Resolvin E1 Reduces Tumor Growth in a Xenograft Model of Lung Cancer

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Inflammation plays a significant role in carcinogenesis and tumor growth. We tested the hypothesis that resolvin E1 (RvE1) and overexpression of the receptor for RvE1 (ERV1) will prevent and reverse tumor generation in a gain-of-function mouse model of tumor seeding with lung cancer cells. To measure the impact of enhanced resolution of inflammation on cancer pathogenesis, ERV1-overexpressing transgenic (TG) and wild-type FVB mice were treated with cisplatin or cisplatin plus RvE1. RvE1 significantly prevented tumor growth and reduced the tumor size, cyclooxygenase-2, NF-κB, and proinflammatory cytokines; these results were significantly greater in TG animals. A significant decrease in Ki-67, vascular endothelial growth factor, angiopeptin (Ang)-1, and Ang-2 was observed. Because ERV1 is only overexpressed on myeloid cells, we measured the effects of the transgene on tumor-associated neutrophils and macrophages, which were significantly reduced by RvE1 (P < 0.001). RvE1 treatment led to a significant reduction of tumor volume and reduced expression of cyclooxygenase-2, NF-κB, vascular endothelial growth factor-A, Ang-1, and Ang-2 when administered together with cisplatin. The data suggest that RvE1 prevents inflammation and vascularization, reduces tumor seeding and tumor size, and, when used as an adjunct to chemotherapy, enhances tumor reduction at significantly lower doses of cisplatin. (Am J Pathol 2022, 189:1–15; https://doi.org/10.1016/j.ajpath.2022.07.004)
Materials and Methods

Reagents

RvE1 was purchased from Cayman Chemicals (Ann Arbor, MI). The LA-P0297 lung cancer cell line was a gift from Dr. P. Huang (Massachusetts General Hospital, Boston, MA). Ki-67, vascular endothelial growth factor (VEGF)-A, CD34, and CD31 primary antibodies were purchased from Abcam (Cambridge, MA). Culture media and TriZol reagent were obtained from Invitrogen (Carlsbad, CA). Primers for NF-κB, cyclooxygenase-2 (COX-2), angiopoietin (Ang)-1, and Ang-2 were purchased from Life Tech (Grand Island, NY). Milliplex kits for multiplex cytokine analysis were obtained from Millipore (Billerica, MA). An inverted microscope (Zeiss Axiostar 200) from Carl Zeiss Microimaging Inc. (Thornwood, NY) was used for imaging.

Animal Model and Generation of ERV1 Transgenic Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Forsyth Institute (Cambridge, MA) and were performed in conformance to the standards of the Public Health Service Policy on Humane Care and Use of Laboratory Animals. FVB background ERV1-transgenic mice were engineered as previously described. In brief, full-length hCD11b promoter cDNA was cloned upstream of the full-length ERV1 cDNA (http://www.ncbi.nlm.nih.gov/nuccore/NM_004072; GenBank accession number). For genotyping, genomic DNA was collected from ear punch biopsies and screened by PCR with primers directed to mouse ERV1 (forward primer, 5'-CTCGGTCTCTTAGGAAC-3' and human ERV1 (forward primer, 5'-GTCTTCTTCAATCCATC-3'). The mouse and human ERV1 amplicons share the same reverse primer (5'-TAGAAAGCAGGAGGAGC-3'). FVB wild-type (WT) mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were housed under specific pathogen-free conditions and received a standard laboratory Chow diet and water ad libitum.

The LA-P0297 cell line is a well-established line of murine lung adenocarcinoma with a high incidence of distant lung metastasis that was kindly provided by Dr. Huang. This line was derived from a spontaneous lung tumor in an FVB/N mouse and used for preclinical studies of anti-angiogenesis therapy in spontaneous autochthonous tumors, and their isografts were implanted in mice to simulate the clinical conditions that affect many human cancer patients more accurately. Because we have generated the ERV1 TG mouse on the FVB background, we specifically chose the LA-P0297 cell line to match the background.

In the first experiment (prevention), after 1 week of acclimatization, all animals (WT and ERV1 TG) received an injection of $1 \times 10^6$ LA-P0297 lung cancer cells subcutaneously (right flank). WT ($n = 18$) and transgenic ($n = 18$) animals were randomly divided into three experimental groups: untreated controls; 100 ng of RvE1 diluted in 100 μL phosphate-buffered saline (PBS) injected intraperitoneally daily for the first 6 days and weekly thereafter for 3 weeks; and injection of 100 μL of vehicle (5% ethanol diluted in PBS). RvE1 was stored in 100% ethanol and diluted in PBS immediately before use. RvE1 and vehicle were administered 30 minutes before the injection of cancer cells. In the second experiment (adjunct to cisplatin chemotherapy), the same
protocol for developing xenograft tumors was used. After xenograft tumors developed, a widely used chemotherapeutic (cisplatin\textsuperscript{35,36}) was administered alone (5 mg/kg body weight intraperitoneally two times/week in 5% ethanol in PBS) or in combination with the RvE1 (100 ng; intraperitoneally) for 4 weeks. Animals were checked routinely for body weight and growth of tumors on the skin.

Tumor volume was measured using a Vernier caliper (Mitutoyo, Japan) and calculated using the following formula: \( V = \frac{1}{2} (L \times W^2) \), where \( L \) = length and \( W = \) width. At the end of each study arm, the animals were euthanized by CO\(_2\) asphyxia; tumor tissues were collected and stored for histologic and molecular analyses.

**Histologic and Immunohistochemical Analysis**

To examine microscopic alterations in the tumor, tissues were fixed in 10% formalin, embedded in paraffin, divided into sections (5 \( \mu \)m thick), and stained with hematoxylin and eosin. Images were photographed and evaluated.

To measure tumor cell proliferation and angiogenesis, we analyzed the expression of Ki-67 as a marker of proliferation and CD31, CD34, and VEGF-A as markers of vascularization by immunohistochemistry. Briefly, sections (5 \( \mu \)m thick) were deparaffinized and rehydrated with serial alcohol solutions. Antigen retrieval was accomplished by incubating sections in 10 mmol/L sodium citrate (pH 6.0) for 10 minutes in a microwave oven at the lowest setting. Endogenous peroxidase activity was quenched by incubation with 3% H\(_2\)O\(_2\) (in methanol) for 20 minutes at 4°C, followed by blocking with 2% bovine serum albumin for 30 minutes at room temperature. The sections were incubated with antibodies to Ki-67, CD31, CD34, and VEGF (1:100 dilution) overnight at 4°C in a humidified chamber. Sections were incubated with ABC reagent VECTASTAIN Elite ABC Standard Kit (PK-6100 midi, Vector Laboratories, Inc, Burlingame, CA), according to the manufacturer’s instructions. The avidin-biotin-peroxidase complex was applied as a second layer and incubated for 30 minutes. The endogenous peroxidase was quenched by incubation with 3% H\(_2\)O\(_2\) (in methanol) for 20 minutes at 4°C, followed by blocking with 2% bovine serum albumin for 30 minutes at room temperature. The sections were then washed, passed through a 70%-ethanol and xylene series, and coverslipped.

**Quantitative Measurement of Inflammatory and Angiogenesis Gene mRNA Expression**

To analyze the impact of RvE1 on inflammation and angiogenesis, we examined NF-\(\kappa\)B, COX-2, VEGF, Ang-1, and Ang-2 mRNA expression by quantitative real-time PCR. Total cellular RNA was extracted from tumor tissue (stored in RNAlater solution) using TriZol reagent. RNA was quantified with a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE). RNA samples were stored at \(-80^\circ\)C until use. The first-strand cDNA was synthesized with High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. For quantitative RT-PCR analysis, oligonucleotides for NF-\(\kappa\)B, COX-2, VEGF, Ang-1, Ang-2, and glyceraldehyde-3-phosphate dehydrogenase were chosen from predesigned assays. Thermal cycling included initial steps at 95°C for 2 minutes and at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The fluorescence of the double-stranded products was monitored in real time, cDNA was amplified and quantified using the Sequence Detection System 7000 (Applied Biosystems). The 2\(^{-}\Delta\Delta\text{Ct}\) (Ct value of target gene minus Ct value of housekeeping gene) method was used to calculate relative quantity fold change. Glyceraldehyde-3-phosphate dehydrogenase was used as the housekeeping gene. The data are from three independent experiments performed in triplicate.

**Identification of TANs and TAMs in Tumor Tissues**

To measure the impact of pretreatment with RvE1 on tumor-associated phagocytes (neutrophils and macrophages), first, the tumors were excised, cut into small pieces, and digested with a mixture of bacterial collagenases I and IV, dispase, and DNase I for 2 hours at 37°C. Dissociated tumor cells were then washed, passed through a 70-\(\mu\)m nylon mesh strainer, and incubated with biotin-conjugated F4/80- or Ly6G-specific monoclonal antibodies for TAMs and TANs, respectively. After incubating with streptavidin-conjugated magnetic microbeads at a 9:1 (v/v) ratio, Miltenyi MACS separation LS columns were used. Columns were washed five times with 1 mL of PBS—bovine serum albumin, and the fraction was collected for further analyses under fluorescent microscopy.

**Quantification of Inflammatory Cytokines**

Tumor inflammation is driven by soluble cytokines produced by tumor cells and cells recruited to the tumor microenvironment. Cytokine levels were measured using a multiplex bead array assay (Millipore) for granulocyte colony-stimulating factor, IL-1\(\alpha\), IL-6, IL-8, IL-12, and tumor necrosis factor-\(\alpha\), per the manufacturer’s instructions, on a Luminex 100 instrument. Appropriate dilutions in assay diluent were made as required. Each sample was assayed in duplicate, and cytokine standards supplied by the manufacturer were used to calculate concentration. Cytokine levels are expressed in pg/mL.

**Statistical Analysis**

The results are expressed as means \(+\) SEM. The differences between the groups were assessed by analysis of variance after ascertaining normality by Q-Q plot. Statistical significance was determined by one-way analysis of variance with Bonferroni multiple comparison post hoc tests, and differences were considered significant at \(P \leq 0.05\).
Figure 1  Tumor volume is reduced by pretreatment with resolvin E1 (RvE1) in wild-type (WT) and receptor for RvE1—overexpressing transgenic (TG) mice. 
A: Morphologic differences between WT and TG groups with or without RvE1 pretreatment at the macroscopic level. The central portion of tumors shows clear necrosis in TG animals treated with RvE1 (arrow). B: In response to pretreatment with RvE1, tumor volume was reduced significantly in both WT and TG animals compared with vehicle-treated and control groups. The reduction was significantly greater in the RvE1-treated TG group than in the RvE1-treated WT group at 14 days. C: RvE1 reduced the progression of tumor development after 28 days. ** P < 0.001 versus control; **** P < 0.001 versus vehicle.
Figure 2  Impact of resolvin E1 (RvE1) on tumor tissue micro-architecture in wild-type (WT) and receptor for RvE1—overexpressing transgenic (TG) mice. A: Tumor architecture was intact and clear in the WT control (untreated) group. Tumor nests are marked by their blood supply. Lymphatic and vascular invasion was present in untreated control samples. Tumor cells showed nucleolus cleavage and a high extent of malignancy. Similar architecture was observed in transgenic animals in the untreated control group, but tumor nests and blood supply are less apparent. Vehicle treatment did not have any impact on the microscopic architecture of the tumors. Tumors in RvE1-treated animals showed a central area with reduced tissue staining and patches of disintegrated tumor tissues indicative of extensive necrosis. Necrosis was more prominent in RvE1-treated transgenic mice than WT mice (arrows show necrosis in the tumor). B: Quantification of tumor area presented as mm². RvE1 treatment significantly reduces tumor area in both WT and TG, and tumor area in TG is significantly less than in WT. C: Necrosis of the tumor area is presented as a percentage of the total area of the tumor. ***P < 0.001 versus control; ****P < 0.001 versus vehicle; **P < 0.01 compared with WT RvE1. Scale bars = 100 μm (A). Original magnifications, ×2.5 (A, main images); ×40 (A, insets).
Results

Pretreatment with RvE1 Reduces Tumor Size

Animals were monitored every day, and none of the animals exhibited any signs of toxicity in response to RvE1, as determined by behavioral changes, changes in eating and drinking habits, and movement. RvE1 pretreatment reduced the number of mice developing tumors to four of six WT mice and three of six transgenic mice. One animal in the vehicle-treated WT group developed metastases. There was no significant difference in body weight between WT and transgenic mice with or without RvE1 injection.

Tumor volume is a simple way to assess the progression of a tumor; therefore, tumor volume was calculated to analyze the impact of RvE1 in both WT and transgenic mice. Figure 3 illustrates the reduction in tumor cell proliferation, cyclooxygenase-2 (COX-2) expression, and NF-κB expression in wild-type (WT) and receptor for RvE1 overexpressing transgenic (TG) mice. A: Ki-67⁺ cells in tumor tissues. Arrows indicate Ki-67⁺ cells. B: Quantification of Ki-67⁺ cells reveals that RvE1 significantly reduces the cell proliferation marker in both WT and TG, and TG expression is significantly lower than WT. C: COX-2 was significantly decreased in response to treatment with RvE1 compared with untreated controls and vehicle-treated groups. The reduction in TG mice was significantly greater than in WT mice. D: The expression of NF-κB was reduced in response to RvE1 in both WT and TG, and the TG reduction was significantly greater than in WT. *** \textit{P} < 0.001 versus control; ⋅⋅⋅ \textit{P} < 0.01 compared with WT RvE1. Scale bars = 100 μm (\textit{A}). Original magnification, ×10 (\textit{A}).
mice. Significant morphologic differences were observed between WT and transgenic groups with or without RvE1 pretreatment at the macroscopic level (Figure 1A). The progression of tumors was exponential from day 7 in control WT and day 11 in control transgenic mice without added RvE1. There was a significant reduction in tumor size with RvE1 treatment, which was significantly \( P = 0.007 \) higher in the RvE1-treated TG group than in the RvE1-treated WT group at 14 days (Figure 1B). The delay in tumor progression in transgenic animals suggests a response to increased endogenously produced RvE1. From the 19th day, necrosis was seen on the surface of tumors in the RvE1-treated groups. The progression of tumor development was significantly reduced in transgenic animals \( P < 0.001 \) compared with WT. In response to pretreatment with RvE1, tumor size was reduced significantly \( P < 0.001 \) in both WT and transgenic animals compared with vehicle and control groups (Figure 1C). Tumors of mice receiving RvE1 appeared soft and pulpos with tumors in animals not treated with RvE1, which were solid and rigid.

### Histopathologic Analysis of Tumor Tissue in Response to RvE1 Pretreatment

Microscopic analyses of the tumor tissues revealed that tumor architecture was intact and clear in the WT control (untreated) group (Figure 2A). Cells formed a tumor nest with reduced tissue staining and patches of disintegrated tumor indicative of extensive necrosis. Tumor area was reduced significantly \( P = 0.004 \), and necrosis was more prominent \( P = 0.006 \), in RvE1-treated transgenic mice compared with RvE1-treated WT mice (Figure 2, B and C).

### Inhibition of Cell Proliferation by RvE1 Pretreatment in Tumor Tissue

Ki-67 protein was used as a cell proliferation marker. Ki-67 is associated with active phases of the cell cycle and is absent from resting cells. The percentage of Ki-67-positive cells in cancer tissues was 73.5% and 66% in WT and transgenic untreated control animals, respectively (Figure 3, A and B). RvE1 treatment resulted in a significant reduction \( *^{8} \) of Ki-67-positive cells in both WT and transgenic groups compared with both vehicle and control groups \( P < 0.001 \). The reduction in the expression of Ki-67 was significantly higher in RvE1-treated transgenic mice than in WT mice treated with RvE1 \( P = 0.003 \).

### RvE1 Pretreatment Suppresses the Expression of Inflammation Genes in Tumor Tissue

Then, the expression of mRNA for inflammatory molecules and targeted COX-2 and NF-κB in tumor tissues was measured using real-time quantitative PCR (Figure 3, C and D). COX-2 was decreased threefold in WT mice and eightfold in TG mice in response to RvE1 compared with untreated controls and vehicle-treated groups \( P < 0.001 \). Likewise, the expression of NF-κB mRNA in response to RvE1 was reduced fourfold in WT mice and 7.6-fold in transgenic mice compared with untreated control and vehicle-treated animals \( P < 0.001 \).

### Modulation of Cytokine Levels in Tumor Tissues with RvE1 Pretreatment

RvE1 pretreatment resulted in a significant decrease in the levels of proinflammatory cytokines (granulocyte colony-stimulating factor, IL-1α, IL-6, IL-8, and IL-12) compared with untreated control and vehicle-treated WT and transgenic mice (Table 1). Tumor necrosis factor-α decreased in response to pretreatment with RvE1 in both WT and TG animals, although the reduction was significant only in RvE1-treated TG mice compared with untreated TG controls.

### Table 1 Cytokine Levels in Tumor Tissues in WT and TG Mice in Response to Pretreatment with RvE1 or Vehicle

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>WT mice Control (untreated)</th>
<th>WT mice RvE1</th>
<th>WT mice Vehicle</th>
<th>TG mice Control (untreated)</th>
<th>TG mice RvE1</th>
<th>TG mice Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>2494.6 ± 248.9</td>
<td>1624.3 ± 170.8*</td>
<td>2581.9 ± 191.5</td>
<td>2110.5 ± 170.6</td>
<td>1156.3 ± 145.3*</td>
<td>2029.3 ± 220.3</td>
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<tr>
<td>IL-1α</td>
<td>249.2 ± 17.1</td>
<td>177.9 ± 15.7*</td>
<td>234.1 ± 15.3</td>
<td>225.6 ± 29.5</td>
<td>146.4 ± 32.4*</td>
<td>225.6 ± 36.7</td>
</tr>
<tr>
<td>IL-6</td>
<td>186.0 ± 29.2</td>
<td>52.1 ± 12.9*</td>
<td>200.4 ± 9.1</td>
<td>186.7 ± 22.2</td>
<td>62.2 ± 3.1*</td>
<td>192.7 ± 21.1</td>
</tr>
<tr>
<td>IL-12</td>
<td>62.2 ± 3.2</td>
<td>46.7 ± 4.7*</td>
<td>63.6 ± 7.4</td>
<td>52.8 ± 3.7</td>
<td>38.6 ± 2.7*</td>
<td>52.9 ± 8.3</td>
</tr>
<tr>
<td>IL-8</td>
<td>3248.6 ± 193.6</td>
<td>2629.4 ± 263.3*</td>
<td>3476.7 ± 344.9</td>
<td>2643.9 ± 191.7</td>
<td>692.6 ± 101.1*</td>
<td>2910.4 ± 216.3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>17.6 ± 1.7</td>
<td>13.2 ± 0.9</td>
<td>15.6 ± 1.7</td>
<td>17.4 ± 2.1</td>
<td>10.1 ± 2.1*</td>
<td>17.2 ± 1.8</td>
</tr>
</tbody>
</table>

*\( P < 0.05 \) compared with WT control.  
\( ^{1} P < 0.05 \) compared with TG control.  
\( ^{2} P < 0.01 \) compared with WT RvE1.  

G-CSF, granulocyte colony-stimulating factor; RvE1, resolvin E1; TG, transgenic; TNF-α, tumor necrosis factor-α; WT, wild type.
Down-Regulation of Vascularization and Angiogenesis Markers by RvE1 Pretreatment

To assess whether RvE1 has an impact on vascularization, the expression of CD31 and CD34 was measured by immunohistochemistry. VEGF-A expression was analyzed by both immunohistochemistry and PCR. Ang-1 and Ang-2 expression was evaluated by real-time quantitative PCR. Staining intensity for CD31 and CD34 was strikingly higher in tumors of WT and transgenic animals in untreated control groups (Figure 4, Aa and B). Vehicle treatment did not impact the expression of CD31 and CD34. The intensity was significantly reduced in both WT and TG mice in response to RvE1, which corresponds with the number of CD31- and CD34-positive cells (Figure 4, C and D). ***P < 0.001 versus control; **P < 0.01 compared with WT RvE1.
CD34-positive cells (Figure 4, C and D). RvE1 reduced the expression of CD34 in tumor tissues in TG animals significantly more than in the WT animals ($P < 0.01$).

Immunohistochemical staining showed that VEGF-A expression was reduced in response to RvE1 compared with untreated control and vehicle-treated animals ($P < 0.001$) (Figure 5A). PCR further confirmed these results; expression of VEGF was decreased fivefold and sevenfold in WT and transgenic mice, respectively (Figure 5B). The decrease was greater in TG mice than in WT mice treated with RvE1 ($P < 0.001$). In parallel, Ang-1 and Ang-2 were significantly decreased in WT and transgenic animals treated with RvE1 compared with untreated controls and vehicle-treated groups ($P < 0.001$) (Figure 5, C and D). The decrease in Ang-1 expression was greater in TG mice than in WT animals ($P < 0.001$).
Pretreatment with RvE1 Reduces TANs and TAMs

To determine the impact of RvE1 on neutrophils and macrophages associated with the tumor microenvironment as a measure of cellular contribution to the inflammatory response, TANs and TAMs were quantified. As shown in Figure 6, RvE1 significantly prevented the infiltration of TAMs and TANs in both WT and TG animals compared with vehicle control ($P < 0.001$). The impact was significantly greater in TG animals compared with the WT controls ($P < 0.001$).

Cisplatin-Induced Reduction in Tumor Volume, Vascularization, and Inflammation Is Enhanced by RvE1

To identify the therapeutic impact of the RvE1 on established tumor growth, the impact of RvE1 on tumors that had
been growing for 4 weeks was tested. RvE1 alone did not significantly impact the tumor volume or size (data not shown). However, when combined with a well-established oncotherapeutic (cisplatin), tumor size was significantly reduced compared with the cisplatin-alone—induced reduction of the tumor size (Figure 7). In addition, RvE1 prevented the weight loss observed in mice treated with cisplatin alone.

In parallel, expression of COX-2 and NF-κB was significantly reduced in both WT and TG groups treated with cisplatin. RvE1-alone significantly reduced NF-κB expression in both WT and TG animals. Cisplatin-mediated reduction of COX-2 and NF-κB was significantly enhanced when administered with RvE1, which was also greater in the TG group than in the WT group (Figure 8). VEGF-A, Ang-1, and Ang-2 expression showed a similar reduction, and RvE1 potentiated the impact of cisplatin. There was a more profound impact on the TG animals.

Inflammatory cytokine levels in tumor tissues from both WT and TG animals were significantly reduced in response to cisplatin (Table 2). RvE1 further enhanced this reduction for all cytokines tested. Treatment in TG animals led to a significant decrease in granulocyte colony-stimulating factor tissue levels compared with WT mice when animals were treated with cisplatin and RvE1. IL-12 levels were not detectable in any group treated with cisplatin with or without RvE1.

**Discussion**

Experimental studies have shown that inflammation plays a significant modifying role in the microenvironment of carcinogenesis through cellular and molecular mechanisms. In the present study, RvE1, an anti-inflammatory and proresolving lipid mediator derived from the ω-3 fatty acid EPA, prevented and decreased tumor growth in both WT and ERV1-overexpressing transgenic mice and resulted in reduced cancer cell proliferation and angiogenesis. TANs and TAMs were significantly reduced in parallel, suggesting the direct involvement of innate immune cells in the tumor-associated inflammatory microenvironment. RvE1 treatment led to spontaneous necrosis of the center of the solid tumor, with viable tumor cells observed only at the periphery. The space between tumor cells was enlarged, and the tumor cells exhibited shrinking spindle-shaped nuclei. There was a marked increase in the extent of necrosis of the tumor core. The impact of RvE1 administration was more pronounced in transgenic animals compared with WT, with a higher degree of necrosis and decreased vascularization. Treatment of both WT and transgenic animals with RvE1 resulted in fewer animals developing tumors in each group. These observations suggest a profound preventive impact of RvE1 treatment and support the hypothesis that increased availability of ERV1 receptor enhances RvE1 anti-cancer activity. Data from the therapeutic arm of this study demonstrated that RvE1 potentiated the oncotherapeutic actions of cisplatin, with a parallel and significant reduction of inflammatory cytokines and markers of angiogenesis. Taken together, the data suggest that the mechanism of RvE1 actions involves the resolution of inflammation in the micro-environment of the tumor tissue, limiting angiogenesis, tumor growth, and viability.

The tissue expression of proinflammatory cytokines to quantify the actions of RvE1 was studied. Granulocyte colony-stimulating factor, IL-1α, IL-6, IL-8, IL-12, and tumor necrosis factor-α were elevated in cancer tissues of both WT and transgenic mice. Infiltrating neoplastic and stromal cells can contribute to the tumor microenvironment by secretion of growth factors, cytokines, and chemokines. These results are in concordance with other reports as well. RvE1 treatment significantly reduced these cytokines in both WT and transgenic mice, with a more pronounced impact in transgenic animals. In animals with established cancer lesions, RvE1 treatment significantly reduced inflammation when used with cisplatin and resulted in a clinical reduction of tumor size, suggesting that the inflammatory resolution was directly involved in tumor resolution. Enhanced resolution of inflammation was an anticipated outcome with RvE1 administration and in transgenic mice. However, the impact on tumorigenesis and...
tumor growth would not necessarily be expected. More important, the observation in the wild-type mice that administration of exogenous RvE1 profoundly improves tumor-associated pathologies and the role of inflammatory processes in pathogenesis is confirmed by the experiments with transgenic mice. Furthermore, animals treated with a combination of cisplatin and RvE1 exhibited weight gain. Thus, agonists for the resolution of inflammation have the potential to reduce the dose and adverse effects associated with mostly toxic chemotherapeutics widely used in cancer treatment.

The source of these local cytokines has been reported to be TAMs or TANs, which dominate the immune infiltrate in tumors, representing key cell types linking inflammation and cancer.9 TAMs in established tumors are generally skewed toward the M2-like phenotype, promoting tumor survival, progression, and dissemination through enhanced angiogenesis, epithelial-mesenchymal transition, and immune suppression.42 Although macrophage polarization has been suggested to play a role in tumor progression and varies with the stage of various cancers, inflammatory changes in the microenvironment and interaction with genetic factors seem to be the driving force for oncogenesis.9 Taken together with the previous observations that CD68+ macrophages were detected in both stroma and tumor cell islets with an inverse relationship between survival and stromal macrophage density,43 the role of the macrophage can be biphasic, where macrophages can be part of both the initiation and the resolution of the inflammatory process. This study demonstrates that both TAMs and TANs were significantly reduced in response to RvE1. The increased impact in the TG group further supports the receptor-mediated actions of RvE1 and SPMs. Although not focused on identifying phagocyte phenotypes and their role in cancer, the data suggested that RvE1 suppressed proinflammatory cytokine generation. Cytokine suppression was
enhanced in transgenic animals overexpressing the receptor for RvE1. Overall, these activities limiting inflammation were in parallel with tumor shrinkage and increased tumor core necrosis.

To further study the mechanism of RvE1 reduction of inflammation, alterations in the molecular regulators of inflammation, COX-2 and NF-kB, were measured. Both were increased in cancerous tissues. COX-2 converts arachidonic acid in the cytoplasmic membrane into prostaglandin E2, regulating cell proliferation, differentiation, and apoptosis through several autocrine and paracrine pathways. Inflammatory cytokines cause the activation of NF-kB, which increases proinflammatory cytokine production and regulates the expression of target genes with critical roles in the inhibition of apoptosis and promotion of tumor growth. Pretreatment with RvE1 resulted in a significant decrease in the expression of NF-kB and COX-2. This observation was in line with a study where supplementation with docosahexaenoic acid, an ω-3 fatty acid and precursor of D-series resolvins, inhibited COX-2 expression and induced apoptosis in the WM266-4 metastatic melanoma cell line. Dietary supplementation with fish oil also decreased COX-2 expression and inhibited the pathogenesis of colon cancer in rats. Hence, regression of the expression of NF-kB and COX-2 in response to pretreatment with RvE1 suggests preventive actions by RvE1. The decrease in the expression of these genes was more marked in transgenic animals, suggesting that overexpression and, therefore, the bioavailability of ERV1 resulted in increased resolution of inflammation and cancer.

Cell proliferation was measured by the expression of Ki-67 in tumor tissues. Ki-67 is present in all dividing cells and defines the increased cell proliferation in cancerous tissue. Abnormal epithelial proliferation is a hallmark of tumorigenesis, and increased Ki-67 expression is observed in cancer. In this study, animals developing tumors demonstrated an increased number of Ki-67–positive cells. In response to RvE1, the percentage of Ki-67–positive cells was decreased in both WT and transgenic animals. The decrease was more marked in transgenic mice, suggesting a direct impact of RvE1 on cancer cell proliferation. A recently published study links the reduction of cancer cell proliferation in lung cancer to the activity of TAMs, where TAM depletion resulted in reduced tumor growth.

The histologic data further document that RvE1 resulted in the necrosis of the central core of tumors. We hypothesized that this phenomenon is associated with decreased vascularization/angiogenesis and analyzed the extent of microvessel density by immunohistochemical staining of CD31 and CD34. Strong CD31 and CD34 staining in tumor tissues suggested an increase in neovascularization in parallel with previous reports. Angiogenesis is essential for the growth and maintenance of solid tumors; without vascularization, tumors cannot grow beyond a few millimeters in size and become necrotic. In response to pretreatment with RvE1, the extent of microvessel density was decreased in both the WT and transgenic animals. Ang-1 and Ang-2, ligands for the Tie-2 receptor expressed on endothelial cells, play a critical role in angiogenesis in the presence of VEGF. Intense immunohistochemical staining and increased VEGF, Ang-1, and Ang-2 expression were observed in the cancer tissue. This observation was well correlated with increased microvessel density data. Ang-1 and Ang-2 are functionally antagonistic molecules regulating angiogenesis. Ang-1 stabilizes blood vessels by promoting the interaction between endothelial cells and the surrounding extracellular matrix, and Ang-2 antagonizes the stabilizing action of Ang-1 by binding to Tie-2 competitively, which destabilizes vessels. However, the vessels destabilized by Ang-2 do not regress but undergo angiogenic changes in the presence of angiogenic factors, such as VEGF. The increased expression of both Ang-1 and Ang-2 in the presence of VEGF suggested the involvement of increased vascularization. Pretreatment with RvE1 significantly attenuated VEGF, Ang-1, and Ang-2 expression, suggesting that RvE1 abrogates the angiogenic response in cancer tissue by down-regulating VEGF. This decrease was more marked in transgenic mice, indicating that overexpression of ERV1 amplifies the activity of RvE1. These results also indicate that the attenuation of vascularization may represent a novel mechanism for the preventive activity of RvE1 in cancer pathogenesis.

Anti-inflammatory compounds can be protective and therapeutic; they decrease the toxicity of common chemotherapeutic drugs, allow for higher doses of those drugs, and...
sensitize cancer cells, making them more likely to respond to chemotherapeutics. The data on cisplatin strongly support that RvE1 enhances the chemotherapeutic actions of cancer drugs. It has already been shown that nonsteroidal anti-inflammatory drugs have anti-cancer effects in colorectal, esophageal, breast, lung, and bladder cancers,57 and corticosteroids are used to ameliorate the adverse effects of chemotherapy and radiation or as a direct treatment.58 However, all currently available anti-inflammatory drugs, except aspirin, have potent adverse effects as their mechanism of action involves inhibition of inflammation. The actions of resolvins are mediated through specific receptors, and they restore homeostasis by resolving inflammation actively, not through inhibition. To date, there are no known adverse effects.59 This strategy may apply to all cancers as the inflammatory process and angiogenesis are inherent to all. Because cancer therapeutics cannot target cancer cells exclusively, resolution-phase agonists have the potential to control the inflammation-mediated expansion of cancer, which is a critical factor that determines invasion and progression. An additional benefit appears to be a reduction of the dosage needed for toxic cancer therapeutics.

Several limitations to this study should be noted. Intrapulmonary growth of tumors was not assessed. Our hypothesis-driven experiments were addressed with a quantitative RT-PCR approach to target specific genes rather than an RNA-sequencing–based transcriptomics approach. To address cell specificity and ontogeny, future exploratory experiments need to be performed using single-cell RNA-sequencing experiments and protein-level expression of targets. One important aspect of our study is to note that we have not used ERV1 knockout mice. Instead, we used a gain-of-function model to increase the availability of the receptor for RvE1 and assess the impact of the enhanced resolution of inflammation (the phenotype of the ERV1 transgenic) on different aspects of disease pathogenesis. In this TG model, ERV1 is only overexpressed on myeloid cells. Thus, we focused on the impact of innate immunity in the resolution of inflammation and vascularization.

In conclusion, treatment with RvE1 reduces inflammation and vascularization that changes the tumor tissue microenvironment, resulting in tumor necrosis. Overexpression of the ERV1 receptor increased the bioavailability and anti-inflammatory activity of RvE1 and increased its impact on tumor growth. In established tumors, RvE1 reduces inflammation, slows tumor growth, and improves the clinical response to chemotherapy.

References


30. Lee HJ, Park MK, Lee EJ, Lee CH: Resolvin D1 inhibits TGF-beta1-


