Tumor and Stem Cell Biology

Alternatively Spliced RAGEv1 Inhibits Tumorigenesis through Suppression of JNK Signaling

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Abstract

Receptor for advanced glycation end products (RAGE) and its ligands are overexpressed in multiple cancers. RAGE has been implicated in tumorigenesis and metastasis, but little is known of the mechanisms involved. In this study, we define a specific functional role for an alternate splice variant termed RAGE splice variant 1 (RAGEv1), which encodes a soluble endogenous form of the receptor that inhibits tumorigenesis. RAGEv1 was downregulated in lung, prostate, and brain tumors relative to control matched tissues. Overexpressing RAGEv1 in tumor cells altered RAGE ligand stimulation of several novel classes of genes that are critical in tumorigenesis and metastasis. Additionally, RAGEv1 inhibited tumor formation, cell invasion, and angiogenesis induced by RAGE ligand signaling. Analysis of signal transduction pathways underlying these effects revealed marked suppression of c-jun-NH2-kinase (JNK) pathway signaling, and JNK inhibition suppressed signaling through the RAGE pathway. Tumors expressing RAGEv1 were significantly smaller than wild-type tumors and displayed prominently reduced activation of JNK. Our results identify RAGEv1 as a novel suppressor, the study of which may offer new cancer therapeutic directions. Cancer Res; 70(13): 5628-38. ©2010 AACR

Introduction

Tumorigenesis is a multistep process involving the alteration of a number of key cellular properties, including uncontrolled proliferation, evasion of cell death (apoptosis and necrosis), vascularization (angiogenesis), and subsequent invasion and migration of tumor cells into the surrounding tissues (1). Understanding the molecular processes underlying these cellular phenotypic changes is critical in developing novel therapies. Central to mediating these changes is the interaction between cell surface receptors and their cognate ligands, which through intracellular signaling induce alterations in gene expression. In this context, recent studies have identified that the receptor for advanced glycation end products (RAGE) and its ligands are overexpressed in multiple cancers. RAGE has been implicated in tumorigenesis and metastasis, but little is known of the mechanisms involved. In particular, RAGE splice variants have been suggested to play a role in the regulation of tumorigenesis. Notably, soluble forms of RAGE are readily secreted from cells (23, 24). Intriguingly, soluble forms of RAGE have been detected in human plasma, and these levels correlate with the presence and/or extent of RAGE-mediated diseases (29). In cancer, specifically, a number of studies have reported that levels of soluble RAGE were lower in subjects afflicted with breast or lung cancer compared with controls (30, 31). Yet, no experiments to date have elucidated a functional role of soluble RAGE isoforms detected in human subjects. Therefore, our goal here was to establish the mechanistic effects of RAGEv1 on RAGE ligand–mediated tumorigenesis and to test if RAGEv1 might suppress tumor-provoking signaling pathways. Here, we therefore report a novel mechanism by which the soluble splice variant of RAGE (RAGEv1) inhibits tumorigenesis and may represent a novel therapeutic target in the treatment of cancer.
**Materials and Methods**

**Cell culture, antibodies, and reagents**

Rat C6 glioma cells were obtained from American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen). Human primary aortic endothelial cells were purchased from Lonza and maintained in EGM2 medium (Lonza). The RAGEv1 rabbit polyclonal antibody was raised against the purified peptide sequence (Ac-CGEQFDKVREADSPQHM-amide) and affinity purified by QCB. This antibody recognizes the unique COOH-terminal region of RAGEv1 but not full-length RAGE.

**Plasmid engineering and RAGEv1 stable clone generation**

RAGEv1 (Genbank no. AY755620) was cloned as previously described (24). The RAGE ligand S100A12 was cloned in frame with the 6× His-tag (COOH-terminal tag) in the vector pET101 (Invitrogen) from lung cDNA, using the primers 5′-CTCCATGACAAAACCTGAGAGC-3′ and 5′-TTCTTTGGGTTGTTGTAATG-3′. C6 cells were stably transfected with pcDNA3.1-RAGEv1 or empty pcDNA3.1 (mock-transfected) constructs, screened for RAGEv1 expression by Western blotting of cell lysates, and conditioned using anti-RAGE IgG and anti-RAGEv1 IgG. RAGEv1 expression was further verified by measurement of conditioned medium using sRAGE Quantikine ELISA (R&D Systems).

**RAGEv1 in vitro binding assay**

Binding of RAGEv1 to RAGE ligand was tested using pull-down assays with the Pierce His Protein Interaction Pull-Down Kit (Pierce) according to the manufacturer’s instructions, using His-tagged s100A12 (bait) and cultured medium from RAGEv1 (prey).

**Cancer PathwayFinder PCR array**

RAGEv1 and mock C6 cells were incubated with or without 10 μg/mL S100B for 2 hours, and total RNA was extracted using Trizol (Invitrogen). Gene expression of 84 genes representative of the six biological pathways involved in tumorigenesis (including five housekeeping genes for normalization: Rplp1, Hprt1, Rpl13a, Ldh1a, and Actb) was assessed using the Rat Cancer PathwayFinder RT2 Profiler PCR Array (SA Biosciences) according to the manufacturer's instructions and analyzed on an MX3005P Real-time PCR System (Stratagene). Only genes demonstrating a 1.5-fold or greater change were considered for further analysis. PCR array data were validated using a combination of Taqman quantitative PCR (QPCR) and Western blot analysis as described in the Supplementary Methods.

**In vitro tumorigenic assays**

**In vitro** angiogenesis assays were performed by seeding endothelial cells on top of Matrigel (BD Biosciences) and incubated with 50% EGM-2 medium: 50% conditioned medium from RAGEv1 and mock cells incubated with or without S100B. After 2 hours, tube formation was assessed and images were taken from four independent fields. Tumor cell adhesion assays were performed by incubating cells with or without 10 μg/mL S100B for 24 hours, seeded into tissue culture plates for 2 hours. Attached cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet, and absorbance was measured at 590 nm. Cellular apoptosis was assessed using Annexin V/propidium iodide (PI) staining. Cells stimulated with or without 10 μg/mL S100B for 24 hours were stained with the ApoTarget Annexin V FITC Apoptosis Kit and analyzed by flow cytometry. Invasion assays were performed using the BD BioCoat BD Matrigel Invasion Chambers (BD Biosciences) by seeding cells into the upper chamber; after 24 hours, invading cells were assessed by staining with 0.1% crystal violet solution. In vitro tumor growth was performed using the Cell Transformation Detection Assay (Millipore) by plating cells in a layer of 0.4% agarose in DMEM. After 14 days, colonies were visualized using Cell Stain solution (Millipore).

**In vivo tumor growth**

All animal studies were performed with the approval of the Institutional Animal Care and Use Committee of Columbia University and conform to the Guide for the Care and Use of Laboratory Animals published by the NIH. Weanling female severe combined immunodeficient mice were s.c. injected in the right flank with 1 × 105 of mock or RAGEv1-expressing C6 cells suspended in 0.1 mL PBS. After 31 days, tumors were measured with calipers and tumor volume was calculated using the following formula: \( V = \frac{a \times (h^2 + 3a)}{6} \), where \( h \) = height of the tumor section, \( a = \) (length + width of the tumor)/4, and \( V \) = volume of the tumor. Excised tumor sections were snap frozen for protein analysis.

**Cell signaling assays**

Activation of cell signaling proteins were performed by Western blotting using antibodies to phosphorylated/total MEK1/2, stress-activated protein kinase/c-jun-NH2-kinase (SAPK/JNK), p38, and AKT (Cell Signaling). Inhibition of these pathways was performed with chemicals for SAPK/JNK (SP600125, 10 μmol/L), MEK1/2 (U0126, 10 μmol/L), p38 (SB203580, 10 μmol/L), and AKT (triciribine, 10 μmol/L) or control (0.1% DMSO), using stimulated cells (10 μg/mL S100B for 2 h). QPCR analysis was performed for Apaf1, Pdgfb, and Tnf as described above. Invasion assays were performed by incubating cells with either 2.5 μmol/L SP600125 or 0.1% DMSO as vehicle, and invasion was performed with ligand stimulation as described above.

**Statistical analyses**

In all experiments, unless otherwise indicated, data are reported as mean ± SEM in at least three replicates per group. Data were analyzed by post hoc comparisons using two-tailed t test, and a P value of <0.05 was considered significant.

**Results**

RAGEv1 is downregulated in various human cancers

Previous studies have monitored the presence of RAGEv1 in human plasma and the relationship with various inflammatory

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disease states. To analyze the expression profile of RAGEv1 directly in human tumor tissue samples, we studied normal and tumor-matched tissue retrieved from the same subject, and examined the expression of RAGEv1 by Western blot analysis (Supplementary Fig. S1). Interestingly, these experiments suggested that levels of RAGEv1 tended to be lower in tumors versus normal matched adjacent tissue. These data suggest for the first time that RAGEv1 levels may be downregulated in the tumor but not in adjacent nonmalignant tissue, and thus we sought to test the hypothesis that RAGEv1 might act as a molecular decoy to block tumorigenesis by preventing the cellular effects of RAGE ligands.

**The ectopic expression of RAGEv1 by tumor cells**

To test these concepts, we first generated rat C6 glioma cells that stably expressed RAGEv1. C6 rat glioma cells are an established model system for both in vitro and in vivo analysis of tumorigenesis, and we have previously seen RAGE ligand-dependent responses in this cell line (s100B/CML; refs. 13, 32). Western blot analysis confirmed the expression of RAGEv1 in transfected cells compared with control empty vector–transfected (mock) cells, by using antibodies to the unique COOH-terminal sequence of RAGEv1 and antibodies to the extracellular domain of human RAGE (Fig. 1A). Furthermore, Western blot analysis of cell culture medium confirmed that RAGEv1 protein was actively secreted by the C6 cells (Fig. 1A). Measurement of RAGEv1 in cell culture medium using a sRAGE ELISA showed levels of ∼1,500 to 2,000 pg/mL, which is in the physiologic range detected in human plasma (7). To confirm that RAGEv1 binds RAGE ligands, pull-down experiments were performed with recombinant RAGE ligand (GST-s100A12), using the conditioned cell culture medium containing RAGEv1. Western blot analysis of pull-down elutant showed a clear interaction between RAGEv1 and s100A12, but not with GST alone (Fig. 1B).

**RAGEv1 expression affects protumorigenic gene expression profiles**

Next, we tested the impact of RAGEv1 on RAGE/ligand–activated tumorigenic pathways. We hypothesized that RAGE activation regulates the expression of genes that promote tumor cell properties, and that RAGEv1 might block these effects. To test this hypothesis, we performed QPCR array analysis on the control- and RAGEv1-transfected cells, and assessed the impact of RAGE ligand stimulation using the Cancer PathwayFinder array. This array consists of 84 genes representing six major biological pathways involved in tumorigenesis. We identified several classes of genes that were altered by RAGEv1 expression, including angiogenesis, adhesion, apoptosis, invasion/metastasis, cell cycle control, and signaling (Supplementary Table S1). Of the 84 cancer pathway focused genes, 19 showed at least a 1.5-fold difference in gene expression between ligand-stimulated cells expressing either RAGEv1- or mock-transfected cells. Interestingly, genes implicated in angiogenesis represented the major class altered both in number and expression level, which includes Fgf1, Ifna1, Pdgfb, Thbs1, Tnf, and Vegfc. As shown in Fig. 2 (and Supplementary Fig. S2), we validated several of these genes for changes in expression by Taqman QPCR and Western blot analysis. Taqman QPCR analysis confirmed that
RAGEv1 impaired RAGE ligand–induced expression of Pdgfb (Fig. 2A) and Tnf (Fig. 2B), and increased expression of the apoptotic gene Apaf-1 (Supplementary Fig. S2A). Western blot analysis showed that RAGEv1 blocked RAGE ligand (s100B)–induced expression of cyclin D1 (Fig. 2C) and increased expression of Apaf-1 (Fig. 2D). Similar changes in protein expression (cyclin D1) were seen for other RAGE ligands (CML-HSA; data not shown). Together, these data indicate that expression of RAGEv1 inhibits expression of genes critically involved in tumorigenesis through blocking RAGE ligand interaction.

RAGEv1 affects the mechanisms of tumorigenesis in vitro

As RAGE has been reported to have a role in promoting cancer cell survival, migration, and invasion, we hypothesized that RAGEv1 may impair these functions. To test this, we analyzed in vitro whether RAGEv1 affects RAGE-mediated tumor cell angiogenesis, adhesion, apoptosis, and invasion. We first evaluated angiogenesis, as the gene expression data–suggested pathways involved in this process were most affected by RAGEv1 blockade of RAGE signaling. Assays were performed by culturing endothelial cells on Matrigel in conditioned medium from RAGEv1-transfected or mock-transfected tumor cells. We observed markedly reduced tube formation of endothelial cells exposed to conditioned medium from RAGEv1 cells versus mock (Fig. 3A). Consistent with these findings, conditioned medium from RAGE ligand–stimulated mock cells induced a 2-fold increase in tube formation compared with unstimulated mock conditioned medium (Fig. 3A), whereas conditioned medium from RAGEv1 failed to stimulate tube formation (Fig. 3A). We next analyzed the effect of RAGEv1 expression on cellular adhesion. As shown in Fig. 3B, RAGEv1-expressing cells adhered more to the substratum than mock cells. RAGE ligand stimulation did not affect tumor cell adhesion in either mock or RAGEv1 cells (Fig. 3B). To determine the effect of RAGEv1 on tumor cell survival, we next studied cellular apoptosis. Using the Annexin V/PI fluorescence-activated cell sorting assay, compared with control cells, RAGEv1-expressing cells displayed more apoptotic

Figure 2. RAGEv1 inhibits RAGE ligand–induced changes in tumorigenic gene expression. A and B, relative gene expression of platelet-derived growth factor β (PDGFB) and tumor necrosis factor (TNF) were determined by QPCR on mock and RAGEv1-expressing cells stimulated with RAGE ligand (s100B); gene expression was normalized to GAPDH levels. C and D, Western blot analysis of cyclin D1 and Apaf1 performed on cell lysates from mock and RAGEv1 cells, stimulated with RAGE ligand (s100B), normalized to GAPDH levels. Data are means ± SEM from three independent experiments. *, significant differences (P ≤ 0.05).
cells (4% versus 8%, Fig. 3C). However, RAGE ligand stimulation did not seem to alter the percentage of apoptotic cells in either control or RAGEv1-expressing cells. To confirm that RAGEv1 expression affects tumor cell survival, we performed cell viability and caspase-3/7 activation assays. We observed a significant decrease in cellular viability of RAGEv1-expressing cells compared with controls, independent of ligand stimulation (Supplementary Fig. S3A). This was further confirmed by an increase in caspase-3/7 activation in RAGEv1-expressing cells compared with control (Supplementary Fig. S3B). These data suggest that by blocking RAGE activation, RAGEv1 prevents tumor cell evasion of apoptosis and cell death.

Ultimately in the tumorigenic process, cells invade the surrounding tissues and metastasize (33). Therefore, we tested whether RAGEv1 blocks RAGE ligand–induced cellular invasion. RAGE ligand stimulation of control cells induced a ~2.5-fold increase in cell invasion (Fig. 3D); in contrast, RAGEv1 expression blocked cell invasion (Fig. 3D).

Previous studies showed that RAGE activation leads to an increased invasive phenotype through activation of matrix metalloproteinases (MMP; ref. 2). To investigate this, we analyzed both protein levels and activity of MMP-2/9 in control and RAGEv1-expressing cells. MMP-9 protein levels were upregulated in control cells in response to RAGE ligand stimulation (Supplementary Fig. S3C), whereas MMP-9 was not detectable in RAGEv1-expressing cells. Furthermore, activity levels of MMP-9 by gelatin zymography in control cell conditioned medium revealed increased MMP-9 activation in response to RAGE ligand, whereas active MMP-9 was not detected in RAGEv1-expressing cells (Supplementary Fig. S3D).

Figure 3. RAGEv1 inhibits mechanisms of tumorigenesis in vitro. A, RAGEv1 blocks RAGE ligand–induced angiogenesis. Matrigel tube formation assay of endothelial cells exposed to conditioned medium from mock- and RAGEv1-transfected tumor cells exposed to vehicle or RAGE ligand (s100B). Tube number was counted in three random fields from three independent experiments. B, RAGEv1 expression increases cellular adhesion. Serum-starved cells were incubated with vehicle or RAGE ligand (s100B) for 24 h, seeded into plates, and cultivated for 2 h. Bound cells were fixed, stained with crystal violet, and quantified by absorbance at 570 nm (OD 570 nm). C, RAGEv1 expression increases cellular apoptosis. Serum-starved cells were incubated with vehicle or RAGE ligand (s100B) for 24 hours, and apoptosis was assessed by staining for Annexin V/FITC (Y axis) and Annexin V/PI (Y axis) and flow cytometry. D, RAGEv1 blocks RAGE ligand–induced tumor cell invasion. Matrigel invasion assays were performed with mock- and RAGEv1-transfected cells in response to vehicle or RAGE ligand (s100B). Data are means ± SEM from three independent experiments. *, significant differences (P ≤ 0.05) between groups.
MMP-2 (antigen or activity) was not detected in either control or RAGEv1-expressing cells (data not shown). Together, these data show that RAGEv1 acts to block RAGE ligand–induced tumor invasion.

RAGEv1 suppresses tumorigenic signaling

Previous studies have suggested that RAGE signals through these MAPK pathways to regulate cellular processes (2, 8, 9). To explore the specific signaling mechanisms modulated by RAGEv1, we examined the MAPK signaling pathway, including MEK1/2, p38, and SAPK/JNK. RAGE ligand stimulation (s100B) resulted in ∼2-fold activation of MEK1/2, p38, and SAPK/JNK in control cells (Fig. 4A–C). In contrast, in cells expressing RAGEv1, activation of MEK1/2, p38, and SAPK/JNK was markedly reduced (Fig. 4A–C). Interestingly, AKT was not activated by RAGE ligand (s100B), nor did activity levels differ between control and RAGEv1-expressing cells (Fig. 4D). Experiments with mock/RAGEv1–expressing cells using the CML-HSA RAGE ligand (13) revealed similar changes in MEK1/2, p38, SAPK/JNK, and AKT (data not shown). To evaluate the specific signaling pathway(s) responsible for the tumorigenic changes observed in these cells, we tested the effect of various inhibitors. Control cells were incubated with inhibitors of MEK1/2 (U0126), p38 (SB203580), SAPK/JNK (SP600125), and AKT (triciribine), followed by RAGE ligand stimulation. Analysis of gene expression by QPCR revealed that in the presence of the SAPK/JNK inhibitor SP600125, RAGE ligand stimulation induced Apaf1 and blocked Pdgfb and Tnf expression, compared with control stimulated cells (Fig. 5A–C). Pretreatment of cells with U0126, SB203580, or triciribine had little or no effect on RAGE ligand–dependent expression of Apaf1, Pdgfb, and Tnf (Fig. 5A–C). To test the functional role of JNK inhibition in RAGE ligand–mediated tumorigenesis, we analyzed its effects on RAGE ligand–driven cellular invasion. Stimulation of control cells induced an ∼3-fold increase in cell invasion (Fig. 5D), whereas treatment of cells with the JNK inhibitor blocked RAGE ligand–stimulated cellular invasion (Fig. 5D).

RAGEv1 suppresses tumorigenesis

Finally, we investigated whether RAGEv1 expression affected tumor cell growth in vitro and in vivo, and if these effects are linked to SAPK/JNK signaling. To determine whether RAGEv1 modulates tumor formation, we performed the soft agar assay. This is perhaps the most consistent in vitro method to replicate the in vivo tumorigenic environment. Control and RAGEv1 cells were assayed for anchorage-independent growth for 14 days. Compared with mock cells, RAGEv1 expression drastically reduced the ability of tumor cells to form colonies in soft agar (Fig. 6A). Athymic nude mice were injected with mock or RAGEv1 cells, and tumor growth was measured after 31 days. Compared with control cells, there was an ∼25-fold lower volume in tumors developed from RAGEv1-expressing cells (Fig. 6B). In parallel with these effects, Western blotting revealed that expression of RAGEv1 significantly reduced SAPK/JNK activation by ∼2-fold compared with control tumors (Fig. 6C).

Discussion

The generation of soluble receptor isoforms represents an important mechanism to regulate aberrant receptor signaling in biological systems and a potential therapeutic strategy for cancer. Here, we have identified for the first time a functional role for the endogenous soluble RAGEv1 isoform as a molecular decoy for RAGE, and as an inhibitor of key cellular properties that facilitate tumorigenesis. First, RAGEv1 expression in tumor cells inhibited RAGE-induced gene expression profiles favoring tumor cell–mediated angiogenesis, invasion, cell cycle progression, and evasion of cell death. Second, functional studies in vitro showed that RAGEv1 strongly inhibited RAGE ligand–induced angiogenesis, tumor cell invasion/metastasis, and JNK activation. Finally, and importantly, in vitro and in vivo studies firmly establish that expression of RAGEv1 strongly suppresses tumor growth. We conclude that RAGEv1 acts as a molecular decoy for RAGE to block the tumorigenic process, and therefore strategies to increase RAGEv1 levels could be useful in the treatment of cancer.

A growing body of evidence suggests that the inhibition of RAGE has the potential to be a specific and effective anticancer therapeutic strategy. In particular, RAGE and its diverse ligands have been shown to be overexpressed in numerous tumorigenic states, and blocking their expression/interaction inhibits tumor growth and metastasis (2, 4). In this regard, these means of inhibition have included the use of RAGE-blocking antibodies, dominant-negative receptor constructs, and the recombinant soluble ligand-binding extracellular domain (sRAGE; refs. 2, 4, 13). However, the occurrence of an endogenous soluble receptor system (RAGEv1) suggests the possibility of a natural means to block RAGE signaling. Furthermore, serum levels of RAGEv1 have been shown to be inversely correlated with various inflammatory disease states, suggesting the role of RAGEv1 as a potentially useful biomarker (26–28). These findings indicate that RAGEv1 may affect the clinical course of RAGE-driven pathologies, including various tumorigenic states, by blocking RAGE ligand interaction. However, to date, no studies have investigated the molecular and cellular significance of RAGEv1 in vascular and tumor biology. Here, we tested the hypothesis that RAGEv1 acts as a molecular decoy for RAGE, blocking tumor-promoting signaling, and in turn inhibiting the tumorigenic processes both in vitro and in vivo.

To explore the molecular mechanisms through which RAGEv1 may affect RAGE-induced tumorigenesis, we analyzed gene expression profiles in tumor cells. Our data revealed that compared with control cells, RAGEv1 altered expression of genes associated with invasion/metastasis, apoptosis, and cell cycle control. However, intriguingly, the genes most dramatically altered were associated with angiogenesis. In vitro cellular assays revealed that RAGEv1 strikingly inhibited RAGE ligand induction of angiogenesis and cellular invasion. However, although RAGEv1 induced tumor cell adhesion and cell death, we were unable to show any further effects of exogenous RAGE ligand stimulation on these processes, therefore suggesting a role for endogenous ligand
generation by these cells. These results suggest that RAGEv1 inhibits multiple RAGE ligand–mediated processes, leading to tumor formation and progression. The surprising finding that RAGEv1 blocks tumor cell expression of genes influencing angiogenesis implicates RAGE/RAGEv1 in multiple facets of tumor development. The recent finding that RAGE influences tumor development through inflammatory cell recruitment (5) and our angiogenesis data in this study suggest that the
RAGE/RAGEv1 system can influence not only tumor cells but also cells of the tumor microenvironment. Furthermore, previous studies have shown in human tumor tissue a correlation between RAGE expression levels and tumor vessel density (34, 35). This, therefore, further implicates an essential role for RAGEv1 in countering the angiogenic effects of RAGE in the process of tumor angiogenesis.

To identify the signaling pathways eliciting these RAGEv1-mediated effects, we studied various components of the MAPK pathway. Work from both our group and others have definitely shown that RAGE predominantly acts as a signal transduction receptor for its ligands, and interruption of RAGE signaling abrogates cellular dysfunction and disease pathogenesis (2, 8–13). Furthermore, numerous studies have shown a central role of MAPK signaling in mediating RAGE ligand effects (9, 36). In agreement with these data, we observed that RAGEv1 blocked RAGE ligand activation of the major MAPK pathways, including MEK, p38, and JNK. Notably, neither RAGE ligand stimulation nor RAGEv1 affected AKT signaling in these cells. Further investigation of the specific MAPK pathway responsible for driving protumorigenic gene expression revealed JNK signaling to predominantly play a role. The JNK pathway has been shown to be crucially involved in cancer development by affecting a wide range of cellular processes, including proliferation, survival, and invasion (37, 38). Moreover, our results show that JNK activation is responsible for the increased tumor cell invasion seen through activation by RAGE ligand. Together, these data suggest that RAGEv1 acts to block tumorigenesis by inhibiting JNK signaling induced by RAGE ligand interaction.

To examine whether RAGEv1 may play a role in tumor formation, we used an in vitro tumor formation assay and an in vivo ectopic murine tumor model. In our experiments, RAGEv1 significantly inhibited tumor growth, which was consistent with previous observations using approaches to block RAGE ligand signaling (2). In agreement with the in vitro signaling data, JNK activation was inhibited in these cells.

Figure 5. RAGEv1 inhibits tumorigenesis by blocking a JNK-dependent signaling mechanism. A to C, cells were incubated with control (DMSO) or inhibitors for SAPK/JNK (SP600125), MEK1/2 (U0126), p38 (SB203580), and AKT (triciribine) for 1 h before stimulation with RAGE ligand (s100B). RNA was then extracted, and QPCR was performed for APAF1, PDGFB, and TNF, normalized to GAPDH levels. D, JNK inhibition blocks RAGE ligand–induced tumor cell invasion. Matrigel invasion assays were performed with mock-transfected cells, treated with control (DMSO) or SAPK/JNK (SP600125), and in response to vehicle or RAGE ligand (s100B). Data are means ± SEM from three independent experiments. *, significant differences \( (P \leq 0.05) \) between groups.
downregulated in tumors expressing RAGEv1, suggesting that JNK may be involved in RAGE ligand–mediated tumorigenesis in vivo. However, due to the highly significant inhibition of tumorigenic growth in the RAGEv1-expressing cells, the very small size of the resulting tumors rendered it difficult to perform extensive histologic analyses, such as quantification of blood vessel density.

Intriguingly, to support these findings, our studies of human tumor versus unaffected tissue suggest that RAGEv1 expression levels are decreased in tumor tissues. These findings suggest that at some point in the tumorigenic process, shutdown of RAGEv1 expression may facilitate tumor growth and invasion. Whether RAGEv1 is downregulated in the process preceding the tumor state, or in the course of tumorigenesis, is a question that future studies need to address. Certainly, however, findings in human tumor versus adjacent normal tissue indicate that RAGEv1 levels are mutable. Recent data from multiple human cohort studies suggest not only that RAGEv1 levels inversely correlated with inflammatory disease states but also that therapies targeting these pathologies lead to increased production of RAGEv1 (39, 40). These data provide further evidence that not only are RAGEv1 levels modulatable but they may also be changed as a consequence of altering the inflammatory state during the course of disease pathology. Understanding the mechanism(s) regulating expression of cell surface RAGE and the production and/or release of RAGEv1, including the potential impact of ligand RAGE interaction itself in these processes, will need to be investigated in future experimentation. Furthermore, what role RAGE ligand signaling plays in their regulation will be interesting to investigate. This, therefore, raises the tantalizing prospects that RAGEv1 may act as a biomarker for inflammatory disease states and targeted strategies to increase RAGEv1 levels may prove to be a useful therapy in the treatment of cancer by countering the effects of RAGE ligand signaling.

In conclusion, our results suggest for the first time that RAGEv1 is likely not only a potential tumor biomarker but also possesses innate functions. Here, we show that RAGEv1...
modulates tumor cell properties not only by affecting tumor cell survival, migration, and invasion, but also by regulating molecular pathways that influence the microenvironment to support tumor growth such as angiogenesis. We therefore propose that strategies to either suppress RAGE ligand signaling in malignant tissue and/or to boost endogenous production and release of RAGE1 in the tumor bed might represent highly potent strategies in the control of tumor growth and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


