S100A9 Is a Novel Ligand of EMMPRIN That Promotes Melanoma Metastasis

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Abstract

The calcium-binding proteins S100A8 and S100A9 can dimerize to form calprotectin, the release of which during tissue damage has been implicated in inflammation and metastasis. However, receptor(s) mediating the physiologic and pathophysiologic effects of this damage-associated "danger signal" are uncertain. In this study, searching for candidate calprotectin receptors by affinity isolation-mass spectrometry, we identified the cell surface glycoprotein EMMPRIN/BASIGIN (CD147/BSG). EMMPRIN specifically bound to S100A9 but not S100A8. Induction of cytokines and matrix metalloproteases (MMP) by S100A9 was markedly downregulated in melanoma cells by attenuation of EMMPRIN. We found that EMMPRIN signaling used the TNF receptor-associated factor TRAF2 distinct from the known S100-binding signaling pathway mediated by RAGE. S100A9 strongly promoted migration when EMMPRIN was highly expressed, independent of RAGE, whereas EMMPRIN blockade suppressed migration by S100A9. Immunohistologic analysis of melanomas revealed that EMMPRIN was expressed at both the invasive edge of lesions and the adjacent epidermis, where S100A9 was also strongly expressed. In epidermal-specific transgenic mice, tail vein–injected melanoma accumulated in skin expressing S100A9 but not S100A8. Together, our results establish EMMPRIN as a receptor for S100A9 and suggest the therapeutic use in targeting S100A9–EMMPRIN interactions. Cancer Res; 73(1); 1–12. ©2012 AACR.

Introduction

There is a well-established link between chronic inflammation and cancer development (1–3). A number of molecules have been identified as contributing to this process: these include TNF-α, interleukin (IL)-1, IL-6, chemokines, matrix metalloproteases (MMP), angiogenic factors, and anti-apoptotic proteins. We have shown that many of these molecules are upregulated in cultured keratinocytes stimulated with S100A8/A9 (4). Furthermore, S100A8/A9-induced cytokines and chemokines, in turn, stimulate keratinocytes to synthesize and secrete S100A8/A9, suggesting a positive feedback mechanism between S100A8/A9 and these proinflammatory factors. In addition to the inflammatory processes, recent investigations have revealed that S100 proteins play important roles in malignancy, especially in cancer cell metastasis (5–8).

Regarding receptors, RAGE (receptor for AGE, advanced glycation end products) is known to be a general and inflammation-related S100 protein receptor (9–11). However, RAGE is a multiligand receptor of the immunoglobulin superfamily of cell surface proteins that acts as pattern recognition receptors. Ligands of RAGE include AGE, S100/calgranulins, high mobility group box-1 (HMGB1), amyloid beta peptide, and beta-sheet fibrils. In addition, S100A9, S100A11, S100A12, S100A13, and S100P were also thought to interact with RAGE and transduce signals (12, 13).

Although this variety of ligands implicates RAGE in a wide spectrum of pathophysiologic conditions, it would be inappropriate to postulate that all the S100-mediated reactions are totally dependent on their interaction with RAGE. Indeed, some S100 proteins seem to signal by engaging not only RAGE but also non-RAGE receptors. Other putative S100-binding proteins include CD36 (14), heparan sulfate proteoglycan (15), and carboxylated glycans (16).

In the present study, we tried to identify the S100A8/A9 receptor involved in the positive feedback mechanism of inflammation. We used Liquid chromatography coupled with electrospray tandem mass spectrometry (LC/MS-MS)-based shotgun proteomics and succeeded in identifying the extracellular matrix metalloprotease inducer (EMMPRIN) as a novel receptor for S100A9. Interestingly, EMMPRIN is a well-known cell surface molecule that is associated with cancer cell malignancy (17, 18). We explored the S100A9–EMMPRIN relationship in the context of melanoma cell metastasis in vitro and in vivo. Our data provide new and important insights into the molecular mechanism of...
S100A9-EMMPRIN–mediated inflammation and cancer cell metastasis.

Materials and Methods

Liquid chromatography coupled with electrospray tandem mass spectrometry

Proteins were identified via a shotgun-type protein identification approach described previously (19, 20). Briefly a lab-made nano-flow liquid chromatography-mass spectrometry platform consisting of a Nanospace SI-2 high-performance liquid chromatograph (Shiseido Co.), a Yates lab-designed nano-electrospray ionization source, and an LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Inc.) were used to obtain tandem mass spectra of tryptic peptides. The resulting tandem mass spectrometry spectra were analyzed using the SEQUEST algorithm against a nonredundant human protein database (NCBI, Feb2007) for putative protein identifications.

Preparation of expression vectors

We prepared various expression vectors for soluble (sol) EMMPRIN, dominant-negative (dn) EMMPRIN, cytoplasmic domains of N-terminal (RAGE-cyt; ref. 13) and C-terminal (EMMPRIN-cyt), EMMPRIN variants (Mut-Traf and Mut-Cter), TIRAP, MyD88, TRAF2, TRAF6, dnTRAF2, dnTRAF6, S100A8, and S100A9. Details of constructions are provided in the Supplementary Methods.

Coimmunoprecipitation analysis

Transient transfection of each plasmid to the HEK293 cells was conducted using FuGENE-HD (Promega Biosciences). After 48 hours, conditioned medium was subjected to coimmunoprecipitation experiments, using anti-HA tag (clone HA-7) agarose (Sigma-Aldrich) and anti-Myc tag (clone 1G4) Agaroses (MBL). The tag-Agarose beads were mixed with conditioned mediums and incubated for 3 hours at 4°C. The precipitated proteins were subjected to SDS-PAGE and detected by subsequent Western blotting using mouse anti-HA (clone 6E2; Cell Signaling Technology) and mouse anti-Myc tag (clone 9B11; Cell Signaling Technology) antibodies. GFP-bound form of cdc42 was also determined using a Rac/cdc42 Activation Assay Kit (Millipore).

S100A9-EMMPRIN binding assay

Recombinant EMMPRIN was immobilized on a 96-well ELISA plate by incubation at 4°C overnight. After washing with PBS containing 0.1% Tween20 (PBS-T), the plate was incubated with PBS containing 5% bovine serum albumin (BSA) at 37°C for 1 hour for blocking. Various concentrations of recombinant S100A9 were added to each well, and the plate was incubated at 37°C for 1 hour. After washing with PBS-T, anti-S100A9 antibody (1:2,000 in PBS) was added, and bound S100A9 was detected with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (1:2,000 in PBS). Color was developed using TMB Peroxidase EIA Substrate Kit (Bio-Rad Laboratories) and read at 630 nm.

Melanoma cell lines

SK-MEL-2 (HTB-68), SK-MEL-3 (HTB-69), SK-MEL-5 (HTB-70), MeWo (HTB-65), and A2058 (CRL-11147) were purchased from American Type Culture Collection in 2002, and after expanded culture, 1 × 10⁶ cells/tube were kept in liquid nitrogen B16-BL6 cells (1), a highly invasive variant of a mouse melanoma B16 cell line, was obtained from Dr. Isaiah J. Fidler (M. D. Anderson Cancer Center, Houston, TX). All cell lines were used from these stocks or within 3 passages. Melanoma cells were cultured with Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum.

Establishment of stable clones

For isolation of clones stably expressing dnEMMPRIN, B16-BL6 cells were subjected to conventional transfection method with the plasmids (pDNRII-CMVi-dnEmmp and pMSCV-puro; Clontech TAKARA) using the FuGENE-HD reagent (Promega Biosciences) and then selected with puromycin.

Microarray analysis

Whole human genome (4 × 44K) Oligo Microarray Kit (Agilent) was used. We prepared the target RNA from SK-MEL-2 and primary keratinocytes in the presence or absence of recombinant S100A8, S100A9, or S100A8/A9 (10 µg/mL each) for 3 or 24 hours as described. cDNAs from total RNA (1.65 µg starting material) isolated from cell culture harvests were used for generation of Cy3-labeled cRNA by in vitro transcription. The cRNA was fragmented before hybridization, and a hybridization cocktail was prepared. Microarrays were hybridized, scanned and image analysis was conducted as described by the manufacturer.

Real-time quantitative reverse transcription PCR

Real-time PCR was carried out with an ABI 7900HT Real time PCR system (Applied Biosystems) using the Sybr Green PCR mix (Invitrogen). Samples were cycled at 95°C for 10 minutes, 40 cycles of: 95°C for 15 seconds, 60°C for 1 minute. Primer sets used are listed in the Supplementary Materials and Methods.

Melanoma tissues

Human skin specimens were obtained under informed consent from the patients with melanoma. A total of 20 melanoma tissues were analyzed immunohistochemically. The study was approved by the Institutional Review Board of Tokyo Medical University (Tokyo, Japan) and the Shiseido Committee on Human Ethics.

Knockdown of EMMPRIN by siRNA silencing

Recombinant S100A8 and S100A9 were prepared as previously described (4). SK-MEL-2 cells were plated at 50% to 60% confluence and transfected with 50 nmol/L siRNA against EMMPRIN or nonspecific control (Santa Cruz) using Lipofectamine RNAi MAX (Invitrogen). After 24 hours post-transfection, 10 µg/mL recombinant S100A8, S100A9, or S100A8/A9 were added and incubated for an additional 3 or 24 hours.
In vivo metastatic assay

Migration of human melanoma cells was assayed using a 96-well Disposable Chemotaxis System (8-μm pore size; Funakoshi). The lower wells of the chamber were loaded with DMEM containing recombinant S100A8, S100A9, or S100A8/A9 (10 μg/mL). Melanoma cells (10,000 cells per 25 μL in DMEM) were placed in the upper wells. After incubation for 48 hours at 37°C, cells on the lower surface of the filter were stained with CellTracker Red CMTPX (5 μm, Molecular Probes), and fluorescence intensity was measured and compared with that of the medium control.

Immunohistochemical procedures

Primary antibodies used are listed in the Supplementary Materials and Methods. HRP-conjugated, goat anti-rabbit IgG or anti-mouse IgG (Nichirei) were used as secondary antibodies and reacted with 3,3′-diaminobenzidine (DAB). For double detection, Alexa Fluor 555 or 488 (Molecular Probes Inc.) was used. 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes) was used to visualize nuclei.

Results

EMMPRIN is identified as an S100A8/A9-binding protein

We carried out affinity purification-mass spectrometry using keratinocyte membrane fractions, as S100A8/A9 is strongly upregulated in the epidermis in inflammatory skin disorders (22). We analyzed interactions between 2 kinds of membrane extracts (from proliferating and differentiated cells) and glutathione S-transferase (GST)-fused S100 proteins. Overlapping proteins interacting with GST were removed and finally we identified total of 13 proteins from the analyses (Table 1). S100A9 showed the highest probability and GST followed next in each sample, indicating high fidelity of these analyses. Interestingly, we found 2 membrane proteins, leucine zipper-EF-hand transmembrane protein 1 (LETM1) and basigin. As LETM1 serves as an anchor protein for complex

Table 1. Summary of LC/MC-MC analysis of S100A8/A9-interacting proteins

<table>
<thead>
<tr>
<th>Description</th>
<th>Protein ID Probabilitya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S100A9</td>
</tr>
<tr>
<td>S100 calcium-binding protein A9 [Homsapiens]</td>
<td>1.00E-302</td>
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<tr>
<td>Glutathione S-Transferase (E.C.2.5.1.18)</td>
<td>4.00E-13</td>
</tr>
<tr>
<td>Xeroderma Pigmentosum Group E Complementing protein</td>
<td>7.29E-13</td>
</tr>
<tr>
<td>Glutathione transferase [Homo sapiens]</td>
<td>1.21E-12</td>
</tr>
<tr>
<td>Tubulin alpha 6 [Homo sapiens]</td>
<td>1.27E-08</td>
</tr>
<tr>
<td>Acyl-CoA synthetase long-chain family</td>
<td>5.94E-08</td>
</tr>
<tr>
<td>Leucine zipper-EF-hand containing transmembrane</td>
<td>1.07E-07</td>
</tr>
<tr>
<td>B Chain B, Human Glutathione S-Transferase</td>
<td>2.93E-07</td>
</tr>
<tr>
<td>Eukaryotic translation elongation factor 1 beta 2</td>
<td>1.19E-05</td>
</tr>
<tr>
<td>Basigin isoform 1 [Homo sapiens]</td>
<td>1.62E-05</td>
</tr>
<tr>
<td>Ribosomal protein P2 [Homo sapiens]</td>
<td>2.71E-05</td>
</tr>
<tr>
<td>Myosin, light polypeptide 6, alkali, smooth muscle</td>
<td>3.86E-04</td>
</tr>
</tbody>
</table>

NOTE: Bold indicates notable molecules including S100A8, S100A9, and basigin (EMMPRIN).

Abbreviation: N.D., not defined.

*aStringent search criteria: S' score > 0.85; peptide probability < 0.001; number of top matches: 1; precursor mass tolerance: < 5 ppm; minimum number of peptides to identify proteins: 1; enzyme specificity: half-tryptic or fully tryptic (peptides only).
formation with the mitochondrial ribosome (23), a cell membrane protein, basigin, was selected as a candidate molecule. Basigin is also known as EMMPRIN or CD147 (24). EMMPRIN was found to be an S100A9 interactor in extracts from both proliferating and differentiated cells.

**S100A9 specifically binds with EMMPRIN**

Extracellular domain of EMMPRIN (soluble form), S100A8, and S100A9 were expressed in HEK293 cells and immunoprecipitated with appropriate antibodies. We found that S100A9 but not S100A8 was coimmunoprecipitated with EMMPRIN, suggesting that EMMPRIN binds to S100A9 (Fig. 1A). In addition, when S100A8 and S100A9 were coexpressed and pulled down with EMMPRIN, both proteins were detected in the precipitated fraction. Thus, it is likely that the S100A8/A9 heterodimer (calprotectin) is also capable to bind with EMMPRIN. Binding of S100A9 was further confirmed with EMMPRIN-immobilized ELISA assays. S100A9 bound with immobilized soluble form of EMMPRIN in a dose-dependent manner (Fig. 1B). We next examined EMMPRIN expression in 5 melanoma cell lines and primary human keratinocytes. The highest expression was seen in SK-MEL-2, which was 3.3 times higher than in SK-MEL-5 (Fig. 1C). The gene expression profile of SK-MEL-2 cells was investigated after stimulation with S100A8, S100A9, or S100A8/A9 (Table 2). We used 10 µg/mL of each S100 protein, as this concentration showed the highest stimulation of cytokine induction in keratinocytes (Supplementary Fig. S1A). Only 16 genes were upregulated more than 2-fold at 3 hours in S100-stimulated SK-MEL-2 cells, including CCL1, CCL2, CCL3, IL-8, TNF-α, BIRC3 (cIAP2), and EFNNA1 (ephrinA1). Components of NF-κB as well as associated factors were also induced by these S100 proteins. Interestingly, most of these factors are related to inflammation and cell survival. Our previous study showed that similar genes were also upregulated in keratinocytes stimulated with S100A8/A9 (4), indicating common and essential features of S100A9 function. As we already knew that MMP10, MMP1, MMP2, and MMP9 were induced at 24 hours after S100A8/A9 stimulation in keratinocytes (see Supplementary Table S1), we tested expression of these MMPs in SK-MEL-2, a high EMMPRIN expressor. MMP1 was always upregulated after stimulation with S100A9, S100A8, or S100A8/A9 (Fig. 1D and E), although induction of MMP9 and MMP2 was inconsistent in SK-MEL-2 (data not shown).

To elucidate the possible involvement of EMMPRIN in these changes, we used 2 approaches, siRNA-mediated knockdown of EMMPRIN (Supplementary Fig. S1B) and treatment with a soluble form of EMMPRIN. Under conditions of EMMPRIN knockdown, induction of cytokines was not detected (Fig. 1D). Induction by S100A8 and S100A8/A9 was also suppressed. As S100A8 and S100A9 are thought to make a stable heterodimer in vivo (25, 26), these results may, at least in part, be dependent on binding with EMMPRIN via S100A9. Furthermore, addition of soluble EMMPRIN to the medium downregulated the induction of cytokines and MMPs in SK-MEL-2 (Fig. 1E; Supplementary Fig. S1C). Collectively these results suggest that S100A9 is a novel ligand of EMMPRIN and that S100A9–EMMPRIN interaction is the key to inducing proinflammatory factors and MMPs both in keratinocytes and in melanoma cells.

**Signal transduction pathways are distinct between EMMPRIN and RAGE**

As RAGE is a well-known receptor of multiple S100 proteins (12), we compared signaling pathways for EMMPRIN and RAGE. First, we identified adaptor molecules for signal transduction. A motif search for EMMPRIN (http://elm.eu.org/) indicated a TRAF-binding site in its cytoplasmic domain (Supplementary Fig. S2). Furthermore, our previous work (13) suggested that RAGE required MyD88 and TIRAP for the signal transduction. Thus, we include MyD88, TIRAP, TRAF2, and TRAF6 as candidate molecules. Each cytoplasmic domain of EMMPRIN or RAGE tagged with hemagglutinin (HA) was expressed in HEK293 cells. Coexpression analysis indicated that cytoplasmic domain of EMMPRIN only bound with TRAF2, whereas that of RAGE trapped MyD88, TIRAP, and TRAF6, suggesting different signal transduction pathways between EMMPRIN and RAGE (Fig. 2A). Mutation of TRAF-binding motif in EMMPRIN (Supplementary Fig. S2) abolished the binding ability with TRAF2 (Fig. 2B). We confirmed that inherent TRAF2 was recruited by the stimulation with externally added S100A9 (Fig. 2C). We next examined whether EMMPRIN and RAGE were able to form heterodimer each other. Figure 2D clearly showed that they were unable to make up heterodimers but present as homodimers. Overexpression of dominant-negative TRAF2 in SK-MEL-2 cells showed significant reduction of S100A9-induced cdc42 activation (Fig. 2E), further supporting the involvement of TRAF2 in S100A9–EMMPRIN signaling pathway.

**Immunohistochemical analysis shows that EMMPRIN and S100A9 are differentially expressed**

We next examined the localization of S100A9 and EMMPRIN. These proteins were hardly detectable in normal human skin (Fig. 3A) or in benign pigmented nevi (Fig. 3B). In contrast, heavy staining of S100A9 was always observed in the epidermis adjacent to HMB–45–positive melanoma-burdened skin (Fig. 3C; Supplementary Fig. S3A). Interestingly, EMMPRIN was detected at the invasive edge of melanoma lesions (Fig. 3C and D) but was absent in the mass of melanoma tissue (Supplementary Fig. S3B). In the epidermis, EMMPRIN was also present in the upper layer of melanoma tissue (Fig. 3C and D). Proximity ligation assay (PLA) using anti-S100A9 antibody and anti-EMMPRIN antibody indicated a positive reaction at the invasive edge of melanoma cells as well as the upper epidermal tissue exposed to the melanoma (Supplementary Fig. S4). These results suggest colocalization of these molecules and possible interaction in vivo. For melanoma cell metastasis, it is necessary to degrade the basement membrane at the dermal–epidermal junction. MMP2 and MMP9 are thought to play an important role in the degradation of basement membrane components (27). Loss of the basement membrane structure was obvious at the invasive edge of melanoma as verified by the absence of type VII collagen (28; Fig. 3E and F). Immunohistochemical data indicate that melanoma cells and...
Figure 1. S100A9 specifically binds with EMMPRIN. A, immunoprecipitation study showing binding of S100A9 with EMMPRIN in HEK293 cells. S100A8 and S100A9 were expressed with HA-tag. Soluble form of EMMPRIN was expressed with both HA-tag and Myc-tag. Pull-down with anti-HA antibody showed sufficient expression of these proteins (lanes 1–3). When S100A8 or S100A9 was expressed together with EMMPRIN and pulled down with anti-Myc antibody, only S100A9 was seen to bind with EMMPRIN as verified with anti-HA antibody. Coexpression of S100A8 and S100A9 resulted in the detection of both proteins. B, ELISA assay showing S100A9 binding with extracellular domain of EMMPRIN in a dose-dependent manner. C, EMMPRIN mRNA expression in 5 melanoma cell lines and normal human keratinocytes. D, effect of EMMPRIN knockdown on the expression of S100-induced TNF-α, IL-8, and MMP1. S100 proteins were added to the SK-MEL-2 culture, and total RNA samples were obtained 3 hours after incubation. E, effect of soluble EMMPRIN (sol. EMMPRIN) on the expression of S100-induced TNF-α, IL-8, and MMP1 in SK-MEL-2. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
keratinocytes respond to each other strongly at the invasive edge, where S100A9–EMMPRIN interaction takes place.

Chemotactic activity of S100A9 is dependent on EMMPRIN expression
Some S100 family members are known to function as chemotactants to neutrophils (29) and cancer cells (30). We analyzed the chemotactic activity of S100A8 and S100A9 on melanoma cell lines. S100A9 showed a strong effect on SK-MEL-2 migration (Fig. 4A). S100A8 and S100A8/A9 also showed chemotactic activity on SK-MEL-2 to some extent. Knockdown of EMMPRIN markedly suppressed the chemotactic activity of S100A9, as well as S100A8 and S100A8/A9 on SK-MEL-2. Quantitative analysis further supported the above results (Fig. 4B). Either S100A9 or S100A8 had little effect on the migration of SK-MEL-3 and SK-MEL-5, low EMMPRIN expressers (Fig. 4C). These results indicate that chemotactic activity of S100A9 is closely related to the expression level of EMMPRIN (Fig. 1C).

In addition, we established a clone of B16-BL6 melanoma cells that constitutively expresses the dominant-negative (dn) form of EMMPRIN (dnEMMPRIN B16) with 1,400 times higher than intact EMMPRIN. This clone lacks the cytoplasmic domain of EMMPRIN and thus is unable to transduce S100A9 signals. S100A9 showed significant chemotactic activity on control B16 melanoma cells (Fig. 4D). On the other hand, S100A9 did not show any effect on the migrating activity of dnEMMPRIN B16. Quantitative analysis clearly showed strong effect of S100A9 on B16 cell migration (Fig. 4E). To assess the involvement of RAGE in melanoma metastasis, we examined expression of RAGE in these cell lines. Expression of RAGE was 40-fold higher in dnEMMPRIN B16 than in B16 control, although that of mouse EMMPRIN is rather suppressed in dnEMMPRIN B16 (Fig. 4F). We also checked expression levels of RAGE in human melanoma cell lines. Quantitative PCR (qPCR) analysis showed it was 1.5 times higher in SK-MEL-2 than in SK-MEL-5 (Fig. 4G). Collectively, these data suggest that S100A9-induced cell migration is strongly dependent on EMMPRIN.

Suppression of migration activity caused by knockdown of EMMPRIN or blocking of signal transduction may be due to cell aggregation (Supplementary Fig. S5A). dnEMMPRIN B16 showed a very compact cell shape and formed dense cell aggregates, although control B16 or vector-transfected cells grew with a rather uniform spread and an elongated cell shape.

Overexpression of S100A9 induced melanoma cell migration in vivo
Finally, we tested whether S100A8 or S100A9 contributed to the metastatic ability of cancer cells. For this purpose, we generated involucrin promoter–driven S100A8 and S100A9 Tg mice (Fig. 5A). We chose the involucrin promoter, as these molecules were expressed in the upper epidermis of inflammatory skin. We used SK-MEL-2 and SK-MEL-5 for the metastasis assay as high and low EMMPRIN expressers, respectively. SK-MEL-2 or SK-MEL-5 cells were injected into the tail vein of wild-type and Tg mice, and their presence was examined in the skin after 4 weeks. Skin specimens were scanned with a confocal microscope in an area 1 × 2 cm² or immunostained with HMB-45 and MART-1 for the detection of melanoma cells. We used SK-MEL-2– and SK-MEL-5–driven S100A8 and S100A9 Tg mice, we found very few HMB-45–positive cells (n = 6; Fig. 5B). However, considerable numbers of positive cells were always detected in the skin of SK-MEL-2–injected S100A9 Tg mice (n = 6; Fig. 5C). Presence of melanin in the skin of both types of mice was confirmed with Fontana–Masson staining (Fig. 5D). Immunohistochemical

Table 2. Summary of microarray analysis for S100-stimulated SK-MEL-2 at 3 hours

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<tr>
<th>Gene symbol</th>
<th>+S100A8</th>
<th>+S100A9</th>
<th>+S100A8/A9</th>
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<tr>
<td>CXCL3</td>
<td>7.30 ± 0.81</td>
<td>14.77 ± 0.56</td>
<td>12.57 ± 0.78</td>
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<tr>
<td>IL-8</td>
<td>6.20 ± 0.68</td>
<td>14.36 ± 2.12</td>
<td>10.69 ± 0.47</td>
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<tr>
<td>CCL2</td>
<td>5.35 ± 0.85</td>
<td>13.62 ± 0.62</td>
<td>10.03 ± 0.35</td>
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<tr>
<td>BIRC3</td>
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<td>ZC3H12A</td>
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<td>7.81 ± 0.37</td>
<td>6.82 ± 0.22</td>
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<tr>
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<td>2.74 ± 0.05</td>
<td>2.59 ± 0.09</td>
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NOTE: Expressed as mean values ± SD from n = 3 microarray analysis.
analyses showed that these cells were strongly positive for HMB-45 and MART-1 (Fig. 5E). Many melanoma cells were found scattered around an artery that were verified with anti-CD31 and anti-smooth muscle antibodies (Fig. 5E; Supplementary Fig. S6). In some cases, melanoma cells were embedded within the vessel wall. Relatively large numbers of melanoma cells were also found near hair follicle–like structures (Fig. 5E). HMB-45–positive cells were not found in the skin of SK-MEL-5–injected S100A8 or S100A9 Tg mice (Supplementary Fig. S7).

To elucidate further involvement of EMMPRIN in cancer cell metastasis, we carried out limited attempts using highly invasive melanoma cell line, B16-BL6 and dnEMMPRIN B16. When these cells were injected via tail vein, only the control B16 cells metastasized to lung in both wild and S100A9 Tg mice after 6 weeks observation (Supplementary Table S2 and Supplementary Fig. S5B). We could not find any incidence for dnEMMPRIN B16 cell metastasis in the lung. Interestingly, we observed 2 tumor nodules in the skin of S100A9 Tg mouse that were tail vein–injected with B16-BL6 cells (Supplementary Fig. S5C).

Discussion

There have been many reports that both S100 proteins and EMMPRIN are involved in malignancy, especially in cancer cell...
metastasis (7, 31, 32). To date, the metastatic ability of these proteins is considered to be an independent phenomenon. In the present study, we clarified for the first time the ligand-receptor relationship between S100A9 and EMMPRIN. Binding of S100A9 to EMMPRIN was confirmed with (i) affinity isolation-mass spectrometry analysis, (ii) immunoprecipitation study in cellular level, and (iii) dose-dependent binding to immobilized EMMPRIN. The activity of EMMPRIN as a functional receptor for S100A9 was further shown by the fact that induction of both proinflammatory cytokines and MMPs was markedly suppressed in the EMMPRIN-knockdown cells. In vivo association between S100A9 and EMMPRIN was also shown with PLA at the invasive edge of melanoma and the adjacent epidermis.

EMMPRIN, also known as CD147, is able to induce production of several MMPs (33). It is overexpressed in various cancerous tissues (18, 34, 35). Although induction of MMPs has been regarded as evidence of the homophilic interaction between EMMPRIN molecules, including a soluble form, our results clearly show that the soluble EMMPRIN is able to bind to S100A9 and prohibits S100A9-mediated MMP induction. Thus, MMP induction by EMMPRIN is, at least in part, caused by the binding of the S100A9 or S100A8/A9 heterodimer to EMMPRIN. In malignant melanoma, both MMP-9 and

Figure 3. Immunohistochemical analysis shows that EMMPRIN is expressed in the invasive edge of melanomas. A, normal skin showing no expression of S100A9 and EMMPRIN. B, pigmented nevus. C, immunohistochemistry for S100A9 (red) and HMB-45 (green) in primary melanoma. Nuclear staining with DAPI (blue) is also shown. D, magnified view of invasive region of melanoma from boxed area of C. E, immunostaining for collagen type VII (Col VII) of normal human skin. F, loss of basement membrane in EMMPRIN-expressing melanoma lesion. An example of melanoma tissue stained with collagen type VII (Col VII, red) and EMMPRIN (green). Dotted line shows the edge of cornified layer. Scale bars, 100 μm.
EMMPRIN were variably expressed in the invasive radial growth phase but not in the vertical growth phase, and early invasion of melanoma is associated with de novo expression of MMP-9 and EMMPRIN (36). Our observation also supports these findings and suggests that peripheral keratinocyte–derived S100A9 promotes MMP production in neighboring cells.
cancer cells via EMMPRIN signal transduction. Apparently, MMP production is required for efficient cancer cell metastasis by degrading basement membrane and surrounding matrix proteins. We showed the disappearance of the basement membrane just in the area where these proteins are coexpressed.

Some cancer cells, including melanoma cells, seem to influence distant tissues to establish a premetastatic niche before metastasis occurs (8). Primary tumor cells secrete pro-inflammatory factors such as VEGF-A, TGF-β, and TNF-α, which induce selective expression of chemoattractants S100A8 and S100A9, thereby making it easier for tumor cells to home in on the premetastatic sites. Our metastasis assays using S100A8 and S100A9 Tg mice revealed that only the high EMMPRIN-expressing SK-MEL-2 cells migrated into the S100A9-expressing skin. Low EMMPRIN expresser (SK-MEL-5), EMMPRIN-knocked down SK-MEL-2, and dnEMMPRIN B16 all failed to metastasize or to migrate in response to S100A9. Furthermore, S100A9–EMMPRIN interaction induced cdc42 activation (Fig. 2E). cdc42 is known to promote filopodia formation, cell polarity, and migration (37). This may be the reason why EMMPRIN is expressed at the invasive edge of melanomas.
and not in the denser mass of melanoma cells. Indeed, when we used the established B16 clone that overexpressed dnEMMPRIN failed to migrate after S100A9 stimulation. Our limited attempts to assess the metastatic ability in vivo also showed that only the EMMPRIN-expressing B16 cells, but not dnEMMPRIN B16, metastasized to lung in mice. These lines of evidence further suggest the critical role of EMMPRIN in melanoma cell metastasis.

It is thought that S100 proteins actively participate in the modulation of inflammation and immune responses by signaling via RAGE (32, 38, 39). On the other hand, EMMPRIN is also known as a signaling receptor for cyclophilines A and B (40). Recent studies have shown a role for cyclophilin–EMMPRIN interactions in the regulation of inflammatory responses (41). Although RAGE and EMMPRIN seem to have multiple ligands, S100A9–EMMPRIN interaction is of particular relevance to cancer cell biology.

In the present study, we clearly showed that EMMPRIN and RAGE have distinct properties in S100A9-induced signal transduction. EMMPRIN recruited TRAF2 as an adaptor molecule in its signaling pathway, whereas RAGE required MyD88, TIRAP, and TRAF6 (Fig. 2). The fact that EMMPRIN failed to form a heterodimer with RAGE further supports the difference in signal transduction and physiologic functions in these molecules.

Because of the involvement of EMMPRIN in the invasion and metastasis processes of many types of cancers, its downregulation would seem to be a logical method of preventing cancer cell invasion (42, 43). Indeed this idea is supported by reports that transfection of EMMPRIN cDNA into breast cancer cells resulted in increased incidence of metastasis (44) and that EMMPRIN-targeting siRNA inhibited the metastatic activity of malignant melanoma in a nude mouse model of pulmonary metastasis (35). However, S100A9 and EMMPRIN are both pleiotropic molecules that play a critical role in various physiologic reactions. S100A9 also works as an anti-inflammatory and anti-inflammatory factor (45), although it is a Janus-faced molecule functioning as a potent amplifier of inflammation in autoimmunity as well as in cancer development and tumor spread (46). EMMPRIN is also involved in fetal development, retinal function, nervous system development, and plaque formation in Alzheimer disease (33). Thus, although knockdown of these molecules might be an effective therapeutic option, there might be limitations to directly targeting S100A9 or EMMPRIN. Interfering with the S100A9–EMMPRIN interaction would be a novel strategy to block metastatic activity of malignant melanomas.

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

Authors’ Contributions

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