RAGE Mediates S100A7-Induced Breast Cancer Growth and Metastasis by Modulating the Tumor Microenvironment

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Abstract

RAGE is a multifunctional receptor implicated in diverse processes including inflammation and cancer. In this study, we report that RAGE expression is upregulated widely in aggressive triple-negative breast cancer (TNBC) cells, both in primary tumors and in lymph node metastases. In evaluating the functional contributions of RAGE in breast cancer, we found that RAGE-deficient mice displayed a reduced propensity for breast tumor growth. In an established model of lung metastasis, systemic blockade by injection of a RAGE neutralizing antibody inhibited metastasis development. Mechanistic investigations revealed that RAGE bound to the proinflammatory ligand S100A7 and mediated its ability to activate ERK, NF-κB, and cell migration. In an S100A7 transgenic mouse model of breast cancer (mS100a7a15 mice), administration of either RAGE neutralizing antibody or soluble RAGE was sufficient to inhibit tumor progression and metastasis. In this model, we found that RAGE/S100A7 conditioned the tumor microenvironment by driving the recruitment of MMP9-positive tumor-associated macrophages. Overall, our results highlight RAGE as a candidate biomarker for TNBCs, and they reveal a functional role for RAGE/S100A7 signaling in linking inflammation to aggressive breast cancer development. Cancer Res; 75(6); 974–85. © 2015 AACR.

Introduction

Receptor for advanced glycation endproducts (RAGE) is the signal transduction receptor that senses a variety of signaling molecules (1). The variety of ligands allows RAGE to be implicated in a wide spectrum of pathologic conditions such as inflammation and cancer (1, 2). Epidemiologic and molecular studies, including mouse models, have shown that if inflammation is prolonged, it promotes cancer development (3–6). It is now believed that most solid tumors, including those in the breast, have an inflammatory microenvironment (4, 5). RAGE expression and activation have been shown to be associated with chronic inflammation, which in turn enhances the malignant transformation of various cancers (1, 2, 7–11). However, its role in breast cancer, especially in the modulation of breast cancer microenvironment, is unknown.

RAGE was first described as a receptor for advanced glycation end products (AGE), but it has since been shown to be the receptor for several other molecules involved in innate immunity, including high mobility group box 1 peptide (HMGB-1), amyloid-β peptide, and the S100 family of proteins (1, 2). Phorbol ester 12-O-tetradecanoylphorbol-13-acetate-induced proinflammatory mediators were shown to be decreased in RAGE-deficient mice (7), which suggests that RAGE expression is involved in sustaining inflammation and cancer (1, 2, 7, 9). It has also been well-documented that RAGE ligands bind to RAGE and activate its downstream signaling mechanisms that sustain chronic inflammatory conditions, leading to neoplastic stage (1, 12, 13). It is interesting to note that there is very low or no RAGE expression in normal tissues but enhanced expression in chronic inflammation and cancer (2, 10). Although, these features of RAGE make it an ideal candidate for therapeutic strategies against chronic inflammation, not much is known about its role in breast cancer.

RAGE has been shown to bind to its ligand S100A7 in keratinocytes and leukocytes (14, 15). S100A7 has been shown to be highly expressed in estrogen receptor (ER)α− breast cancer (16, 17). It is believed that S100A7 mediates breast cancer growth and metastasis by recruiting proinflammatory cell infiltrates (18, 19). Also, proinflammatory cytokines enhance triple-negative breast cancer (TNBC) growth and metastasis (20). However, very little is known about mechanisms through which RAGE/S100A7 axis modulates tumor microenvironment and enhances breast cancer growth and metastasis.

Macrophages can be divided into subtypes M1 and M2, where M1 macrophages are associated with an anti-cancer phenotype and M2 macrophages express a pro-cancer phenotype (21, 22). Cytokines/chemokines and growth factors modulate the tumor microenvironment, which could directly/indirectly polarize
macrophages toward the M2 tumor-associated macrophages (M2-TAM) phenotype (22, 23).

In this investigation, we for the first time show that RAGE is expressed in a panel of aggressive breast cancer cell lines, TNBC, and metastatic lymph node deposits. We also demonstrate that blocking of RAGE reduces tumor metastasis and that RAGE ablation inhibits breast cancer growth. In addition, we show that RAGE mediates its tumor-promoting effects in breast cancer through binding to S100A7. Our findings also uncovered that the RAGE/S100A7 pathway enhanced breast cancer growth and metastasis. These studies further demonstrate that RAGE neutralizing antibodies/soluble RAGE could be used to inhibit breast cancer growth and metastasis, especially in S100A7-expressing invasive cancers. Furthermore, these studies suggest that RAGE could be used as novel biomarker and therapeutic strategy against TNBCs.

Materials and Methods

Patients

Institutional Review Board of the Ohio State University (OSU; Columbus, OH) has approved protocol for the constructed TNBC tissue microarrays (TMA; n = 173). The clinical and pathologic characteristics of TNBC TMAs have been recently described (24). TMA for lymph node metastasis (BR1008) was obtained from US Biomax, Inc.

Immunohistochemistry, immunofluorescence, and ELISA

Samples from mammary glands and tumors were formalin-fixed and paraffin-embedded (18). Standard IHC techniques were used according to the manufacturer’s recommendations (Vector Laboratories) using antibodies against RAGE (Abcam, 1:400) Ki67 (Neomarkers, 1:100), CD31 (Santa Cruz 1:100), F4/80 (AbD Serotec, 1:50), arginase 1 (Santa Cruz, 1:200), and iNOS (Abcam, 1:200) for 60 minutes at room temperature. Vectastain Elite ABC reagents (Vector Laboratories), using avidin DH:biotinylated horseradish peroxidase H complex with 3,3’-diaminobenzidine (Polysciences) and Mayer’s hematoxylin (Fisher Scientific) were used for detection of bound antibodies. Staining of TMAs was graded as previously described (25). Immunofluorescence was performed on paraffin-embedded tissues. Briefly, sections were stained with F4/80 (1:75), and MMP9 (R&D Systems, 1:150). Alexa Fluor–conjugated secondary antibodies (Life Technologies) were used to detect primary antibody. Sections were mounted by VECTASHIELD mounting media containing DAPI (Vector Laboratories, Inc.). Images were analyzed by confocal microscopy. Binding of RAGE with human recombinant S100A7 was performed as described (26).

Cancer patient data analysis

High RAGE and S100A7 expressions were defined as overexpression of ager mRNA being greater than 0.5-fold and overexpression of s100a7 being greater than 1.0-fold of SD above the mean, respectively. Association of gene expression alterations was performed on the basis of The Cancer Genome Atlas (TCGA) database by Fisher exact test. Analysis of RAGE expression between basal and non-basal breast cancer samples was based on a subtype-specific breast cancer study (GEO accession GDS2250; ref. 27). For Kaplan–Meier survival analysis, patient samples with RAGE expression values greater than its median were grouped as high RAGE and the other half as low RAGE.

Cell culture

Murine macrophage cell line RAW264.7 and human breast carcinoma cell lines MDA-MB-231, MDA-MB-453, MCF7, T47D, BT-474 were obtained from ATCC. SCG2 cells were kindly provided by Dr. Massague (28). MVT-1 cells (derived from MMTV-c-Myc; MMTV-VEGF bitransgenic mice) were obtained from Dr. Johnson and PyMT cells derived from MMTV-PyMT C57BL/6 mice were obtained from Dr. Hai (OSU; ref. 29). MVT-1 highly metastatic clone, PyMT, Met1, and 4T1 cells were cultured as described (18, 29).

Chemotaxis

Chemotactic assays were performed using Transwell chambers (Costar; 8-μm pore size) as described (18, 30).

Mice

Nude mice were obtained from Charles River. C57B6/6 background RAGE−/− mice were kindly provided by Dr. Schmidt (New York University, New York, NY), and TetO-mS100a7a15 mice were kindly provided by Dr. Yuspa (NIH; Bethesda, MD). TetO-mS100a7a15 mice (15) were cross-bred with MMTV-rtTA mice to generate bitransgenic MMTV-mS100a7a15 mice. Knockout and transgenic littermates were genotyped by PCR. Female MMTV-mS100a7a15 mice were fed with doxycycline-chow 1 g/kg (BioSerd), and mice with normal diet served as controls. All mice were kept in The OSU’s animal facility in compliance with the guidelines and protocols approved by the OSU Institutional Animal Care and Use Committee.

Orthotopic injection assay

MVT-1 or PyMT cells were injected into the mammary glands of transgenic or knockout mice. Transgenic mice injected with MVT-1 cells were either fed with doxycycline-chow 1 g/kg or normal diet (control). Tumors were measured weekly with external calipers and volume was calculated according to the formula V = 0.52 × a² × b, where a is the smallest superficial diameter and b is the largest superficial diameter. Orthotopically injected animals were sacrificed and tumors were excised (18). RAGE neutralizing antibody (human or murine) and soluble RAGE (human or murine) were purchased from R&D Systems.

FACS analysis

Freshly prepared single-cell suspensions of tumor-infiltrating cells were incubated with anti-F4/80 PE or anti-CD11b APC (18). RAGE expression was analyzed by staining with RAGE antibody (Abcam) followed by Alexa Fluor 488 antibody. After staining, cells were analyzed by FACS Calibur using CellQuest software (BD Biosciences).

Western blot and coimmunoprecipitation

Western blot analysis of cell or tumor lysates was done as described (30). Coimmunoprecipitation was carried out using protein G plus A-agarose beads as described (31), with S100A7 rabbit (Novus Biologicals) and RAGE mouse (Santa Cruz Biotechnology) antibodies.

Luciferase reporter assay

NF-κB activity was determined using NF-κB luciferase reporter assay (Promega) per manufacturer’s protocol.
Figure 1.
RAGE expression in breast cancer cell lines and patient samples. A, FACS analysis of RAGE in human TNBC, ERα−, and highly metastatic murine mammary tumor cell lines. B, quantification of RAGE expression obtained by FACS. C, RAGE expression in basal and non-basal type breast cancer. RAGE (ager) expression values are normalized to β-actin (actb). D, RAGE expression in normal and invasive breast cancer. E, representative photographs of RAGE expression in TNBC TMA. F, bar graph showing RAGE expression in TNBC (n = 80) and HER2 (n = 33) TMA samples. G, bar graph showing RAGE expression in metastatic breast cancer. We used TMA (n = 100) that contained n = 40 lymph node metastasis, n = 50 malignant, and n = 10 normal tissues. H, expression level of RAGE predicts survival differences by Kaplan–Meier analysis using R2 microarray dataset. Scale bar, 100 μm. #, P < 0.05; ##, P < 0.01.
Statistical analysis
To test the association between two categorical variables, $\chi^2$ tests or Fisher exact tests were used. For continuous variables, 2-sample $t$ tests were used if two groups were compared, and ANOVAs were used if more than two groups were compared. * or # indicates $P < 0.05$; ** or ## indicates $P < 0.01$.

Results
RAGE is expressed in highly metastatic breast cancer cells and its expression correlates with worse clinical prognosis
We analyzed RAGE expression in a panel of breast cancer cell lines. RAGE expression was higher in metastatic TNBC cell lines, whereas low or no RAGE expression was observed in ER$^+$ breast cancer cell lines (MCF7, T47D, and BT474; Fig. 1A and B), which are weakly metastatic (32–34). These data suggest that RAGE is predominantly expressed in ER$^-$ and highly metastatic breast cancer cell lines. To test the correlation of RAGE with ER status, we analyzed open-access Gene Expression Omnibus (GEO) datasets for the expression of RAGE. In a subtype-specific breast cancer study (GEO accession GDS2250), RAGE expression is significantly enhanced in basal type (majorly TNBC) invasive breast cancer patient tumor samples compared with non-basal type tumors (majorly ER$^+$ cancer; Fig. 1C). Next, we analyzed open-access dataset for RAGE expression. We found that high RAGE expression was observed in invasive breast cancer (IBC) compared with normal control (Fig. 1D). Furthermore, we analyzed the expression of RAGE in breast cancer TMAs with accompanying outcome data and other clinical information by IHC. We found that 92% of the samples showed high RAGE expression in TNBC tissues (Fig. 1E and F). However, RAGE was expressed only in 29% HER2-positive TMAs (Fig. 1E and F). Using another TMA that contained metastatic and malignant patient samples, we found that metastatic tissue tends to have higher RAGE expression than malignant tissue ($P < 0.0001$; Fig. 1G). Next, we analyzed open-access dataset for clinical outcome of RAGE expression. We found that high RAGE expression was associated with poor prognosis (Fig. 1H). Taken together, these results show that RAGE expression is associated with highly aggressive and metastatic breast cancers, including TNBC.

Blockade of RAGE inhibits tumor growth and metastasis in vivo
To investigate the role of RAGE in metastasis, we used the IVIS imaging system to analyze the metastatic potential of RAGE-expressing SCP2 cells. We injected highly metastatic single-cell progeny clone 2 (SCP2) of MDA-MB-231 cell lines intracardially into nude mice and blocked RAGE activity with RAGE neutralizing antibody (naRAGE). As shown in Fig. 2A and B, naRAGE treatment significantly reduced the metastatic potential of SCP2 cells as compared with IgG control-treated mice. Next, we elucidated the role of host RAGE on mammary cancer progression

Figure 2.
Inhibition of RAGE reduces breast cancer growth and distant metastasis. A, luciferase-positive SCP2 cells (1 $\times$ 10$^6$/100 µL) were injected intracardially into nude mice ($n = 6$) pretreated with mouse RAGE neutralizing (naRAGE) or IgG antibody (20 µg/mouse). Representative bioluminescent images (BLI) show comparative metastases of naRAGE- or IgG-treated mice. B, normalized photon flux of mice treated with naRAGE or IgG. C, RAGE expression was analyzed in PyMT cell line by FACS. Red, IgG; black, RAGE. D, PyMT (1 $\times$ 10$^5$) cells were injected into the mammary gland of RAGE$^{+/+}$ and RAGE$^{-/-}$ mice ($n = 5$) and tumor volume was measured every week. E, after 5 weeks, tumors were excised from mice and weighed. F, representative photograph of mice showing tumors dissected from different experimental groups. G, PyMT cell line–derived tumors from RAGE$^{+/+}$ and RAGE$^{-/-}$ mice were subjected to IHC staining for CD31 and Ki67 expression. H, tumor lysates (50 µg) from RAGE$^{+/+}$ and RAGE$^{-/-}$ mice were subjected to Western blotting using phospho-ERK (P-ERK), total ERK (T-ERK), cyclin D1, and MMP-2 antibodies. GAPDH served as loading control. Data represent mean ± SD of three independent experiments. Scale bar, 100 µm. * $P < 0.05$. 

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and development using the RAGE−/− model. First, we analyzed surface expression of RAGE on PyMT cells by FACS analysis. As shown in Fig. 2C, PyMT cells expressed high RAGE expression. Next, mice were injected with PyMT cells and observed for tumor growth for 35 days (Fig. 2D). Interestingly, PyMT-derived tumor growth was significantly inhibited in RAGE−/− mice as compared with RAGE+/− mice (P = 0.0125; Fig. 2E and F). To identify the signaling molecules that are associated with breast cancer tumor growth, we examined the expression of ERK, cyclin D1, and MMP2 in PyMT-derived tumor tissues obtained from RAGE−/− mice. We observed reduced phospho-ERK, MMP2, cyclin D1, CD31, and Ki67 expression in RAGE−/− mice compared with RAGE+/− mice (Fig. 2G and H). Cyclin D1 and Ki67 are markers for active proliferation (35, 36). These data demonstrate that RAGE blockade significantly inhibits mammary tumor development and progression.

RAGE mediates its effect in breast cancer cells through S100A7

RAGE has been shown to bind to S100A7 in various immune cells (14). When analyzing the RAGE and S100A7 expression in breast cancer (TCGA), we discovered that RAGE and S100A7 are often simultaneously upregulated in breast tumors among patients of the IBC cohort (P = 0.0055; Fisher exact test), whereas an irrelevant gene ERK (mapk1) showed no correlation with RAGE or S100A7 (Fig. 3A). This co-currency of gene upregulation implies a functional link between RAGE and S100A7 in breast cancer. Furthermore, we observed enhanced expression of RAGE in S100A7-overexpressing MDA-MB-231 cells by Western blot, FACS, and immunofluorescent analyses (Fig. 3B–D). This suggests that RAGE could be a receptor for S100A7 as ligand in breast cancer cells. We further verified the direct interaction between human RAGE and human S100A7 protein using an ELISA-based binding assay, in which EGFR, an irrelevant receptor, was used as a
negative control. As shown in Fig. 3E, the total binding was dependent on RAGE-Fc concentration, whereas no binding was observed between EGFR-Fc and S100A7 protein. To further confirm the association of RAGE with S100A7, we performed a coimmunoprecipitation assay. S100A7 coimmunoprecipitated with RAGE in S100A7-overexpressing MDA-MB-231 cells (Fig. 1F). Next, we analyzed the effect of human S100A7 on MDA-MB-231 and SC2 cell migration and that of its murine paralog mS100A7a15 on MVT-1 cell migration. S100A7 enhanced the migration of MDA-MB-231 and SC2 cells, respectively, and these effects were significantly abrogated by blocking RAGE using neutralizing antibodies (Fig. 3G and H). We also showed that its murine ortholog mS100a7a15 enhanced migration of MVT-1 cells (Supplementary Fig. S1A). Next, we analyzed the S100A7-induced wound-healing capacity of MDA-MB-231 cells and observed that RAGE neutralizing antibodies significantly abrogated this effect (Supplementary Fig. S1B and S1C). In addition, we observed that S100A7 or mS100a7a15 significantly enhanced the invasion of SC2 and MVT-1 cell lines (Supplementary Fig. S1D and S1E). To examine whether activation of RAGE/S100A7 enhanced downstream signaling in breast cancer cells, we analyzed ERK activation. We showed that S100A7-induced ERK activation was inhibited by blocking S100A7 with soluble RAGE (sRAGE) in MDA-MB-231 and SC2 cells (Fig. 3I and J). To further confirm these effects, we used RAGE neutralizing antibody to block S100A7-induced ERK activation in MDA-MB-231 cells (Supplementary Fig. S1F). In addition, we observed that sRAGE inhibits S100A7-induced MMP9 secretion in SC2 cells (Supplementary Fig. S1F). NF-kB has also been shown to be the downstream target of RAGE (37). Using NF-kB reporter assay, we showed that S100A7 significantly increased NF-kB activity and this effect was inhibited by nA-RAGE (Fig. 3K). Taken together, these results imply that the RAGE/S100A7 signaling axis is necessary for breast cancer cell migration and invasion.

**Blockade of RAGE inhibits mammary tumor growth and metastasis in inducible MMTV-mS100a7a15 mice**

To analyze the relevance of RAGE/mS100a7a15 in a mammary tumor growth and metastasis model, we used an MVT-1 syngeneic orthotopic model that recapitulates the stages of human primary tumors. We injected MVT-1 cells into MMTV-mS100a7a15 transgenic mice and blocked RAGE with neutralizing RAGE antibody. Mice were treated with doxycycline-chow (1 g/kg) 1 week before MVT-1 injection to switch on the expression of mS100a7a15. When tumors grew as large as 100 mm³, the doxycycline-treated group was given nA-RAGE (20 µg/mouse) or IgG (20 µg/mouse) intraperitoneally 3 times a week for 20 days. MMTV-mS100a7a15 mice fed with normal chow were used as a negative control. Inducible mice treated with nA-RAGE showed reduced tumor progression compared with the IgG-treated group (Fig. 4A–C). To determine whether a blocking ligand of RAGE inhibits mammary tumor progression, we used soluble RAGE (sRAGE). Mice were fed with doxycycline-chow 1 week before the injection of MVT-1 cells into the #4 mammary gland. After day 1 of doxycycline induction, mice were injected intraperitoneally with murine sRAGE (2 µg/mouse) or PBS. MMTV-mS100a7a15 mice who received normal chow were used as a negative control. As shown in Fig. 4D–F, sRAGE treatment significantly reduced tumor progression in the doxycycline-induced group as compared with the PBS doxycycline-induced group. Furthermore, we observed that blocking RAGE or mS100a7a15 substantially decreased proliferation and angiogenesis in MVT-1–derived tumors obtained from inducible MMTV-mS100a7a15 mice (Fig. 4G and H). Next, we investigated whether RAGE inhibition reduces surface lung metastases in inducible MMTV-mS100a7a15 mice. We observed a significant decrease in surface lung metastases in the mice treated with nA-RAGE (Fig. 4I and J) or sRAGE (Fig. 4K and L) in MVT-1–derived tumors obtained from inducible MMTV-mS100a7a15 mice as compared with control groups. Taken together, these results suggest that RAGE plays an important role in mS100a7a15–induced breast cancer progression and metastasis.

**RAGE/S100A7 axis modulates the tumor microenvironment**

Macrophages, especially M2-TAMs, have been shown to enhance tumor growth and metastasis (21, 22, 38, 39). To identify the molecular mechanism of RAGE-mediated breast tumor growth and metastasis, we analyzed macrophage recruitment in the PyMT-derived tumors of RAGE−/− and RAGE+/− mice. As shown in Supplementary Fig. S2, F4/80/Arg1-positive macrophages were substantially reduced in RAGE−/− PyMT–derived tumors as compared with RAGE+/− tumors. Next, we observed that mS100a7a15-overexpressing mice significantly increased the recruitment of F4/80+/Arg1 macrophages and RAGE blockage by nA-RAGE treatment significantly reduced the recruitment of F4/80+/Arg1 macrophages compared with IgG control, as analyzed by IHC (Fig. 5A). In addition, we observed substantial decrease in the recruitment of M2 macrophages in MVT-1–derived tumors pretreated with sRAGE in inducible mice compared with PBS–treated mice (Fig. 5B). We also observed reduced CD11b/F4/80+ TAMs by FACS (Fig. 5C). We further showed decreased expression of iNOS (M1 marker) in primary tumors compared with IgG control (Fig. 5A and B). We also analyzed the infiltrations of macrophages in the lung tissues and observed reduced expression of F4/80+ macrophages and Arg1 expression in nA-RAGE- or sRAGE-treated MMTV-mS100a7a15–inducible mice when compared with control mice (Supplementary Fig. S3). These studies suggest that blockade of RAGE in mS100a7a15 transgenic mice inhibits tumor growth and metastasis through inhibition of M2 macrophage recruitment.

Because macrophage recruitment to primary tumors plays an important role in promoting S100A7/mS100a7a15–induced metastasis (18), we wanted to know whether recombinant mS100a7a15 might affect macrophage activity in a RAGE-dependent manner. We analyzed the migration of RAW264.7 (RAW), a macrophage cell line, in the presence of mS100a7a15 recombinant protein. We showed that mS100a7a15 significantly enhanced the migration of RAW and that pretreatment with nA-RAGE significantly abrogated mS100a7a15–induced migration compared with IgG control (Fig. 6A and B). Although S100A7 has been shown to regulate MMPs in cancer cells (17), its role in the macrophage is not known. We analyzed MMP9 secretion in the presence of mS100a7a15 with or without sRAGE treatment. mS100a7a15–induced MMP9 secretion was enhanced in RAW cells and this effect was diminished in the presence of sRAGE (Fig. 6C). Using double immunofluorescence, we also observed enhanced recruitment of MMP9–expressing F4/80+ macrophages in the MVT-1 tumors of MMTV-mS100a7a15–inducible mice compared with noninducible mice. The recruitment of MMP9+/F4/80+ macrophages...
was substantially decreased in the naRAGE-treated group compared with IgG-treated MMTV-mS100a7a15-inducible mice (Fig. 6D). We further analyzed the molecular mechanism of the mS100a7a15-induced migration of RAW cells. As shown in Fig. 6E and F, mS100a7a15-induced ERK activation was significantly reduced in the presence of sRAGE treatment. These studies suggest that RAGE receptor regulates mS100a7a15-induced ERK activation in macrophages.

Discussion

Emerging data have implicated importance of RAGE in the pathogenesis of various human disorders including cancers (2, 10). The interactions between RAGE and its ligands trigger the activation of MAPK, JAK/STAT, and NF-κB in various cancers (1, 2). In this work, we identified that RAGE plays a critical role in promoting breast cancer growth and metastasis. We documented that RAGE is highly expressed in human TNBC and murine breast tumors (2, 10). The interactions between RAGE and its ligands trigger the activation of MAPK, JAK/STAT, and NF-κB in various cancers (1, 2). In this work, we identified that RAGE plays a critical role in promoting breast cancer growth and metastasis. We documented that RAGE is highly expressed in human TNBC and murine breast tumors.
Figure 6.
RAGE/mS100a7a15 regulates MMP9+ macrophages. A, RAGE expression was analyzed on RAW cells by FACS. B, RAW cells were subjected to mS100a7a15 (100 ng/mL)-induced migration in presence of murine RAGE neutralizing (naRAGE) or control IgG antibodies. C, RAW cells were stimulated with mS100a7a15 (100 ng/mL) in the presence or absence of sRAGE (SR, 50 ng/mL) for 24 hours. Conditioned media were analyzed for MMP activity. C, bottom, quantification.
D, tumors excised from (-Dox) MMTV-mS100a7a15 mice were subjected to double immunofluorescence for MMP9 (green), F4/80 (red), or DAPI (blue). E, RAW cells were pretreated with naRAGE or IgG antibodies for 1 hour, stimulated with mS100a7a15 (SA15, 100 ng/mL), and subjected to Western blotting for P-ERK. Scale bar, 63 um. F, quantification of Western blot analyses. Data represent mean ± SD per experimental group. * or #, P < 0.05 * versus con; # versus SA15 IgG.
cancer cell lines and weakly expressed in low metastatic ER+ cells. However, there are some conflicting reports regarding the expression of RAGE in the MCF7 cell line (40, 41). It could be due to the difference in the techniques that were used. We detected RAGE expression by FACS analysis. However, other studies detected RAGE expression by Western blot analysis. There is possibility that RAGE detected by Western blotting could be a truncated or soluble form of RAGE. In addition, we showed that RAGE is preferentially expressed in invasive and metastatic tumor deposits. This is consistent with a recent report that demonstrated that high RAGE expression was observed in lymph node and distant metastases patients samples (42). Moreover, we observed that RAGE expression was associated with poor prognosis in breast cancer. We also characterized the role of RAGE in breast cancer progression and metastasis. Using a genetic approach, we showed that RAGE ablation significantly reduced PyMT cell–derived tumor growth while blocking RAGE with neutralizing antibodies inhibits breast cancer visceral metastasis in preclinical mouse models. It has been shown recently that RAGE knockdown by siRNA significantly inhibited tumorigenic potential of MDA-MB-231 cell line (40). RAGE ablation in a triple transgenic model of spontaneous pancreatic cancer has also been shown to delay pancreatic cancer development (9, 43).

Moreover, we demonstrated that RAGE binding to S100A7 enhanced RAGE expression in breast cancer cells. In addition, we provide evidence that S100A7 coimmunoprecipitated with RAGE in S100A7-overexpressing MDA-MB-231 cells. Our bioinformatics data also showed that RAGE is co-overexpressed with S100A7 in human breast cancer tissue. Proinflammatory RAGE ligands, such as S100B, S100A4, and S100A8/A9, have been shown to enhance RAGE expression, and continuous activation of RAGE maintains an inflammatory milieu at the tumor microenvironment (2, 44). Our data suggest that S100A7 enhances activation of NF-kB through RAGE activation. We also showed that blocking RAGE or inhibiting S100A7/mS100a7a15 binding to RAGE by soluble RAGE (sRAGE) inhibits breast cancer cell migration and ERK activation. The sRAGE acts as a decoy that prevents ligands from interacting with the cell surface receptor. The application of sRAGE in vivo resulted in an effective blockade of RAGE, in accordance with this decoy mechanism, in a range of animal models (11). It is well documented that S100A8/A9-RAGE axis plays a significant role in breast and colon cancer growth and metastasis by modulating its downstream targets such as ERK1/2 (P44/p42), MAPK, and NF-kB signaling pathways (7, 42, 45).

In the current study, we have shown that RAGE deficiency in the host reduced breast cancer growth by decreasing recruitment of TAMs and tumor angiogenesis. We have shown previously that mS100a7a15 overexpression in mammary glands enhanced mammary tumor growth metastasis through macrophage recruitment (18). To further analyze molecular mechanism of these effects, we blocked RAGE activation in MMTV-mS100a7a15–inducible model by naRAGE or sRAGE. Blocking RAGE reduced macrophage recruitment into the MVT–1–derived tumors in the MMTV-mS100a7a15–inducible model. Our study further revealed that blocking the RAGE/mS100a7a15 axis inhibits M2 marker arginase expression. M2-polarized TAMs are known to drive tumor progression by stimulating angiogenesis and metastasis (21, 38, 46). We did not observe a significant change in CD4/CD3/CD8-positive T cells and other immune cells such as natural killer cells, as detected by FACS in MVT–1–derived tumors obtained from MMTV-mS100a7a15–inducible mice treated with RAGE neutralizing antibody or sRAGE. We showed that blocking of RAGE inhibits MmS100a7a15–induced recruitment and MMP9 activation in macrophages. MMP9 has been shown to degrade the extracellular matrix and release growth factors to enhance angiogenesis (47, 48). Furthermore, it has been shown that MMP9 induction by primary tumors in macrophages and the lung endothelium promotes metastasis, especially to lung (48, 49).

In summary, our study shows that RAGE is highly expressed in basal-type breast cancer, especially TNBC, and is preferentially expressed in invasive and lymph node metastasis tissues. As depicted by our model (Fig. 7), elucidation of the molecular mechanism behind enhanced breast cancer growth and metastasis shows that this is likely due to binding of RAGE to S100A7. In turn, the RAGE/S100A7 axis is responsible for enhanced recruitment of MMP9-positive TAMs. We have also shown that RAGE neutralizing antibody and soluble RAGE significantly decrease tumor growth and metastasis in an inducible mS100a7a15 transgenic mouse model. These data imply that the RAGE/S100A7 signaling axis could be used to inhibit TNBC growth and metastasis. Furthermore, these studies demonstrate that RAGE could be used as a novel biomarker and that neutralizing antibodies/soluble RAGE could be used to develop novel therapeutic strategies against TNBC.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.W. Nasser, N.A. Wani, D.K. Ahirwar, C.A. Powell, J. Ravi, M. Elbazz, K. Shilo, C. Shapiro, R.K. Ganju


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