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RAGE signaling significantly impacts tumorigenesis and hepatic tumor growth in murine models of colorectal carcinoma

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Abstract

Background—The receptor for advanced glycation end-products (RAGE) is a cell surface receptor implicated in tumor cell proliferation and migration. We hypothesized that RAGE signaling impacts tumorigenesis and metastatic tumor growth in murine models of colorectal carcinoma.

Materials and Methods—*Tumorigenesis*: *Apc*^{1638N/+} mice were crossed with *Rage*^{−/−} mice in the C57BL/6 background to generate *Apc*^{1638N/+}/*Rage*^{−/−} mice. *Metastasis*: BALB/c mice underwent portal vein injection with CT26 cells (syngeneic) and received daily soluble (s)RAGE or vehicle. *Rage*^{−/−} mice and *Rage*^{+/+} controls underwent portal vein injection with MC38 cells (syngeneic). *Rage*^{+/+} mice underwent portal vein injection with MC38 cells after stable transfection with full-length RAGE or mock transfection control.

Results—*Tumorigenesis*: *Apc*^{1638N/+}/*Rage*^{−/−} mice had reduced tumor incidence, size, and histopathologic grade. *Metastasis*: Pharmacological blockade of RAGE with sRAGE or genetic deletion of *Rage* reduced hepatic tumor incidence, nodules, and burden. Gain of function by transfection with full-length RAGE increased hepatic tumor burden compared to vector control MC38 cells.

Conclusion—RAGE signaling plays an important role in tumorigenesis and hepatic tumor growth in murine models of colorectal carcinoma. Further work is needed to target the ligand-RAGE axis for possible prophylaxis and treatment of primary and metastatic colorectal carcinoma.

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Keywords

RAGE; receptor for advanced glycation end-products; colorectal carcinoma; colon cancer; sRAGE; *Rage* knockout mice

Introduction

Colorectal carcinoma is the most common gastrointestinal malignancy and the third-leading cause of cancer-related deaths in the United States. [1] Colorectal carcinoma commonly metastasizes to the liver, after which 5-year patient survival is approximately 30%. [2] There is a need to identify novel targets for intervention in colorectal carcinoma tumorigenesis and metastasis; however, the cellular and molecular mechanisms involved are incompletely understood. Mutations in the *Apc* gene play a crucial, early role in the development of familial and sporadic intestinal tumors, [3] and there is mounting evidence that an inflammatory microenvironment supports tumorigenesis and metastasis by promoting cancer cell proliferation, invasion, and migration. [4,5,6]

The receptor for advanced glycation end-products (RAGE) is broadly implicated in both inflammation and cancer. [7,8,9,10] RAGE is a multi-ligand, transmembrane cell surface receptor of the immunoglobulin superfamily. Increased expression of RAGE and its ligands has been documented in various inflammatory diseases such as sepsis, diabetes, and inflammatory bowel disease. [7,8,11] Furthermore, up-regulation and co-localization of RAGE and many of its ligands in a range of human tumors, including colorectal tumors, suggest that the ligand-RAGE axis plays an important role in tumorigenesis and metastasis. RAGE ligands interact in complex autocrine and paracrine manners within the tumor microenvironment to promote cell survival, invasion, and migration. [9]

Among the RAGE ligands, two are widely implicated in tumorigenesis and metastasis: S100 proteins and high-mobility group box 1 (HMGB1). S100 proteins are small, calcium-binding molecules that can interact with RAGE and promote inflammation by activating endothelial cells, macrophages, and lymphocytes. [9] Increased expression of S100P has been documented in human colorectal carcinoma, and S100P has been shown to stimulate colon cancer cell proliferation and migration *in vitro*. [12] HMGB1, in part via its interaction with RAGE, can act as a potent pro-inflammatory cytokine to promote a microenvironment that is conducive to tumor growth, invasion, and metastasis. [13,14,15] Increased expression of HMGB1 has been demonstrated in colon adenomas and carcinomas, [16] and co-expression of RAGE and HMGB1 has been associated with tumor invasion, metastasis, and poor prognosis in colorectal cancer. [17,18,19]

Ligand-RAGE interactions activate multiple signaling pathways that are implicated in tumor proliferation and progression, including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and nuclear factor (NF)- κ B pathways. [13,18] Depending on the cell type and biological context, RAGE-mediated activation of NF- κ B primes cells for pro-inflammatory and anti-apoptotic signaling. [20,21] For example, NF- κ B is known to play a critical role in the molecular pathogenesis of colon cancer associated with inflammatory bowel disease. [22] Other transcriptional targets of RAGE signaling include vascular cell

adhesion molecule-1 (VCAM-1) and tissue factor, which contribute significantly to tumor cell interactions with the endothelium. [23,23] Finally, increased matrix metalloproteinase (MMP) activity in cells over-expressing RAGE has been shown to correlate with metastatic potential in colorectal and other tumor cells. [13,18,19]

In the current study, we examined the effects of RAGE signaling in murine models of colorectal carcinoma. We employed an established model of familial adenomatous polyposis (FAP) to test the impact of *Rage* gene deletion on tumorigenesis in *Apc*^{1638N/+} mice. We then used pharmacological blockade and *Rage* gene deletion to evaluate the impact of loss of RAGE function on metastatic colorectal carcinoma cells. Finally, we used cell transfection with full-length RAGE to test the impact of cell-specific gain of RAGE function on metastatic tumor growth. Taken together, these data suggest that the ligand-RAGE axis plays an important role in the development of primary and metastatic colorectal carcinoma in mice.

Materials and Methods

Animals

Apc^{1638N/+} mice in the C57BL/6 background were kindly provided by Howard L. Kaufman, MD (Rush University Medical Center, Chicago, IL). *Rage* knockout (*Rage*^{-/-}) mice were generated in the C57BL/6 background as described previously. [25,26] *Rage*^{-/-} mice develop normally and are reproductively fit. Absence of RAGE expression in *Rage*^{-/-} mice has been documented previously at our institution. [27] Wild-type BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in a specific pathogen-free facility of Columbia University (New York, NY), housed in a temperature-controlled room with alternating 12-hour light/dark cycles in transparent cages with free access to food and water. Mice were acclimatized for at least 72 hours prior to experimentation. Pups were weaned at 21 days. *Apc*^{1638N/+} mice, *Rage*^{-/-} mice, and their offspring were genotyped by using tail sample DNA extraction (Qiagen, Valencia, CA) for allele-specific polymerase chain reaction. Mice were euthanized with isoflurane followed by cervical dislocation at the time of autopsy and organ procurement. All animal experiments were approved by the Institutional Animal Care and Use Committee of Columbia University and conformed to the guidelines outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Apc^{1638N/+} model of tumorigenesis

Apc^{1638N/+} mice develop intestinal tumors that progress in an adenoma-carcinoma sequence similar to human FAP. [28] *Apc*^{1638N/+} mice were crossed with *Rage*^{-/-} mice to generate *Apc*^{1638N/+}/*Rage*^{-/-} mice. In parallel, *Apc*^{1638N/+} mice were bred with C57BL/6 mice to generate *Apc*^{1638N/+}/*Rage*^{+/+} mice for controls. Mice were aged to 30 weeks and euthanized to harvest the intestine from duodenum to rectum. The lumen of the intestine was flushed with phosphate buffered saline (PBS) to remove fecal debris followed by 10% buffered formalin to preserve mucosal architecture. The intestine was divided into two halves of equal length, rolled into Swiss roll formations in tissue cassettes, and fixed for 24 hours in 10% buffered formalin. Fixed intestine was then embedded in paraffin block, and 5-μm

sections were cut at three successively deeper levels, discarding 100 μ m between levels. Hematoxylin and eosin (H&E) stained sections at each level were viewed by two pathologists (H. Rotterdam and F. Bao) who were naïve to the treatment or genotype groups. Numbers of tumors per mouse were counted and sized by measurement of tumor greatest diameter in mm. Histopathology was graded as adenoma, adenoma with high-grade dysplasia, intramucosal carcinoma, or invasive adenocarcinoma. By definition, adenoma shows low-grade epithelial dysplasia, intramucosal carcinoma (carcinoma *in situ*) shows invasion of the lamina propria without extension through the muscularis mucosae, and invasive adenocarcinoma shows invasion beyond the muscularis mucosae into the submucosal tissue.

Tumor cell lines

CT26 murine colon adenocarcinoma cells (BALB/c syngeneic) and MC38 murine colon adenocarcinoma cells (C57BL/6 syngeneic) were purchased from American Type Culture Collection (Manassas, VA). CT26 cells were maintained in RPMI-1640 medium and MC38 cells were maintained in DMEM medium, both supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin. Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. To establish a full-length-RAGE-transfected MC38 cell line, complementary DNA for human full-length RAGE (FL-RAGE) was inserted into the pcDNA3 vector (Life Technologies, Carlsbad, CA). Purified plasmids and control vector (pcDNA3) were introduced into MC38 cells using Lipofectamine (Life Technologies). Cells were selected in the presence of Geneticin (G418) 1.5mg/mL (Life Technologies), and individual clones were isolated by limiting dilution to obtain stable transfectants (MC38/FL-RAGE and MC38/mock). On the day of experiment, cells were harvested in their logarithmic growth phase using 0.25% trypsin-EDTA, washed with PBS three times prior to counting, and reconstituted in Hank's balanced salt solution (HBSS) at a cell concentration of 2.0×10^5 cells per mL. Cell viability exceeded 95% when assessed by trypan blue exclusion of cell suspensions before and after experiments.

Hepatic metastasis model

Intrahepatic tumors were generated by direct portal vein injection of tumor cells using a standardized technique. Mice were anesthetized with a single intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg) prior to abdominal shaving with clippers and prepping with betadine and alcohol. An upper midline incision was made, and the intestines were eviscerated and reflected to the right to expose the portal vein. A 30-gauge needle was used to cannulate the portal vein and inject 100 μ L of the cell suspension, delivering a total inoculum of 2.0×10^4 cells per mouse. Hemostasis was achieved by gentle compression of the injection site with a cotton swab prior to closing the abdomen with clips.

Livers were excised, weighed, and assessed in a blinded manner without knowledge of treatment for tumor incidence, nodule count, and tumor burden. Tumor incidence was defined as the presence or absence of tumor by gross inspection of the liver. Individual tumor nodules were counted on the liver surface. To calculate tumor burden, the expected weight of the liver was subtracted from the actual weight of the liver. The expected liver weight was calculated using the ratio of average liver to body weight from 25 normal mice

of equivalent age, multiplied by the body weight at the time of sacrifice of the experimental mouse ($[\text{average liver weight}_{\text{normal mice}} / \text{average body weight}_{\text{normal mice}}] \times \text{body weight}_{\text{experimental mouse}}$).

Pharmacological blockade of RAGE in the CT26 model

Pharmacological blockade of RAGE was achieved by treating mice with a soluble form of the receptor which lacks the transmembrane and cytosolic components of the molecule. Despite these deletions, the truncated receptor maintains its ability to bind ligands and functions as a competitive inhibitor. Soluble RAGE (sRAGE) was prepared in a baculovirus expression system as previously described. [29] Prior pharmacokinetic experiments have demonstrated effective receptor blockade without toxicity at a dose of 100 μg daily. [30] The agent is dissolved in PBS and delivered by intraperitoneal injection in a total volume of 100 μL . BALB/c mice were randomly assigned to treatment with sRAGE (experimental group) or vehicle (control group). Mice then underwent portal vein injection with 2.0×10^4 CT26 cells as described above. Beginning on the day of portal vein inoculation, the experimental group received daily intraperitoneal injections of sRAGE and the control group received daily intraperitoneal injections of PBS. The initial treatment with sRAGE was administered after portal vein inoculation, but before the mice awoke from anesthesia. Six mice from each group were euthanized on postoperative days 21 and 28 for evaluation of hepatic tumors as described above.

Host *Rage* deletion in the MC38 model

Twenty *Rage*^{-/-} mice and 20 C57BL/6 controls underwent portal vein injection of 2.0×10^4 MC38 wild-type cells. Ten mice from each group were euthanized on postoperative days 21 and 28 for evaluation of hepatic tumors as described above.

Tumor cell *Rage* up-regulation in the MC38 model

Thirty C57BL/6 mice underwent portal vein injection of 2.0×10^4 MC38/FL-RAGE, MC38/mock cells, or MC38 wild-type cells, 10 mice in each group. Mice were euthanized on postoperative day 28 for evaluation of hepatic tumors as described above.

Western blot analysis

Protein extracts were prepared from tumors harvested from the livers of the above mice and from CT26 wild-type, MC38 wild-type, MC38/FL-RAGE, and MC38/mock cells using cell lysis buffer (Cell Signaling, Beverly, MA). Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were placed in each lane and separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. Nonspecific binding was blocked by incubation of membranes with nonfat dry milk (5%) in Tris-buffered saline containing Tween 20 (0.1%) (blocking buffer) for 1 hour at room temperature or overnight at 4°C. RAGE was detected by incubating the transferred membrane overnight at 4°C with rabbit polyclonal antibody (Gene Tex, Irvine, CA) at 1:500 dilution. HRP-conjugated donkey anti-rabbit IgG secondary antibody (1:2,000; Amersham Biosciences) was used to identify sites of binding of primary

antibody. Final detection of immunoreactive bands was performed using the enhanced chemiluminescent Western blotting system (Amersham Biosciences).

Immunohistochemical analysis

Intestine and liver tumors from the above mice were harvested and fixed in 10% buffered formalin, followed by paraffin-embedding and generation of sections (5 μ m thick). The sections were de-paraffinized and rehydrated in graded alcohols. Certain sections were stained with H&E. Sections to be stained with the antibodies to RAGE or HMGB1 were pretreated with trypsin for 20 minutes. Sections to be stained with the antibody to S100 were heated by boiling in 10 mM citrate buffer, pH 6.0 for 10 minutes followed by cooling at room temperature for 20 minutes before immunostaining. After blocking with 10% normal goat serum (Vector Laboratories, Burlingame, CA), serial sections were stained with the rabbit polyclonal antibodies to RAGE (1:100), [13] HMGB1 (1:50; ProteinTech Group, Chicago, IL), S100 (1:300; Abcam, Cambridge, MA) and were incubated overnight at 4°C in a humidified chamber. After washing with PBS, sections were stained with biotinylated secondary goat anti-rabbit antibody (1:200; Vector Laboratories) followed by incubation with Texas Red–avidin D. Sections were mounted. The signals of images for antigen detection were performed using a Zeiss Fluorescent Scope equipped with a filter specific for Texas Red. Negative controls consisted of serial sections stained with equivalent concentrations of preimmune IgG in place of the primary antibody.

Statistics

Continuous variables were compared using Student's t-test or Mann-Whitney U test. Group means were compared using ANOVA followed by Student's t-test where indicated. Categorical variables were compared using Fisher's exact test. A p-value of less than 0.05 was considered statistically significant.

Results

RAGE and RAGE ligand expression in *Apc*^{1638N/+}, CT26, and MC38 models

Histologic examination of H&E-stained sections of intestine from *Apc*^{1638N/+} mice showed a spectrum of neoplasia, ranging from benign adenoma to invasive carcinoma. Representative sections of an adenoma with high-grade dysplasia demonstrated strong staining for RAGE and its ligands, S100 and HMGB1 (Figure 1a). Representative sections of intrahepatic CT26 and MC38 tumors also demonstrated strong staining for RAGE, S100, and HMGB1 (Figure 1b and 1c). Western blot analysis of CT26 and MC38 cells *in vitro* and *in vivo* demonstrated RAGE protein expression (Figure 1d).

Rage deletion inhibits intestinal tumor development and progression in *Apc*^{1638N/+} mice

At 30 weeks, *Apc*^{1638N/+}/*Rage*^{-/-} mice (n=6) had fewer tumors compared to *Apc*^{1638N/+}/*Rage*^{+/+} mice (n=7), though these results were not statistically significant (1.00 $\pm$ 1.10 tumors vs. 1.86 $\pm$ 1.68 tumors, p=0.31). However, mean tumor diameter was significantly smaller in *Apc*^{1638N/+}/*Rage*^{-/-} mice (1.62 $\pm$ 0.45 mm vs. 2.81 $\pm$ 0.83 mm, p<0.001). Most importantly, no *Apc*^{1638N/+}/*Rage*^{-/-} mouse displayed pathological evidence of

carcinoma, whereas there was a significantly higher 46.7% incidence of carcinoma noted in *Apc*^{1638N/+}/*Rage*^{+/+} mice ($p=0.03$) (Figure 2).

Pharmacological blockade of RAGE inhibits intrahepatic CT26 tumor growth

To further establish the role of RAGE in tumor growth, we treated mice inoculated with CT26 tumors with sRAGE or vehicle as described above. On day 21, 16.7% of sRAGE-treated mice ($n=6$) versus 83.3% of control mice ($n=6$) had intrahepatic tumors ($p=0.08$). There was a greater than 10-fold lower nodule count in sRAGE-treated mice compared to control mice (0.17 ± 0.41 nodules vs. 2.17 ± 1.72 nodules, $p=0.02$). sRAGE-treated mice had significantly lower mean tumor burden compared to control mice (0.19 ± 0.05 g vs. 0.29 ± 0.06 g, $p=0.02$). On day 28, 66.7% of sRAGE-treated mice ($n=6$) had tumors whereas 100% of control mice ($n=6$) had tumors ($p=0.45$). Finally, there was a 4-fold lower nodule count (1.17 ± 1.17 nodules vs. 4.17 ± 2.40 nodules, $p=0.02$) and significantly lower mean tumor burden in sRAGE-treated mice compared to control mice (0.30 ± 0.18 g vs. 0.49 ± 0.11 g, $p<0.05$) (Figure 3).

Rage deletion inhibits intrahepatic MC38 tumor growth

On day 21, *Rage*^{-/-} mice ($n=8$) had a lower incidence of intrahepatic tumors compared to *Rage*^{+/+} mice ($n=9$), though this difference was not statistically significant (75% vs. 100%, $p=0.21$). Mean nodule count was significantly lower in the *Rage*^{-/-} mice compared to *Rage*^{+/+} mice (3.88 ± 7.83 nodules vs. 30.00 ± 28.92 nodules, $p=0.03$). There was a 30-fold reduction in mean tumor burden in *Rage*^{-/-} mice compared to *Rage*^{+/+} mice (0.03 ± 0.06 g vs. 0.94 ± 0.94 g, $p=0.02$). On day 28, significantly fewer *Rage*^{-/-} mice ($n=10$) had tumors compared to *Rage*^{+/+} mice ($n=9$) (50% vs. 100%, $p=0.03$). Mean nodule count was significantly lower in *Rage*^{-/-} mice compared to *Rage*^{+/+} mice (6.70 ± 12.00 nodules vs. 31.00 ± 13.70 nodules, $p<0.01$). Finally, there was a 5-fold lower mean tumor burden in *Rage*^{-/-} mice compared to controls (0.70 ± 1.29 g vs. 3.44 ± 1.93 g, $p<0.01$) (Figure 4).

RAGE gain of function increases intrahepatic MC38 tumor growth

Western blot analysis confirmed RAGE protein over-expression *in vitro* in the MC38/FL-RAGE cells compared to MC38/mock and MC38 wild-type cells. On day 28 after intraportal injection of transfected cells, mice in all experimental groups ($n=9$ /group) developed tumors. There were no statistically significant differences in mean nodule count between groups ($p=0.13$). Mice injected with MC38/FL-RAGE cells had significantly increased mean tumor burden compared to mock transfected controls (1.33 ± 1.34 g vs. 0.46 ± 0.37 g, $p=0.04$) and MC38 wild-type cells (1.33 ± 1.34 g vs. 0.27 ± 0.25 g, $p=0.02$). There was no difference in tumor burden between mock transfected controls and MC38 wild-type cells ($p=0.23$) (Figure 5).

Discussion

Colorectal carcinoma is a leading cause of cancer-related deaths worldwide. The liver is the most frequent site of metastasis, and patients with metastatic disease have significantly worse survival. [2] The molecular mechanisms of tumorigenesis and metastasis in colorectal carcinoma are incompletely understood, although genetic mutation and inflammation are

known potentiating factors. Mounting evidence suggests that the ligand-RAGE axis is a link between inflammation and the initiation and progression of intestinal neoplasia. Fuentes and colleagues [12] documented expression of S100P in human colon tumor tissue. They further showed that the S100P-RAGE interaction stimulated cell proliferation, cell migration, and NF- κ B activation in *in vitro* studies employing human colon cancer cell lines. Using a competitive RAGE ligand antagonist, amphoterin peptide, they demonstrated that blockade of RAGE significantly inhibited cell proliferation. Turovskaya and colleagues [22] demonstrated that the ligand-RAGE axis mediated inflammation-associated colon cancer through activation of NF- κ B. Using a murine model of colitis-associated cancer (CAC), they found increased expression of S100 proteins in the tumor microenvironment and dramatically reduced incidence of CAC in *Rage*^{-/-} compared to *Rage*^{+/+} mice.

In the current study, we used the *Apc*^{1638N/+} mouse model of FAP to evaluate the effects of RAGE signaling on the development of intestinal neoplasia. Mutation of the *Apc* gene is a known early event in the progression from normal intestinal mucosa to adenoma to carcinoma. Various *Apc* mutant mouse models exist, and we chose *Apc*^{1638N/+} model for several reasons. *Apc*^{1638N/+} mice have a reduced tumor burden and consequently increased lifespan compared to other *Apc* mutant mice. This increased lifespan allows time for more advanced tumors to develop and provides a spectrum of benign to malignant intestinal neoplasia. The progression of tumors in *Apc*^{1638N/+} mice thus more accurately models the development of colorectal carcinoma in humans. [3]

We observed the complete spectrum of intestinal neoplasia in *Apc*^{1638N/+} mice, ranging from benign adenoma to invasive adenocarcinoma. Similar to prior reports, [3,28] we observed a majority of lesions in the small intestine. We first documented the presence of RAGE and its ligands in these lesions by immunofluorescent staining. We then examined how intestinal neoplasia develops and progresses in the absence of RAGE by breeding the *Rage*^{-/-} locus into the *Apc*^{1638N/+} mouse. The *Apc*^{1638N/+}/*Rage*^{-/-} mice tended to have fewer tumors of markedly decreased size. Strikingly, the tumors in the *Rage*^{-/-} mice had more benign histopathologic grade with no *Rage*^{-/-} mouse harboring carcinoma compared to a nearly 50% incidence of carcinoma in control mice. Our findings support the hypothesis that RAGE signaling plays an important role in the initiation and progression of intestinal neoplasia. In future work, it will be interesting to examine the intestines of *Apc*^{1638N/+}/*Rage* heterozygous (*Rage*^{+/-}) mice to study the effects of partial allelic loss on tumorigenesis in the model.

The role of the ligand-RAGE axis in the development and growth of metastatic tumors is becoming increasingly evident. It is known that an influx of tumor cells into the liver causes an acute inflammatory response characterized by ligand-RAGE interactions and release of TNF- α . [31] Expression of RAGE and its ligands has been correlated with metastatic disease in colorectal carcinoma. Kuniyasu and colleagues [17] observed that RAGE expression increased in parallel with Dukes' stage. Over-expression of RAGE was observed in 19%, 81%, and 100% of the Dukes' B, C, and D cases, respectively. In addition, the authors reported significantly reduced survival in Dukes' B and C cases with co-expression of RAGE and HMGB1 compared to those without co-expression. Similarly, Kostova and

colleagues [32] observed intense signal for RAGE and HMGB1 in immunohistochemical studies of primary and metastatic human colorectal carcinoma specimens.

These data led us to hypothesize that blockade of the RAGE signaling pathway would reduce tumor growth in mouse models of colorectal liver metastasis. First, we demonstrated expression of RAGE and its ligands in CT26 and MC38 cells *in vitro* by western blot. *In vivo* CT26 and MC38 cells also expressed RAGE on western blot and stained strongly for RAGE and its ligands by immunohistochemical analysis of hepatic tumors. Having established RAGE expression in these tumor cells, we tested the impact of pharmacological blockade of RAGE by administering sRAGE after intraportal injection of syngeneic CT26 tumor cells in BALB/c mice. sRAGE is the extracellular domain of RAGE and acts a competitive inhibitor of receptor activation by binding RAGE ligands. [29] Treatment with sRAGE had a potent protective effect as development of liver metastases was delayed and tumor burden was significantly reduced. A limitation of long-term pharmacological blockade is the potential for tumor burden to overwhelm the competitive inhibitory effects of sRAGE via the increased release of RAGE ligands by necrotic cells. [32]

To overcome the limitations of long-term pharmacological blockade, we performed intraportal injections with syngeneic MC38 cells in *Rage*^{-/-} mice. At the early time point, *Rage*^{-/-} mice had a similar incidence of hepatic disease, but significantly fewer nodules and lower tumor burden compared to *Rage*^{+/+} mice. Interestingly, these differences became more pronounced at the later time point. Intraportal injection in *Rage*^{-/-} mice thus allowed us to examine host effects on tumor growth in the liver. Previous work by Liang and colleagues [31] demonstrated similar host effects on tumor growth in *Rage*^{+/+} mice. They showed that administration of ethyl pyruvate prior to intraportal MC38 injection significantly reduced serum levels of inflammatory cytokines and resulted in reduced number of tumor nodules. A potent anti-inflammatory agent, ethyl pyruvate exerts its effects in part via inhibition of inflammatory cytokines such as TNF- α and HMGB1. In light of that work, our current data suggest that absence of RAGE in the host liver dampens the deleterious effects of the inflammatory response elicited by metastatic tumor cells.

As our findings indicated that RAGE loss of function inhibited tumor growth, we then assessed the impact of RAGE gain of function on tumor growth. We stably transfected MC38 clones with full-length RAGE to mediate over-expression and injected C57BL/6 mice with syngeneic full-length RAGE-transfected or mock-transfected control MC38 cells. We noted significantly increased tumor burden compared to mock and wild-type MC38 cells. These results mirror earlier work with full-length RAGE-transfected C6 glioma, which exhibited markedly increased tumor growth compared to mock-transfected glioma. [13] The full-length RAGE-transfected C6 glioma also demonstrated enhanced proliferation, invasion, and migration *in vitro*. In future work, it will be interesting to examine the *in vitro* effects of RAGE over-expression in the full-length RAGE-transfected MC38 cells. Assays measuring cell proliferation, invasion, and apoptosis will help characterize the mechanisms by which RAGE signaling impacts tumor growth in this model.

Our data thus show the key finding that both host and tumor cell RAGE expression contribute significantly to tumor growth in a murine model of colorectal carcinoma

metastasis. Further work is needed to evaluate the relative contributions of host and tumor cell RAGE interactions and to elucidate the mechanisms by which RAGE signaling influences tumor development and progression.

Conclusion

These studies provide further evidence that RAGE signaling plays an important and complex role in the biology of intestinal neoplasia. Using an established murine model of intestinal neoplasia, we demonstrated significant inhibition of tumor growth and delay of progression to carcinoma in *Apc*^{1638N/+}/*Rage*^{-/-} mice. We showed that loss of function via pharmacological blockade of RAGE and genetic deletion of the *Rage* gene had profound effects on growth of colorectal carcinoma cells in murine models of metastasis. Finally, we showed that RAGE gain of function by direct manipulation of murine colorectal carcinoma cells significantly increased tumor growth in the liver. Further cellular and molecular work is needed to target the ligand-RAGE axis for possible prophylaxis and treatment of primary and metastatic colorectal carcinoma.

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Figure 1. RAGE and its ligands, S100 and HMGB1, are expressed in intestinal neoplasia in *Apc*^{1638N/+} mice and in CT26 and MC38 murine colorectal carcinoma cells *in vitro* and *in vivo*

a. An H&E-stained section of intestine from a 30-week-old *Apc*^{1638N/+} mouse demonstrates an adenoma with high-grade dysplasia (arrow) adjacent to normal mucosa. Immunofluorescent staining of representative sections demonstrates the presence of RAGE, S100, and HMGB1 in the neoplasm. **b.** and **c.** H&E-stained sections of livers from *Rage*^{+/+} mice demonstrate metastatic CT26 and MC38 colorectal carcinoma tumors. An asterisk marks MC38 tumor bounded by normal liver. Immunofluorescent staining demonstrates the presence of RAGE, S100, and HMGB1 in the metastatic tumors. Magnification scale bars are indicated. **d.** Expression of the RAGE protein (~55 kDa) is demonstrated by immunoblotting of CT26 and MC38 cells *in vitro* and *in vivo*. Murine lung tissue serves as the control.

Figure 2. *Rage* deletion inhibits tumor development and progression in *Apc*^{1638N/+} mice
Apc^{1638N/+}/*Rage*^{-/-} mice (n=6) had reduced tumor count, smaller tumor size, and more benign histopathologic grade of intestinal neoplasia compared to *Apc*^{1638N/+}/*Rage*^{+/+} mice (n=7) at 30 weeks of age. **a.** Mean and median tumor count are shown. **b.** Mean and median tumor diameter are shown. **c.** The incidences of adenoma, high-grade dysplasia, intramucosal carcinoma, and invasive carcinoma as seen on histopathologic examination are shown. *p<0.0001, ** p=0.01, NS=not significant.

Figure 3. Pharmacological blockade of RAGE inhibits intrahepatic CT26 tumor growth
a. Twenty-one days after portal vein injection of 2.0×10^4 CT26 colorectal carcinoma cells, sRAGE-treated mice (n=6) had lower tumor incidence and significantly lower hepatic nodule count and tumor burden compared to controls (n=6). **b.** This difference persisted on day 28 with lower tumor incidence and significantly lower nodule count and tumor burden in sRAGE-treated (n=6) versus control (n=6) mice. *p=0.02, **p<0.05, NS=not significant.

Figure 4. Host *Rage* deletion significantly inhibits intrahepatic MC38 tumor growth

a. After portal vein injection of 2.0×10^4 MC38 colorectal carcinoma cells, *Rage*^{-/-} mice (n=8) had lower tumor incidence and significantly lower nodule count and tumor burden on day 21 compared to *Rage*^{+/+} mice (n=9). **b.** This difference was more pronounced on day 28 with significantly lower tumor incidence, nodule count, and tumor burden in *Rage*^{-/-} mice (n=10) compared to controls (n=9). *p=0.03, **p=0.02, ***p<0.01, NS=not significant.

Figure 5. RAGE gain of function increases intrahepatic MC38 tumor growth

a. Stably transfected MC38/full-length RAGE (FL-RAGE) cells demonstrated increased expression of RAGE protein (~55kDa) *in vitro* compared to MC38/mock (M) and MC38 wild-type (WT) cells examined by immunoblot analysis. **b.** Intraportal injection (n=9 mice/group) of 2.0×10^4 MC38/FL-RAGE cells resulted in increased mean tumor burden compared to MC38/M and MC38 WT cells. *p=0.02, **p=0.04.