S100P-Derived RAGE Antagonistic Peptide Reduces Tumor Growth and Metastasis

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Abstract

Purpose: The receptor for advanced glycation end products (RAGE) contributes to multiple pathologies, including diabetes, arthritis, neurodegenerative diseases, and cancer. Despite the obvious need, no RAGE inhibitors are in common clinical use. Therefore, we developed a novel small RAGE antagonist peptide (RAP) that blocks activation by multiple ligands.

Experimental Design: RAGE and its ligands were visualized by immunohistochemical analysis of human pancreatic tissues, and siRNA was used to analyze their functions. Interactions between RAGE and S100P, S100A4, and HMGB-1 were measured by ELISA. Three S100P-derived small antagonistic peptides were designed, synthesized, and tested for inhibition of RAGE binding. The effects of the peptide blockers on NFκB-luciferase reporter activity was used to assess effects on RAGE-mediated signaling. The most effective peptide was tested on glioma and pancreatic ductal adenocarcinoma (PDAC) models.

Results: Immunohistochemical analysis confirmed the expression of RAGE and its ligands S100P, S100A4, and HMGB-1 in human PDAC. siRNA silencing of RAGE or its ligands reduced the growth and migration of PDAC cells in vitro. The most effective RAP inhibited the interaction of S100P, S100A4, and HMGB-1 with RAGE at micromolar concentrations. RAP also reduced the ability of the ligands to stimulate RAGE activation of NFκB in cancer cells in vitro and in vivo. Importantly, systemic in vivo administration of RAP reduced the growth and metastasis of pancreatic tumors and also inhibited glioma tumor growth.

Conclusion: RAP shows promise as a tool for the investigation of RAGE function and as an in vivo treatment for RAGE-related disorders.

Introduction

The receptor for advanced glycation end products (RAGE) is a multiligand receptor of the immunoglobulin superfamily that is expressed in all tissues (1–3). First described as a receptor for advanced glycation end products (AGE), which are products of the nonenzymatic glycation and oxidation of proteins, RAGE also responds to several molecules released during periods of acute and chronic stress (1–3). Non-AGE ligands of RAGE include members of the S100 family, high mobility group box 1 peptide (HMGB-1, also called amphoterin), and amyloid-β peptide (1–4). Binding of these ligands to RAGE activates key cell signaling pathways, including mitogen-activated protein (MAP) kinase and nuclear factor NFκB pathways (1, 5). Consistent with its interactions with this group of disease-related ligands, RAGE has been confirmed to contribute to the pathogenesis of cancer, diabetes mellitus, Alzheimer’s disease, arthritis, and other acute and chronic inflammatory disorders (1–4).

In preclinical models, RAGE has been inhibited using several approaches, including expression of a dominant-negative truncated receptor (6), treatment with a RAGE-blocking antibody (7), treatment with a 33-amino acid antagonistic peptide derived from HMGB-1 (8), treatment with cromolyn (9), gene silencing with antisense oligonucleotides, and treatment with a truncated form of RAGE called sRAGE (10). sRAGE, a synthetic version of a naturally occurring secreted form of the receptor that sequesters RAGE ligands, has been most studied in this regard. Administration of sRAGE was shown to suppress the growth of tumor cells in animal studies (6), improve outcomes in diabetes (11, 12), and block the transport of amyloid-β across the blood–brain barrier (13). These preclinical studies support the hypothesis that blocking RAGE activation will be useful as a treatment in a variety of pathologies. However, none of these current strategies has become available clinically.

As an approach to developing a RAGE inhibitor that would be useful in vivo, we tested small peptide antagonists. In this study, we show that an approximately 1 kDa peptide RAP binds RAGE and blocks its activation by a variety of
**Translational Relevance**

Receptor for advanced glycation end products (RAGE) is a key molecule in several important pathologies, including diabetes, Alzheimer’s disease, sepsis, arthritis, and cancer. Blocking RAGE–ligand interaction is clinically beneficial, but there are currently no drugs available for this purpose. We have developed a small peptide antagonist, RAGE antagonistic peptide (RAP), that prevents RAGE from binding with several of its most important ligands, including HMGB-1, S100P, and S100A4. This study also evaluated whether RAP could achieve relevant levels of inhibition in vivo. We showed that systemic administration to orthotopic tumors resulted in the inhibition of tumor growth and metastasis. Therefore, this peptide should be useful for the study of RAGE in various diseases. RAP may itself be a useful treatment for several diseases involving RAGE, although more study is needed to verify this.

Peptide antagonists

Previously, the specific amino acid sequence between 150 to 183 of COOH-terminal motif in HMGB-1 was reported to be similar to the sequence of some members of the S100 family and responsible for RAGE binding (8). This 33-amino acid (a.a.) peptide competitively blocked the binding of HMGB-1 with RAGE and had anticancer effects in an experimental metastasis study. In this study, we compared the HMGB-1–blocking peptide sequence with the sequence of S100P and observed a similarity in amino acid sequence and pattern between the first 40 a.a. of S100P and the 33 a.a. RAGE-binding peptide derived from HMGB-1. On the basis of this similarity, we designed and synthesized 3 peptides from the sequence of S100P (elkvelmekel, kelpgflqsgkdkd, and glkhdavdkld) with 95% purity (CPC Scientific). These peptides were designed with the amino terminus blocked by amidation and the carboxyl terminus by acetylation to improve peptide stability.

**Materials and Methods**

**Cell lines**

All cell lines were obtained from the American Type Culture Collection. Cell line identities were verified using DNA fingerprinting (Powerplex16 system; Promega). Cells were routinely cultured in recommended media. All cells were maintained at 37°C in a humidified atmosphere of 5% CO2.

**siRNA transfection**

Cells in 6-well plates were grown to 70% to 80% confluence and transfected with double-stranded siRNA (Qiagen Inc.) for RAGE (SI00022393) S100P (SI03246677), S100A4 (SI00709667), and HMGB-1 (SI02627828) or with a control siRNA (SI03650318) in serum-free medium without antibiotic supplements using HiPerFect Transfection Reagent (Qiagen Inc.). Cells were incubated under these conditions for 72 hours and silencing was then confirmed by Western blotting.

**Western blotting**

Protein lysate from control and target siRNA-transfected cells were prepared, and protein concentrations were measured by Bio-Rad reagent. Protein (50 µg) was loaded onto 10% (RAGE) and 15% (S100P, S100A4, and HMGB-1) SDS-PAGE gels, and Western blot was conducted using primary antibody against S100P (0.1 µg/mL, R&D Systems), S100A4 (1:100 dilution; DakoCytomation), HMGB-1 (1:1,000 dilution; Abcam) and RAGE (1:200 dilution; Millipore). The same blot was probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 0.5 µg/mL; R&D Systems) and E-Cadherin (1:100 dilutions; BD Biosciences), which served as loading control for the experiment. Precision plus protein standards (Bio-Rad) served as molecular weight markers. Western blot imaging and processing were done with Odyssey machine (LiCor Bioscience).

**Immunohistochemical staining**

Unstained 4-µm sections of clinical specimens were deparaffinized with xylene and rehydrated with ethanol. Immunohistochemistry used RTU Vectastain Elite ABC Universal kit (Vector Laboratories) according to the manufacturer’s instructions. Primary antibodies against S100P, RAGE (R&D Systems), S100A4, and HMGB-1 (Abcam) were diluted 1:250 in 2% bovine serum albumin (BSA)/0.02% Triton in PBS. Finally, slides were developed with 3,3-diaminobenzidine substrate counterstained with hematoxylin, dehydrated with ethanol, fixed with xylene, and mounted.

**Cell growth and migration studies**

Pancreatic cancer cells (MPanc96, MOH, HPAF II, and BxPC-3) were plated on 100-mm dishes and transiently transfected with siRNAs against RAGE, S100P, S100A4, HMGB-1, and siControl at a final concentration of 10 nmol/L (Qiagen) with HiPerfect transfection reagent.
S100P, S100A4, and HMGB-1–binding study

Recombinant S100P (14), S100A4, and HMGB-1 proteins (R&D Systems), S100P monoclonal antibody (R&D Systems), S100A4 and HMGB-1 antibody (Abcam), and an ELISA kit (KPL) were used in to develop an ELISA-based assay to determine the binding of S100P with its receptor RAGE. Briefly, sRAGE (ref. 6; 5 μg/mL) was coated onto an ELISA plate by using an antigen coating solution for 1 hour at room temperature; nonspecific binding sites were blocked by incubating the plate in 1% BSA solution. Different concentrations of ligands with and without RAP were incubated in sRAGE-coated wells for 1 hour, and unbound molecules were removed by washing with wash solution. Bound S100P, S100A4, and HMGB-1 were detected with horseradish peroxidase–labeled anti-S100P, S100A4, and HMGB-1 secondary antibodies and 3,3',5,5'-tetramethylbenzidine substrate. Color development was blocked with 1 mol/L phosphoric acid and read at 450 nm.

Luciferase assay for NFκB activity

In vitro, BxPC-3 cells stably expressing a Lenti-NFκB-luc reporter construct (9) were treated with S100P alone or in combination with RAP for 5 hours. β-Luciferin (150 μg/mL) was added to the cells, and luciferase activity was measured using an IVIS bioluminescence system (Caliper Life Sciences). To measure NFκB promoter activity in vivo, BxPC-3 cells (200,000/50 μL) stably expressing an NFκB-luc reporter were injected orthotopically into the pancreas of 4-week-old male nude mice. After 2 weeks, mice were injected intraperitoneally with β-luciferin (150 mg/kg), and basal NFκB activity was determined using the IVIS system. Subsequently, mice were injected with RAP (100 μg/d) intratumorally or intraperitoneally, and NFκB luciferase activity was reanalyzed after 5 hours.

Tumor growth and invasion study in nude mice

Subcutaneous glioma model. The antitumorogenic capability of RAP was assessed in mice by using a luciferase gene stably expressing rat C6-glioma cells. Luciferase-labeled C6-glioma cells (1 × 10^5/100 μL) were injected subcutaneously, and control and RAP peptide (100 μg/d) were injected for another 3 weeks. At the end of the experiment, tumor growth was analyzed by bioluminescence imaging.

Pancreatic orthotopic model. The antitumorogenic capability of RAP was assessed in the mice by using a luciferase gene stably expressing highly aggressive MPanc96 cells. Luciferase-labeled MPanc96 cells (2 × 10^7/50 μL) were injected into the pancreas. Bioluminescent imaging was used to estimate tumor volume after 1 week, and the animals were divided into 2 groups of 5 animals per group such that the mean tumor size was equal among the groups. For the next 4 weeks, group 1 animals were treated intraperitoneally with control peptide (100 μg/d) and group II animals were treated with RAP (100 μg/d). At the end of the experiment, the animals were sacrificed, tumor weight was measured, and tumor volume and invasion of the liver were assessed by bioluminescence imaging.

Statistical analysis

Data are presented as mean ± SEM. All in vitro experiments were repeated at least 3 times (n = 3). For in vivo studies, we used 5 animals per group (n = 5). Statistically significant differences were determined by using the unpaired t test. When more than 2 groups were analyzed, ANOVA was used to analyze the data and the Newman–Keuls multiple comparison test was used to check the postest significance. Statistical significance was defined as P < 0.05. Results were compared using GraphPad Prism 4 software.

Results

Pancreatic cancer cells express and are stimulated by RAGE and its ligands S100P, S100A4, and HMGB-1

We and others have previously reported that mRNAs for RAGE, S100P, S100A4, and HMGB-1 are specifically expressed in pancreatic ductal adenocarcinoma (PDAC) tissue (1, 5). In this study, RAGE, S100P, S100A4, and HMGB-1 protein expression in human tissues was evaluated by immunohistochemical analysis. S100P was very specifically present in cancer cells but not in normal cells; 92% (92 of 100) of pancreatic cancer patient tissue samples possessed S100P (Fig. 1A). S100A4 was also specifically expressed in cancer when compared with normal ducts, but immune and stromal cells in cancer tissue also expressed S100A4. RAGE and HMGB-1 were expressed in both normal and cancer cells. siRNAs were used to reduce the protein levels for RAGE and its ligands in PDAC cells (Fig. 1B). Silencing of the constitutively released RAGE ligands, S100P and S100A4, significantly reduced the growth of PDAC cell...
lines (Fig. 1C). In contrast, silencing of HMGB-1, which is not constitutively released by these cells, had no effect on their growth in vitro.

Small peptides based on the structure of S100P block the binding of multiple ligands to RAGE

A cell-free ELISA was developed to screen peptides for the ability to block ligand binding with RAGE. Recombinant sRAGE, a secretory form of the extracellular portion of RAGE, was coated onto ELISA plates, and the binding of specific RAGE ligands was quantified using an anti-IgG-HPR antibody. In this assay, S100P, S100A4, and HMGB-1 bound with RAGE in a concentration-dependent manner with HMGB-1 showing the highest affinity of binding (Fig. 2A–C).

We then analyzed the effects of 3 small peptides (elkvlmekel, kelpgflqsgkdkd, and gkdkdavdkllkd), designed on the basis of the structure of S100P, to inhibit the binding of RAGE ligands with sRAGE. Elkvlmekel and kelpgflqsgkdkd, but not gkdkdavdkllkd, reduced S100P binding (Fig. 2D).

Elkvlmekel was the most efficacious at blocking S100P and was therefore selected as the lead molecule and is henceforth referred to as RAP (RAGE antagonistic peptide). Kelpgflqsgkdkd, which did not inhibit RAGE binding, was used as a control peptide for further studies. The effects of RAP on the binding of RAGE with S100P, S100A4, and HMGB-1 were then assessed (Fig. 2E). RAP inhibited the binding of each these RAGE ligands, suggesting that RAP competes for a site on RAGE that is required for receptor binding by each of these ligands.

Next we examined the ability of RAP to inhibit RAGE-activated cell functions and signaling. We compared the effects of RAP treatments to those of siRNA-mediated RAGE silencing on the growth of 3 different cancer cell lines (MPanc96, MOH, and HPAF II) in vitro. Each of these cell lines expresses both RAGE and RAGE ligands (data not shown). RAP treatment and siRNA-mediated silencing of RAGE both reduced the growth of each of the cancer cell lines (Fig. 3A). We also observed an inhibition of migration after RAP treatment (Fig. 3B). Ligand binding with RAGE...
activates a number of intracellular pathways, including NFkB (14). NFkB signaling is of particular interest in PDAC in which it is constitutively high in PDAC cells (9). Therefore, in this study, we examined the effects of RAP on RAGE-mediated NFkB activity. Cells expressing NFkB-luc were treated with S100P, with or without RAP, and NFkB activity was analyzed after 5 hours. RAP greatly reduced the ability of S100P to stimulate RAGE-mediated NFkB activation (Fig. 3C).

**RAP inhibits RAGE-mediated basal NFkB activity in PDAC cells in vivo**

To examine the utility of RAP as a RAGE antagonist *in vivo*, we used cancer cells expressing the NFkB-luc reporter implanted into immune-deficient mice. Intratumoral delivery of both siRNA (Fig. 4A) against RAGE or RAP (100 µg; Fig. 4B) to subcutaneously implanted pancreatic cancer cells bearing NFkB-luc caused a dramatic reduction in NFkB activity. To determine the effectiveness of RAP delivered systemically, we next administered RAP (100 µg) intraperitoneally to mice bearing orthotopic PDAC tumors formed with NFkB-luc reporter cancer cells (Fig 4C). We observed that RAP systemic administration caused a substantial reduction ($P < 0.05$) in the NFkB signal 5 hours after injection.

**RAP treatment reduces PDAC tumor growth and metastasis in vivo**

RAP showed significant *in vivo* activity on RAGE signaling. Therefore, we next wished to examine its ability to inhibit *in vivo* tumor growth. It has previously been shown that inhibition of RAGE using sRAGE reduced C6-glioma tumors. So, initially to confirm the efficacy of RAP, we next analyzed its effects on rat C6-glioma cells bearing luciferase injected subcutaneously into nude mice. Mice with glioma tumors were treated systemically with RAP or a control peptide (each 100 µg/d i.p. for 3 weeks). RAP treatment significantly reduced glioma tumor growth, as assessed by bioluminescence imaging, compared with the control peptide (Fig. 5A).

To examine the effects of RAP in a more difficult tumor model, pancreatic cancer Mpanc96 cells bearing luciferase were implanted orthotopically into the pancreas and tumors were established. The effects of RAP treatments on tumor burden and metastasis were analyzed by weekly bioluminescent imaging. Control peptide–treated animals showed extensive tumor growth and metastasis to the liver. In contrast, RAP-treated animals did not show any toxicity as evidenced by unaltered body weight when compared with control peptide–treated animals (Fig. 5D).

**Discussion**

In this study, we provide evidence that a small peptide developed from the structure of S100P can block the binding of several key RAGE ligands that are each involved in inflammation and cancer. This peptide was functional *in vivo* and provided therapeutic benefit against glioma and
pancreatic cancer in preclinical models. This represents the first RAGE-competitive antagonist shown to be effective in vivo. The data shown suggest that this peptide may be useful as a treatment for cancer. On the basis of its ability to block several diverse RAGE ligands, it is also likely that it will be useful in other diseases in which RAGE is involved.

A peptide consisting of residues 150 to 183 of HMGB-1 (the end of the B-box and its linker to the acidic tail) was previously shown to successfully compete with HMGB-1 binding to RAGE in vitro (8). Pretreatment of cancer cells with this peptide in vitro successfully blocked their subsequent migration and their metastasis in an experimental in vivo model (8). This HMGB-1–derived peptide also blocked the binding and activation of RAGE by S100P (15). The sequence of the HMGB-1 RAGE antagonist is similar to the first 40 amino acids (the first EF-hand helix-loop-helix sequence) of several S100 proteins (8). The peptides examined in this study were designed on the basis of the structural and pattern similarity of S100P within this same region. The RAP amino acid sequence occurs between amino acids 32 to 41 of S100P. RAP blocked the binding of not only S100P but also HMGB-1 and S100A4 to RAGE. Other ligands were not evaluated, but it is likely that this peptide will block a wide variety of RAGE ligands, as the S100s and HMGB-1 seem to share a common binding domain. These data support the further development of antagonistic peptides to block RAGE activation.

RAGE has previously been shown to transduce the extracellular effects of many different ligands (1–5). RAGE is expressed ubiquitously in many tissues and cell types; in our immunohistochemical data, we observed RAGE expressed in both cancer cells and in normal tissue. Despite its wide spread distribution, RAGE-deficient mice developed normally (1). Physiologically, RAGE plays a crucial role in embryonic neuronal outgrowth. However, in the adult, RAGE seems to act in pathologic responses as a receptor for a very broad range of ligands that fall into the category of damage-associated molecular pattern (DAMP) molecules. Expression of RAGE ligands is elevated in a variety of inflammatory conditions and in most cancers the levels of these ligands determine the activity of RAGE (1–5). RAGE has 3 immunoglobulin-like regions: one "V-type" domain and 2 "C-type" domains, a short transmembrane domain,
and a 43-a.a. cytoplasmic tail (1; 16). The V-type domain has been found to confer ligand binding (1–3, 16). However, the specific basis of RAGE binding has yet to be determined. Comparisons of amino acid sequences in different RAGE ligands do not indicate a high conservation of structure. Narrowing the list of ligands to the numerous members of the S100 family of proteins indicates that these molecules also do not share a high level of sequence homology outside of the Ca$^{2+}$-binding EF-hand domains. Nevertheless, these molecules, as well as other non-S100 molecules, act as ligands for RAGE. In this study, experimental peptides elkvlmekel and kelpgflqsgkdkd possess little sequence homology, but both were inhibitory to RAGE binding. Taken together, these observations suggest that RAGE binding is not determined by a specific sequence of amino acids but rather a general pattern. For this reason, RAGE is considered a pattern recognition receptor (17–20).

The use of peptides as therapeutics is often limited by their short half-life in vivo and the potential for generation of an immune response. To reduce the degradation of RAP by exoproteases, we blocked the amino and carboxyl termini. The half-life of RAP was not measured in this study. However, strong inhibition of both NFkB activity and tumor growth were observed when RAP was administered intra-peritoneally. This indicates that RAP has a sufficient half-life in vivo to provide therapeutic benefit. Future improvements in the molecule, especially with regard to its stability, are likely to increase this in vivo effectiveness.

Activation of RAGE by S100P stimulates several cellular signaling pathways, including MAP kinase and NFkB (5, 14). These 2 pathways are constitutively active in many cancer cell lines (21) and influence tumor growth and chemotherapeutic drug resistance (22, 23). This and previous studies indicate that inhibiting S100P–RAGE
interactions significantly reduces basal levels of NFκB activity in PDAC and support the existence of an autocrine loop involving RAGE ligands and RAGE in PDAC (9). This characteristic provides the explanation for the ability of RAP to inhibit basal NFκB activity in cancer cells in vivo.

High mobility group box 1 (HMGB-1) is another well-known ligand for RAGE. HMGB-1 is the prototypic DAMP molecule and has been implicated in several inflammatory disorders and cancer (1, 4, 24). HMGB-1 is a DNA-binding nuclear protein expressed in the nucleus of most normal cells, but it is secreted after a variety of stresses including cancer, inflammation, necrosis, and chemo- or radiotherapy. Once released, HMGB-1 binds with RAGE and activates signaling (1–5). HMGB-1 release from damaged cells used acts as a ‘necrotic marker’ used by the immune system to recognize tissue damage, initiate reparative responses, and promote maturation of lymphocytes. Extracellular HMGB-1 also acts as a potent proinflammatory cytokine, contributing to the pathogenesis of a wide variety of inflammatory disorders (1). HMGB-1 expression and secretion is elevated in melanoma, colon cancer, prostate cancer, pancreatic cancer, and breast cancer (24). HMGB-1 expression in cancer is associated with increased angiogenesis, migration, invasion, and metastasis. Previous in vivo studies showed that blockade of HMGB-1 and RAGE interaction using treatments with sRAGE suppresses tumor growth and metastasis in a murine model of lung cancer (8). HMGB-1 was also found to drive the aggressive growth of glioma tumors (6). In this study, RAP was able to block HMGB-1 binding to RAGE in vitro and to inhibit glioma growth in vivo. These data support RAP as a useful HMGB-1 inhibitor. Apart from binding with RAGE, HMGB-1 can also act through toll-like receptors (TLR-2 and TLR-4), especially in immune cells including macrophages and act as late mediator of inflammatory responses (25). We did not find higher expression of TLRs in PDAC cells compared with normal pancreas (data not shown). Nevertheless, future studies blocking both RAGE and TLRs may be useful to understand further the roles of HMGB-1 in PDAC.

S100A4 is a member of the S100 family of proteins, which has roles in inflammation and cancer. Originally cloned from highly metastatic murine mammary carcinoma cells, S100A4 has well-established importance in the process of cancer metastasis (26). S100A4 has also been reported to influence pancreatic cancer resistance to therapy (27). In this study, RAP blocked S100A4 binding to RAGE. The activities promoted by S100A4 that are mediated by RAGE remain uncertain. However, RAP should be a useful tool for the determination of these functions.

In summary, our study identifies RAP as a peptide antagonist that binds with RAGE and blocks its activation by several clinically relevant RAGE ligands. This peptide should be useful for studies of the roles of RAGE in a variety of diseases. This peptide will also be a starting point for the further development of a therapeutic for the many diseases involving RAGE.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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