiveness and in vivo aggressiveness typical of myoblast neoplastic counterparts.

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