Regulation of monocyte migration by amphoterin (HMGB1)

Ari Rouhiainen, Juha Kuja-Panula, Erika Wilkman, Jukka Pakkanen, Jan Stenfors, Raimo K. Tuominen, Mauri Lepäntalo, Olli Carpeñ, Jaakko Parkkinen, and Heikki Rauvala

Amphoterin (HMGB1) is a 30-kD heparin-binding protein involved in process extension and migration of cells by a mechanism involving the receptor for advanced glycation end products (RAGE). High levels of amphoterin are released to serum during septic shock. We have studied the expression of amphoterin in monocytes and the role of amphoterin and RAGE in monocyte transendothelial migration. Unactivated monocytes in suspension did not reveal amphoterin on their surface, but adherent monocytes exported amphoterin to the cell surface. Immunohistochemical staining of arterial thrombi in vivo revealed amphoterin in mononuclear cells and in surrounding extracellular matrix. Amphoterin was secreted from phorbol ester and interferon-γ (IFN-γ)-activated macrophages, and the secretion was inhibited by blocking the adenosine 5′-triphosphate (ATP)–binding cassette transporter-1, a member of the multidrug resistance protein family. Amphoterin was specifically adhesive for monocytes in peripheral blood leukocyte adhesion assay. Adhesion caused an extensive spreading of cells, which was inhibited by the dominant-negative RAGE receptor (soluble ectodomain of RAGE), and adhesion up-regulated chromogranin expression in monocytes, also suggesting a RAGE-dependent interaction. Monocyte transendothelial migration was efficiently inhibited by anti-amphoterin and anti-RAGE antibodies and by the soluble RAGE. We suggest that amphoterin is an autocrine/paracrine regulator of monocyte invasion through the endothelium.

Introduction

Circulating monocytes adhere to sites of vascular injury where they participate together with other cells in the regulation of blood clotting, inflammation, and wound healing. Adhesion to other cells and extracellular matrix components is a prerequisite for migration and tissue recruitment of monocytes.1,2 The knowledge of molecules involved in monocyte transendothelial migration is rapidly increasing. However, the overall picture of the transendothelial migration mechanism is not completely understood.2

Amphoterin is a 30-kD heparin-binding protein widely expressed in humans and other organisms, and it is abundantly expressed in the developing brain as well as in various immature and transformed cell lines.3,4 It was isolated as an extracellular neurite outgrowth-promoting protein, but its amino acid sequence turned out to be identical to high-mobility group–1 protein.5,7 In a new nomenclature of high-mobility group proteins amphoterin and other proteins identical in the cDNA sequence are called as HMGB1 (high-mobility group B-1).8 We have used the designation amphoterin for the protein occurring in the extracellular space and interacting with the cell surface.5

Surface-bound amphoterin is adhesive for neural cells and platelets, and it induces extension of membrane processes in adherent cells.3,9,10 Amphoterin binds to plasma membrane lipids, mainly to phosphatidylserine and sulfatide, and enhances and localizes plasminogen activation.5,9,11-13 In neurons, neurite outgrowth on amphoterin surface is mediated by receptor for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily transmembrane receptors.14,15 Consistent with a potential role of RAGE-amphoterin interaction, RAGE and amphoterin were shown to colocalize during brain development, and their signaling induces neurite outgrowth by way of the guanosine triphosphatases (GTPases) rac and Cdc42 and up-regulates expression of chromogranins.14-16 Neurite outgrowth and tumor cell migration can be inhibited with sRAGE (soluble RAGE), antibodies against amphoterin, and by antisense oligonucleotides transfected into cells.14,17,18

Amphoterin can be detected on the cell surface and substratum-attached material under cell culture conditions and in vivo.4,6,9,19-21 Because amphoterin lacks a classical signal sequence, its export mechanism is currently poorly understood. It has been shown that amphoterin is secreted by way of an endolysosomal vesicle-mediated pathway and not by way of the classical endoplasmic reticulum (ER)/Golgi-mediated pathway.22,23 Amphoterin resembles in this respect other proteins, which lack a classical signal sequence but are considered to have an extracellular function, like basic fibroblast growth factor and interleukin-1β (IL-1β).24

Macrophages and monocytes have been shown to secrete amphoterin after induction with cytokines or endotoxin, and high amounts of amphoterin have been detected in sera from patients...
with endotoxic or hemorrhagic shock. Exogenous amphoterin causes cultured human umbilical vein endothelial cell (HUVEC) activation, inducing intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin up-regulation, and increased adhesion of neutrophils, which can be partly inhibited by anti-RAGE antibodies.

Although recent studies have highlighted the role of amphoterin/ HMGBl protein as a monocyte-activating molecule, the physiological function of amphoterin/HMGBl in monocyte biology is not fully understood. Because adhesive interactions, motility, and plasminogen activation are important for the physiologic function of monocytes and macrophages, we have studied the expression of amphoterin and its receptors in monocyte/macrophages. Further, we have studied mechanism of amphoterin secretion and the role of extracellular amphoterin and RAGE in transendothelial migration of monocytes. Our results suggest that amphoterin is secreted by way of a pathway requiring a multidrug resistance protein, and amphoterin-RAGE interactions play an essential role in the migration of monocytes through the endothelium.

Materials and methods

Materials

Recombinant amphoterin (recAtn), affinity-purified polyclonal anti-recombinant amphoterin (anti-recAtn), and antipeptide antibodies I to V were produced as described before. The recAtn preparations did not contain detectable endotoxin (< 0.05 IU/μg) as studied by the Luminex Amelobocyte Lysate assay Pyrogen (BioWhittaker, Walkersville, MD). Anti-Atn was produced in chicken with use of recAtn (AgriSera AB, Vännäs, Sweden). Immunoglobulin Y (IgY) was isolated, and antibodies were affinity purified with use of a recAtn column. Ficoll-Hypaque and Dextran T 500 were from Pharmacia Biotech (Uppsala, Sweden). Alkaline phosphatase–conjugated (AP) rabbit antichicken and goat antitumor immunoglobulins, antiproin, low-molecular-weight (LMW) heparin, heparin, bovine serum albumin (BSA), poly-L-lysine, sulphobromophthalein (BSP), 4-phorbol-12-myristate-13 acetate (PMA), 4,4′-diiodohexanoanilinephthalein-2,2′-disulfonic acid (DIDS), and formyl-methionyl-leucyl-phenylalanine (FMLP) were from Sigma (St Louis, MO). Glyburide reagents were from Sigma and ICN Biomedicals (Aurora, OH). Fibronection was from Sigma and Alexis Corporation (Läufel-ingen, Switzerland). Vitronectin was from Alexis Corporation. Alkaline phosphatase–conjugated swine antirabbit and the rhodamine isothiocyanate (TRITC)–conjugated antimouse antibodies were from Dakopatts (Glostrup, Denmark). Horseradish peroxidase (HRP)–conjugated goat antirabbit IgG and rabbit antigo IgG were from Bio-Rad (Heracles, CA). HRP–conjugated rabbit antichicken IgY was from Zymed (San Francisco, CA). Mouse monoclonal anti-CD14 was from CLB (Amsterdam, the Netherlands). Fluorescein isothiocyanate (FITC)–conjugated antichicken was from Jackson Immuno Research (West Grove, PA). Goat anti-RAGE antisera was from Chemicon International (Temecula, CA). Human plasma albumin (HSA) was from the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland). Bovine lung soluble RAGE was purified as described by using hydroxylapatite-, heparin-Sepharose, Mono S, and gel filtration chromatographies. Anti-P300 and anti-P301 were produced as described.

RAGE-N16 was from Santa Cruz Biotechnology (Santa Cruz, CA). Interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) were from Roche (Basel, Switzerland).

Production of recombinant fusion proteins

The entire extracellular coding region of mouse RAGE was amplified by using mouse RAGE specific primers (5′ primer, 5′-TACTAGCTAGCCCGAATTTGGAAGACCCATTG-3′; 3′ primer, 5′-ATAGTTTAGAAGC- CGCCACAGCTCAACCCACAGG-3′), and the RAGE V1 domain was amplified with use of human RAGE specific primers (5′ primer, 5′-TACATGCTAGCCCGAATTTGGAAGACCCATTG-3′; 3′ primer, 5′-ATAGTTTAGAAGC-GCCACAGCTCAACCCACAGG-3′).

Cells

RAW264.7, U937, K562, and HL-60 cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD), and cells were grown according to ATCC’s guidelines. HUVECs were obtained from BioWhittaker and grown as described.

Rat leukocytes were isolated from blood in ethylenediaminetetraacetic acid (EDTA) by Ficoll-Hypaque centrifugation for 30 minutes at 500g. Glial cells were isolated from postnatal rat brain (P5-7) hypothalamus and midbrain areas as described previously. The cells were cultured for 2 to 6 weeks, and microglia was isolated by using extensive overnight shaking. Detached microglia was used for protein or RNA isolations or cell-spreading assays within 24 hours.

For immunofluorescence staining assays monocytes were isolated from whole blood oruffy coats with use of the Nycoprep 1.068 (Nycomed Pharma AS, Oslo, Norway) density centrifugation method or by dextran sedimentation (0.55% [wt/vol] dextran for 0.5 hours) to obtain leukocyte-rich plasma. The amount of platelets was minimized by centrifuging the whole blood or buffy coats with use of the Nycoprep 1.068 (Nycomed Pharma AS, Oslo, Norway) density centrifugation method or by dextran sedimentation (0.55% [wt/vol] dextran for 0.5 hours) to obtain leukocyte-rich plasma. For immunostaining in vivo thrombi

This study was approved by an institutional ethical committee of the Department of Surgery, Helsinki University Central Hospital, and informed consent was obtained from the study subjects. Embolectomy samples were obtained from patients treated for an acute lower limb arterial occlusion at the Department of Vascular Surgery of Helsinki University Central Hospital. The embolectomy specimens were taken within 24 hours of the onset of the symptoms, snap-frozen, and stored under liquid nitrogen. Frozen sections were fixed with cold acetone (−20°C) for 10 minutes and
rinsed in PBS. The sections were incubated with affinity-purified anti-
recAtn or nonspecific chicken IgY, both at 3 μg/mL, and the bound 
antibodies were visualized with the ABC complex/HRP method (Dakopatts).

**Macrophage secretion assay**
Trypsinized RAW 264.7 cells in 10% fetal calf serum (FCS) RPMI were plated 
(1.5 × 10^6 cells/well) to 6-well plates (Corning, Corning, NY) for 1 hour. 
Nonadherent cells were washed away. The cells were cultured in 1.5 mL 
OPTI-MEM 1 (Invitrogen Corporation, Carlsbad, CA) containing 10 μg/mL 
aprotinin and possible activators and inhibitors. At different time points medium 
was collected, 1 mL of 10 μg/mL LMW heparin in PBS was added to the cells, 
incubated for 1 minute, and pooled to the medium. The medium was centrifuged 
for 5 minutes at 700g, and the supernatants were used for lactate dehydrogenase 
(LDH) and amphoterin quantifications. LDH activity was detected with Cytotox-
fi(LDH) and amphoterin quantified for 5 minutes at 700g.

**Inhibition of IL-1β secretion by ABC-1 inhibitors**
RAW 264.7 cells (1.5 × 10^6 cells/well) to 6-well plate were activated with 
INF-γ (20 ng/mL) for 3 hours. In some wells BP, DIDS, and glyburide were 
used as inhibitors. IL-1β content in the culture medium was analyzed with 
enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, 
MN) by using recombinant mouse IL-1β (Roche) as a standard. Amphoterin was detected by Western blotting. A 1.5-mL sample of the 
medium was concentrated with Microcon 10 (Millipore, Billerica, Spain), 
lyophilized, and analyzed with use of anti-recAtn Western blotting. Optical 
density of the amphoterin bands was analyzed by using Tina 2.0 software 
(Raytest Isotopenmessgerate GmbH, Staufenhardt, Germany).

**Cell adhesion ELISA**
The cell ELISA assay was performed as described before37,38 with the 
following modifications. Peripheral blood mononuclear cells in RPMI 
containing 0.1% HSA were incubated in the protein-coated microwells for 1 
hour. The microwells were washed, and the bound cells were fixed and 
blocked. The cells were incubated with anti-CD14, washed, and incubated 
with AFOS-conjugated antinitr for 1 hour. The color development with 
AFOS substrate was measured.

**Cell spreading studies**
Central areas of plastic plates PD-LD, 3.5 cm in diameter (Greiner Bio-One, 
Longwood, FL) were coated with 20 μg/mL recAtn or fibronectin for 2 
hours and blocked with 1% BSA-PBS. Monocytes were isolated by using 
RosetteSep-method (StemCell Technologies, Vancouver, Canada), and 
platelets were depleted with 10% BSA-PBS centrifugation. Monocytes or 
microglial cells were suspended in 0.1% BSA-RPMI and adhered for 1 
hour, and nonadherent cells were washed away. The cell area was measured 
by taking digital pictures (Olympus IX70 microscope, 40 x objective, 
DP-10 digital camera; Olympus Optical, Tokyo, Japan) from adherent cells, 
which were analyzed using BioRad Quantity One software (Bio-Rad).

**Expression of chromogranins**
Human peripheral blood monocytes (isolated with the RosetteSep-method) 
or rat brain microglia in OPTI-MEM I were adhered to recAtn or fibronectin 
(20 μg/mL) coated plastic. Expression of chromogranin cDNA amplifications 
was matched to different exons to avoid genomic DNA amplification. The primer sequences were as follows: 5’- CTCTTITCAGGATAGCACTGCTCA-3’ and 5’- CGCTGCTATATT- 
GAGGCCTCTC-3’ for human chromogranin B; 5’- AGGAAATATGCTGTG- 
GGAGCCCTCCT-3’ and 5’- CTGTTTGGTCAGACTGAGGGTCAT-3’ for human 
chromogranin C; 5’- CTGCTCTGGAATGCCCTATCAGTC- 
CAGT-3’ and 5’- TATCTTCTGCTTGGGACATTGTTGTT-3’ for rat 
chormogranin B; and 5’- CTTGAGGAGCCTCTACAAATAAAGA-3’ and 
5’- TTCACTGCTGAAACCAGCTAAGT-3’ for rat chromogranin C. 
Chromogranin B and C cDNAs were amplified by using program 
containing 45 cycles. cDNA content of rat samples was equalized 
according to RT-PRC results of porphobilinogen deaminase cDNA.40 All 
cDNA amplifications were made using DynaZyme DNA polymerase 
(Finnzymes, Espoo, Finland).

**Monocyte chemotaxis assay**
Human peripheral blood monocytes (5 × 10^6 cells; isolated with the 
RosetteSep-method) in 10% FCS-RPMI were placed to the upper compart-
ment of the Transwell chambers (3-μm pore size). ILMP (100 nM) or 
recAtn (40 nM) was added to the lower well when indicated, and the cells 
were allowed to migrate for 3 hours. Migrated cells in the lower wells were 
quantified by using CyQuant (Molecular Probes, Leiden, The Netherlands).

**Transendothelial migration**
HUVECs (5 × 10^5/well) were grown overnight on fibronectin-coated (50 
μg/mL) porous membranes in a Transwell chamber (Corning) of 6.5-mm 
diameter and 5-μm pore size and activated for 1 day with 0.5 ng/mL 
TNF-α.41 Unadherent HUVECs were washed away with PBS, and the 
Transwells were placed in RPMI 1640 + 10% FCS + 25 mM HEPES 
(N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), pH = 7.2. Mono-
cytes (5 × 10^6) in 100 μL medium, with inhibitors when indicated, 
were added to the upper chamber. In transendothelial chemotaxis assay serum-
free Dulbecco modified Eagle medium (DMEM) was used as a medium in 
both compartments, and fMPL was used as a positive control.45 After 
3 hours the plates were transferred to 4°C for 30 minutes, and the migrated 
cells were counted microscopically.

**Statistics**
P values were calculated using Student unpaired t test in Microsoft 
Excel2000 program (Microsoft Corporation, Redmond, WA).

**Results**

**Identification of amphoterin and RAGE**
Western blotting of cell lysates using affinity-purified anti-recAtn 
 antibodies revealed amphoterin in U937, HL-60, polymorphonuclear 
cells, and monocytes (Figure 1A). Amphoterin was detected in similar 
amounts in monocytes as in the immature myeloid cell lines HL-60 and 
U937 (Figure 1A). According to semiquantitative Western blotting 
amphoterin corresponded to 0.5% of total cellular protein in these cells 
and in K562 cells. In granulocytes the amount of amphoterin per total 
protein was about half of that in monocytes (data not shown). Thus, the 
proportion of amphoterin of total protein was about 50-fold higher in 
leukocytes than in platelets. No detectable amphoterin was present in 
erthrocytes (data not shown). The 30-kD protein in the monocyte lysate 
was also recognized with affinity-purified polyclonal antibodies raised 
against 5 synthetic peptides covering different parts of the amphoterin 
sequence (Figure 1B). All antibodies recognized the 30-kD band in the 
monocyte lysate (Figure 1B). Further, the 30-kD protein from the 
monocyte lysate bound to Heparin-Sepharose and eluted at a similar salt 
concentration (0.7-0.9 M NaCl) as the recombinant protein and was 
recognized by anti-recAtn (data not shown). Anti-recAtn and anti-
peptide II, which have been shown to detect only amphoterin and not 
any other highly homologous 28- to 29-kDa proteins, detected a 30-kD 
band in rat brain microglia lysate (Figure 1C). 

RAGE was detected in human and rat monocytes by immuno-
blotting of cells after adhesion to amphoterin. Functionality of the 
antibodies was tested by detecting sRAGE from bovine lung 
(Figure 1D). RAGE in human monocytes was detected with 
anti-RAGE and anti-P300 against human RAGE and with anti-
P301 in rat monocytes (Figure 1E). Anti-RAGE detected an 
additional band of 45-kD size (Figure 1E).
Figure 1. Western blotting analysis of amphoterin and RAGE. (A) Leukocytes were lysed with 1% SDS, and 10-μg samples of total cellular protein or 40 ng recAtn were run under reducing conditions on SDS-PAGE and transferred to a nitrocellulose filter. The filter was immunostained with anti-recAtn antibodies. PMN indicates polymorphonuclear leukocytes. (B) Monocyte lysates (40 μg protein) and recAtn (80 ng) were run in SDS-PAGE and transferred to nitrocellulose filters. The filters were immunostained with 5 affinity-purified antibodies raised against different amphoterin peptides (I-V). (C) Rat brain microglia lysates were analyzed in Western blotting with anti-recAtn and antipeptide II. Both antibodies recognized a single 30-kD band from the lysate. (D) sRAGE was purified from bovine lung acetone powder. Purified sRAGE migrated as a single band in 12% SDS-PAGE stained with Coomassie blue (lane i). sRAGE was detected by 4 different anti-RAGE antibodies in 10% to 20% SDS-PAGE and Western blotting experiment: anti-P300 (lane ii), anti-P301 (lane iii), anti-RAGE (lane iv), and anti-RAGE N-16 (lane v). (E) SDS-PAGE and Western blotting experiment: anti-P300 (lane ii), anti-recAtn and antipeptide II. Both antibodies recognized a single 30-kD band from the lysate. (F) sRAGE was purified from bovine lung acetone powder. Puriﬁed sRAGE recognized a single 30-kD band from the lysate. (G) sRAGE was puriﬁed and sRAGE migrated as a single band in 12% SDS-PAGE stained with Coomassie blue (lane i). sRAGE was detected by 4 different anti-RAGE antibodies in 10% to 20% SDS-PAGE and Western blotting experiment: anti-P300 (lane ii), anti-RAGE (lane iv), and anti-RAGE N-16 (lane v). (E) Western-blotting of monocyte RAGE. RAGE was detected from 1-hour amphoterin adherent human monocytes or rat leukocytes using anti-P300 (lane i), anti-RAGE (lane ii), or anti-P301 (lane iii). Lanes i-ii: human cells. Lane iii: rat cells.

Cell surface immunostaining of amphoterin in monocytes

The surface localization of amphoterin in monocytes was studied by double-immunofluorescence staining with anti-recAtn and anti-CD14 antibodies. When freshly isolated peripheral blood leukocytes were ﬁxed in solution, CD14 cells were not stained with anti-recAtn antibodies (Figure 2A-B). When the cells were kept on serum-coated coverslips for 1 hour, a minor proportion of the adherent cells were stained (data not shown), and after culture for 18 hours more than half of the CD14 cells were stained with anti-recAtn antibodies (Figure 2C-D). Staining was more intense for cells cultured for 18 hours than after 1-hour attachment (data not shown). Characteristic for the staining was the occurrence of 1 or 2 intense patches localized mainly at the edges of the cells and staining of cellular processes. Control stainings using anti-α-actinin or nonspeciﬁc chicken IgG antibodies conﬁrmed that the plasma membrane remained impermeable, and that the staining was speciﬁc for amphoterin (data not shown).

Expression of amphoterin in mononuclear cells and extracellular matrix of arterial thrombi

Because adhesion induced amphoterin secretion from monocytes, we studied amphoterin secretion in vivo using an easily available model of adherent monocytes. Frozen sections of arterial thrombi, which contained adherent monocytes, were immunostained with...
anti-amphoterin antibodies. The presence of amphoterin in monocellular cells in vivo was analyzed from thromboemboli obtained from lower limb arteries. Immunohistochemical staining of the thrombi demonstrated typical morphology with layers of tangled fibrin strands intermingled by platelet aggregates. Strong amphoterin reactivity was seen in monocellular cells visible among the fibrin strands (Figure 2E). Fainter extracellular reactivity was seen in regions populated by monocellular cells (Figure 2F-G). The staining pattern differed from that seen with the platelet-specific markers GP IIbIIIa and P-selectin which stained large platelet aggregates (Figure 2E). Measurement of amphoterin and fibronectin adherent monocellular areas. Monocytes were adhered to recAtn or fibronectin-coated plastic for 1 hour, and adherent cells were fixed. Digital microscopy pictures were taken, and cell areas were measured. Mean of areas was calculated. Amphoterin-adherent cells spread strongly compared with fibronectin-adherent cells, and spreading was inhibited with 100 ng/mL sAMIGO but not with amphoterin. Error bars represent ± SD (n = 3). (E) Peripheral blood monocellular cells in serum-free medium were incubated in microwells coated with recAtn, fibronectin (Fn), or albumin (BSA) for 1 hour, and the bound CD14+ cells were detected with anti-CD14 and ELISA. (F-G) Peripheral blood leukocytes were incubated on recAtn or Fn-coated microwells for 1 hour in serum-free medium, and the bound cells were immunostained with anti-CD14 and TRITC-labeled second antibody. The number of CD14+ cells (F) and their proportion of total cells (G) were determined by fluorescence and phase-contrast microscopy. The mean and SEM of 3 different experiments (E) or 12 fields of 2 different experiments (F-G) are shown.

**Figure 3. Adhesion and spreading of monocytes on amphoterin and extracellular matrix proteins.** (A-C) Peripheral blood leukocytes were kept on coverslips coated with vitronectin (A), fibronectin (B), or recAtn (G) for 1 hour in serum-free medium. The coverslips were washed, fixed with 2% paraformaldehyde, and immunostained with anti-CD14 and TRITC-labeled antimouse immunoglobulins. Scale bar (A-C) 10 μm. (D) Measurement of amphoterin and fibronectin adherent monocellular areas. Monocytes were adhered to recAtn or fibronectin-coated plastic for 1 hour, and adherent cells were fixed. Digital microscopy pictures were taken, and cell areas were measured. Mean of areas was calculated. Amphoterin-adherent cells spread strongly compared with fibronectin-adherent cells, and spreading was inhibited with 100 ng/mL sAMIGO but not with amphoterin. Error bars represent ± SD (n = 3). (E) Peripheral blood monocellular cells in serum-free medium were incubated in microwells coated with recAtn, fibronectin (Fn), or albumin (BSA) for 1 hour, and the bound CD14+ cells were detected with anti-CD14 and ELISA. (F-G) Peripheral blood leukocytes were incubated on recAtn or Fn-coated microwells for 1 hour in serum-free medium, and the bound cells were immunostained with anti-CD14 and TRITC-labeled second antibody. The number of CD14+ cells (F) and their proportion of total cells (G) were determined by fluorescence and phase-contrast microscopy. The mean and SEM of 3 different experiments (E) or 12 fields of 2 different experiments (F-G) are shown.

**Secretion of amphoterin from monocellular cells**

The mouse macrophage cell line RAW 264.7 secreted amphoterin after treatment with 20 ng/mL IFN-γ or 10 nM PMA. Secretion did not correlate to LDH-leakage (Figure 2H). Amphoterin secretion was dose dependently inhibited by the ABC-1 inhibitor DIDS (Figure 2H). At 100 μM concentration DIDS inhibited amphoterin secretion totally (Figure 2D). IL-1β secretion was as well, inhibited by DIDS (1.57 ± 0.03 ng/mL and 0.55 ± 0.28 ng/mL, noninhibited and 50 μM DIDS inhibited [± SD], respectively (n = 3, P = .034). Another ABC-1 inhibitor, glyburide, inhibited both amphoterin (Figure 2H) and IL-1β (1.23 ± 0.05 ng/mL, n = 3, P = .006) release. However, even at high concentrations of glyburide, inhibition of amphoterin release was only partial (Figure 2H). BSP inhibited IL-1β secretion at 300 μM concentration (0.54 ± 0.09 ng/mL, n = 3, P = .0007), but it did not inhibit amphoterin secretion (Figure 2H-I).

**Adhesion and spreading of monocytes to immobilized amphoterin**

Morphology of the CD14+ cells adhering to amphoterin-coated coverslips was strikingly different when compared with cells adhering to fibronectin- or vitronectin-coated coverslips. On the latter proteins, CD14+ cells exhibited little spreading during 1 hour (Figure 3A-B). In contrast, cells adhering to amphoterin demonstrated remarkably flattened morphology and large lamellipodia already after 1 hour (Figure 3C). In many cases the lamellipodia surrounded the cells in a halofish fashion. Staining with rhodamine-phalloidin revealed the presence of actin filaments in the cytoplasm of cells kept on amphoterin and fibronectin for 1 hour. The surface area of amphoterin-adherent cells was clearly larger than that of fibronectin-adherent cells (Figure 3C). sRAGE inhibited monocyte spreading on amphoterin, whereas the control immunoglobulin superfamily protein, sAMIGO, had no effect (Figure 3D). Rat brain microglial cells studied as a representative of tissue-derived macrophages did not display differential spreading on amphoterin and fibronectin (217 ± 69 versus 258 ± 63 mm² × 10⁻⁶ cellular area in cells adhering on amphoterin and fibronectin, respectively [mean ± SD], n = 3, P = NS).

When peripheral blood leukocytes were allowed to adhere to immobilized amphoterin, fibronectin, or albumin, CD14+ cells bound to a similar extent to microwells coated with amphoterin or fibronectin, whereas only low binding was observed to wells coated with albumin (Figure 3E). When the cells were kept for 1 hour on protein-coated coverslips, about 30% more CD14-expressing cells were seen on fibronectin-coated coverslips than on amphoterin-coated coverslips (Figure 3F). Interestingly, almost all cells adhering to recAtn were CD14+, whereas half of the cells adhering to fibronectin were CD14− (Figure 3G). The total number of adhered leukocytes was 2- to 3-fold higher on fibronectin than on amphoterin.

**Amphoterin-induced chromogranin B up-regulation**

Up-regulation of chromogranins has been identified as a hallmark of amphoterin-RAGE interactions in neural cells. Recently, Tasiemski et al showed that monocytes express mRNA coding for chromogranin B but not for chromogranin A. We, therefore, studied whether mononuclear cells also express chromogranin C (secretogranin II) and whether the expression of chromogranins is up-regulated by amphoterin. RT-PCR revealed a band of expected size for chromogranin C in both monocytes and microglial cells (Figure 4A-B). Chromogranin B expression in amphoterin adherent monocytes was not altered after 1 hour, but it was strongly up-regulated after 20 hours (Figure 4A-C). In addition, chromogranin B was up-regulated in amphoterin-adherent microglia (Figure 4B-C). The expression level of chromogranin B in fibronectin-adherent cells remained unaltered (Figure 4A-C). Chromogranin C mRNA was not up-regulated in monocytes or microglia during adhesion (Figure 4A-C).

**Amphoterin mediates transendothelial migration of monocytes**

Expression of amphoterin and RAGE in monocytes and the extensive cell spreading effect of surface-bound amphoterin on monocytes suggested that amphoterin mediates monocyte migration in an autocrine/paracrine manner. We, therefore, tested whether amphoterin and RAGE are involved in migration of monocytes across porous filters. Amphoterin was not chemotactic in migration assays across uncoated or endothelium-coated polycarbonate filters (Table 1).
The number of transmigrated cells was determined (n = 3). Amphoterin and fMLP concentrations in the polycarbonate filter assay were 40 nM and 100 nM, respectively. Cells were allowed to migrate for 3 hours, and the number of transmigrated cells was determined (n = 3).

Table 1. Amphoterin is not chemotactic for monocytes

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<tr>
<th>Condition</th>
<th>Endothelial cell monolayer, %</th>
<th>Polycarbonate filter, %</th>
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<tbody>
<tr>
<td>Noninduced</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Amphoterin</td>
<td>98.9 ± 15.9</td>
<td>88.9 ± 24.4</td>
</tr>
<tr>
<td>IMLP</td>
<td>681.2 ± 123.2*</td>
<td>152.0 ± 13.3*</td>
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Monocyte migration toward recAtm or IMLP was studied in Transwell chamber assay. The lower compartment containing chemotactic stimuli and the upper compartment containing monocytes were separated by either the endothelial monolayer-coated porous polycarbonate filter or uncoated porous polycarbonate filter. Amphoterin and IMLP concentrations in the polycarbonate filter assay were 40 nM and 100 nM, respectively. Cells were allowed to migrate for 3 hours, and the number of transmigrated cells was determined (n = 3).

However, transendothelial migration was significantly and dose dependently inhibited by anti-amphoterin and anti-RAGE antibodies and by sRAGE (Figure 5B-D). Furthermore, ligand-binding distal immunoglobulin domain (V1 domain) of RAGE significantly inhibited transendothelial migration of monocytes (Figure 5E). It, thus, appears that amphoterin/RAGE has an autocrine/paracrine role in transendothelial migration of monocytes rather than a chemotactic role.

![Image](https://example.com/image.png)
Discussion

In the present paper we show that amphoterin is secreted from mononuclear cells, specifically induces monocyte spreading as compared with peripheral leukocytes, and mediates transendothelial migration of monocytes. Amphoterin is one of at least 3 closely homologous proteins occurring in tissues.6 The same sequence as amphoterin has been cloned in studies of the DNA-binding HMGB1-protein.7 One highly homologous molecule existing in rat brain is HMGB2.48 The third member of the HMGB family, HMGB3, appears to be highly similar to rat brain heparin-binding protein P28 according to peptide sequences of digested fragments of P28 and results of Western blotting experiments with antipeptide antibodies.6,49 To discern amphoterin from the other homologous proteins, we have used affinity-purified antibodies against recombinant amphoterin and synthetic amphoterin peptides. In an earlier study we have demonstrated that amphoterin can be specifically recognized with these antibodies.6 The findings that all anti-amphoterin antibodies tested recognized the 30-kD monocyte protein and that the 30-kD heparin-binding protein was detected with anti-recAtn confirmed its identity with amphoterin.

The localization of amphoterin in the surrounding matrix of mononuclear cells observed in arterial thrombi suggests that amphoterin is exported from monocytes in vivo. This suggests that monocytes are at least one source of amphoterin in serum of septic patients. We have previously shown that platelets contain amphoterin mRNA and protein, and they release amphoterin to the extracellular space. However, the concentration of amphoterin in platelets is several fold lower than in monocytes. This may explain why platelet-rich areas in our staining of arterial thrombi were negative for amphoterin. Platelets have been shown to synthesize IL-1β from their mRNA during clot formation.50 Whether platelets use the same mechanism to synthesize new amphoterin polypeptide inside thrombi and, thus, enhance monocyte migration into the areas where platelet-rich fibrin clots are formed remains to be clarified.

Various cell-activating agents have been shown to induce amphoterin secretion in culture.19,23,25 However, nicotine and acetylcarnine may inhibit the release of proinflammatory cytokines, such as amphoterin/HMGB1, from macrophages expressing α-bungarotoxin-sensitive acetylcarnine receptors.51,52 In this study we show that in addition to IFN-γ and PMA activation, adhesion induces amphoterin secretion from monocytes. Inhibition of IFN-γ and PMA-induced secretion by DIDS suggests that ABC-1 is involved in the secretion process. However, another ABC-1 inhibitor, BSP, did not inhibit amphoterin secretion. Recently, Gardella et al25 showed that IL-1β and amphoterin/HMGB1 are not secreted by an identical mechanism. These results indicate some similarity, but not identity, in IL-1β and amphoterin secretion routes. Secretion of some other leaderless secreted proteins, annexin I and macrophage migration inhibitory factor, are inhibited with ABC-1 inhibitors.53,54 These data suggest that ABC-1 is involved in the secretion of some unrelated leaderless proteins.

The finding that monocytes but not other leukocytes adhered and rapidly spread on immobilized amphoterin suggests that extracellular amphoterin mediates cell-to-cell and cell-to-matrix interactions in monocytes. As the other leukocytes and tissue macrophages did not show corresponding adherence and spreading on amphoterin, this property appears to be specific for mature monocytes. However, in this study inability of rat brain microglia to spread on amphoterin could be due to the selection of poorly adherent microglia to the experiments, because microglia was isolated as a loosely adherent cell population detached by shaking. RAGE has been shown to mediate firm adhesion of monocytes to immobilized glycated albumin.55 Amphoterin-induced monocyte spreading that is inhibited by sRAGE suggests that RAGE is a monocyte receptor for amphoterin.

Our results show that anti-amphoterin and anti-RAGE antibodies and the dominant-negative RAGE receptor effectively inhibit transendothelial migration of monocytes. The finding that amphoterin is highly expressed in monocytes and also secreted by these cells suggests that amphoterin/RAGE belongs to the autocrine machinery enhancing monocyte migration rather than acting through a chemotactic mechanism, which inference is supported by our chemotaxis assays. Taken into account that HUVECs, in addition to monocytes, may release amphoterin, amphoterin-mediated transendothelial migration may also involve a paracrine interaction because of HUVEC-derived amphoterin.

The finding that the tissue-derived sRAGE is a more potent inhibitor than the recombinant sRAGE in the invasion assays may be due to different glycosylation of RAGE in tissue, which was recently shown to affect amphoterin binding.56 The strong inhibition hints at the possibility that blocking of amphoterin/RAGE interactions may inhibit some of the key regulators in the signaling cascade involved in transendothelial migration of monocytes. Rho family of small GTPases, including rac and Cdc42, is involved in RAGE-mediated amphoterin signaling.15 These GTPases are critical mediators of leukocyte invasion through the endothelium.15,60 This suggests that the inhibition mechanism of monocyte transendothelial migration by blocking amphoterin/RAGE may involve inhibition of the small GTPase signaling.

Anti-amphoterin antibodies and soluble fragments of RAGE cause a 60% to 70% inhibition of monocyte migration through endothelium. Furthermore, the anti-RAGE antibodies used in our study display some function-blocking activity. On the basis of these experiments the possibility still remains that other amphoterin-binding molecules in addition to RAGE, like proteoglycans11 or sulfoglycolipids,12 could play a role as amphoterin receptors in transendothelial migration.

The ability of amphoterin to bind plasminogen and effectively enhance its activation suggests that it may function as a nidus for plasminogen activation on the cell surface and enable directed proteolysis on the surface of monocyte/macrophages during cell migration and tissue repair. Expression of amphoterin at the cell surface of spreading monocytes is compatible with this inference.

Chromogranin B was found to be an amphoterin/RAGE-induced gene in differential display assays, and ligation of hippocampal neuron surface RAGE with amphoterin or with anti-RAGE antibodies up-regulated chromogranin B mRNA expression.16,34 In this study we found that this same gene is up-regulated during monocyte adhesion to amphoterin. This finding, supported by our other results, suggests that interactions of monocytes with amphoterin are mediated by RAGE. Induction of chromogranin B gene because of amphoterin interactions in monocytes suggests a possible role of amphoterin in innate immune response mediated by secretolytin which is an antibacterial peptide derived from the C-terminus of chromogranin B.62 In addition, chromogranin B has been shown to be a neurite outgrowth-enhancing heparin-binding protein.63 It might, thus, act as a cell migration and motility factor such as amphoterin.
Whether amphoterin induces release of chromogranin B or secretolytin from monocytes is not currently known.

In this study we show that both monocytes and microglia express RNA coding for chromogranin C. According to our knowledge this is the first time that chromogranin C is identified in mononuclear cells. Chromogranin C is a precursor for the monocyte migration-enhancing peptide secretoneurin. Expression of chromogranin C in monocytes and microglia brings out an interesting possibility that chromogranin C and peptides derived from it might act as endogenous migration-enhancing substances in monocytes and microglia.

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AMPHOTERIN MEDIATES MONOCYTE MIGRATION 1181

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Regulation of monocyte migration by amphoterin (HMGB1)

Ari Rouhiainen, Juha Kuja-Panula, Erika Wilkman, Jukka Pakkanen, Jan Stenfors, Raimo K. Tuominen, Mauri Lepäntalo, Olli Carpén, Jaakko Parkkinen and Heikki Rauvala