The Receptor for Advanced Glycation End-products (RAGE) Sustains Autophagy and Limits Apoptosis, Promoting Pancreatic Tumor Cell Survival

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Abstract

Activation of the induced receptor for advanced glycation endproducts (RAGE) leads to initiation of NF-κB and MAP kinase signaling pathways resulting in propagation and perpetuation of inflammation. RAGE knock out animals are less susceptible to acute inflammation and carcinogen induced tumor development. We have reported that most forms of tumor cell death result in release of the RAGE ligand, HMGB1. We now report a novel role for RAGE in the tumor cell response to stress. Targeted knockdown of RAGE in the tumor cell, leads to increased apoptosis, diminished autophagy and decreased tumor cell survival. In contrast, overexpression of RAGE is associated with enhanced autophagy, diminished apoptosis and greater tumor cell viability. RAGE limits apoptosis through a p53 dependent mitochondrial pathway. Moreover, RAGE-sustained autophagy is associated with decreased phosphorylation of mTOR and increased Beclin-1/VPS34 autophagosome formation. These findings demonstrate that the inflammatory receptor RAGE plays a heretofore unrecognized role in the tumor cell response to stress. Furthermore, these studies establish a direct link between inflammatory mediators in the tumor microenvironment and resistance to programmed cell death. Our data suggest that targeted inhibition of RAGE or its ligands may serve as novel targets to enhance current cancer therapies.

INTRODUCTION

A growing body of literature supports the notion that many of the phenotypic alterations observed in cancer cells are a result of inflammatory signals found within the tumor microenvironment. Recent efforts have focused on identifying the responsible molecular pathways in cancer associated inflammation that result in promotion of carcinogenesis and
One such molecular target is the inflammatory receptor, Receptor for Advanced Glycation End products or RAGE. Expression of RAGE enhances tumor formation in an inflammatory carcinogen model of cancer and has been demonstrated to be associated with increased resistance to chemotherapy (3, 4, 5).

RAGE, an evolutionarily recent type I transmembrane receptor, is a member of the immunoglobulin superfamily. It is encoded within the gene-dense major histocompatibility Class III region, adjacent to TNF-α and several complement components. The ligands identified for RAGE include high mobility group protein 1 (HMGB1), advanced glycation endproducts and some but not all members of the S100 protein family (6). RAGE/RAGE ligand interactions are associated with survival of cells expressing this receptor, inflammation, increased levels of phosphorylated ERK and increased levels of NF-κB p65 (6, 7). Stressed cells markedly enhance RAGE expression which has been implicated in the pathogenesis of a variety of inflammatory diseases including sepsis, diabetes, atherosclerosis, and Alzheimer’s disease (6, 8, 9). RAGE has also been linked to the development and progression of cancer (10). Mice deficient for RAGE expression are resistant to DMBA/TPA-induced skin carcinogenesis demonstrating increased apoptosis in the tumor microenvironment and diminished inflammatory responses (11); however, the mechanism by which this occurs was not previously delineated. Inhibition of RAGE and its ligands with antibodies or sRAGE expression causes decreased growth and metastases of both implanted tumors and tumors developing spontaneously in susceptible mice (12). RAGE mediates resistance to hypoxia and consequently, RAGE knockdown in murine hepatomas slowed tumor growth (13). Inhibition of RAGE interaction with S100p leads to enhanced anti-tumor activity of conventional chemotherapy in a xenograft model of pancreatic cancer (14). In humans, RAGE and RAGE splice variants are overexpressed in adenocarcinomas of the pancreas and correlate with proliferation and invasiveness (15). Polymorphisms in the RAGE gene have also been linked to increased risk of gastric cancer (16).

We have previously demonstrated that one of the principle ligands for RAGE, the inflammatory cytokine HMGB1, is released following most forms of pancreatic tumor cell death (17, 18). We demonstrate here that expression of RAGE correlates with tumor cell survival following cytotoxic insult. RAGE-mediated tumor cell survival is associated with increased ‘programmed’ cell survival (autophagy) and decreased programmed cell death (apoptosis). RAGE limits apoptosis through a p53 dependent mitochondrial pathway. Moreover, RAGE-sustained autophagy is associated with decreased phosphorylation of mTOR and increased Beclin-1/VPS34 autophagosome formation. These findings suggest that RAGE promotes tumor cell survival in stressed cells and provides the framework for the development of novel clinical approaches targeting the RAGE pathway.

RESULTS

RAGE expression promotes tumor cell survival following genotoxic or metabolic stress

We analyzed RAGE expression in several cultured tumor cell lines. RAGE was expressed in a variety of human and mouse tumors under in vitro growth conditions; including pancreatic (Panc1.28, Panc2.03, Panc3.27 and Panc02), breast (4TI), and colonic (MC38, CT26).
carcinomas, a fibrosarcoma (MCA205) and melanoma (B16V1, B16M05) (Figure S1A). Interestingly, RAGE expression was greatest in the tumor cell lines derived from murine and human adenocarcinoma of the pancreas. RAGE and its ligand, HMGB1, were also expressed in murine pancreatic tumor cell lines growing in vivo. (Figure S1B). We chose to focus on these pancreatic tumors for our subsequent studies. Since RAGE expression is associated with increased nuclear translocation of NF-κB, a known tumor survival factor, we sought to better understand the impact of RAGE expression on tumor cell response to stress. We utilized a target-specific short hairpin RNA (shRNA) against RAGE to knockdown expression of RAGE and a full length plasmid expressing the murine or human RAGE gene (pUNO1-RAGE) to force overexpression in murine (Panc02) and human (Panc2.03) pancreatic tumor cell lines. Transfection of RAGE-shRNA decreased RAGE protein levels by nearly 80% (Figure 1A). Similarly, transfection with a full length RAGE plasmid increased the RAGE protein levels by two-fold in our cell lines (Figure 1B). One of the earliest events following activation of RAGE is phosphorylation of the extracellular signal-regulated kinase (ERK) (19). Consistent with this notion, we observed that knockdown of RAGE almost completely abrogated the ability of our tumor cell lines to phosphorylate ERK in response to chemotherapy induced stress (Figure S2). Next, we measured the effect of RAGE knockdown on cell viability following exposure to genotoxic or metabolic stress. We observed that depletion of RAGE significantly increased cell death of both murine and human pancreatic tumor cell lines following exposure to cytotoxic chemotherapy, UV radiation, and hypoxia (Figure 1C and S3). In contrast, forced overexpression of RAGE promoted survival of tumor cells following exposure to these stressors (Figure 1C). Measurement of long term cell viability by colony formation assays confirmed that RAGE depletion decreased tumor cell viability and RAGE overexpression increased tumor cell survival following exposure to cytotoxic chemotherapy (Figure S4). Increased cytotoxicity in RAGE knockdowns was observed with several chemotherapeutic agents that utilize varying mechanisms of action including oxaliplatin (platinating/DNA crosslinking agent), melphalan (alkylating agent) and gemcitabine (nucleoside analogue). Re-expression of RAGE with delivery of a full-length plasmid vector restored a ‘protective’ phenotype confirming the specificity of RAGE in promoting tumor cell survival (Figure 1D).

**RAGE promotes tumor cell survival by enhancing autophagy and limiting apoptosis**

To better understand the mechanisms of RAGE-mediated pro-survival signals, we measured caspase-3 activity as a measure of the terminal effector phase of apoptotic cell death. Knockdown of RAGE led to significantly greater induction of cleaved caspase-3 in these murine tumor cell lines following treatment with chemotherapeutic agents (Figure 2A and 2B). Consistent with this observation, addition of the pan-caspase inhibitor ZVAD-FMK reversed the increased caspase 3 activity and enhanced tumor cell death observed with RAGE knockdown (Figure 2A). This suggests that depletion of RAGE enhances apoptotic cell death following cytotoxic or metabolic stress. As autophagy is an increasingly recognized mechanism for tumor cell survival and resistance to apoptosis in response to stress (20), we examined levels of autophagy in murine pancreatic tumor cell lines treated with chemotherapeutic agents. Detection of LC3-II by immunoblotting or immunofluorescence is a widely used method for demonstrating increased autophagy (21). We observed a significant increase in LC3-II levels and an aggregation of LC3 into punctae...
in both human and murine pancreatic tumor cells treated with the LD50 of our chemotherapeutic agents (Figure 2B and 2C). Knockdown of RAGE limited this increase in markers of autophagy (Figure 2B and 2C). An alternative method for detecting autophagy is measuring enhanced degradation of p62 (SQSTM1/sequestosome), a long lived scaffolding protein involved in transport of ubiquitinated proteins destined for proteosomal digestion (22, 23). Indeed, knockdown of RAGE limited degradation of p62 induced by chemotherapeutic agents (Figure 2B). Consistent with the decreased autophagy observed in RAGE knockdown tumor cells lines, we found an increase in markers of apoptosis (cleaved PARP and cleaved caspase-3). Wortmannin, a Class III phosphoinositol-3 kinase [PI3K] inhibitor that blocks autophagy, significantly diminished LC3 punctae formation in both control shRNA and RAGE shRNA treated Panc02 cells (Figure 2C). Consistent with these findings, we observed that forced expression of RAGE led to decreased levels of cleaved caspase 3 and LC3 punctae in tumor cells treated with chemotherapeutic agents (Figure S5). To confirm that this observation was due to targeted knockdown of RAGE rather than nonspecific factors following transfection with shRNA vectors, we examined re-expression of RAGE through transfection with the pUNO1-mRAGE construct. Re-expression of RAGE in the knockdown tumor cell lines restored apoptosis and autophagy to basal levels (Figure 2D). We also examined markers of autophagy and apoptosis in fibroblasts from RAGE knockout animals. Primary short term cultures of lung fibroblasts from wild-type and RAGE knock out controls were propagated in short term cultures. We observed significant reduction in the levels of LC3-II protein expression in the RAGE KO fibroblasts following exposure to chemotherapeutic agents compared with their wild type counterparts (Figure S6). The RAGE ligand, HMGB1, is released following several methods of inducing tumor cell death including chemotherapy (17, 18). We hypothesize that HMGB1 released upon tumor cell death interacts with RAGE leading to increased resistance to programmed cell death. Consistent with this hypothesis we observed that depletion of the pool HMGB1 by shRNA in panc02 cells rendered them significantly more sensitive to melphalan-induced apoptotic cell death as demonstrated by increased cleaved PARP. We also observed decreased LC3-II, consistent with the lower levels of autophagy (Figure 2E). We have also observed that exogenous provision of rHMGB1 can promote autophagy in pancreatic tumor cell lines through a RAGE dependent pathway [Tang DL et al, submitted]. These findings strongly support a critical role for HMGB1/RAGE pathway in the regulation of apoptosis and autophagy in stressed or dying tumor cells.

**Increased apoptosis in RAGE knockdown tumor cells is associated with phosphorylation of p53 and its translocation to the mitochondria**

To further characterize the molecular events associated with the regulation of apoptosis by RAGE, we examined a panel of apoptotic markers. Depletion of RAGE by shRNA increased phosphorylation of p53 at Ser392, but not Ser6 and Ser 15 (Figure 3A and S7) following treatment with chemotherapeutic agents. At the same time, RAGE shRNA had no effect on the level of acetylation of p53 at Lys379 (Figure 3A). Expression levels of anti-apoptotic members of the mitochondrial pathway including Bcl-2 and Bcl-XL, were reduced by ~40–70% in RAGE knockdown Panc02 cells when compared with control cells in response to chemotherapy treatment. However, there was no significant difference in expression levels of the cytosolic pro-apoptotic Bcl-2 family members Bax and PUMA when comparing
control shRNA and RAGE shRNA treated Panc02 cells. This suggests that RAGE expression limits the p53-dependent mitochondrial pathway of apoptosis. To confirm this we also used pifithrin alpha (PFT-α), a pharmacological antagonist of p53 (24), to block p53 expression and function. Pretreatment with PFT-α blocked phosphorylation of p53 at ser392 and cleavage of caspase 3 in RAGE knockdown Panc02 cells following oxaliplatin treatment (Figure 3B). Furthermore, inhibition of p53 by PFT-α decreased the oxaliplatin-induced activation of caspase-3 and cell death in RAGE knockdown Panc02 cells (Figure 3B). Similarly, we observed that knockdown of RAGE had no effect on oxaliplatin induced tumor cell death in p53 knockout HCT116 cell lines (Figure 3C). Next, we observed that depletion of RAGE increased translocation of p53 to the mitochondria and promoted cytochrome c release from the mitochondria to the cytosol following oxaliplatin treatment (Figure 3D). RAGE shRNA, however, did not influence the p53 reporter activity induced by chemotherapeutic drugs such as oxaliplatin and melphalan (Figure 3E). Together, these results suggest that when RAGE is present it limits translocation of p53 to the mitochondria, in turn inhibiting apoptosis and enhancing survival following treatment with chemotherapy.

**Decreased autophagy in RAGE knockdown tumor cells is associated with increased levels of phosphorylated mTOR and decreased autophagosome formation**

Autophagy is controlled by several kinases including the mammalian target of rapamycin (mTOR), which promotes active protein translation and suppresses autophagy (25). To gain insight into the molecular mechanism by which RAGE regulates autophagy in pancreatic cancer cells, we examined the effect of RAGE expression on mTOR. mTOR is autophosphorylated at Ser2481 and phosphorylated at Ser2448 via the class I PI3 kinase/Akt signaling pathway (26). Phosphorylation of mTOR was increased in RAGE shRNA treated Panc02 cells when compared with control shRNA treated cells (Figure 4A). Unexpectedly, the phosphorylation of the mTOR downstream target 4E-BP1 (p-4E-BP1) was inhibited following depletion of RAGE (Figure 4A). This discrepancy suggests that a more complex signaling pathway regulates p-4E-BP1. Furthermore, p-4E-BP1 is also positively regulated by the ERK pathway in lung endothelial cells (27). Consistent with this finding, we observed that knockdown of RAGE almost completely abrogated the ability of our tumor cell lines to phosphorylate ERK in response to chemotherapy induced stress (Figure S2). At the same time, RAGE shRNA had no effect on the expression level of Raptor and Rictor, two molecular components of the mTORC1 and mTORC2 complexes (28) (Figure 4A).

Treatment with rapamycin, a specific inhibitor of mTOR (29) limited phosphorylation of mTOR and increased autophagy as demonstrated by increased LC3-II in RAGE knockdown tumor cell lines treated with oxaliplatin (Figure S8). These observations suggest that targeted knockdown of RAGE limits autophagy and tumor cell survival following treatment with chemotherapy through an mTOR dependent pathway.

The Beclin-1/VPS34 (the yeast homolog of the class III PI3K) complex is important in mediating the localization of other autophagy-related proteins to the preautophagosomal membrane (30). Thus, we evaluated the effects of RAGE shRNA on Beclin-1/VPS34 complex formation and activity in Panc02 cells. Panc02 RAGE knockdown cells expressed similar levels of Beclin-1, but less VPS34 when compared with control shRNA treated cells.
Notably, less Beclin-1 co-immunoprecipitated with VPS34 in RAGE knockdown Panc02 cells when compared with control shRNA treated Panc02 cells (Figure 4B). This suggests that in addition to inhibiting autophagy through phosphorylation of mTOR, knockdown of RAGE also limits autophagy by limiting the formation of the Beclin-1/VPS34 complex.

**Increased sensitivity to chemotherapy, increased apoptosis and decreased autophagy in RAGE knockdown tumor cells is dependent on ATG5**

ATG5 is a critical link between autophagy and apoptosis (31). Since we have demonstrated that RAGE knockdown cells exhibit a shift from autophagy to apoptosis following treatment with chemotherapy, we examined the role of ATG5 in this pathway. We transfected pancreatic tumor cell lines with shRNA specific for RAGE, ATG5 or Beclin-1 (Figure 5A). Consistent with a role for ATG5 in both apoptotic and autophagic pathways, we observed that its knockdown decreased both LC3 punctae formation and caspase 3 activity in pancreatic tumor cells following treatment with chemotherapeutic agents. Interestingly, depletion of ATG5 but not Beclin-1 reversed the increased chemosensitivity of RAGE knockdown tumor cell lines (Figure 5B). Furthermore, depletion of ATG5 reversed the proapoptotic effects of RAGE knockdown as demonstrated by decreased caspase 3 activity. As expected, knockdown of Beclin-1 and RAGE resulted in marked decreases in apparent autophagy. Wortmanin, an autophagic inhibitor, increased or restored apoptosis and cell death following treatment with chemotherapy in both wild type cells and cells overexpressing RAGE (Figure 5B and C). Together these data support the notion RAGE though ATG5 plays an important role in the balance between autophagy and apoptosis and ultimately tumor cell survival following treatment with chemotherapy.

**Targeted knockdown of RAGE increases sensitivity to chemotherapy in vivo and is associated with increased apoptosis and decreased autophagy**

To test if targeted knock down of RAGE also increased sensitivity to chemotherapy in vivo we inoculated C57/BL6 mice with $10^6$ Panc02 tumor cells with stable transfection of control or RAGE specific shRNA and treated with oxaliplatin. In vivo, growth of the RAGE knockdown tumor cells was significantly slower than the controls, at all tumor cell injection concentrations (range $10^5$–$10^6$; data not shown). Growth of RAGE knockdown tumor cells was significantly inhibited and in some cases completely cleared at a dose of oxaliplatin (7mg/kg) that was clinically ineffective on control shRNA transfected tumors (Figure 6A). We observed that tumor cells transfected with RAGE specific shRNA demonstrated decreased autophagy and increased apoptosis in vivo when treated with oxaliplatin as compared with control shRNA transfected tumor cells (Figure 6B–C). Together, these results demonstrate that RAGE is critical in modulating apoptosis and autophagy in stressed cancer cells in vivo.

**DISCUSSION**

The receptor for advanced glycation endproducts [RAGE] is an induced receptor on both inflammatory and endothelial cells. We and others have now demonstrated that this receptor can also be constitutively expressed on many murine and human epithelial tumor cell lines.
Interestingly, we observed the highest levels of RAGE expression in murine and human pancreatic adenocarcinoma tumors, a tumor type notoriously resistant to conventional therapies. Genotoxic and/or metabolic stress led to modest but reproducible increases in overall expression of RAGE on murine pancreatic and colorectal adenocarcinoma cell lines. We observed minimal RAGE expression in murine lung and renal tumor cell lines. This is consistent with recent reports demonstrating that human lung carcinomas, unlike most other cancer types, often down-regulate RAGE expression [both at the mRNA and protein level] (27).

Remarkably, we observed that RAGE expression correlated directly with the ability of both murine and human pancreatic tumor cell lines to survive cytotoxic insult. Targeted knockdown of RAGE significantly increased cell death, while forced overexpression promoted survival. The enhanced sensitivity to cell death in the setting of RAGE knockdown was associated with increased apoptosis and could be reversed in part by treatment with pan-caspase inhibitors. In cells treated with a pharmacologic inhibitor of p53, pifithrin α, and p53 knock out tumor cell lines we observed abrogation of the increased cell death observed with RAGE knockdown. p53 induces apoptosis through a mechanism that does not depend solely on its nuclear role but rather involves translocation of p53 to the mitochondria and induction of mitochondrial outer membrane permeability [MOMP] (35). Stressed tumor cells with RAGE knockdown demonstrated increased phosphorylation of p53. This was associated with a decrease in cytosolic and increase in mitochondrial translocation, rather than transactivation. This suggests that when present, RAGE protects tumor cells from cytotoxic insult in part by limiting progression through a mitochondrial, p53 mediated pathway of programmed cell death.

Our observations that the proapoptotic effects of RAGE knockdown were p53 dependent led us to examine the role of autophagy in the setting of RAGE knockdown or overexpression. It has been reported that p53 regulates autophagy in a dual fashion. The pool of cytoplasmic p53 protein represses autophagy in a transcription-independent fashion (36, 37), while the pool of nuclear p53 stimulates autophagy through the transactivation of specific genes (38). Although autophagy was initially described as a non-apoptotic pathway of programmed cell death, it now appears that in most instances it serves as a survival strategy by which stressed cells limit apoptosis (39, 40). Autophagy is increased in pancreatic cancer cells in resected tumors and correlates with poor patient outcome (41). Both murine and human pancreatic tumor cell lines demonstrated clear evidence of enhanced autophagy following treatment with chemotherapeutic agents. Knockdown of RAGE limited induction of this autophagic response. Similarly, overexpression of RAGE led to greater induction of autophagy and improved tumor cell survival. Autophagy in mammalian cells is predominately under the control of the serine kinase, mammalian target of rapamycin (mTOR), which suppresses autophagy and enhances transcriptional activities in response to nutrient availability (29, 39, 40). Phosphorylation of mTOR released the suppression of autophagy allowing for formation of a multiprotein complex that includes class III phosphatidylinositol 3-kinase (PI3K), Beclin-1 and vacuolar protein sorting factor protein 15 (VPS15) (42). We demonstrated that knockdown of RAGE enhanced mTOR phosphorylation in response to chemotherapy thus preventing induction of a survival response. RAGE knockdown also prevented stabilization of the Beclin-1/VPS34 complex, further limiting the autophagic pathway.
response. The induction of autophagy in RAGE knockdown tumor cell lines was also partly dependent upon the activity of ATG5. ATG5 has been demonstrated to be an important molecule in both apoptosis and autophagy (43). Our data suggests that under stress, the cell balances apoptotic and autophagic responses through ATG5. Autophagy has become a dominant theme in cancer research and target for emergent therapies as it enables survival within the tumor microenvironment under stress (20). An understanding of how various inflammatory signals promote these survival pathways is increasingly being explored. Our findings suggest that RAGE is a potential mediator of enhanced autophagy in the tumor microenvironment.

The endogenous ligand for RAGE in the setting of cancer is not known, although it is likely that there are multiple ligands for this promiscuous receptor. The RAGE ligand S100p has been shown to promote pancreatic and colorectal tumor cell survival, invasiveness and resistance to chemotherapy through RAGE interactions (32). We have previously demonstrated that one of the RAGE ligands, HMGB1, is released following several methods of inducing tumor cell death including chemotherapy (17, 18). We have also observed that exogenous provision of rHMGB1 can promote autophagy in pancreatic tumor cell lines through a RAGE dependent pathway [Tang DL et al, submitted]. Here we now report that reduction of the intracellular pool of HMGB1 through targeted knockdown, enhanced tumor cell death by increasing programmed cell death (apoptosis) and diminishing programmed cell survival (autophagy). Together, these observations suggest a new paradigm in tumor biology, whereby stressed or dying tumor cells release pro-inflammatory factors that in aggregate are termed Damage Associated Molecular Pattern molecules or DAMPs. When released, DAMPs interact with receptors (DAMP-R) such as RAGE on surviving, stressed cells in the tumor microenvironment. This favors tumor survival in part by increasing programmed cell survival (autophagy) and decreasing programmed cell death. Based on our findings and an emerging literature regarding the effects of DAMPS in the tumor microenvironment, we hypothesize that cancer is fundamentally a disorder of cell death (44). The findings presented here are consistent with this hypothesis.

In conclusion we have demonstrated a novel role for RAGE in enhancing tumor cell survival through increased autophagy and diminished programmed cell death in pancreatic tumor cells both in vitro and in vivo. These data support the development of novel clinical therapies targeting the RAGE pathway.

MATERIALS AND METHODS

Reagents

The antibodies to cleaved-caspase 3, cleaved-PARP, Bax, PUMA, Bcl-XL, Bcl-2, p-p53 (Ser392), p-p53 (Ser6), p-p53 (Ser15), Ace-p53 (Lys379), p53, p-4E-BP1 (Thr70), p-4E-BP1 (Thr37/46), 4E-BP1, p-mTOR (Ser2448), mTOR, Raptor, Rictor, VPS34, p-ERK (Thr202/Tyr204) and ERK were obtained from Cell Signaling Technology (USA); The antibodies to α-Tubulin and actin, were from Sigma (USA); The antibody to TLR4 was from Abcam (USA); The antibodies to LC3, ATG5 and p62 were from Novus (USA); Anti-RAGE antibody was from Sigma (USA) or Abcam (USA); Anti-Beclin-1 antibody was from Cell Signaling Technology (USA) or Novus (USA); Anti-cytochrome c antibody was from
Santa Cruz Technology (USA); Alexa Fluor 488 or 647-conjugated anti-rabbit or anti-mouse Ig were from Invitrogen (USA). ZVAD-FMK was purchased from Calbiochem (USA). Gemcitabine was from Eli Lilly (USA). Oxaliplatin, melphalan, and wortmannin were obtained from Sigma.

**Cell culture**

Cell lines were derived from the Hillman Cancer Institute. All cell lines were cultured in RPMI or DMEM medium 1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and antibiotic-antimycotic mix in a humidified incubator with 5% CO$_2$ and 95% air.

**Cell viability assay**

Cells were plated at a density of $10^4$ cells/well in 96-well plates in 100 µl RPMI. Cell viability was evaluated using the CCK8 assay kit (Dojindo Laboratories, Tokyo, Japan) according to the manufacturer’s instructions. Long term cell viability by colony formation assay was also performed. Cells were treated for 24 h with chemotherapeutic agents (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml; and gemcitabine, “GM”, 100 nM) and plated at a density of $2 \times 10^3$ cells/well in 24 well culture plates. Colonies were visualized by crystal violet staining 3 weeks later.

**Subcutaneous tumor models**

To generate murine subcutaneous tumors, $10^6$ Panc02 WT or RAGE knockdown cells were injected subcutaneously to the right of the dorsal midline in C57/Bl6 mice. Tumors were measured twice weekly, and volumes were calculated using the formula $length \times width^2 \times \pi/6$. The procedures for performing animal experiments were approved and in accordance with the principles and guidelines of the University of Pittsburgh Institutional Animal Care and Use Committee.

**Western blotting**

Proteins in the whole-cell lysate were resolved on 4–12% Criterion XT Bis-Tris gels (Bio-Rad, USA) and transferred to a nitrocellulose membrane as previously described (45, 46). After blocking, the membrane was incubated for 2 h at 25°C or overnight at 4°C with various primary antibodies. After incubation with peroxidase-conjugated secondary antibodies for 1 h at 25°C, the signals were visualized by enhanced chemiluminescence (Pierce, USA) according to the manufacturer’s instruction. The relative band intensity was quantified using the Gel-pro Analyzer® software (Media Cybernetics, USA).

**Gene transfection and shRNA**

RAGE-shRNA, Beclin-1-shRNA, ATG5-shRNA, HMGB1-shRNA (Sigma, USA) or pUNO1-RAGE (Invivogen, USA) were transfected into cells using Lipofectamine 2000 reagent (Life Technologies, USA) according to the manufacturer’s instructions. At the end of the shRNA treatment (48 h), the medium was change before the addition of the chemotherapy agent.

*Cell Death Differ.* Author manuscript; available in PMC 2012 August 12.
**Immunofluorescence analysis**

Cells were cultured on glass cover-slips and fixed in 3% formaldehyde for 30 min at room temperature prior to detergent extraction with 0.1% Triton X-100 for 10 min at 25°C. Cover slips were saturated with 2% BSA in PBS for 1 h at room temperature and processed for immunofluorescence with primary antibodies followed by Alexa Fluor 488 or 647-conjugated anti-rabbit or anti-mouse Ig respectively. Nuclear morphology was analyzed with the fluorescent dye Hoechst 33342 (Sigma, USA). Between all incubation steps, cells were washed three times for 3 min with 0.5% BSA in PBS. Images were taken with a fluorescence microscope.

For tissue immunofluorescence analysis, tissues were embedded in optimum cutting temperature cryomedium (Sakura, the Netherlands) and subsequently, cut into 8 µm sections as described previously (11). Tissue sections were stained with LC3, RAGE, or HMGB1 antibody followed by Alexa Fluor 488-conjugated anti-rabbit Ig. Nuclear morphology was analyzed with the fluorescent dye Hoechst 33342 (Sigma, USA). Actin was imaged with phalloidin-binding (Sigma, USA). TUNEL assay was performed using the In Situ Cell Death Detection Kit, according to the manufacturer’s recommendations (Roche, Sweden).

**Immunoprecipitation analysis**

Cells were lysed at 4°C in ice-cold RIPA lysis buffer (Millipore, USA), and cell lysates were cleared by centrifugation (12000 g, 10 min). Concentrations of proteins in the supernatant were determined by BCA assay. Prior to immunoprecipitation, samples containing equal amount of proteins were pre-cleared with Protein A or protein G agarose/sepharose (Millipore, USA) (4°C, 3 h) and subsequently incubated with various irrelevant IgG or specific antibodies (5 µg/mL) in the presence of protein A or G agarose/sepharose beads for 2 h or overnight at 4°C with gentle shaking (47). Following incubation, agarose/sepharose beads were washed extensively with PBS, and proteins were eluted by boiling in 2× SDS sample buffer before SDS-PAGE electrophoresis.

**Caspase-3 activity assay**

Caspase-3 activity was assayed using the Caspase-3 Colorimetric Assay Kit (Calbiochem, USA) according to manufacturer’s instructions.

**p53 reporter assay**

p53 reporter activity was assayed using the p53 reporter (luc) kit (SABiosciences Corporation, USA) according to the manufacturer’s instructions.

**Analysis of autophagy by imaging cytometry**

Cells were seeded in 96-well plates and cultured in the presence of stimulus for given time, then fixed with 3% paraformaldehyde and stained with LC3 antibody. Secondary antibodies were Alexa Fluor 488 conjugated anti-rabbit Ig. Nuclear morphology was analyzed with the fluorescent dye Hoechst 33342 (Sigma, USA). Images data were collected with an ArrayScan HCS 4.0 imaging cytometer with a 20x objective (Cellomics, Pittsburgh, USA). Arrayscan is an automated fluorescent imaging microscope that collects information about...
the spatial distribution of fluorescently labeled components in cells placed in 96 well microtiter plates. The Spot Detector BioApplication was used to acquire and analyze the images after optimization. Images of 1,000 cells for each treatment group were analyzed to obtain LC3 fluorescence spot number per cell as previously described (45, 48).

Statistical analysis
Data are expressed as means ± SEM of three independent experiments performed in triplicate. One-way ANOVA was used for comparison among the different groups. When the ANOVA was significant, post hoc testing of differences between groups was performed using LSD test. A P-value < 0.05 was considered significant.

Supplementary Material
Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS
This project was funded by a grant from the NIH 1 PO1 CA 101944-04 (Lotze, Michael T) Integrating NK and DC into Cancer Therapy National Cancer Institute and a grant with the Pennsylvania Department of Health. The department specifically disclaims responsibility for any analyses, interpretations or conclusions derived from this work.

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Cell Death Differ. Author manuscript; available in PMC 2012 August 12.
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Figure 1. RAGE promotes tumor cell survival following genotoxic or metabolic stress
(A) Targeted knockdown of RAGE protein following transfection with RAGE shRNA. RAGE, TLR4 and actin protein levels in Panc02 (murine) or Panc2.03 (human) cells transfected with nonspecific shRNA (Ctrl shRNA, “1, 3”) or RAGE shRNA (“2, 4”) at 48 h post transfection (* p < 0.05 versus Ctrl shRNA).
(B) RAGE protein was overexpressed following transfection with a RAGE plasmid. RAGE, TLR4 and actin protein levels in Panc02 or Panc2.03 cells transfected with empty vector

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(pUNO1, “1, 3”) or RAGE plasmids (pUNO1-RAGE, “2, 4”) at 48 h post transfection (* p < 0.05 versus pUNO1).

(C) RAGE expression correlates with tumor cell survival. Knockdown of RAGE or overexpression of RAGE in pancreatic cancer cell lines is indicated. These cells were then treated with the LD50 of individual chemotherapeutic agents (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml; or gemcitabine, “GM”, 100 nM), UV irradiation (5 min after 50 mJ/cm2) or hypoxia (1% O2). Cell viability was examined at 24 h (n=3, * p < 0.05 RAGE shRNA versus ctrl shRNA or pUNO versus pUNO1-RAGE).

(D) Re-expression of RAGE with full length plasmid restored the chemotherapy-protective phenotype. RAGE was overexpressed in RAGE knockdown Panc02 tumor cells as indicated and then treated with chemotherapeutic agents (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml; and gemcitabine, “GM”, 100 nM). Cell viability was examined at 24 h (n=3, * p < 0.05).
Figure 2. Targeted knockdown of RAGE in murine and human pancreatic tumor cell lines results in increased apoptosis and decreased autophagy following treatment with chemotherapeutic agents

(A) A pan-caspase inhibitor reverses the increased caspase 3 activity and tumor cell death observed in RAGE knockdown cells. Panc02 tumor cells were pretreated with the pan-caspase inhibitor (ZVAD-FMK, 20 µM) for 1 h and then treated with chemotherapeutic agents (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml; and gemcitabine, “GM”, 100 nM). Caspase 3 activity and cell viability were examined at 24 h (n=3, * p <
0.05 RAGE shRNA versus ctrl shRNA, # p < 0.05 RAGE shRNA+ZVAD-FMK versus RAGE shRNA).

(B) Knockdown of RAGE decreases markers of autophagy and increases markers of apoptosis. Panc02 tumor cells were treated with chemotherapeutic agents (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml) for the indicated time. Western blot analysis of protein levels is indicated. Left panel is a representative picture of western blots (n=3, * p < 0.05 RAGE shRNA versus ctrl shRNA).

(C) An autophagy inhibitor limits LC3 punctae in control and RAGE knockdown cells. Panc02 tumor cells were pretreated with the PI3K/autophagy inhibitor, wortmannin (WM) 100 nM for 1 h and then treated with chemotherapeutic agents (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml) for the indicated time. Cells were immunostained with LC3-specific antibodies. Images of 1,000 cells were analyzed to obtain the average LC3 spots by imaging cytometric [Arrayscan] analysis. Left panel is a representative picture of each condition. Inset shows a higher magnification of LC3 stain.

(D) Re-expression of RAGE with full-length plasmid restores relative autophagy and apoptosis levels to baseline. RAGE was overexpressed in RAGE knockdown Panc02 tumor cells and cells were treated with chemotherapeutic agents (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml). Caspase 3 activity and LC3 spot formation were examined at 24 h (n=3, * p < 0.05).

(E) HMGB1 knockdown cells demonstrate increased resistance to melphalan-induced apoptotic cell death. Panc02 cells were knocked down by HMGB1 shRNA and Ctrl shRNA for 48 h and then stimulated with melphalan (“ME”, 320 µg/ml) for 24 h. Representative western blot analysis of protein levels is presented.
Figure 3. Increased apoptosis in RAGE knockdown tumor cells is p53 dependent

(A) RAGE knockdown significantly increases phosphorylation of p53 at ser392 and decreases anti-apoptotic Bcl-2 family proteins following treatment with anti-cancer agents. Panc02 cells were treated with chemotherapeutic agents (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml) for the indicated time. Western blot analysis of protein levels is presented. Left panel is a representative picture of western blots (n=3, * P < 0.05 RAGE shRNA versus ctrl shRNA group).

(B) A small molecule inhibitor of p53 reverses chemotherapy-induced apoptosis in RAGE knockdown cells. RAGE knockdown Panc02 cells were pretreated with a p53 inhibitor (PFT-α, 20 µM) for 1 h and then treated with oxaliplatin (“OX”, 160 µg/ml) for 24 h. Western blot analysis of protein levels are indicated. Caspase 3 activity and cell death were examined at 24 h (n=3, * P < 0.05).

(C) p53−/− reverses anti-cancer drug-induced cell death in RAGE knockdown cells. RAGE was knocked down by shRNA transfection in p53 WT HCT116 cell or p53−/− HCT116 cells, and then these cells were treated with oxaliplatin (“OX”, 160 µg/ml). Cell death was assayed at 24 h (n=3, * p < 0.05).

(D) RAGE knockdown increases mitochondrial translocation of p53 in cells following treatment with chemotherapeutic agents. RAGE knockdown Panc02 cells were treated with oxaliplatin (“OX”, 160 µg/ml) for 12 h. Western blot analysis of p53 and cytochrome c (Cyt c) was then assessed in the cytoplasmic and mitochondrial fractions of these cells. The successful separation of cytoplasmic (“Cyt”) and mitochondria (“Mit”) fraction was confirmed by western blot analysis of each fraction for known cytoplasmic (tubulin) and mitochondrial (COX IV) proteins.

(E) RAGE shRNA does not influence chemotherapy drug-induced p53 reporter activity. The indicated Panc02 cells were transfected with a p53 reporter. After 24 hours of transfection, cells were treated separately with the chemotherapeutic agents (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml). A luciferase assay was performed 12 hours following treatment.
Figure 4. Decreased autophagy in RAGE knockdown tumor cells is associated with increased levels of phosphorylated mTOR and decreased autophagosome formation

(A) RAGE knockdown results in increased phosphorylation of mTOR and decreased phosphorylation of 4E-BP1. The indicated Panc02 cells were treated with anti-cancer drugs (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml) for the given times. Western blot analysis of protein levels is shown.

(B) RAGE knockdown blocks Beclin-1/VPS34 complex formation during autophagy. RAGE knockdown Panc02 cells were treated with oxaliplatin (“OX”, 160 µg/ml) for 3 h. Cell lysates were prepared for IP with anti-Beclin-1 or anti-VPS34 or IgG. The resulting immune complexes were analyzed by western blotting using antibodies to Beclin-1 or VPS34.
Figure 5. Increased sensitivity to chemotherapeutic agents, increased apoptosis and decreased autophagy in RAGE knockdown tumor cells is dependent on ATG5

(A) Western blot analysis of protein levels in Panc02 cells as indicated after knockdown of RAGE, Beclin-1, and ATG5 by shRNA.

(B) Depletion of ATG5, but not Beclin-1 reverses the increased chemosensitivity in RAGE knockdown cells. The indicated Panc02 cells were treated with chemotherapeutic drugs (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml) with or without wortmannin (100 nM) and then assayed for cell death, caspase 3 activity and LC3 punctae formation (n=3, * p < 0.05 versus ctrl shRNA group).
(C) An autophagy inhibitor increases cell death following chemotherapy in cells overexpressing RAGE. The indicated Panc02 tumor cells were pretreated with wortmannin (100 nM) for 1 h and then treated with chemotherapeutic agents (oxaliplatin, “OX”, 160 µg/ml; melphalan, “ME”, 320 µg/ml). Cell viability was examined at 24 h (n=3, * p < 0.05).
Figure 6. Expression of RAGE mediates chemoresistance in vivo and is associated with decreased apoptosis and increased autophagy

(A) RAGE knockdown tumor cells are more sensitive to oxaliplatin in vivo. C57/BL6 mice were inoculated with $10^6$ Panc02 tumor cells following stable transfection of control (WT) or RAGE specific shRNA (KD) and treated with oxaliplatin (“OX”, 7mg/kg) or PBS beginning at day 11 (day 11 normalized to 1). Tumors were measured twice weekly, and volumes were calculated for 38 days (* p < 0.05 KD+OX versus WT+OX).

(B) RAGE knockdown increases apoptosis following treatment with oxaliplatin (7 mg/kg) in vivo. Included here are representative images of implanted WT (control shRNA) and KD (RAGE shRNA) pancreatic tumors sections that were analyzed by TUNEL assay (green signal). Nuclear staining was done with Hoechst 33342 (blue signal). Inset shows a higher magnification of TUNEL stain.

(C) RAGE knockdown decreases autophagy after treatment with oxaliplatin (7 mg/kg) in vivo. Included here are representative images of implanted WT (control shRNA) and KD (RAGE shRNA) pancreatic tumors sections that were analyzed by indirect IF staining of LC3 protein (green signal). Nuclear staining was done with Hoechst 33342 (blue signal). Inset shows a higher magnification of cells following staining with LC3.