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S100A8/A9 at low concentration promotes tumor cell growth via RAGE ligation and MAP-kinase dependent pathway

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Abstract

The complex formed by two members of the S100 calcium-binding protein family, S100A8/A9, exerts apoptosis-inducing activity against various cells, especially tumor cells. Here, we present evidence that S100A8/A9 also has cell growth-promoting activity at low concentrations. Both RAGE gene silencing and co-treatment with a RAGE-specific blocking antibody revealed that this activity was mediated via RAGE ligation. In order to investigate the signaling pathways, MAP-kinase phosphorylation and NF- κ B activation was characterized in S100A8/A9 treated cells. S100A8/A9 caused a significant increase in p38-MAP and p44/42 kinase phosphorylation while the status of SAPK/JNK phosphorylation remained unchanged. Treatment of cells with S100A8/A9 also enhanced NF- κ B activation. RAGE siRNA pretreatment abrogated the S100A8/A9-induced NF- κ B activation. Our data indicate that S100A8/A9-promoted cell growth occurs through RAGE signaling and activation of NF- κ B.

Keywords

S100A8/A9; RAGE; NF- κ B; proliferation; MAP-Kinase

Introduction

The S100 protein family is a multigenic group of non-ubiquitous cytoplasmic EF-hand Ca^{2+} -binding proteins, which are expressed in a wide variety of cell types [1]. In recent years they have been linked to human pathologies due to their differential expression in chronic diseases and critical involvement in pivotal signal transduction pathways, including the receptor of

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advanced glycation end products (RAGE) [2]. An additional important indication for their involvement in inflammatory and neoplastic disorders is that most S100 genes are found near a break-point region on human chromosome 1q21, which if affected, is responsible for a number of genetic abnormalities related to autoimmune pathologies or cancer [3,4]. Although the function of S100 proteins in cancer cells in most cases is still unknown, the specific expression patterns of these proteins are a valuable prognostic tool [5].

Two S100 proteins, S100A8 and S100A9, have been linked to neoplastic disorders. Although they are predominantly expressed in myeloid cells, S100A8 and S100A9 are also found in the epidermis upon response to stress [6,7] and in several tumor cell types. Immunohistochemical investigations have shown that these proteins are expressed in hepatocellular carcinomas, pulmonary adenocarcinoma, and invasive ductal carcinomas of the breast [8-10]. In these tumors, elevated expression is correlated with poor differentiation. S100A8 and S100A9 are also found to be enriched in cystic fluid and serum of patients with ovarian cancer [11]. Furthermore, their expression is enhanced in gastric cancer [12]. In contrast, S100A8 and S100A9 are frequently down regulated in poorly differentiated esophageal squamous cell carcinomas [13,14].

Recently, we have reported a novel pro-apoptotic effect of the S100A8/A9 protein complex formed by the two calcium-binding proteins S100A8 and S100A9 [15]. The S100A8/A9 protein complex is released from activated phagocytes and exerts apoptosis-inducing activity through a dual mechanism: one associated with zinc extraction from the target cells, and the other through binding to the cell surface of the target cells, possibly via ligand-induced receptor activation. This finding is of great interest as S100A8 and S100A9 are abundant in cells of the innate immune system, and S100A8/A9-positive cells accumulate along the invasive margin of cancer [16].

Several members of the S100 protein family have been reported to bind to the receptor for advanced glycation end products (RAGE) [17-19]. RAGE is a multiligand receptor belonging to the immunoglobulin superfamily. It transduces inflammatory responses and the effects of neurotrophic and neurotoxic factors, plays a role in tumor growth [20,21], and as shown recently, is involved in the pathogenesis of several diseases, including neurodegeneration, inflammation and cancer [20,21]. Although direct interaction of S100 proteins with RAGE has been shown only for S100A12 (ENRAGE), S100B, S100A1, and S100P [17,19,22], it has been suggested that RAGE may serve as a common extracellular S100 receptor because the S100 proteins have common structural features and display sequence homology [17].

Huttunen et al. [18] communicated recently that nanomolar concentrations of S100B induce trophic effects in RAGE-expressing cells, whereas micromolar concentrations of S100B induce apoptosis in an oxidant-dependent manner. Therefore, we explored the effects of S100A8/A9 at low concentrations (<25 µg/ml) on tumor cells and signal transduction pathways. In this study we showed that S100A8/A9 also displays a bimodal function and its cell growth-promoting effect is mediated by RAGE-dependent signaling.

Materials and Methods

Materials and reagents

Cell culture media were purchased from either Sigma Co. (Canada, Oakville, ON) or Gibco (Canada). Cell culture plastic ware was obtained from Nunc Co. (Canada), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), BrdU incorporation ELISA kit from Roche Applied Science (Canada), Insulin-Transferrin-Selenium supplements (ITS) from Invitrogen (USA), rabbit polyclonal anti-human, -murine, and -rat RAGE from Abcam (Cambridge, MA, USA), U0126 and SB203580 from Cell Signaling (USA), FITC labeled

monoclonal 27E10 antibody to human S100A8/A9 from Acris (Germany), MAPK family antibody, sampler kit, and phospho-MAPK family antibody sampler kit from Cell Signaling (USA), anti-human, mouse, rat RAGE antibodies, human RAGE siRNA and siRNA negative control from Santa Cruz Biotechnologies (USA), anti-human RAGE antibody, goat anti-human RAGE blocking antibody from R&D Systems (Hornby, ON, CA), anti-human, rat-, murine-HMGB1 from Abcam (Cambridge, MA, USA), Transbinding NF- κ B assay kit from Panomics (Redwood City, CA, USA).

Purification of S100A8 and S100A9 from human neutrophils

Human neutrophils were prepared from leukocyte-rich blood fractions ("Buffy coat"). S100A8/A9 was purified as described earlier [23]. Prior to use, the proteins were rechromatographed by anion exchange using a UnoQ column (BioRad, Munich, Germany). Recombinant protein was produced by bacterial over-expression as previously described [24].

Cell culture

MCF-7 (human estrogen receptor positive breast cancer), MDA-MB231 (human estrogen receptor negative breast cancer), Jurkat (human T-cell leukemia), BJAB (murine B-cell leukemia), L929 (murine fibrosarcoma), HEK-293 (human embryonic kidney), SHEP and KELLY (human neuroblastoma) were cultured in either RPMI-1640 or DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were maintained under logarithmic growth conditions.

Cell proliferation assay (MTT- and BrdU assay)

Cells were starved in 1% ITS medium without FBS for three days. Then, various concentrations of S100A8/A9 were added for different time intervals as indicated, and cell viability was determined by MTT assay as previously described [10,11]. The percentage cell viability was calculated using the equation: (mean OD of treated cells/mean OD of control cells) $\times$ 100. For confirmation, BrdU incorporation ELISA assay was performed according to manufacturer's instruction.

Determination of S100A8/A9 binding sites on the cell surface

Harvested cells were washed three times with PBS containing 3% bovine serum albumin (BSA) and 0.05% sodium azide (NaN₃) (B-PBS). A total of 2×10^6 cells were incubated with 10 μ g of human S100A8/A9 for 1 h, washed three times with B-PBS, and then incubated for 30 min in the dark with 200 μ l FITC-labeled anti-S100A8/A9 antibody (1:50) (murine IgG1 clone 27E10) containing 20 μ g/ml propidium iodide in order to gate out dead cells. Finally, they were washed three times with B-PBS. In order to determine nonspecific binding of the FITC-labeled anti-S100A8/A9, the cells were incubated with FITC-labeled antibody in the absence of human S100A8/A9. The stained cells were analyzed on a FACS machine at an excitation wavelength of 488 nm. Automated analyses were performed using CellQuest Pro software.

Protease Protection Assay

Limited proteolysis was performed at room temperature on proteins dialyzed against 10 mM HEPES-NaOH, 75 mM NaCl at pH 7.0. The preparation of the tandem VC1 fragment of RAGE has been described previously [19]. For each experiment, 50–100 μ g of purified protein was incubated with chymotrypsin at an enzyme to protein ratio of 1:500 (w/w) in a volume of 100 μ l. The reaction was stopped at indicated time points by aliquoting 14 μ l of reaction mix into 14 μ l 2 $\times$ SDS loading buffer and heating at 90°C for 5 min. For the VC1-S100A8/A9 complex, S100A8/A9 was mixed with VC1 at a 2:1 molar stoichiometry and CaCl₂ was added to a final

concentration of 1 mM prior to the addition of enzyme. Control experiments with S100A8/A9 alone and VC1 were performed under identical conditions (i.e., in the presence of Ca^{2+}) [19].

Immunoblotting

RAGE expression was analyzed by Western blot. The same method [25] was used to detect p38 MAPK, p44/p42 MAPK, SAPK/JNK, phospho-p38 MAPK, phospho-p44/42 MAPK, and phospho-SAPK/JNK in MCF-7 and MDA-MB231 cells that had been treated with 10 $\mu\text{g}/\text{ml}$ S100A8/A9 for different time intervals. Cell lysates were prepared. Briefly, the harvested cells were washed once with cold PBS and re-suspended for 20 min on ice in a lysis buffer: 20 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 0.5 mM PMSF and 0.5% protease inhibitor cocktail (Sigma). The high-speed supernatant (10,000g) was collected. Proteins (30 μg) were separated by SDS-PAGE and then transferred onto nylon membranes. The membranes were blocked in 5% non-fat dried milk in Tris-buffered saline-Tween 1% (TBS) (0.05 M Trizma base, 0.9% NaCl and 1% Tween-20), then incubated overnight with the primary antibodies at 4°C. The membranes were then incubated at room temperature for 1 h with the relevant secondary antibodies conjugated with HRP, and developed by enhanced chemiluminescence (ECL) detection (Amersham-Pharmacia Biotech). In experiments detecting sRAGE and HMGB1, cells were treated with S100A8/A9 (15 $\mu\text{g}/\text{ml}$) for different time intervals as indicated. In other experiments, cells were treated with different concentration of S100A8/A9 (0, 5, 20, 25 $\mu\text{g}/\text{ml}$) for 36 h. The cell culture medium were collected and centrifuged at 10,000g for 10 min to precipitate the floating cells and cells debris. Then the supernatants were collected and centrifuged in 50 ml tubes which were equipped with filters that kept the protein over 15 kDa. Finally, protein concentration was measured by Bradford, and equal amounts of protein were subjected to SDS-PAGE followed by Western blotting.

RNA interference (RNAi)

The target siRNA for RAGE (sc-36374) and a negative control siRNA (sc-37007) with an irrelevant sequence were purchased from Santa Cruz Biotechnologies. The cells were grown to 60–80% confluence and then transfected with the siRNA duplex (final concentration 100 nM) using Lipofectamine (Invitrogen) according to the manufacturer's instructions. RAGE expression was determined by immunoblotting at 0, 24, 48 and 72 h post-transfection. The transfected cells (72 h post-transfection) were then treated with 5 and 10 $\mu\text{g}/\text{ml}$ S100A8/A9 for 48 h and cell proliferation was assessed by MTT-assays.

Blocking of RAGE with specific blocking antibody

Cells were grown in 96-well plates. After 72 h starvation in 1% ITS medium, they were treated with RAGE blocking antibody (166 $\mu\text{g}/\text{ml}$) for 1 h, then treated with S100A8/A9 (5 and 10 $\mu\text{g}/\text{ml}$) for another 24 h. Proliferation was assessed using MTT-assays.

Immunocytochemistry and confocal imaging

Cells were grown overnight on coverslips and then treated with 10 $\mu\text{g}/\text{ml}$ S100A8/A9. After 12 h, they were washed with PBS and fixed in 4% paraformaldehyde, then permeabilized with 0.1% Triton X-100. The cells were blocked with PBS containing 3% BSA (IgG and protease free). To locate either RAGE or NF- κB -p50, the cells were incubated with anti-RAGE rabbit IgG (1:200 dilution) and anti-NF κB -p50 goat IgG (1:100 dilution), respectively. After three washes with PBS, the RAGE and NF- κB -p50-antibody complexes were stained with the corresponding FITC (Sigma, 1:50 dilution) and Cy5-conjugated secondary antibodies (Sigma, 1:1,500 dilution) then washed three times with PBS. The fluorescent images were then observed and analyzed using an Olympus-FV500 multi-laser confocal microscope.

NF- κ B activation in MCF-7 and MDA-MB231 cells after treatment with S100A8/A9

Cells were cultured in 6-well plates and treated with S100A8/A9 (10 μ g/ml) for 8 h. The cells were scraped and the nuclear fractions were prepared. NF- κ B activation was analyzed using the Panomics Transbinding ELISA kit according to the manufactures' instructions

Statistical analysis

The results were expressed as means $\pm$ SD and statistical differences were evaluated by one-way and two-way ANOVA followed by Tukey's post-hoc test, using the software package SPSS 11. $P < 0.05$ was considered significant.

Results

S100A8/A9 at low micromolar concentrations promotes cell growth in different tumor cells

To investigate the putative cell growth-promoting activity of S100A8/A9, MCF-7, MDA-MB231 and SHEP cells were starved in ITS-containing medium for 72 h followed by treatment with increasing S100A8/A9 protein concentrations for different time intervals as indicated. Cell proliferation was determined by MTT assay (Fig. 1A-C).

As obvious from figure 1, S100A8/A9 exerts hypertrophic activity on MCF-7, MDA-MB231, and SHEP cell lines. S100A8/A9 at 10 μ g/ml induced significant cell growth ($P < 0.05$), however, the growth-promoting activity of S100A8/A9 reached a plateau or decreased at increasing protein concentrations and incubation periods of 36 h or 48 h (Fig. 1A-C). Similar results were also obtained with Jurkat, BJAB, L929, HEK-293, and KELLY cells (data not shown). Next, the data was confirmed by using BrdU incorporation assay (Fig. 1DE). In agreement with the MTT assay, S100A8/A9 exerted cell growth-promoting activity. Higher S100A8/A9 protein concentrations did not increase cell proliferation if treatment was extended to 48 h.

S100A8/A9 specifically binds to MCF-7, MDA-MB231, and SHEP cells

Specific binding of S100A8/A9 to MCF-7 and MDA-MB231 cells was analyzed by a binding assay and evaluated by flow cytometry. The cells were incubated in the presence or absence of S100A8/A9, and the amount of bound protein was measured by flow cytometry using the FITC-labeled monoclonal antibody 27E10, which specifically recognizes the S100A8/A9 heterodimer. The fluorescence in the absence of S100A8/A9 (non-specific binding) was subtracted from the fluorescence determined in its presence. The data were analyzed using CellQuest Pro software.

As shown in Fig. 2A-C, MCF-7, MDA-MB231, and SHEP cells specifically bound S100A8/A9. The mean fluorescence intensities are shown in Fig. 2D. Similar results were also obtained with Jurkat, BJAB, HEK-293, L929, SHEP and KELLY cells (data not shown).

RAGE is involved in cell growth signaling by S100A8/A9

Since it has been suggested that RAGE serves as a primary extracellular-membrane receptor of S100 proteins, RAGE expression was analyzed by Western blot. As shown in Fig. 3A, a 50 kDa band was recognized by polyclonal anti-RAGE antibody. These data were confirmed by immunocytochemistry as shown in Fig. 3BC.

In order to confirm that S100A8/A9 exerts its activity through RAGE, a protease protection assays was performed (Fig. 3D) similarly as it was described in a recent study demonstrating that the extracellular region of RAGE (sRAGE) is protected from proteolytic digestion due to interaction with S100B [19]. When the tandem VC1 complex of RAGE was treated with

chymotrypsin, both full length VC1 and the 25 kDa fragment were completely digested within 45–60 min in the absence of S100A8/A9 (**left panel**). In the presence of S100A8/A9, both full length VC1 and the 25 kDa fragment were clearly detectable after 60 min (**right panel**). These results provide direct evidence for a physical interaction between S100A8/A9 and the VC1 structural domain of RAGE.

To explore whether RAGE ligation was responsible for the cell growth-promoting activity of S100A8/A9, RAGE expression was inhibited in MDA-MB231 cells by RAGE-specific siRNA. Fig. 3E shows that RAGE expression decreased with time as cells were treated with the specific, RAGE-targeting siRNA. After 72 h, RAGE protein was nearly undetectable. The specificity of the gene silencing was shown by the negative control siRNA, which had no effect on RAGE expression. Then S100A8/A9 binding was analyzed in MDA-MB231 cells treated for 72 h with either RAGE-specific siRNA or negative control siRNA by flow cytometry (Fig. 3F). Blocking of RAGE expression by the specific siRNA significantly reduced S100A8/A9 binding compared to either untreated or negative control siRNA-treated cells, indicating that S100A8/A9 binds to RAGE.

We then investigated the cell growth-promoting activity of S100A8/A9 in MDA-MB231 cells that were treated with either RAGE-specific siRNA or negative control siRNA. As shown in Fig. 4A, the cell growth-promoting activity of S100A8/A9 was significantly suppressed in cells where the expression of RAGE was also suppressed by the treatment with the RAGE-targeting siRNA ($P < 0.05$). Similar results were obtained with RAGE blockage antibody in MDA-MB231 and SHEP cells, indicating that RAGE-mediated signaling is involved in the S100A8/A9-induced cell growth (Fig. 4B and Fig. 4C).

S100A8/A9 induces sRAGE release from cells but does not induce ‘high mobility group box 1’ (HMGB1) release from the cells

Soluble RAGE (sRAGE) is produced in humans by alternative splicing of RAGE mRNA [26–28], and it has recently been shown that pericytes and endothelial cells produce and release sRAGE, suggesting the presence of a negative feedback mechanism in RAGE signaling [26]. Therefore, we investigated the presence of sRAGE in the supernatant of S100A8/A9-treated cells to verify the possibility that S100A8/A9-induced release of sRAGE might act as a competitive receptor for cellular RAGE, thereby partly blocking the S100A8/A9-mediated cell growth. MDA-MB231 cells were treated either with S100A8/A9 (15 µg/ml) for different time intervals (0–48 h) or increasing protein concentrations of S100A8/A9 (0, 5, 20, 25 µg/ml) for 36 h, and cell supernatants were subjected to Western blot using a sRAGE-specific antibody. As shown in figure 4DE, S100A8/A9 treatment induced sRAGE release in cell culture media of MDA-MB231 cells in a time and protein concentration dependent manner. Similar data was also obtained for MCF-7 cells (data not shown).

Interestingly, S100A8/A9 treatment did not induce the release of HMGB1 from MDA-MB231 cells (Fig. 4F). These data was confirmed by a similar experiment using MCF-7 cells (data not shown). We observed a trace basic level of release of HMGB1 from MDA-MB231 and MCF-7 cells that was not affected by S100A8/A9 treatment (Fig. 4F), and HMGB1 release was not observed in those cells treated with complete medium (data not shown). HMGB1 is a protein with key roles in maintenance of nuclear homeostasis. Surprisingly, a large body of experimental evidence demonstrates that HMGB1 is also endowed with extracellular signaling functions on various cell types using different receptors like RAGE [29]. Thus, the protein has been included to the “alarmin” family, a term used by Oppenheim and coworkers [30], to identify a group of endogenous factors, also known as “endokines”, which once released in the extracellular space interact with membrane receptors on immune cells to activate the inflammatory response [31]. Thus theoretically, extracellular HMGB1 might provide a proliferation signal via RAGE. To test this possibility MDA-MB231 cells were treated with

S100A8/A9 (5 µg/ml) in presence of anti-HMGB1 for 36 h, and cell proliferation was measured using MTT and BrdU-assay. The results showed that anti-HMGB1 co-treatment did not significantly change the growth effect of S100A8/A9 ($P<0.05$) (data not shown).

Impact of S100A8/A9 on MAP kinase phosphorylation

RAGE-ligation can activate multiple signaling pathways, including Ras-Mitogen-Activated Protein Kinase (MAPK), Phosphatidylinositol 3-Kinase (PI3K), Protein Kinase C (PKC), the Janus tyrosine kinases (JAKs), and transcription factors, including STAT3, AP1, and NF-κB [21,32,33]. However, the signaling mechanisms triggered by the ligation of RAGE in MCF-7 and MDA-MB231 cells remain unknown. Therefore, we first examined whether RAGE ligation by S100A8/A9 could activate p38, p44/42 MAP kinase or SAPK/JNK. MDA-MB231 cells were treated with 10 µg/ml S100A8/A9 for different time intervals, and cell lysates were analyzed by immunoblotting using suitable phosphospecific antibodies: GAPDH was included as loading control.

We analyzed by Western blot the amount of phosphorylated and nonphosphorylated kinases (Fig. 5A) along with densitometric semiquantitative assessment of their phosphorylation (Fig. 5B). S100A8/A9 treatment rapidly induced phosphorylation of both p38 (within 30 min) and p44/42 MAP kinases (within 15 min), and phosphorylation was sustained up to 120 min. However, we failed to detect significant phosphorylation of SAPK/JNK. Similar results were obtained with MCF7 cells (data not shown).

The above results implicate signaling through p38 and p44/42 MAP kinase pathway in S100A8/A9 induced cell growth. To verify their involvement, we performed proliferation assays in the presence and absence of specific kinase inhibitors. MCF-7 and MDA-MB231 cells were treated with S100A8/A9 (10 µg/ml, 24h) in the presence of p38 MAP kinase inhibitor (SB203580, 10µM) and p44/42 MAP kinase inhibitor (U0126, 10µM). As shown in figure 5CD, both inhibitors completely reversed the proliferation effect of S100A8/A9 on these cell lines.

NF-κB activation was analyzed in MCF-7 and MDA-MB231 cells. After treatment with S100A8/A9 (10 µg/ml) for 8 h, nuclear extracts were subjected to NF-κB-specific ELISA assay sensitive to NF-κB p50 subunit. Results showed an 8-fold increase of NF-κB activation in MDA-MB231 cells and a 6-fold increase in MCF-7 cells, respectively (Fig. 6A). In contrast, pre-treatment with RAGE-specific siRNA for 72 h abolished S100A8/A9-induced NF-κB activation ($P<0.0001$, Fig. 6B). There was no significant difference in S100A8/A9-induced NF-κB activation ($P>0.05$) between control and those treated with negative control siRNA (Fig. 6C). Finally, we explored the translocation of NF-κB p50 in MDA-MB231 treated with S100A8/A9. As shown in figure 6D, S100A8/A9 induced p50 translocation into the nucleus while p50 remains in the cytosol in non-stimulated cells. These data indicate that S100A8/A9-promoted cell growth occurs through RAGE signaling and NF-κB activation.

Discussion

S100A8 and S100A9 appear to have a dual role in tumor biology. They help cells acquiring migratory activity by stimulating pseudopodia formation for invasion (invadopodia) [34] and exert growth-promoting activity as shown in the present study. On the other hand, we and others have demonstrated the pro-apoptotic effect of S100A8/A9 at higher protein concentrations [15,35-38,43]. The apoptosis-inducing activity was within the range of 80 to 100 µg/ml [15]. However, S100A8/A9 has cell growth-promoting properties at protein concentrations below 25 µg/ml (Fig. 1). This finding is in agreement with a report of Huttunen et al. [18] demonstrating that nanomolar concentrations of another S100-family member, namely S100B, has growth-promoting effects in RAGE-expressing cells, whereas micromolar concentrations of S100B trigger apoptosis in an oxidant-dependent manner. S100B plays a role

in brain trophism by acting as an intracellular regulator and an extracellular signal [33,39]. Extracellular S100B regulates astrocytic, neuronal, and microglial activities, in part via engagement of RAGE [33,39]. Interestingly, several cytokines exhibit a dual role: at low concentrations, they exert a trophic and protective effect, whereas at high concentrations, they cause cell death. Therefore, it could be concluded that S100A8/A9 has a dual function in cell death and survival similar to cytokines.

RAGE has been proposed to serve as a primary receptor for S100 proteins present in the extracellular space [17]. S100A12 and S100B are well established as ligands of RAGE [17], and the members of the S100/calgranulin family have common structural features and display sequence homology. However, the binding of S100A8/A9 to RAGE is still debated [40]. Therefore, we investigated whether RAGE ligation was responsible for the S100A8/A9-promoted cell growth. Among other experiments, we inhibited RAGE expression by pretreatment with RAGE-targeting siRNA. RAGE knock-down experiments provided strong evidence that RAGE is in fact the receptor for S100A8/A9, and RAGE-dependent signaling is involved in the cell growth-promoting activity of S100A8/A9 (Fig. 3). This finding was also confirmed by RAGE-blocking antibody.

The interaction of RAGE with S100A8/A9 was further analyzed in a proteolysis protection assay using the VC1 fragment of RAGE (Fig. 3D). The extracellular region of RAGE consists of one V-type (variable) immunoglobulin domain, followed by two C-type (constant) immunoglobulin domains. It has been recently shown that the V and C1 domains are not fully independent domains, but rather form an integrated structural unit [19]. In contrast, C2 is attached to VC1 by a flexible linker and is fully independent. Our data shows that the presence of S100A8/A9 protects the VC1 fragment against chymotrypsin digestion, thereby confirming our initial hypothesis that S100A8/A9 binds and exerts activity through RAGE.

Furthermore, we could show for the first time that cells treated with S100A8/A9 release the soluble form of RAGE (sRAGE, Fig. 4D). Since it has been assumed that sRAGE competes with binding of ligands to RAGE, we conclude that this competition is responsible for the decrease in growth-promoting activity of S100A8/A9 at concentrations in the range of 25 µg/ml. However, we note that RAGE does not seem to be the sole receptor for S100A8/A9. RAGE down-regulation by RAGE siRNA in MDA-MB231 cells did not completely inhibit the binding of S100A8/A9 to these cells (Fig. 3E). Therefore, we conclude that there is another, yet unknown second receptor. A number of potential candidates for this activity have been reported in the last years, including heparan sulfate proteoglycan [40], carboxylated glycans [41], and FAT/CD36 [42].

Activation of RAGE triggers several signal transduction pathways, including MAP-kinase, Cdc42/Rac, JAKs, and NF-κB signaling pathways, thereby influencing primary cellular events such as survival, motility, and the inflammatory response [21]. Consistent with other reports, we demonstrated that phosphorylation of p38 and p44/42 MAPK was induced after S100A8/A9 treatment (Fig. 5AB). These findings were confirmed by using specific p38 and p44/42 MAPK inhibitors (Fig. 5CD). However, we did not observe a significant phosphorylation for SAPK/JNK. Treatment with S100A8/A9 also caused NF-κB activation as shown by a Transbinding ELISA assay. This finding was confirmed by NF-κB p50 translocation studies, RAGE blockage experiments, and the fact that S100A8/A9-induced NF-κB activation could be blocked by inhibition of RAGE expression (Fig. 6).

In summary, we provide strong evidence that S100A8/A9 exerts cell growth-promoting activity at lower protein concentrations via RAGE-dependent signaling and subsequent phosphorylation of p38- and p44/42-MAP kinase as well as NF-κB activation. In contrast, RAGE knockdown did not reverse the apoptosis-inducing activity of S100A8/A9 at higher

protein concentrations [43], indicating that the bimodal function of S100A8/A9 is mediated by two distinct receptors and signaling pathways.

Notably, primary tumors release soluble factors that induce the expression of members of S100 protein family, primarily in lung and only minimally in liver or kidneys. The selective up-regulation of S100 proteins in lungs could facilitate the survival and proliferation of metastasizing cancer cells [44]. Thus, our findings might be useful for the development of strategies counteracting tumor metastasizing to certain organs. Our novel finding of cell growth-promoting activity of S100A8/A9 supports the concept that S100A8 and S100A9 play an important role in tumor growth and malignancy.

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Abbreviations

RAGE, receptor of advanced glycation end products; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; MAPK, mitogen-activated protein kinase; SAPK/JNK, stress-activated protein kinase/c-Jun NH2-terminal kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; JAKs, Janus tyrosine kinases (JAKs); ITS, insulin-transferrin-selenium supplements; sRAGE, soluble RAGE; HMGB1, high mobility group box 1.

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Figure 1. Growth-promoting effect of S100A8/A9 on MCF-7 (A), MDA-MB231 (B) and SHEP (C) cells

Cells were treated with various concentrations of S100A8/A9 (0–25 μg/ml) for 12–48 h, and proliferation was assessed by MTT (A–C) and BrdU (D, E) assays. S100A8/A9 induced

significant growth in all cell lines at all times ($P<0.05$) while BrdU incorporation decreased significantly after 48 h ($P<0.05$). Results are expressed as percentage of corresponding control and represent the mean $\pm$ SD of four independent experiments.

Figure 2. Specific binding sites for S100A8/A9 on MCF-7 (A), MDA-MB231 (B) and SHEP (C) cells. Cells were incubated with 10 μ g/ml S100A8/A9 for 1 h on ice and washed three times with cold B-PBS. Then they were incubated with FITC-labeled S100A8/A9-specific 27E10 antibody.

for 30 min on ice and finally washed three times with cold B-PBS. Cell-associated fluorescence was measured by flow cytometry. Blue histograms show non-specific binding and red total binding, respectively. Mean fluorescence intensity (defined as mean fluorescence intensity of total binding minus mean fluorescence intensity of non-specific binding) of S100A8/A9 is shown in panel **D**.

D

Figure 3. RAGE expression in MCF-7 and MDA-MB231 breast cancer cells, and its interaction with S100A8/A9

(A) Detection of RAGE expression in MCF-7 and MDA-MB231 cells by Western blot. Cell lysates were analyzed by Western blot using a polyclonal RAGE-specific antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control (for further details see method section). (B,C) Cellular localization of RAGE in MCF-7 (B) and MDA-MB231 (C) cells was visualized by confocal microscopy. Cells were immunostained with a RAGE-specific antibody and corresponding FITC conjugated secondary antibody (green). (D) Protease protection of VC1-sRAGE fragment by S100A8/A9. Reducing SDS-PAGE of sRAGE -VC1 incubated with chymotrypsin in the absence (left panel) or presence (right panel) of S100A8/A9. (*) marks impurity in the S100A8/A9 sample. The arrow indicates the 25 kDa fragment of VC1. (E) siRNA-mediated RAGE gene silencing in MDA-MB231 cells. Cells were treated either with RAGE-targeting siRNA or negative control siRNA for different time intervals as indicated. The inhibition of RAGE expression was then assessed by Western blot, using RAGE-specific antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (F) Flow cytometric assessment of cell surface binding of S100A8/A9 on MDA-MB231 in the absence (green) and presence of either negative control siRNA (blue) or RAGE-targeting siRNA (red). Cells were transfected with indicated

siRNA and after 72 h, the cells were stained with FITC-labeled S100A8/A9-specific 27E10 antibody (see legend to figure 2 for further details).

D

E

F

Figure 4. S100A8/A9-promoted cell growth is RAGE-dependent, and stimulates sRAGE release from cells

(A) MDA-MB231 cells were pretreated with either RAGE-targeting siRNA or negative control siRNA. The proliferative effect of S100A8/A9 was assessed by MTT assay. Results are expressed as difference to corresponding controls and represent the mean $\pm$ SD of four independent experiments. (B,C) The inhibition of growth-promoting activity of S100A8/A9 on MDA-MB231 (B) and SHEP (C) cells by RAGE-blocking antibody. Results are expressed as percentage deviation from corresponding control and represent the mean $\pm$ SD of four independent experiments. For further details see the method section. (D) Time kinetics: MDA-MB231 cells were treated with S100A8/A9 (15 $\mu\text{g/ml}$) for 0, 12, 24, and 48 h, and both cell lysates and cell culture media supernatants were subjected to Western blot analysis with sRAGE-specific antibody. (E) Concentration kinetics: MDA-MB231 cells were treated with S100A8/A9 (0, 5, 20, 25 $\mu\text{g/ml}$) for 36 h, and both cell lysates and cell culture media supernatant were subjected to Western blot analysis with sRAGE-specific antibody. (F) MDA-MB231 cells were treated with S100A8/A9 (0, 5, 20, 25 $\mu\text{g/ml}$) for 36 h, and both cell lysates and cell culture media supernatant were subjected to Western blot analysis with HMGB1-specific antibody.

A **B** **C** **D**

Figure 5. S100A8/A9 treatment activates MAP kinases, but not SAPK/JNK kinase
 (A) MDA-MB231 cells were treated with S100A8/A9 (10 µg/ml) for 0, 15, 30, 60, and 120 min, and cell lysates were analyzed by Western blot using the indicated antibodies. GAPDH was included as a loading control. (B) The quantities of phosphorylated and total kinases were estimated for triplicate of blotting by scanning of the Western blot membranes by “Storm” scanner and subsequent signal quantification by Image Quant software. The intensity of experimental signals were compared to control and then normalized to GAPDH loading control. (C,D) S100A8/A9 proliferative effect was inhibited by SB203580 (p38 inhibitor, 10µM) and U0126 (p44/42 MAPK inhibitor, 10µM).

Figure 6. S100A8/A9 induces NF-κB activation

MCF-7 (black bars) and MDA-MB231 (white bars) cells were treated with S100A8/A9 (10 μg/ml) for 8 h, and nuclear extracts were subjected to NF-κB trans-binding assay (**A**). NF-κB activity was significantly increased in both cell lines ($P < 0.0001$). Panel **B** shows the corresponding experiment performed with cells pretreated for 72 h with RAGE targeting

siRNA. Panel C shows the corresponding experiment using cells pre-treated for 72 h with negative control siRNA. **(D)** Nuclear translocation of NF- κ B p50 subunit after treatment of MDA-MB231 cells with S100A8/A9 was detected by confocal microscopy. MDA-MB231 cells were either left untreated (control panel) or treated with S100A8/A9 (10 μ g/ml) (S100A8/A9 panel). After 8 h, cells were immunostained with an anti-NF- κ B p50 antibody followed by the corresponding Cy-5 conjugated secondary antibody (magenta). S100A8/A9 treated cells show partial NF- κ B translocation from cytosol to nucleus.