

RAGE: developmental expression and positive feedback regulation by Egr-1 during cigarette smoke exposure in pulmonary epithelial cells

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Reynolds PR, Kasteler SD, Cosio MG, Sturrock A, Huecksteadt T, Hoidal JR. RAGE: developmental expression and positive feedback regulation by Egr-1 during cigarette smoke exposure in pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 294: L1094–L1101, 2008. First published April 4, 2008; doi:10.1152/ajplung.00318.2007.—The receptor for advanced glycation end-products (RAGE) is a member of the immunoglobulin superfamily of multiligand receptors. Following ligand binding, mechanisms associated with host defense, tissue remodeling, and inflammation are activated. RAGE is highly expressed in pulmonary epithelium transitioning from alveolar type (AT) II to ATI cells and is upregulated in the presence of ligand; however, the regulation and function of RAGE during development are less clear. Herein, immunohistochemistry demonstrated a temporal-spatial pattern of RAGE expression in pulmonary epithelial cells from *embryonic day 17.5* to *postnatal day 10*. Cotransfection experiments revealed that the mouse RAGE promoter was activated by early growth response gene 1 (Egr-1) and inhibited by thyroid transcription factor-1 (TTF-1) via interaction with specific regulatory elements. A rat ATI cell line (R3/1) with endogenous RAGE expression also differentially regulated RAGE when transfected with TTF-1 or Egr-1. Because Egr-1 is markedly induced in pulmonary epithelial cells exposed to cigarette smoke extract (CSE; Reynolds PR, Hoidal JR. *Am J Respir Cell Mol Biol* 35: 314–319, 2006.), we sought to investigate RAGE induction by CSE. Employing RT-PCR and Western blotting, RAGE and common ligands (amphoterin and S100A12) were upregulated in epithelial (R3/1 and A549) and macrophage (RAW) cell lines following exposure to CSE. Immunostaining for RAGE in cells similarly exposed and in lungs from mice exposed to cigarette smoke for 6 mo revealed elevated RAGE expression in pulmonary epithelium. After the addition of glyoxylated BSA, an advanced glycation end-product that binds RAGE, real-time RT-PCR detected a 200-fold increase in Egr-1. These results indicate that Egr-1 regulates RAGE expression during development and the likelihood of positive feedback involving Egr-1 and RAGE in cigarette smoke-related disease.

development; disease; lung; receptor for advanced glycation end-products; early growth response gene 1

RECEPTORS FOR ADVANCED GLYCATION end-products (RAGE) are members of an immunoglobulin superfamily of cell-surface receptors expressed in many cell types, including endothelial and vascular smooth muscle cells, fibroblasts, neurons, and macrophages/monocytes (40). RAGE contains an extracellular ligand-engaging V-region-like domain and two COOH-region-like domains, a single transmembrane domain, and a short, highly charged cytoplasmic domain essential for signal transduction (13, 14, 24, 33). RAGE expression is most abundant in

the lung, from which it was initially isolated, and was identified to be selectively localized to the basolateral membranes of well-differentiated AT1 cells (34). Identification in these cells has led to the implication of RAGE in important developmental processes such as the spreading, thinning, and adherence that characterize the transitioning of alveolar type (AT) II cells to squamous ATI cells. Although first described as a progression factor in cellular responses induced by advanced glycation end-products (AGEs) that accumulate in hyperglycemia and oxidant stress, other studies have demonstrated RAGE as a pattern recognition receptor that also binds S100/calgranulins, amyloid- β -peptide, and HMGB-1 (or amphoterin) to influence gene expression via activated signal transduction pathways (13, 36, 43).

RAGE expression increases whenever its ligands accumulate (34), and RAGE-ligand interaction leads to physiological and pathological processes, including diabetic complications, neurodegenerative disorders, atherosclerosis, and inflammation (13, 36). Furthermore, unpublished data reveal that RAGE null mice are protected from inflammatory lung disease. Despite upregulation of the receptor in cases of injury and disease, high levels of RAGE expression during lung development and in adult pulmonary tissue suggest a likely beneficial function in lung morphogenesis and homeostasis. Additionally, RAGE expression in other tissues that experience branching morphogenesis, such as the kidney (32) and salivary gland (6), also suggests functions that are likely associated with organogenesis. However, the full extent of RAGE expression and the molecular mechanisms that control it have not been evaluated adequately. Understanding the role of RAGE in development could provide insights to pursue the reduction of RAGE-mediated lung injury.

In a previous investigation, our laboratory demonstrated that early growth response gene 1 (Egr-1) is markedly upregulated in pulmonary epithelial cells during exposure to cigarette smoke and in lungs of mouse models of emphysema (28). Cigarette smoke-induced elevation of Egr-1 expression also directly influences the secretion of proinflammatory cytokines. Egr-1 is a zinc finger-containing, hypoxia-inducible transcription factor. Egr-1 is developmentally regulated and binds the GC-rich sequence GCG(G/T)GGGCG, which often overlaps the DNA-binding sequence of stimulating protein 1. Competition between stimulating protein 1 and Egr-1 leads to either activation or suppression of common target genes (15, 21). Egr-1 is a crucial regulator of cell proliferation, differentiation,

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and survival after induction by several stimuli such as growth factors, cytokines, radiation, apoptosis-promoting factors, injury, and stretch (4, 15, 27). Furthermore, depending on the stimulus and cell type, various signal transduction pathways induce Egr-1, including those mediated by the mitogen-activated protein kinases extracellular signal-regulated kinase 1/2, protein kinase C- β , Rho GTPase, or p38/c-Jun NH₂-terminal kinase (8). Although research aimed at understanding these signal transduction pathways is progressing, studies linking Egr-1 and RAGE to specific developmental processes or mechanisms of tobacco-related diseases are lacking.

In the present study, we test the hypothesis that RAGE is developmentally expressed in differentiating epithelial cells and transcriptionally regulated by Egr-1. We also test the hypothesis that Egr-1, previously demonstrated to be induced by cigarette smoke, controls RAGE expression in tobacco-related environments.

We report that RAGE is expressed in subsets of pulmonary epithelial cells during stages of lung development that correlate with proximal-distal differentiation of the lung. We demonstrate that RAGE is transcriptionally activated by Egr-1, an early transcriptional regulator, and inhibited by thyroid transcription factor-1 (TTF-1), a homeobox-containing transcription factor expressed by differentiated ATII cells that critically influences lung morphogenesis. Additionally, AGE-RAGE interaction culminates in marked upregulation of Egr-1, suggesting a positive feedback loop that must be balanced during development but activated during injury to synergistically augment RAGE expression and elicit additional cellular responses. Further demonstration of such feedback mechanisms is presented in data identifying significant upregulation of both RAGE (current investigation) and Egr-1 (28) in pulmonary epithelial cells exposed to cigarette smoke extract (CSE). In addition to receptor augmentation, CSE induced the synthesis of RAGE ligands, such as S100A12 and HMGB-1, indicating that, while RAGE and its ligands are specifically controlled during lung development and maintenance, stimuli such as cigarette smoke cause marked upregulation of RAGE and its ligands via the likely employment of dysregulated developmental mechanisms. Collectively, these data offer novel insights into potential mechanisms whereby RAGE is controlled developmentally and during exposure to cigarette smoke.

MATERIALS AND METHODS

Antibodies and immunohistochemistry. A goat RAGE polyclonal antibody was developed by ProSci (Poway, CA) against a specific peptide PKKPPQRLEWKLNTGRTE (amino acids 42–59) and was used at a dilution of 1:200. Cells were fixed by incubation in 4% paraformaldehyde for 20 min and then washed in three changes of PBS. Sections (5 μ m) were cut from lungs, inflation fixed with 4% paraformaldehyde, and immunostained for the presence of RAGE using standard techniques, as previously outlined (29). Control cultures were incubated in blocking serum alone.

Plasmid construction and mutagenesis. Primers were designed to retrieve 1.5 and 0.75 kb of the mouse RAGE promoter by high-fidelity PCR using the Expand Long Template PCR System (Roche, Indianapolis, IN). The resulting fragment was directionally cloned into the pGL4-basic luciferase reporter plasmid (Promega, Madison, WI). Site-directed mutagenesis of potential TTF-1 (CAAG \rightarrow CCCC) and Egr-1 (CGGGGGC \rightarrow CGAAAGC) sites was performed by using the reporter construct (pGL4-0.75-RAGE) and following the manufacturer's instructions included in the QuickChange Site-Directed Mutagen-

esis kit (Stratagene, La Jolla, CA). All constructs were verified by nucleotide sequencing.

Transfection and reporter gene assays. Functional assays of reporter gene constructs were performed by transient transfection of R3/1 cells using FuGENE-6 reagent (Roche). R3/1 cells are derived from rat and are characteristic of ATI cells (19). Cells in 35-mm dishes at 40–50% confluence were transfected with 500 ng pRSV- β gal, 100 ng pGL4-0.75-RAGE or pGL4-1.5-RAGE, 200 ng pCMV-TTF-1 and/or pCMV-Egr-1, and pCDNA control vector to bring total DNA concentration to 1.0 μ g. Cells were allowed to grow 48 h, washed, lysed, and snap-frozen for several additional hours. The plates were scraped and centrifuged, and the cleared supernatant was used for both β -gal and luciferase assays. Reporter assays were normalized for transfection efficiency based on β -gal assays performed as previously described (30). Luciferase activity was determined in 10 μ l of extract at room temperature with 100 μ l of luciferase reagent (Promega) for 10 s after a 2-s delay in a MicroLumat LB 96P luminometer.

Expression of RAGE was assessed by RT-PCR in R3/1 cells before and after transfection with either pCMV-TTF-1 or pCMV-Egr-1. As described previously (29), FuGENE-6 was used to transfect a total of 1.0 μ g DNA that included 500 ng of the specific expression vector and 500 ng RSV- β gal.

CSE. CSE was generated as previously described (28). Briefly, two 2RF4 research cigarettes (University of Kentucky) were continuously smoked with a vacuum pump in 20 ml of DMEM. This smoke-bubbled medium was passed through a 0.22- μ m filter (Pall, Ann Arbor, MI) to remove large particles, and the resulting medium was defined as 100% CSE. In each 2RF4 cigarette, the total particulate matter content, tar, and nicotine is 11.7, 9.7, and 0.85 mg, respectively. Dilutions were made using DMEM.

Generation of glyceraldehyde and glyoxylated BSA. AGE-BSAs [glyceraldehyde (GA) and glyoxylated (GO) BSA] were generated as outlined (37). R3/1 cells were incubated with either GA-BSA or GO-BSA at a concentration of 100 μ g/ml for 6 h. The cells were immediately lysed, mRNA was isolated, and the isolated message was subjected to real-time RT-PCR.

Cell culture. R3/1 cells were maintained in DMEM supplemented with 10% FCS, L-glutamine, and antibiotics. Cells were split into six-well dishes and grown to 40–50% confluence. Cultures were exposed to media supplemented with CSE (25%) or media alone for 2 h. At the termination of the experiment, cells were immediately fixed for immunohistochemistry, and RNA was isolated for assessment by RT-PCR or lysed and subjected to Western blot analysis. Additionally, cells were exposed to GA-BSA or GO-BSA for 6 h, after which total mRNA was isolated and subjected to real-time RT-PCR analysis.

RNA isolation and RT-PCR analysis. Total RNA was isolated from cells grown in culture using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene). Reverse transcription of total mRNA and PCR conditions were as previously summarized (29). Primers used for the amplification of RAGE were as follows: 5'-TTCAGCTGTTGGTTGAGCCTGAA-3' and 5'-TCGCCGTTTCTGTGACCCTGAT-3' with an annealing temperature of 58°C. cDNA (100 ng) was used in real-time RT-PCR analyses involving primers for Egr-1 (5'-ACCTGACCGCAGAGTCTTTTC-3' and 5'-GCCAGTATAGGTGATGGGG-3', each 200 nM) and Power SYBR Green PCR Master Mix (Stratagene). Conditions included 40 cycles at 95°C for 15 s and 60°C for 60 s. Gene expression was assessed in three replicate pools, and representative data are shown.

Protein analysis. Whole cell lysates were subjected to Western blot analysis with the goat polyclonal RAGE antibody described previously. Briefly, equal amounts of total protein were analyzed by SDS-PAGE, blocked with 5% nonfat milk, and exposed to the primary antibody diluted at 1:200 at 4°C overnight. Exposure to horseradish peroxidase-conjugated secondary antibodies was followed by development with enhanced chemiluminescence (Amer-

sham Biosciences, Buckinghamshire, UK). Images presented are representative.

Mouse lung samples. Lung tissue from two strains of mice (AKR/J and NZWLac/J) were isolated following exposure to cigarette smoke [two standard research cigarettes (2R1)/day, 5 days/wk] or normal air from age 3 to 9 mo (28). At the conclusion of the exposure experiment, mice were killed, and lungs were inflation fixed with 4% paraformaldehyde and immunostained as outlined above. Animal use and husbandry followed protocols approved by the Institutional Animal Care and Use Committee at the University of Utah and McGill University.

Statistical analysis. Values are expressed as mean \pm SD obtained from at least three separate experiments in each group. Data were assessed by one-way analysis of variance. When ANOVA indicated significant differences, the Student's *t*-test was used with Bonferroni correction for multiple comparisons. Results presented are representative, and those with *P* values <0.05 were considered significant.

RESULTS

Temporal-spatial distribution of RAGE in the mouse lung. RAGE expression was assessed in mouse lung from *embryonic day* (E) 13.5 to *postnatal day* (PN) 10 by immunohistochemical staining. RAGE was only minimally detected in pulmonary epithelial cells at E17.5, the later stage of the canalicular period of lung development (Fig. 1A). Although RAGE was observed on luminal edges of many epithelial cells, a threshold required for immunohistochemical detection may not have been met in all cells. Just before birth, staining of lung tissue at E18.5 revealed persistent RAGE expression in undifferentiated lung parenchyma (Fig. 1B). Neonatal staining at PN1 revealed that RAGE expression was maintained in the distal lung coincident with progressing branching morphogenesis, most prominently in ATI cells with minimal detection in AIIII cells (Fig. 1C). At the onset of murine alveologenesis (PN4), RAGE was robustly expressed in parenchymal epithelial cells (ATI cells) as they morphologically thinned during secondary alveolar septation (Fig. 1D), with only minimal detection in AIIII cells. This pattern of upregulated RAGE expression chiefly in ATI respiratory epithelial cells persisted in the periphery at later stages of alveologenesis (PN10, Fig. 1E). RAGE staining was not detected in bronchiolar epithelial cells or in PN10 respiratory epithelial cells in the absence of primary IgG (Fig. 1F).

Egr-1 and TTF-1 influence RAGE expression in pulmonary epithelial cells. The pattern of developmental RAGE expression correlated with cells that diminish expression of TTF-1, a homeobox-containing transcription factor that is critical in the control of branching morphogenesis and cell differentiation (22). Assessment of the mouse RAGE promoter revealed the presence of four TTF-1 regulatory elements (TREs) in the first 750 bp and a total of nine when the promoter length was doubled (1.5 kb; Fig. 2A). Also included in the regulatory sequence of RAGE are three GC-rich regions that likely bind Egr-1. To determine whether these two important transcription factors control RAGE expression, reporter constructs were generated in which both promoters (0.75 and 1.5 kb) were individually ligated upstream of a luciferase gene and cotransfected in R3/1 cells with varying concentrations of TTF-1 (pCMV-TTF-1) and/or Egr-1 (pCMV-Egr-1). TTF-1 (200 ng) significantly inhibited RAGE expression in R3/1 cells, whereas Egr-1 (200 ng) positively activated RAGE expression (Fig. 2B). These effects were observed in both the 0.75- and 1.5-kb

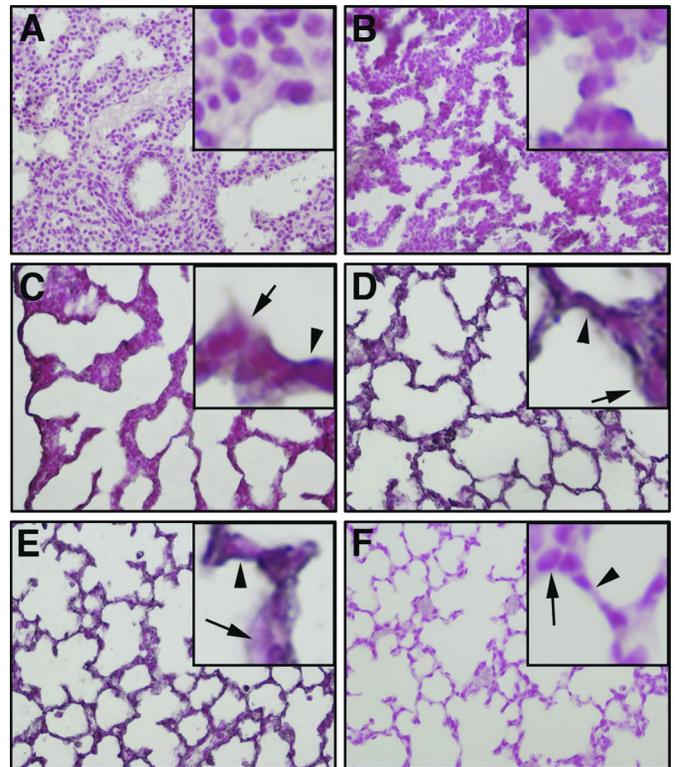


Fig. 1. Immunohistochemical staining identifies receptor for advanced glycation end-product (RAGE) expression in the lung during pulmonary morphogenesis. A: faint RAGE expression was initially detected in pulmonary epithelial cells in the late canalicular period of development [*embryonic day* (E) 17.5]. B: lung parenchymal cells had persistent RAGE expression just before birth (E18.5). C: RAGE expression continued in postalveolar duct epithelial cells at *postnatal day* (PN) 1, localizing predominantly to alveolar type (AT) I cells (arrowhead) and only minimally to AIIII cells (arrow). D: at the start of murine alveologenesis (PN4), RAGE expression was significantly elevated in ATI epithelial cells (arrowhead). E: robust RAGE expression in the peripheral gas exchange portion of the lung persisted in ATI cells (arrowhead) at PN10. F: RAGE was absent in lung tissue not exposed to anti-RAGE IgG. Minor RAGE expression was observed postnatally in AIIII cells (arrows in C–E). Main images and insets are at $\times 100$ and $\times 400$ magnification, respectively.

RAGE promoter constructs. When transfection experiments involving both TTF-1 and Egr-1 were performed, it was discovered that the two transcription factors are additive in nature, and they combine to return luciferase expression to near baseline (Fig. 2B). Site-directed mutagenesis performed on the truncated promoter revealed that the second TRE and first Egr-1 regulatory element were important in the regulation of RAGE expression (Fig. 2C).

To further assess the effects of TTF-1 and Egr-1 on RAGE expression, R3/1 cells, which express basal levels of RAGE, were transfected with 500 ng of exogenous TTF-1 or Egr-1, mRNA was isolated 24 h after transfection, and RT-PCR was performed to assess changes in RAGE transcription. Complementing *in vitro* reporter assay experiments, RAGE was markedly inhibited by TTF-1 and induced by Egr-1 (Fig. 3).

Because of significant upregulation of RAGE by Egr-1, *in vivo* assessment of RAGE was also performed by immunohistochemistry in the lungs of Egr-1 null mice. Egr-1 knockout mice survive normally, and, despite noted reproductive defects that cause sterility, these mice have not been characterized as having adverse developmental effects in the lung (41). Adult

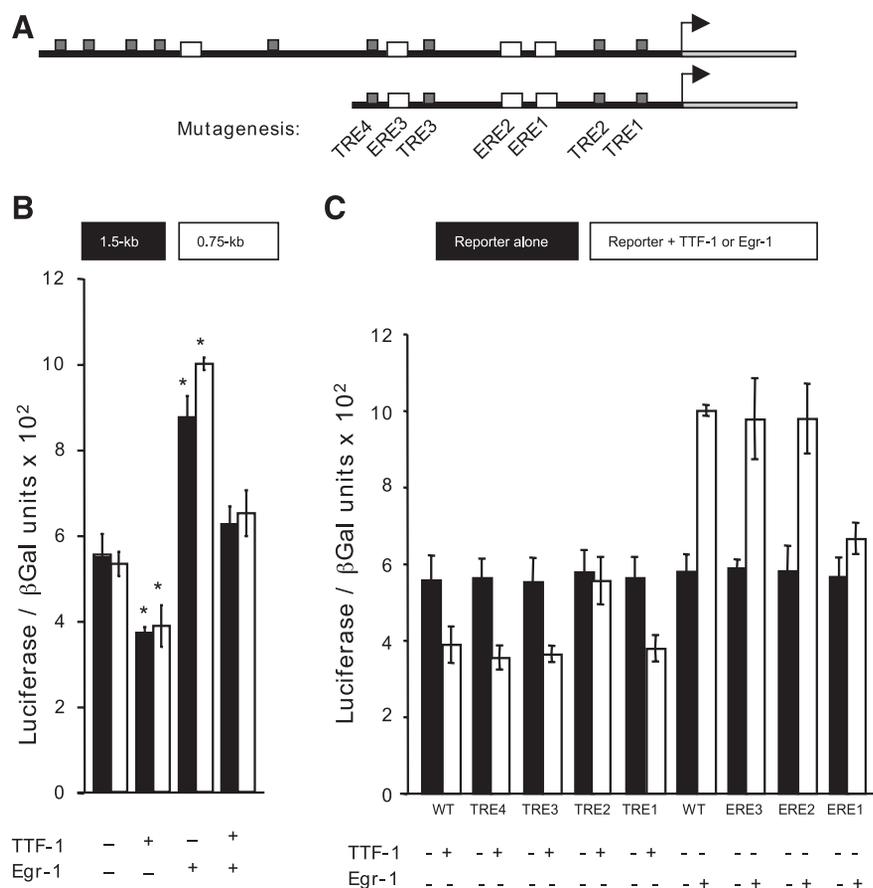


Fig. 2. Early growth response gene 1 (Egr-1) activates and thyroid transcription factor-1 (TTF-1) represses RAGE promoter activity. **A**: genomic DNA of mouse RAGE promoter (-1515 to +50 and -758 to +50). **B**: transfection of R3/1 cells with reporter constructs TTF-1 and/or Egr-1. Cotransfection of RAGE reporter constructs with Egr-1 activated RAGE transcription, whereas TTF-1 significantly inhibited transcription. Both Egr-1 and TTF-1 returned transcription levels to baseline. **C**: mutant constructs were generated by using the 0.75-kb RAGE reporter construct and mutating putative TTF-1 (TREs) and Egr-1 (EREs) regulatory elements. Mutagenesis of ERE1 (CGGGGGGCG) and TRE2 (CAAG) demonstrated that these sites are important in Egr-1- and TTF-1-mediated control of RAGE transcription. Transfection efficiency was accounted for by normalizing luciferase to β -gal levels. Significant differences in luciferase/ β -gal levels are noted at $P \leq 0.05$ (*).

C57BL/6 wild-type mice expressed basal quantities of RAGE in the parenchyma (Fig. 4A), and lungs from Egr-1 null mice with a C57BL/6 background, while phenotypically indistinguishable from wild-type mice, revealed a detectable downregulation of RAGE (Fig. 4B).

CSE induces RAGE and Egr-1. Our laboratory has recently demonstrated that CSE induces Egr-1 expression in pulmonary epithelial cells and that the secretion of proinflammatory cytokines associated with CSE exposure occurs via an Egr-1-mediated mechanism (28). The demonstration that Egr-1 influences RAGE expression led us to assess RAGE expression in a tobacco-related environment. Exposure of R3/1 cells to 25% CSE for 2 h caused significant upregulation of RAGE protein

by Western blot (Fig. 5A). RT-PCR also revealed upregulation of RAGE mRNA in R3/1 (ATI), A549 (ATII), and RAW (mouse macrophage) cell lines (Fig. 5B). Preliminary studies also indicated that the expression of RAGE ligands, including HMGB-1 and S100A12, was increased following 2 h of CSE exposure (Fig. 5C). Immunohistochemical staining revealed elevated RAGE expression in R3/1 cells exposed to CSE for 2 h (Fig. 5D), and confirming data performed in vivo demonstrated that RAGE was induced in the lungs of Akkr/J mice, a strain susceptible to tobacco-induced emphysema (10), after exposure to cigarette smoke for 6 mo (Fig. 5E). Concomitant induction of Egr-1 was previously demonstrated to occur in the lungs of Akkr/J mice after exposure to cigarette smoke (28). Emphysema-resistant strains of mice such as NZW/LacJ (10)

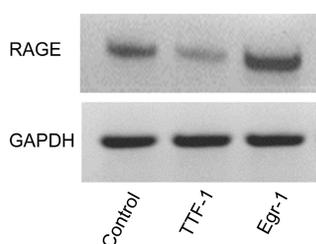


Fig. 3. RAGE expression is induced by Egr-1 and diminished by TTF-1 in R3/1 cells. R3/1 cells were grown to ~40% confluence and transfected with 500 ng of either Egr-1 or TTF-1. After transfection (2 days), whole RNA was isolated and reverse transcribed, and the resulting cDNA was subjected to PCR. R3/1 cells endogenously express RAGE, and its expression increases with Egr-1. RAGE expression was reduced to below basal levels when transfected with TTF-1. RT-PCR of glyceraldehyde-3-phosphate dehydrogenase was performed as an internal loading control.

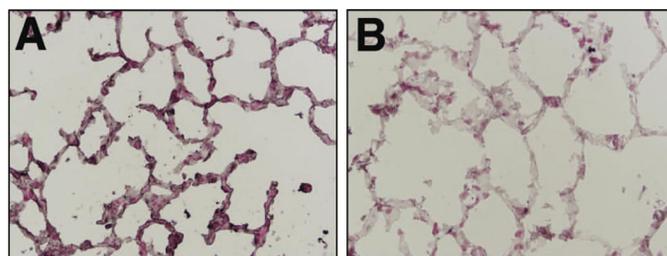


Fig. 4. RAGE Expression is decreased in the lungs of Egr-1 null mice. **A**: immunohistochemistry of lungs from adult C57BL/6 wild-type mice demonstrates detectable RAGE expression in the parenchyma. **B**: morphologically indistinguishable lungs from age-matched Egr-1 null mice had significantly decreased RAGE protein expression in the lung periphery. Images are at $\times 100$ magnification.

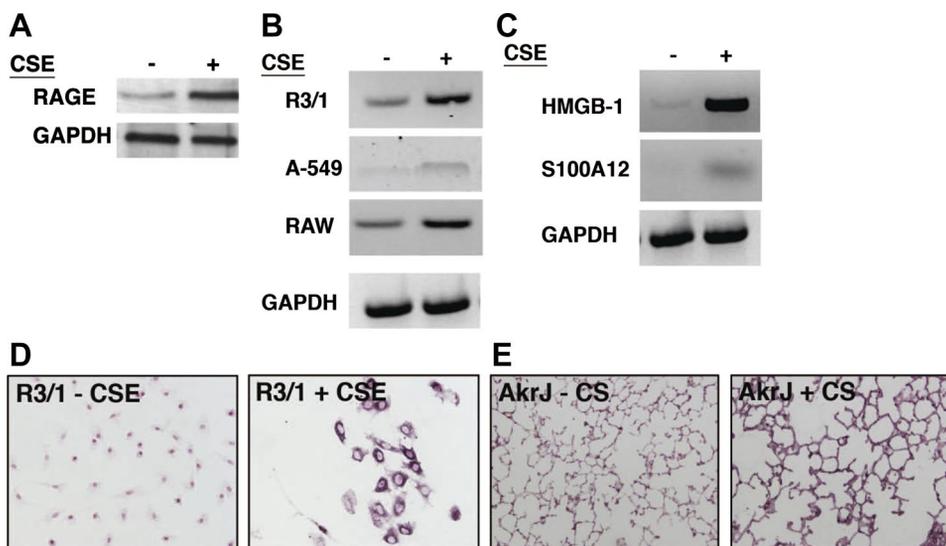


Fig. 5. Cigarette smoke extract (CSE) induces RAGE and ligand expression. *A*: R3/1 cells were exposed to media with 25% CSE or in the absence of CSE for 2 h, lysed, and subjected to Western blot analysis. Cells exposed to CSE had a significant increase in RAGE protein expression. *B*: R3/1, A549 (human pulmonary adenocarcinoma cells similar to ATII cells), and RAW (mouse macrophage) cells were exposed to 25% CSE for 2 h, and mRNA was isolated and subjected to PCR for RAGE. All cell lines upregulated RAGE in the presence of CSE. *C*: R3/1 cells exposed to 25% CSE for 2 h also upregulated the expression of amphoterin (HMGB-1) and S100A12, two RAGE ligands, after exposure to CSE. *D*: immunostaining for RAGE revealed upregulation of the receptor in R3/1 cells exposed to 25% CSE for 2 h. *E*: immunostaining for RAGE was markedly increased in the lungs of emphysema-susceptible mice (AkrJ) exposed to cigarette smoke for 6 mo (+CS), compared with mouse lungs exposed to room air (–CS). Lung images are at $\times 100$.

did not significantly upregulate RAGE when exposed to cigarette smoke for 6 mo (data not shown).

RAGE-ligand interaction induces Egr-1. Although the current research demonstrates that endogenously produced RAGE ligands are upregulated by cigarette smoke (Fig. 5C), cigarette smoke also provides a preponderance of AGE ligands via tobacco-associated oxidative stress that modifies cellular proteins via glycation reactions (25, 26). Generating GO-BSA and GA-BSA results in two distinct proteins with patterned modifications that are recognized by RAGE. These AGE-BSAs were singularly added to R3/1 cells for 6 h, and quantitative real-time RT-PCR was performed to assess changes in Egr-1 transcription. There was a significant upregulation of Egr-1 in cells exposed to GA-BSA (5-fold) and a highly significant increase in Egr-1 activation (200-fold) when cells were incubated with GO-BSA (Fig. 6), revealing a vicious cycle involving CSE-induced Egr-1, RAGE activation, and augmentation of Egr-1 following RAGE-ligand interaction.

DISCUSSION

Developmental expression of RAGE and its role in paracrine signaling during lung morphogenesis. The temporal-spatial pattern of RAGE, a multiligand pattern recognition receptor of the immunoglobulin superfamily, was determined during embryonic and postnatal periods of lung development. RAGE protein was detected in differentiating lung epithelial cells commencing in the later stages of the canalicular period of development, and dramatic changes in expression intensity occurred in the postnatal period. RAGE was expressed initially in undifferentiated parenchymal cells in the saccular stage (E17.5–PN5). Profound upregulation of receptor expression was observed in ATI epithelial cells during the alveolar (PN5–PN20) stage of lung development. Furthermore, RAGE was not noticeably detected in proximal bronchiolar epithelial cells, such as cili-

ated columnar epithelial cells and Clara cells, and was only marginally observed in ATII cells in the periphery, the latter finding likely due to potential ATII-ATI differentiation. RAGE was also detected in subsets of endothelial cells in embryonic vessels and alveolar capillaries, suggesting a potential secondary role for RAGE in the interrelated processes of angiogenesis, vasculogenesis, and the septation of functional alveoli

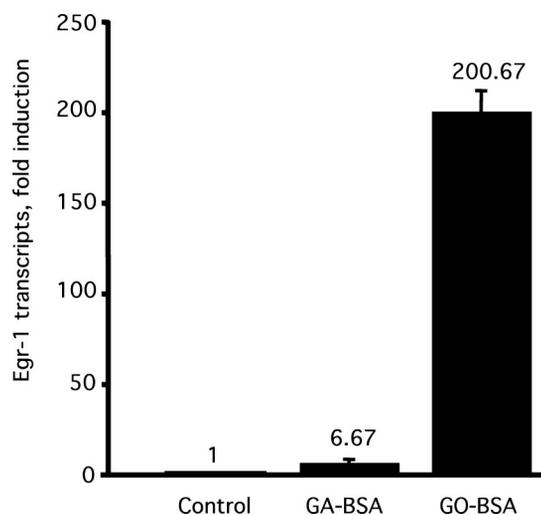


Fig. 6. RAGE-ligand interaction increased Egr-1 expression. R3/1 cells were incubated in the absence (control) or in the presence of advanced glycation end-products (AGEs), including glyceraldehyde-bovine serum albumin (GA-BSA) or glyoxylated-bovine serum albumin (GO-BSA), for 6 h and then subjected to real-time quantitative RT-PCR analysis. A 5-fold upregulation of Egr-1 transcription was observed in cells exposed to GA-BSA, and a highly significant 200-fold induction occurred in cells exposed to GO-BSA. Three separate experiments, each in triplicate, were performed, and significant differences were determined by utilizing Student's *t*-tests with Bonferroni correction.

during alveologenesis (12). Robust RAGE expression in vessels and respiratory epithelium persisted through PN10. In our studies, RAGE frequently localized to luminal edges of airway epithelial cells as well as basolateral membranes previously identified (34), suggesting availability of the receptor to ligands exposed to the lung via respiration and/or maternal transfer in utero and a possible site of protein accumulation important in paracrine signaling between pulmonary epithelium and underlying mesenchymal and/or vascular cells, respectively.

Proper lung development depends on highly ordered and sequential interactions between epithelial and mesenchymal cells (11). Although the functions of RAGE are not completely understood at present, RAGE has been implicated as a receptor involved in tissue remodeling (45), apoptosis (31), and survival (2), as well as epithelial cell spreading and adherence (7). These critical processes are directly involved in perinatal transitioning of the embryonic lung into a mature, functional organ.

Postnatal staining of RAGE intensified in the parenchyma during the sacular period of development, which is a time of decreased proliferation and differentiation of Clara, ciliated, and ATI cells (39). In mice, alveolar development occurs in the postnatal period when mesenchymal thinning and partitioning of the terminal ducts and saccules form true alveolar ducts and alveoli (39). This intense remodeling of the lung saccules requires proliferation, migration, and differentiation of both epithelial and mesenchymal cell types. Therefore, intense staining of RAGE in ATI epithelial cells at PN10 suggests that RAGE plays a role in later stages of lung morphogenesis, when alveolar and vascular remodeling occurs.

Role of Egr-1 and TTF-1 in the regulation of RAGE expression. Egr-1 is a potent regulator that controls the expression of a wide variety of genes (16). Most of the understanding of Egr-1 comes from investigations of Egr-1 null mice or specific molecular suppressors of Egr-1 such as strategies employing antisense or small-interfering RNA; however, only a few of the genes identified as being directly regulated by Egr-1 in vitro (3, 17) have been confirmed in vivo. Study of the Egr-1 knockout mouse, for example, has only revealed that luteinizing hormone- β (20), apo A-1 (44), and tissue factor (46) are regulated by Egr-1. Although our current research demonstrates that Egr-1 is directly induced by cigarette smoke and it influences RAGE expression in vitro, it is likely that a combination of factors function to induce its downstream target genes such as RAGE in vivo.

TTF-1 regulates cytodifferentiation and formation of the respiratory epithelium during early lung development (18). Later (E13.5–E15.5), TTF-1, GATA-6, and Foxa2 are coexpressed in the airway epithelium, where the expression of genes critical to lung function, including surfactant proteins A, B, and C (22), are coordinated. Surfactant protein synthesis is indicative of ATII cells that abundantly express TTF-1 and serve as progenitor cells of ATI cells (42). As TTF-1 expression diminishes in differentiating ATI cells, other factors likely become active in the control of elevated RAGE expression. Data demonstrating inhibition of RAGE expression by TTF-1 in R3/1 cells are consistent with this model. As cells maintain an ATII phenotype, RAGE expression is minimal; however, decreased inhibition by TTF-1 in transitioning cells may indirectly influence RAGE-mediated signaling that leads to thinning and spreading characteristic of differentiated ATI cells.

Site-directed mutagenesis of putative Egr-1- and TTF-1-binding sites demonstrated the importance of these factors in RAGE transcription. Because Egr-1 acts with stimulating protein 1 and TTF-1 acts in concert with other factors such as nuclear factor-1, Foxa2, GATA-6, and activator protein-1, it is likely that the distribution of RAGE is influenced in specific cell types in a complex manner by several transcription factors.

CSE induces Egr-1, RAGE, and RAGE ligands. We have previously demonstrated that Egr-1 is significantly induced in respiratory epithelial cells after exposure to CSE and causes increased secretion of proinflammatory cytokines (28). The current investigation adds to the understanding of Egr-1 by demonstrating that RAGE is one of its transcriptional targets. Because of probable compensation by other transcriptional mechanisms, Egr-1 targeting in mice induces subtle anomalies, yet it is not an essential transcription factor during development (35, 41). De novo induction of Egr-1 by CSE and its transcriptional control of RAGE identify it as an important factor that influences epithelial cellular responses to numerous classes of stimuli, potentially via direct RAGE activation.

Exposure of Akv mice to cigarette smoke for 6 mo resulted in elevated RAGE expression and emphysema as manifested by significant airspace enlargement and decreased lung elastance (10). Interestingly, Nzw/LacJ mice, which did not upregulate RAGE expression in response to cigarette smoke exposure, were resistant to the development of emphysema, revealing no changes in relative alveolar size or elastance when room air and smoke-exposed mice were compared. It is highly likely that RAGE is induced in the lungs of Akv mice after a brief period of smoke exposure that may correspond to RAGE

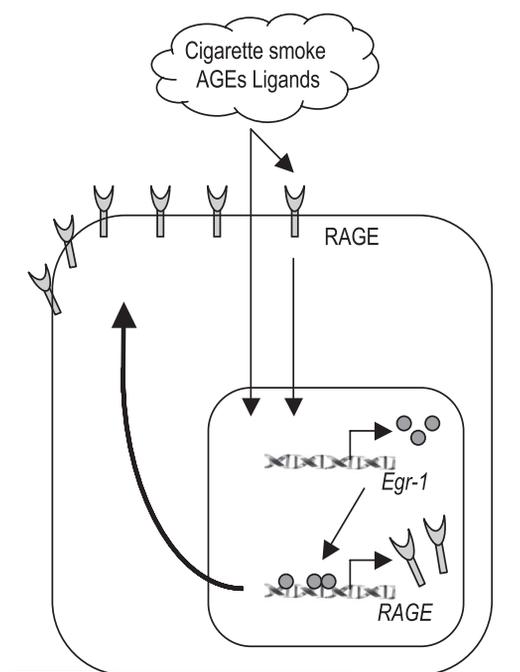


Fig. 7. Model of positive feedback mechanism involving cigarette smoke, RAGE, and Egr-1. In a proposed model of a RAGE-expressing cell, RAGE is transcriptionally regulated by Egr-1. This cell is also susceptible to cigarette smoke-mediated upregulation of RAGE and Egr-1. The increased availability of RAGE that results combines with RAGE ligands induced by or contained within cigarette smoke to significantly elevate Egr-1 transcription, completing a likely potent positive feedback loop.

induction in vitro. Early induction of RAGE in the lungs of smokers likely interacts with elevated ligand availability, particularly AGEs prominently detected in smoke (25, 26), and culminates in persistent pulmonary inflammation and alveolar damage.

Upregulated RAGE binds ligand induced by, or contained within, cigarette smoke and causes rapid and sustained cellular activation and gene transcription (23). Immediate effects include increased inflammation, which has been observed in many studies that identify activated RAGE in inflammatory lesions (1, 5, 9, 38). Although this work identifies CSE-mediated induction of RAGE and its ligands via Egr-1, further work is required that aims to discover potential redundant CSE-mediated mechanisms of RAGE activation in an Egr-1-targeted environment, both in vitro and in vivo.

RAGE-ligand interactions induce Egr-1 expression. Complementing novel data that demonstrate RAGE/ligand induction by CSE were the findings that RAGE receptors expressed on the membranes of rat ATI cells (R3/1) interact with AGE-BSA (GA-BSA and GO-BSA), resulting in profound upregulation of Egr-1. Because CSE (28) and RAGE-ligand interactions markedly upregulate Egr-1, which also activates RAGE transcription, we demonstrate a positive feedback mechanism that likely functions to control RAGE expression during developmental periods in the lung that is also susceptible to tobacco smoke exposure. Once exposed to tobacco smoke, a fierce cycle may augment RAGE expression (Fig. 7). AGEs associated with tobacco smoke (25, 26) combine with upregulated RAGE ligands, such as S100A12 and HMGB-1, to ensure that RAGE and its ligands accumulate in the parenchyma of the lung simultaneously, thus making possible the initiation of signal transduction pathways that lead to downstream deleterious effects (Fig. 7).

Conclusions

The intensity of RAGE expression in respiratory epithelial cells varies during lung morphogenesis. RAGE is minimally expressed during midembryonic periods and is postnatally upregulated in the lung stroma and vasculature. Therefore, RAGE is likely to function in paracrine signaling during pulmonary remodeling of both mesenchymal- and endoderm-derived compartments of the lung. Our data further demonstrate that RAGE is influenced by differential expression of transcription factors such as TTF-1 and Egr-1 and that activation of RAGE and Egr-1 by cigarette smoke synergistically elaborates both RAGE and its ligands to maintain RAGE signaling. Positive activation of RAGE-mediated signaling pathways in a postdevelopmental period likely influences the inflammatory response and perturbed alveolar remodeling characteristic of pulmonary disease.

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