

Receptor for Advanced Glycation End Products-binding COOH-terminal Motif of Amphoterin Inhibits Invasive Migration and Metastasis¹

Henri J. Huttunen,² Carole Fages, Juha Kuja-Panula, Anne J. Ridley, and Heikki Rauvala

Program of Molecular Neurobiology, Institute of Biotechnology, and Department of Biosciences, University of Helsinki, Helsinki FIN-00014, Finland [H. J. H., J. K.-P., H. R.], and Ludwig Institute for Cancer Research, Royal Free and University College School of Medicine, London W1W 7BS, United Kingdom [C. F., A. J. R.]

ABSTRACT

Amphoterin has been suggested to regulate invasive process extension and cell migration in tumor cells and embryonic neurons through binding to receptor for advanced glycation end products (RAGE), a multiligand transmembrane receptor belonging to the immunoglobulin superfamily. In this study, we identify a COOH-terminal motif in amphoterin (amino acids 150–183) that is responsible for RAGE binding. We show that as a surface-bound ligand, this part of amphoterin is sufficient to induce RAGE-dependent process extension, suggesting a role in the regulation of cell motility. When applied in solution, the RAGE-binding COOH-terminal motif of amphoterin efficiently inhibits process extension and transendothelial migration of tumor cells. Furthermore, in an *in vivo* model, the corresponding synthetic peptide significantly suppresses formation of lung metastases. Taken together, these results suggest that amphoterin binds to RAGE through a COOH-terminal motif that can be used as an efficient inhibitor to block invasive migration of tumor cells.

INTRODUCTION

The acquisition of invasively motile phenotype is a key step in the development of malignant tumors and formation of metastases. Alteration of the interactions between the neoplastic cells and their surrounding extracellular matrix plays an important role in the detachment of tumor cells from the primary tumor and in the migration of invasive cells through the endothelium to the vasculature, through which they can reach distant locations throughout the body (reviewed in Ref. 1).

Amphoterin and RAGE³ have been identified as a ligand-receptor pair that plays an important role in regulating the invasiveness of tumor cells (2). Amphoterin (also known as HMGB1) is expressed in a large variety of transformed cells (3) and has been shown to be a general regulator of cell migration (4). On the other hand RAGE, an immunoglobulin superfamily member (5), is known to stimulate multiple signaling pathways crucial for cell migration, such as Ras-extracellular signal-regulated kinase 1/2 (6), Cdc42/Rac (7), stress-activated protein kinase/c-Jun-NH₂-terminal kinase, and p38 mitogen-activated protein kinase pathways (2). Furthermore, several transcriptional targets of RAGE signaling, such as vascular cell adhesion molecule 1 (8) and tissue factor (9), are likely to contribute to the interaction of tumor cells with vascular endothelium. Recent reports have shown that the level of RAGE expression correlates strongly with metastatic potential of gastric and pancreatic carcinomas (10, 11). Interestingly, RAGE activation is known to result in increased expression of RAGE itself, creating a positive feedback loop between RAGE signaling and expression (12).

Received 3/27/02; accepted 6/7/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by grants from the Academy of Finland (Finnish Center of Excellence Programme 2000–2005), Finnish Cancer Society, Sigrid Jusélius Foundation, Emil Aaltonen Foundation, and European Community Grant QLGI-CT-1999-01036.

² To whom requests for reprints should be addressed, at P. O. Box 56 (Viikinkaari 5), University of Helsinki, Helsinki FIN-00014, Finland. Phone: 358-9-19159060; Fax: 358-9-19159068; E-mail: Henri.Huttunen@helsinki.fi.

³ The abbreviations used are: RAGE, receptor for advanced glycation end products; HMG, high mobility group; MMP, matrix metalloproteinase; GST, glutathione S-transferase; HUVEC, human umbilical vein endothelial cell; sRAGE, soluble ectodomain of RAGE.

A general feature associated with invasive and metastatic capacity is the deregulation of extracellular matrix-degrading proteases (1). In addition to the membrane-associated proteins RAGE, heparan sulfate proteoglycan syndecan-1 (13), and chondroitin sulfate proteoglycans phosphacan and neurocan (14), amphoterin binds both plasminogen activators (tissue-type plasminogen activator and urokinase-type plasminogen activator) and plasminogen, resulting in the formation of a ternary complex that facilitates production of active plasmin (3). The ternary complex appears transient because activation of plasminogen results in hydrolysis of amphoterin, creating temporary binding sites for the motile processes. In addition, it has been reported that tumor cells expressing RAGE have increased activity of MMP-2 and MMP-9 correlating with the metastatic potential of these cells (2). These characteristics position both amphoterin and RAGE in a central place in the regulation of invasive migration of tumor cells. Thus, understanding the molecular nature of amphoterin-RAGE interaction will likely provide novel therapeutic approaches for treatment of cancer, especially for suppressing the formation of secondary tumors.

In addition to amphoterin, RAGE serves as a receptor for advanced glycation end products (5), members of the S100 protein family (15, 16), and various amyloids [amyloid- β peptide and amyloid A (17, 18)]. Rational design of RAGE antagonists for practical applications, including inhibition of tumor cell invasion, would require insight into the structural basis of RAGE-ligand interactions, which is currently poorly understood. In this study, we identify a COOH-terminal motif of amphoterin that is responsible for RAGE binding. We show that when immobilized to a surface, this RAGE-binding motif can induce RAGE-mediated process outgrowth, but in solution, it can efficiently block transendothelial migration of tumor cells. Furthermore, a synthetic peptide corresponding to the RAGE-binding region in amphoterin significantly suppressed formation of lung metastases in an *in vivo* model. Interestingly, we found that the sequence of the RAGE-binding COOH-terminal motif of amphoterin is homologous with S100 proteins.

MATERIALS AND METHODS

Expression and Purification of Proteins and Peptides. The production and purification of baculovirus-expressed wild-type amphoterin have been described previously (3). Amphoterin fragments (Fig. 1A) were synthesized by PCR and cloned in-frame with the GST of pGEX-6P1 vector (*Bam*HI-*Eco*RI site; Amersham Biosciences). The following oligonucleotides (Sigma-Genosys) were used: amphoterin 1–185 For, AGAGGATCCATGGGCAAAGGAGATCCT; amphoterin 1–185 Rev, GGCGAATTCTCACTCTTTTCTTGCT; amphoterin 1–140 For, AGAGGATCCATGGGCAAAGGAGATCCT; amphoterin 1–140 Rev, GTCGAATTCTTAGTCATCCGACAGAGTGT; amphoterin 141–190 For, TCTGGATCCAAGCAGCCCTATGAAAAG; and amphoterin 141–190 Rev, GGCGAATTCTTAGTCGCTTCTCTCTCTTCTT.

The authenticity of cloned constructs was verified by sequencing. GST-amphoterin fragment fusion proteins were expressed in *Escherichia coli* and purified on glutathione-Sepharose columns (Amersham Biosciences). Some of the proteins were used as GST fusion proteins, whereas some were used with the GST moiety cleaved off. To obtain amphoterin fragments without GST, fusion proteins were cleaved with PreScission Protease (Amersham Biosciences), and the cleaved GST was then removed with a glutathione-Sepha-

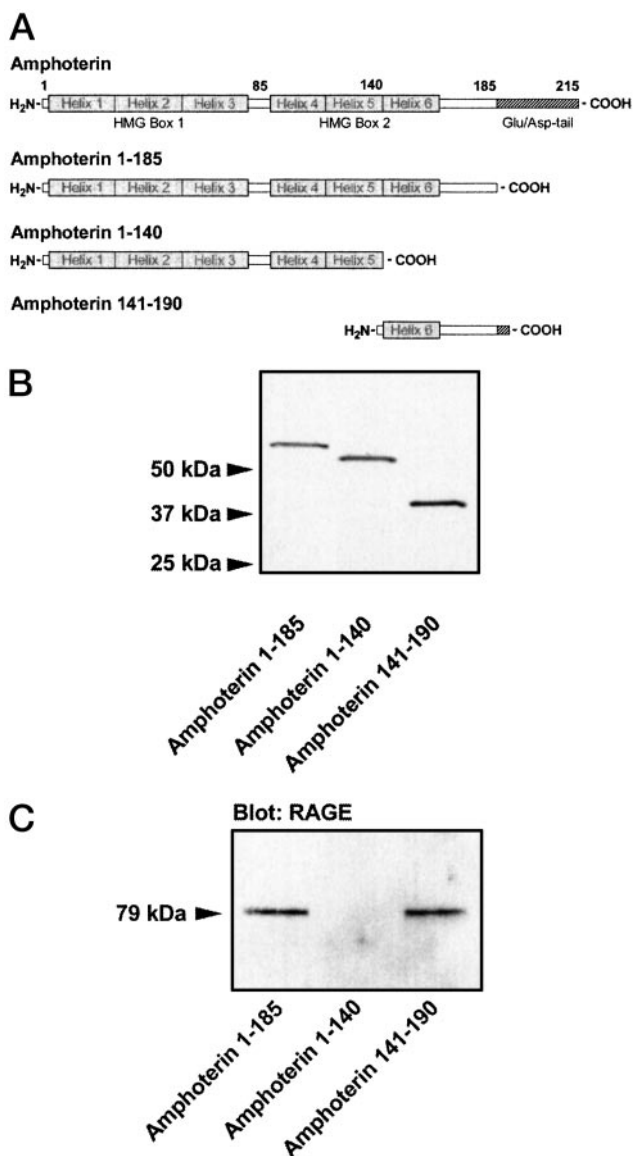


Fig. 1. Production and RAGE binding capacity of recombinant amphoterin fragments. **A**, schematic representation of amphoterin fragments produced as GST fusion proteins. **B**, Coomassie Blue-stained gel of purified GST fusion proteins. **C**, amphoterin fragment-GST fusion proteins were attached to glutathione-Sepharose beads, and purified sRAGE-Fc protein was run through the column. After extensive washing, the columns were eluted with 1.0 M NaCl. RAGE binding to the amphoterin fragments immobilized to the columns was detected by Western blotting with an anti-RAGE antibody.

rose column. Recombinant proteins were characterized on SDS-PAGE gels and Western blots (Fig. 1B).

The following oligonucleotides were used to clone mouse RAGE for production of recombinant protein: RAGE For, TACTAGCTAGCGCCCGGAT-TGGAGAGCCACTTG; and RAGE Rev, ATAGTTTAGCGGCCCGCCCA-GACTCACCCACAGAG. Mouse sRAGE was cloned to Signal pIgPlus vector (*NheI-NotI* site; R&D Systems) to generate a fusion protein with a COOH-terminal human Fc part. 293T cells were transfected with FuGENE 6 reagent according to the manufacturer's instructions (Roche Molecular Biochemicals). The cells were replated on 130-mm plates and changed to serum-free medium after 24 h. Conditioned medium was collected after 3 days, and sRAGE-Fc was purified with protein A-Sepharose chromatography.

Peptides were synthesized and purified by Sigma-Genosys. The sequences used for peptide synthesis were as follows: amphoterin 150–183, KLKEKY-EKDIAAYRAKGPDAAKKGVVKAEEKSKK; and amphoterin 150–183 (scrambled), KSKKVEAVKKAKAGGPKDRAAYAKEIYDKLEKK. Because the peptide sequences contained difficult areas, the identity of purified peptides was verified by mass spectrometry and sequencing.

In Vitro Binding Assay. Glutathione-Sepharose (Amersham Biosciences) was packed in 0.2-ml columns to which 50 μ g of GST-amphoterin fragment fusion proteins were attached by a 1-h incubation at 4°C. Columns were washed with 5 volumes of cold PBS and then incubated with 10 μ g of sRAGE-Fc protein for 2 h at 4°C. The columns were washed with 5 volumes of cold PBS and eluted with $2 \times 200 \mu$ l of 1.0 M NaCl. Eluates were run on 10–20% SDS-PAGE gels and electrotransferred on nitrocellulose filters. Filters were blocked with 2% BSA, and RAGE was detected with a primary antibody (Chemicon) followed by incubation with a peroxidase-conjugated secondary antibody (Amersham Biosciences). The bands were visualized with enhanced chemiluminescence (Amersham Biosciences).

Neurite Outgrowth Assay. Stably transfected clones of N18 mouse neuroblastoma cells were obtained as described previously (16). Cells were cultured in DMEM supplemented with 0.1 mg/ml streptomycin, 100 units/ml penicillin G, 10% FCS, and 100 μ g/ml G418 (Invitrogen) in 5% CO₂ atmosphere. For neurite outgrowth assays, the cells were detached from culture plates by incubation in PBS + 0.5 mM EDTA, washed with serum-free DMEM supplemented with 1% BSA (Sigma), and plated without serum on 96-well plates that had been coated with either 20 μ g/ml recombinant amphoterin fragments or 50 μ g/ml synthetic peptides. Cells were fixed after 24 h of incubation, and neurite outgrowth was quantified by counting cells bearing neurites longer than 1 diameter of cell soma in five randomly selected fields. Phase-contrast micrographs were taken with an Olympus IX-70 reversed microscope with Hoffman Modulation phase-contrast optics and an Olympus DP-10 digital camera.

Transendothelial Migration Assay. HUVECs were prepared from umbilical veins as follows. The umbilical cords were flushed twice with PBS, filled with 0.1% collagenase (Sigma) in PBS prewarmed to 37°C, and incubated for 20 min at 37°C. The collagenase solution was then collected, and the veins were washed twice with 20 ml of Medium 199 (Invitrogen) containing 20% FCS to collect the remaining cells. The collagenase solution and the washes were pooled and centrifuged. The cell pellet was resuspended in 20 ml of Medium 199/20% FCS, and the cells were plated into a 75-ml culture flask (Corning Life Sciences) coated with 10 μ g/ml fibronectin. After 3 h, the medium was changed to remove nonadherent cells. HUVECs were cultured in Medium 199 containing 20% FCS, 100 μ g/ml endothelial cell growth supplement (Sigma), 100 μ g/ml heparin (Sigma), and 1% Nutridoma NS (Boehringer Mannheim) in flasks coated with 10 μ g/ml human fibronectin.

For transendothelial migration assays, HUVECs were grown on fibronectin-coated polyester Transwell-Clear filters (3 mm, pore size; 12 mm, diameter; Corning Life Sciences). The cells were plated at a density of 3×10^5 cells/well, and after 4 h, nonadherent cells were removed. Filters were used for experiments 4 days after plating. HUVEC monolayers grown on filters were examined by microscopy for confluence before the experiment. HT1080 human fibrosarcoma cells or stably transfected clones of N18 cells (16) were serum-starved and labeled by incubation for 15 min with 6.7 μ M succinimidyl ester of carboxyfluorescein diacetate (CFDA SE; Molecular Probes, Inc.). After a 30-min preincubation with the amphoterin peptides, HT1080 cells were plated on HUVEC monolayers. Cells were incubated for 5 h before they were fixed and stained with TRITC-phalloidin (Molecular Probes, Inc.). Migrated cells were counted in four randomly selected fields in three layers with an Olympus IX-70 reversed fluorescence microscope.

Experimental Pulmonary Metastasis Assay. B16-F1 mouse melanoma cells were grown in DMEM supplemented with 0.1 mg/ml streptomycin, 100 units/ml penicillin G, and 10% FCS in 5% CO₂ atmosphere for 1 week to collect a sufficient number of cells. The cells were detached from culture plates by incubation in PBS + 0.5 mM EDTA and washed with serum-free DMEM. A total of 3.0×10^5 cells exhibiting >90% viability were suspended in 250 μ l of serum-free DMEM and preincubated with the amphoterin peptides for 30 min before injection into the tail veins of isogenic, immunocompromised C57BL/6-*scid-beige* mice (6–8-week-old female mice; Harlan). After 14 days, the animals were sacrificed, and the lungs were collected and analyzed for the presence of metastatic foci. Stably transfected B16-F1 cells expressing either full-length RAGE or the cytoplasmic domain deletion mutant of RAGE (RAGE Δ cyto) were produced as described for N18 cells (16). Photomicrographs were taken with a Leica stereomicroscope and an Olympus DP-10 digital camera.

Sequence Analysis. Amino acid sequence alignments were calculated using Genetics Computer Group Wisconsin Package Version 10.2 (19). Multiple sequence alignment was constructed using the ClustalX program (20).

Statistical Analysis. Student's *t* test was used to analyze the statistical significance of the results. *P* values are shown in the Figs. 2–5.

RESULTS

Identification of RAGE-binding Motif in Amphoterin. Our previous studies have shown that an antibody against a COOH-terminal peptide of amphoterin (amino acids 166–75) inhibits haptotactic cell migration to laminin (4). To study whether this region of amphoterin was involved in receptor binding, we produced and purified GST fusion proteins with various amphoterin fragments (Fig. 1, A and B). Amphoterin 1–185 consisted of the major part of the molecule lacking the 31 last amino acids that make up the highly acidic tail of amphoterin. Amphoterin 1–140 consisted of HMG box 1 and the first two α -helices of HMG box 2, whereas amphoterin 141–190 consisted of the third α -helix of HMG box 2 and the region between the HMG box 2 and the acidic tail of amphoterin (21, 22).

To study the binding of the amphoterin fragments to RAGE, the receptor of amphoterin, GST fusion proteins were attached to glutathione-Sepharose columns through which purified soluble ectodomain of RAGE was passed. After extensive washing, the columns were eluted with 1.0 M NaCl, and bound RAGE was identified by Western blotting. As seen in Fig. 1C, amphoterin 1–185 and amphoterin 141–190 were able to bind to RAGE, whereas amphoterin 1–140 columns did not bind RAGE. This result suggests that RAGE binds to a COOH-terminal region of amphoterin that consists partly of the HMG box 2 and partly of the intermediate region between the HMG box 2 and the acidic tail of the molecule.

RAGE-binding Motif of Amphoterin Induces Neurite Outgrowth. To further characterize the RAGE-binding motif of amphoterin, we tested the amphoterin fragments in a simple bioassay previously used to study the role of RAGE signaling in process outgrowth (7, 16). N18 neuroblastoma cells expressing either full-length RAGE or a signaling-incapable, cytoplasmic domain deletion mutant of RAGE (RAGE Δ cyto) were grown on surfaces coated with amphoterin fragments (the GST moiety had been cleaved off), and neurite outgrowth response was followed for 24 h.

Roughly 25% of RAGE-expressing cells and 5% of RAGE Δ cyto-expressing cells grew neurites on amphoterin surface (Fig. 2G). Amphoterin 1–185 had a similar capacity to promote neurite outgrowth as the full-length protein (Fig. 2, A and G). Remarkably, RAGE-expressing cells plated on amphoterin 1–140 were unable to grow neurites (Fig. 2, B and G), whereas on amphoterin 141–190, >20% of RAGE-expressing cells grew neurites (Fig. 2, C and G). The neurite outgrowth-promoting effect of both amphoterin 1–185 and 141–190 depends on RAGE signaling because RAGE Δ cyto-expressing cells did not display neuritic morphology when grown on these amphoterin fragments (Fig. 2, D, F, and G).

A peptide corresponding to a slightly shorter region of the RAGE-binding motif of amphoterin (amino acids 150–183) was synthesized for *in vivo* assays. In addition, the same sequence was used in a scrambled order to synthesize a control peptide. The amphoterin 150–183 peptide was tested in the neurite outgrowth assay (coated at 50 μ g/ml) and showed a similar capacity to promote RAGE-dependent neurite outgrowth as did the longer recombinant amphoterin 141–190 fragment, whereas cells grown on the scrambled amphoterin 150–183 peptide displayed a significantly lower number of neurites (Fig. 2H). Next we tested whether these peptides could be used to inhibit amphoterin-RAGE interaction when applied in solution (at 500 μ g/ml, \sim 130 μ M). As seen in Fig. 2I, when cells were grown on an

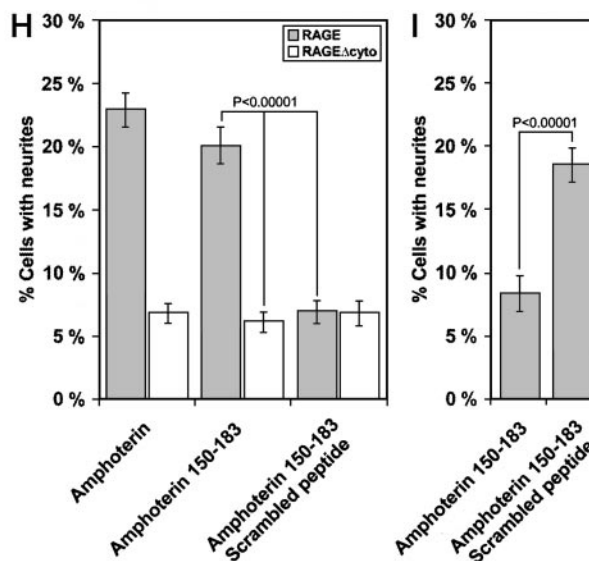
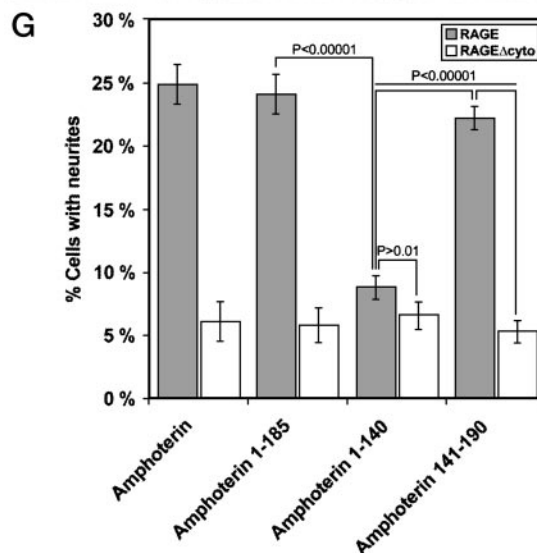
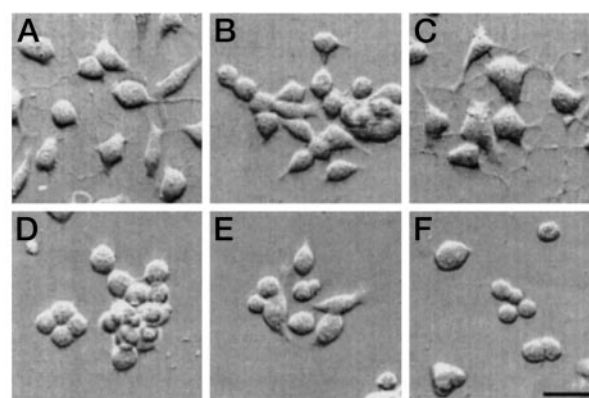


Fig. 2. Effect of recombinant amphoterin fragments and synthetic peptides on RAGE-dependent neurite outgrowth. N18 neuroblastoma cells stably transfected with either full-length RAGE (N18/RAGE; A–C; gray bars in G–I) or a cytoplasmic domain deletion mutant of RAGE (N18/RAGE Δ cyto; D–F; white bars in G and H) were grown overnight on surfaces coated with various amphoterin fragments (20 μ g/ml); amphoterin 1–185 (A and D), amphoterin 1–140 (B and E), and amphoterin 141–190 (C and F) are shown. Neurite outgrowth was quantified by counting cells bearing neurites longer than 1 diameter of cell soma in five randomly selected fields. The proportion of neurite-bearing cells to total number of cells in three replicate experiments \pm SD is shown for recombinant amphoterin fragments (G) and for synthetic peptides (H). Synthetic peptides were used in solution to inhibit neurite outgrowth promoted by surface-bound full-length recombinant amphoterin (I). Bar, 20 μ m.

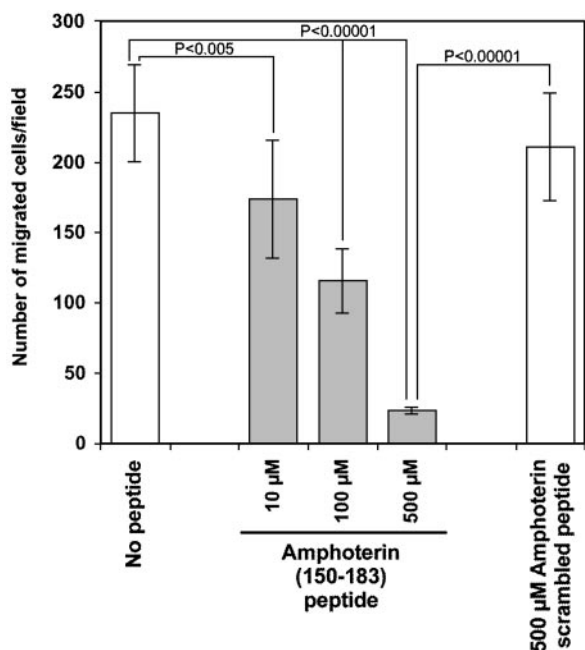


Fig. 3. Effect of amphoterin 150–183 peptide on transendothelial migration. HUVEC monolayers were prepared on fibronectin-coated (10 $\mu\text{g}/\text{ml}$) polycarbonate filters in the transfilter system. HT1080 fibrosarcoma cells were serum-starved and loaded with a fluorescent tracer (succinimidyl ester of carboxyfluorescein diacetate). After a 30-min preincubation with the peptides, HT1080 cells were plated on HUVEC monolayers. Cells were incubated for 5 h before they were fixed and stained with TRITC-phalloidin. Migrated cells were counted in four randomly selected fields in three layers. The number of cells that migrated through the endothelial monolayer in three replicate experiments \pm SD is shown. Scrambled sequence of amphoterin 150–183 was used as the control peptide.

amphoterin-coated surface in the presence of soluble amphoterin 150–183 peptide, the amount of neurites was reduced more than 2-fold in comparison with the scrambled peptide.

These results confirm that the COOH-terminal motif of amphoterin is responsible for RAGE binding and that the RAGE-binding region in amphoterin can be shortened to residues 150–183. In addition, these results show that the RAGE-binding motif of amphoterin can mimic the full-length protein, serving as a RAGE agonist when presented to the cells in a surface-bound form, but when applied in solution, this motif serves as a RAGE antagonist.

RAGE-binding Peptide Inhibits Transendothelial Migration of Tumor Cells. Because previous results have suggested that both amphoterin and RAGE are involved in invasive migration and formation of metastasis (2, 4), we decided to determine whether the RAGE-binding COOH-terminal motif of amphoterin could be used to block migration of tumor cells through the endothelium. We set up an *in vitro* transendothelial migration assay using labeled HT1080 fibrosarcoma cells migrating through an endothelial cell monolayer prepared from HUVECs. A synthetic peptide corresponding to region 150–183 of amphoterin was used as an inhibitor in solution to block the endogenously occurring RAGE in HT1080 fibrosarcoma cells (data not shown). Scrambled sequence of the amphoterin 150–183 peptide was used as a control. As seen in Fig. 3, addition of the amphoterin 150–183 peptide inhibited transendothelial migration of HT1080 cells in a dose-dependent manner, reaching 88.9% inhibition at 500 μM ($\text{IC}_{50} = 144.1 \mu\text{M}$) in comparison with the scrambled peptide. This result shows that blocking amphoterin-RAGE interaction with soluble RAGE-binding amphoterin peptide can efficiently inhibit invasive migration of tumor cells through the endothelial cell monolayer.

Comparison of N18/RAGE and N18/RAGE Δcyto cells in the transendothelial migration assay shows that inhibition of RAGE sig-

naling significantly suppresses the ability of tumor cells to migrate through the endothelial cell monolayer without affecting their ability to adhere to the endothelial cells (Fig. 4A), further supporting the idea of RAGE-amphoterin interaction as a regulator of transendothelial migration of tumor cells.

RAGE-binding Peptide Suppresses Formation of Pulmonary Metastasis. The metastatic potential of tumor cells can be studied *in vivo* in several animal models using immunocompromised mice. We tested the effect of the amphoterin peptides in an experimental pulmonary metastasis model using B16-F1 melanoma cells injected into the tail veins of isogenic C57BL/6-*scid-beige* mice (23, 24). After 2

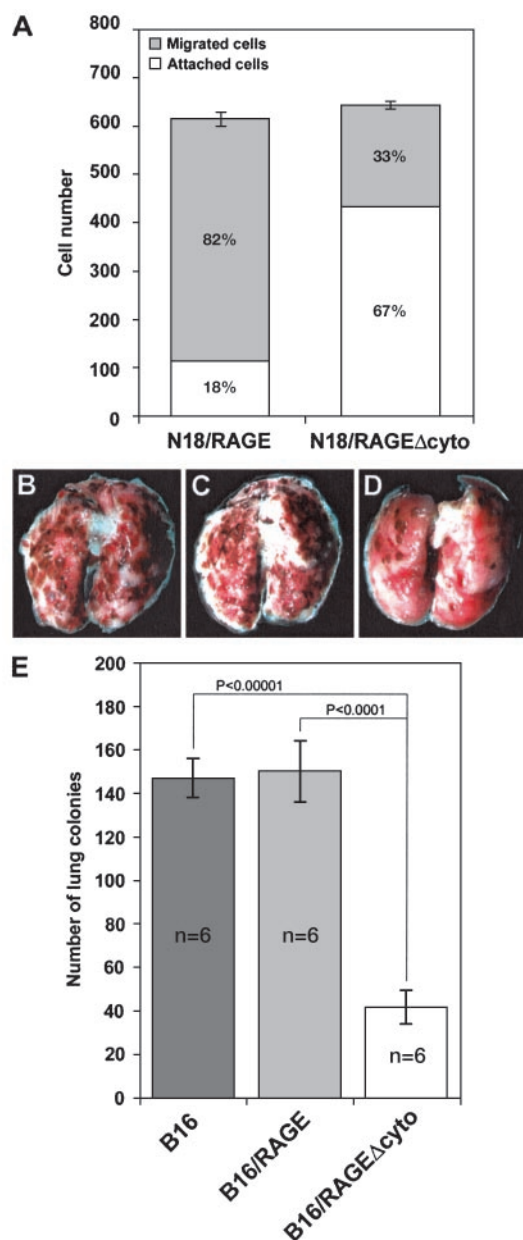


Fig. 4. Blockade of RAGE signaling inhibits transendothelial migration and metastasis of tumor cells. A, N18/RAGE and N18/RAGE Δcyto were tested in the transendothelial migration assay as described in Fig. 3. The proportion of cells that migrated through the endothelial cell monolayer and the proportion of nonmigrated cells attached to the upper surface of the endothelial cell layer are shown for both cell lines. B–E, parental B16-F1 melanoma cells (B) and stably transfected B16-F1 cells expressing either RAGE (B16/RAGE; C) or the cytoplasmic domain deletion mutant of RAGE (B16/RAGE Δcyto ; D) were injected (3×10^5 cells in 250 μl) into the tail veins of C57BL/6-*scid-beige* mice. After 2 weeks, the animals were sacrificed, and the lungs were analyzed for metastatic foci. E, the number of lung colonies \pm SD is shown.

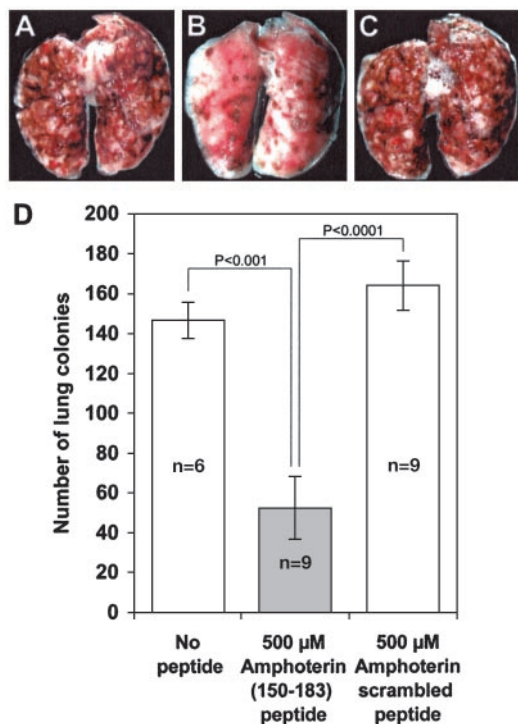


Fig. 5. Effect of amphoterin 150–183 peptide on formation of lung colonies in a pulmonary metastasis assay. B16-F1 melanoma cells (3×10^5 cells in $250 \mu\text{l}$) were preincubated with the peptides (A, without peptide; B, with amphoterin 150–183; C, with amphoterin 150–183, scrambled sequence) in a serum-free medium for 30 min before injection into the tail veins of C57BL/6-*scid-beige* mice. After 2 weeks, the animals were sacrificed, and the lungs were analyzed for metastatic foci. D, the number of lung colonies \pm SD is shown.

weeks, lung colonies can be easily detected because the B16-F1 cell line is pigmented and has been selected on the basis of almost exclusive formation of lung metastasis.

When control B16-F1 cells were injected i.v. to mice, approximately 150 lung colonies formed during the 2-week period (Fig. 4, B and E). Mice receiving injection of B16/RAGE Δ cyto cells displayed 71.6% fewer lung colonies than mice receiving injection of parental B16-F1 cells (Fig. 4, D and E), suggesting that blocking RAGE signaling can efficiently inhibit formation of tumor metastasis. Remarkably, competition of ligand binding to RAGE by injection of cells together with 500 μM amphoterin 150–183 peptide decreased the number of lung colonies by 64.3% (Fig. 5, B and D). Importantly, roughly 160 lung colonies were detected when cells were injected together with 500 μM scrambled control peptide (Fig. 5, C and D). These results show that inhibition of RAGE signaling is an effective way to suppress invasive migration and that the inhibitory activity of amphoterin 150–183 peptide on invasive migration can be extended to an *in vivo* situation.

The COOH-terminal RAGE-binding Motif of Amphoterin Shares Sequence Homology with S100 Proteins. Amphoterin is an extremely well-conserved protein between species, especially between vertebrates. For example, region 141–188 of amphoterin is identical in human, rat, mouse, and bovine sequences. In addition to amphoterin, RAGE is known to serve as a receptor for members of the S100 protein family (15, 16), advanced glycation end products (5), and various amyloids [amyloid- β peptide and amyloid A (17, 25)]. Because both amphoterin and S100 proteins are small RAGE-binding proteins with α -helical secondary structures, we did a sequence comparison to look for similarities between the newly identified COOH-terminal RAGE-binding region of amphoterin and S100 proteins. Multiple alignment of amphoterin residues 141–193 with various

S100 proteins (Fig. 6) shows that the RAGE-binding region in amphoterin has remarkable resemblance with the NH₂ termini of S100 proteins.

DISCUSSION

Tumor cell invasion in general may be a deregulated form of a physiological invasion process required for neuronal wiring in the embryo because many mechanistic features seem to be common for these processes. Both amphoterin and RAGE seem to be involved in regulation of neurite outgrowth of embryonic neurons and invasive migration of tumor cells (reviewed in Refs. 26 and 27). The present study demonstrates that amphoterin can bind to RAGE through a COOH-terminal motif spanning residues 150–183 and that this part of amphoterin can be used to inhibit amphoterin-RAGE interaction-mediated invasive migration both *in vitro* and *in vivo*. Interestingly, a heparin binding consensus sequence can be found in the NH₂ terminus of amphoterin (residues 5–11; Ref. 28). Thus, it is possible that the NH₂ terminus of amphoterin is responsible for proteoglycan binding, whereas the COOH terminus of amphoterin binds to RAGE.

Our results show that the RAGE-binding COOH-terminal motif of amphoterin is sufficient to block transendothelial migration of tumor cells. However, the exact mechanism of RAGE-mediated transendothelial migration is not fully understood. Because RAGE is expressed in endothelial cells (29), it is likely that endothelial RAGE is involved in the transmigration process. In fact, it has been shown that inhibition of endothelial RAGE inhibits amyloid- β peptide-induced migration of monocytes across brain endothelial cells (30). It seems that RAGE activation in the migrating cells is important for invasion, provided that they express RAGE. Several reports have shown that the metastatic potential of tumor cells correlates with the expression level of RAGE (2, 10, 11). Interestingly, our results show that tumor cells expressing a signaling-incapable, cytoplasmic domain deletion mutant of RAGE adhere normally to endothelial cells but are unable to migrate through the endothelial cell monolayer. Furthermore, cells expressing the cytoplasmic domain deletion mutant of RAGE formed 70% fewer lung colonies in the pulmonary metastasis assay.

It is interesting to note that the RAGE-binding COOH-terminal region of amphoterin shares sequence homology with the NH₂ termini of S100 proteins. Structures of several S100 proteins have been resolved, whereas only separate structures of the two HMG boxes of amphoterin are currently known (21, 22). Our homology modeling studies of amphoterin 150–183 with S100A12 (31) and S100B (32) suggest that residues 173–183 of amphoterin could form a seventh α -helix (data not shown) and that, together with the third α -helix of HMG box 2 (residues 142–161), this α -helix could form a helix-loop-helix structure resembling the first EF-hand of the S100 proteins (as illustrated in Fig. 6). It seems likely that the RAGE-binding peptides form a structural motif that is a prerequisite for RAGE binding because two shorter amphoterin peptides (residues 162–177 and 160–183) had greatly reduced activity in our bioassays (data not shown). Interestingly, our preliminary results with the EN-RAGE (S100A12) 3–39 peptide suggest that this peptide possesses even stronger anti-invasive capacity than the amphoterin 150–183 peptide (data not shown), supporting our working hypothesis.

Our present results show that intervening with the amphoterin-RAGE interaction using a synthetic peptide can efficiently suppress formation of metastasis in an *in vivo* model. Blocking amphoterin-RAGE interaction might be an efficient way to inhibit tumor cell invasion for several reasons. Ligation of RAGE is known to activate multiple signaling pathways, including Ras-extracellular signal-regulated kinase 1/2 (6), Cdc42/Rac (7), stress-activated protein kinase/c-Jun-NH₂-terminal kinase, and p38 mitogen-activated protein kinase

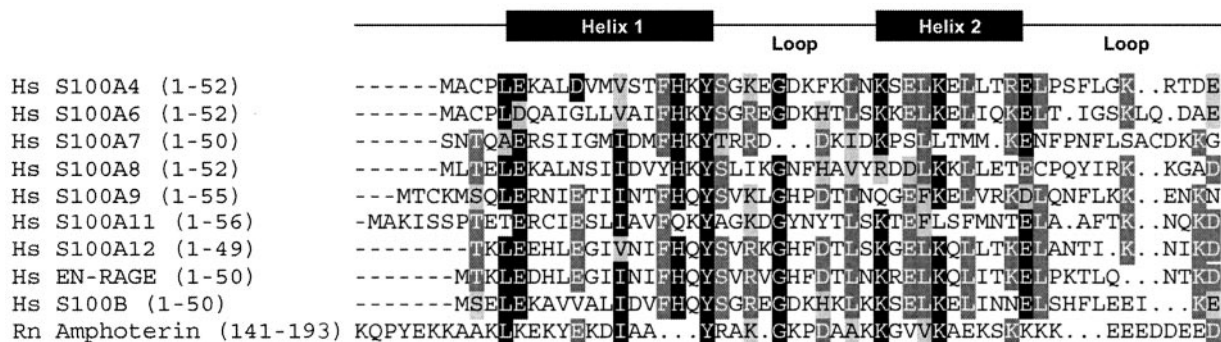


Fig. 6. Sequence comparison of amphoterin COOH terminus with NH₂ termini of S100 family proteins. Alignment of amino acids 141–193 of amphoterin with the first ~50 NH₂-terminal amino acids of nine S100 family proteins is shown. Amino acids that are identical in 80% of the sequences are shown in *black*, and amino acids that are identical in 50% of the sequences are shown in *dark gray*. Amino acids that are similar by nature are shown in *light gray*. Schematic illustration of the helix-loop-helix secondary structure of the first EF-hand of S100 proteins is depicted *above* the sequence alignment.

pathways (2), that regulate both cytoskeletal organization and gene expression required for cell motility. RAGE signaling has been demonstrated to activate several transcription factors, including nuclear factor κ B, Sp1, and cAMP-responsive element binding protein (25, 33).⁴ Interestingly, RAGE has been shown to promote cell survival through increased expression of the antiapoptotic protein Bcl-2 (16). Because there is an inverse correlation between apoptosis and metastatic potential of tumor cells (34), activation of RAGE might augment metastasis also through promoting cell survival. In addition to promoting the invasive phenotype through cytoskeletal and transcriptional regulation, amphoterin-RAGE signaling is linked to local proteolytic activation, as might be expected for a mechanism mediating invasive migration. The activation of plasminogen through interaction with amphoterin might directly promote degradation of surrounding extracellular matrix (3) but might also activate other proteolytic mechanisms, such as MMPs (2).

The results presented in this study provide support for amphoterin-RAGE interaction being an attractive target for therapeutic intervention in antitumor strategies. Therapies based on inhibition of tumor cell invasion may be eventually developed to complement other forms of cancer treatment. Such anti-invasion therapy might be especially valuable in the case of brain tumors that characteristically destroy surrounding tissue by local invasion.

ACKNOWLEDGMENTS

The excellent technical assistance of Nina Nevalainen and Eeva-Liisa Saarikalle is gratefully acknowledged.

REFERENCES

- Hanahan, D., and Weinberg, R. A. The hallmarks of cancer. *Cell*, *100*: 57–70, 2000.
- Taguchi, A., Blood, D. C., Del Toro, G., Canet, A., Lee, D. C., Qu, W., Tanji, N., Lu, Y., Lalla, E., Fu, C., Hofmann, M. A., Kislinger, T., Ingram, M., Lu, A., Tanaka, H., Hori, O., Ogawa, S., Stern, D. M., and Schmidt, A. M. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature (Lond.)*, *405*: 354–360, 2000.
- Parkkinen, J., Raulo, E., Merenmies, J., Nolo, R., Kajander, E. O., Baumann, M., and Rauvala, H. Amphoterin, the 30-kDa protein in a family of HMGI-type polypeptides. Enhanced expression in transformed cells, leading edge localization, and interactions with plasminogen activation. *J. Biol. Chem.*, *268*: 19726–19738, 1993.
- Fages, C., Nolo, R., Huttunen, H. J., Eskelinen, E., and Rauvala, H. Regulation of cell migration by amphoterin. *J. Cell Sci.*, *113*: 611–620, 2000.
- Neeper, M., Schmidt, A. M., Brett, J., Yan, S. D., Wang, F., Pan, Y. C., Elliston, K., Stern, D., and Shaw, A. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J. Biol. Chem.*, *267*: 14998–15004, 1992.
- Lander, H. M., Taurus, J. M., Ogiste, J. S., Hori, O., Moss, R. A., and Schmidt, A. M. Activation of the receptor for advanced glycation end products triggers a p21^{ras}-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. *J. Biol. Chem.*, *272*: 17810–17814, 1997.
- Huttunen, H. J., Fages, C., and Rauvala, H. Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF- κ B require the cytoplasmic domain of the receptor but different downstream signaling pathways. *J. Biol. Chem.*, *274*: 19919–19924, 1999.
- Schmidt, A. M., Hori, O., Chen, J. X., Li, J. F., Crandall, J., Zhang, J., Cao, R., Yan, S. D., Brett, J., and Stern, D. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J. Clin. Invest.*, *96*: 1395–1403, 1995.
- Bierhaus, A., Illmer, T., Kasper, M., Luther, T., Quehenberger, P., Tritschler, H., Wahl, P., Ziegler, R., Muller, M., and Nawroth, P. P. Advanced glycation end product (AGE)-mediated induction of tissue factor in cultured endothelial cells is dependent on RAGE. *Circulation*, *96*: 2262–2271, 1997.
- Takada, M., Koizumi, T., Toyama, H., Suzuki, Y., and Kuroda, Y. Differential expression of RAGE in human pancreatic carcinoma cells. *Hepatogastroenterology*, *48*: 1577–1578, 2001.
- Kuniyasu, H., Oue, N., Wakikawa, A., Shigeishi, H., Matsutani, N., Kuraoka, K., Ito, R., Yokozaki, H., and Yasui, W. Expression of receptors for advanced glycation end-products (RAGE) is closely associated with the invasive and metastatic activity of gastric cancer. *J. Pathol.*, *196*: 163–170, 2002.
- Li, J., and Schmidt, A. M. Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. *J. Biol. Chem.*, *272*: 16498–16506, 1997.
- Salmivirta, M., Rauvala, H., Elenius, K., and Jalkanen, M. Neurite growth-promoting protein (amphoterin, p30) binds syndecan. *Exp. Cell Res.*, *200*: 444–451, 1992.
- Milev, P., Chiba, A., Haring, M., Rauvala, H., Schachner, M., Ranscht, B., Margolis, R. K., and Margolis, R. U. High affinity binding and overlapping localization of neurocan and phosphacan/protein-tyrosine phosphatase- ζ/β with tenascin-R, amphoterin, and the heparin-binding growth-associated molecule. *J. Biol. Chem.*, *273*: 6998–7005, 1998.
- Hofmann, M. A., Drury, S., Fu, C., Qu, W., Taguchi, A., Lu, Y., Avila, C., Kambham, N., Bierhaus, A., Nawroth, P., Neurath, M. F., Slattery, T., Beach, D., McClary, J., Nagashima, M., Morser, J., Stern, D., and Schmidt, A. M. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell*, *97*: 889–901, 1999.
- Huttunen, H. J., Kuja-Panula, J., Sorci, G., Agnelli, A. L., Donato, R., and Rauvala, H. Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. *J. Biol. Chem.*, *275*: 40096–40105, 2000.
- Yan, S. D., Chen, X., Fu, J., Chen, M., Zhu, H., Roher, A., Slattery, T., Zhao, L., Nagashima, M., Morser, J., Migheli, A., Nawroth, P., Stern, D., and Schmidt, A. M. RAGE and amyloid- β peptide neurotoxicity in Alzheimer's disease. *Nature (Lond.)*, *382*: 685–691, 1996.
- Yan, S. D., Zhu, H., Zhu, A., Golabek, A., Du, H., Roher, A., Yu, J., Soto, C., Schmidt, A. M., Stern, D., and Kindy, M. Receptor-dependent cell stress and amyloid accumulation in systemic amyloidosis. *Nat. Med.*, *6*: 643–651, 2000.
- Genetics Computer Group. Program Manual for the Wisconsin Package, Version 10.2. Madison, WI, 2001.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, *25*: 4876–4882, 1997.
- Read, C. M., Cary, P. D., Crane-Robinson, C., Driscoll, P. C., and Norman, D. G. Solution structure of a DNA-binding domain from HMGI. *Nucleic Acids Res.*, *21*: 3427–3436, 1993.
- Weir, H. M., Kraulis, P. J., Hill, C. S., Raine, A. R., Laue, E. D., and Thomas, J. O. Structure of the HMG box motif in the B-domain of HMGI. *EMBO J.*, *12*: 1311–1319, 1993.

⁴ H. J. Huttunen, J. Kuja-Panula, and H. Rauvala. RAGE signaling induces CREB-dependent chromogranin expression during neuronal differentiation, submitted for publication.

23. Humphries, M. J., Olden, K., and Yamada, K. M. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. *Science (Wash. DC)*, *233*: 467–470, 1986.
24. Ohyama, C., Tsuboi, S., and Fukuda, M. Dual roles of sialyl Lewis X oligosaccharides in tumor metastasis and rejection by natural killer cells. *EMBO J.*, *18*: 1516–1525, 1999.
25. Yan, S. D., Schmidt, A. M., Anderson, G. M., Zhang, J., Brett, J., Zou, Y. S., Pinsky, D., and Stern, D. Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J. Biol. Chem.*, *269*: 9889–9897, 1994.
26. Huttunen, H. J., and Rauvala, H. Common mechanistic features in cell-extracellular matrix interactions regulating neurite outgrowth and tumor cell invasion. *In*: J. Heino and V-M. Kahari (eds.), *Cell Invasion*, pp. 108–120. Austin, TX: Eurekah.com/Landes Bioscience, 2002.
27. Schmidt, A. M., Yan, S. D., Yan, S. F., and Stern, D. M. The biology of the receptor for advanced glycation end products and its ligands. *Biochim. Biophys. Acta*, *1498*: 99–111, 2000.
28. Merenmies, J., Pihlaskari, R., Laitinen, J., Wartiovaara, J., and Rauvala, H. 30-kDa heparin-binding protein of brain (amphoterin) involved in neurite outgrowth. Amino acid sequence and localization in the filopodia of the advancing plasma membrane. *J. Biol. Chem.*, *266*: 16722–16729, 1991.
29. Brett, J., Schmidt, A. M., Yan, S. D., Zou, Y. S., Weidman, E., Pinsky, D., Nowygrod, R., Neeper, M., Przysiecki, C., and Shaw, A. Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. *Am. J. Pathol.*, *143*: 1699–1712, 1993.
30. Giri, R., Shen, Y., Stins, M., Du, Y. S., Schmidt, A. M., Stern, D., Kim, K. S., Zlokovic, B., and Kalra, V. K. β -Amyloid-induced migration of monocytes across human brain endothelial cells involves RAGE and PECAM-1. *Am. J. Physiol. Cell Physiol.*, *279*: C1772–C1781, 2000.
31. Moroz, O. V., Antson, A. A., Dodson, G. G., Wilson, K. S., Skibshoj, I., Lukanidin, E. M., and Bronstein, I. B. Crystallization and preliminary X-ray diffraction analysis of human calcium-binding protein S100A12. *Acta Crystallogr. Sect. D Biol. Crystallogr.*, *56*: 189–191, 2000.
32. Drohat, A. C., Baldisseri, D. M., Rustandi, R. R., and Weber, D. J. Solution structure of calcium-bound rat S100B($\beta\beta$) as determined by nuclear magnetic resonance spectroscopy. *Biochemistry*, *37*: 2729–2740, 1998.
33. Li, J. F., Qu, X. Q., and Schmidt, A. M. Sp1-binding elements in the promoter of RAGE are essential for amphoterin-mediated gene expression in cultured neuroblastoma cells. *J. Biol. Chem.*, *273*: 30870–30878, 1998.
34. Wong, C. W., Lee, A., Shientag, L., Yu, J., Dong, Y., Kao, G., Al-Mehdi, A. B., Bernhard, E. J., and Muschel, R. J. Apoptosis: an early event in metastatic inefficiency. *Cancer Res.*, *61*: 333–338, 2001.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Receptor for Advanced Glycation End Products-binding COOH-terminal Motif of Amphoterin Inhibits Invasive Migration and Metastasis

Henri J. Huttunen, Carole Fages, Juha Kuja-Panula, et al.

Cancer Res 2002;62:4805-4811.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/62/16/4805>

Cited articles This article cites 31 articles, 15 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/62/16/4805.full#ref-list-1>

Citing articles This article has been cited by 32 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/62/16/4805.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/62/16/4805>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.