Dependence of Resolvin-Induced Increases in Corneal Epithelial Cell Migration on EGF Receptor Transactivation

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PURPOSE. To determine whether resolvin E1 (RvE1), an endogenous oxygenation product of eicosapentaenoic acid (EPA), induces increases in migration in human corneal epithelial cells (HCECs) and to identify signal pathways mediating this response.

METHODS. Migration was measured with the scratch wound assay. Western blot analysis identified changes in the phosphorylation status of prospective intracellular signal transduction mediators. Immunocytochemistry probed for intracellular paxillin localization and actin reorganization.

RESULTS. RvE1 enhanced HCEC migratory rates to levels comparable to those induced by epidermal growth factor (EGF). These increases were accompanied by increases in the phosphorylation status of epidermal growth factor receptor (EGFR), Akt, p38 MAPK, GSK-3 α/β , and paxillin, which essentially persisted for up to 60 minutes. The EGFR inhibitor AG1478 blocked the subsequent effects of RvE1 to induce increases in phosphorylation status and cell migration. The PI3-K inhibitor LY294002 or wortmannin or the p38 inhibitor BIRB796 blocked resolvin-induced increases in cell migration. Either the matrix metalloproteinase (MMP) inhibitor GM6001 or the specific heparin-bound EGF-like growth factor inhibitor CRM197 suppressed RvE1-induced stimulation of EGFR/PI3-K/Akt phosphorylation and cell migration.

CONCLUSIONS. RvE1 enhances HCEC migration through MMP and shedase-mediated EGFR transactivation. This response is dependent on PI3-K and p38-linked signaling eliciting paxillin (Tyr118) phosphorylation. (*Invest Ophthalmol Vis Sci.* 2010; 51:5601–5609) DOI:10.1167/iovs.09-4468

The corneal epithelium provides an effective barrier against ocular damage by noxious agents, infection, and environmental insults. Because the corneal epithelium may be the first point of contact for such stresses, maintenance of its integrity

is critical for continued protection and retention of optical transparency. To preserve integrity, the corneal layers must undergo continuous renewal in a complex process controlled by a host of growth factors and cytokines that, through activation of their cognate receptors, regulate epithelial cell proliferation, migration, and differentiation.¹

Should corneal epithelial injury occur, some of these mediators undergo upregulation, which hastens wound healing and prevents losses in corneal transparency.^{2,3} In some cases, these responses depend on complex interactions between different receptors and their linked cell signaling pathways. Both mitogen-activated protein kinase (MAPK) and PI3-K/Akt pathways are involved in mediating receptor control of migration and proliferation.⁴ Characterization of the healing response has shown that EGF is one of the most efficacious endogenous mediators for the stimulation of corneal epithelial wound closure.^{5,6} However, during persistent and severe corneal inflammation, wound healing is delayed or even unable to restore epithelial barrier function. Other contributors to an inappropriate healing response include scarification, which leads to loss in tissue transparency.^{7–9} Epithelial growth factor receptor (EGFR) transactivation is one type of receptor interaction contributing to corneal wound healing. It is elicited by endogenous mediators other than EGF, which induce responses underlying reepithelialization through EGFR-linked signaling pathway activation.^{10–21}

RvE1 (5S, 12R, 18R-trihydroxyeicosapentaenoic acid; RX-10001) is an endogenous anti-inflammatory mediator formed as an oxygenation product of eicosapentaenoic acid (EPA), one of the main dietary essential fatty acids.²² This lipid mediator was first identified in vivo during the spontaneous resolution phase of inflammation in exudates collected from inflamed dorsal pouches in mice fed EPA.²³ In acute and chronic models of inflammation, administration of RvE1 either before an insult or during elaboration of inflammation inhibited this response. The models in which this inhibitory effect was demonstrated include TNBS-induced colitis,^{24,25} *Porphyromonas gingivalis*-induced periodontitis,^{26,27} allergy-mediated lung inflammation,²⁸ and suppression of retinal angiogenesis.²⁹ Recently, in a murine model of desiccating stress, RvE1 reduced corneal surface fluorescein staining and inflammation, suppressed goblet cell losses, and restored tear secretion rates (Li N, et al. *IOVS* 2008;49:ARVO E-Abstract 121; Gjorstrup P, et al. *IOVS* 2008;49:ARVO E-Abstract 122). These improvements in ocular surface health may be attributed to the fact that inflammation suppression can hasten reepithelialization. Indeed, in a recent study,³⁰ application to mice of the endogenous lipid autocoids lipoxin A4 (LXA4) and neuroprotectin D1 (NPD1) decreased proinflammatory chemokine production by the stromal cells and accelerated corneal reepithelialization. However, the study did not determine whether stimulation of this response also involved a direct increase in cell migration.³⁰

Collectively, the data from these models of inflammation and tissue stress suggested that resolvins may also act through

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other mechanisms to promote tissue homeostasis. We now show that RvE1 induces in HCECs a dose-dependent increase in cell migration and that this effect is mediated through EGFR transactivation followed by transient activation of the PI3-K/Akt/GSK-3 α / β and p38 MAPK signaling pathways. These responses are associated with increases in both paxillin phosphorylation and apparent heightened wound edge localization.

MATERIALS AND METHODS

Cell Culture

SV40-immortalized HCECs (a generous gift from Kaoru Araki-Sasaki, Ideta Eye Hospital, Kumamoto City, Kumamoto, Japan) were cultured in Dulbecco's modified Eagle's medium/Ham F12 (D/F12; Invitrogen, Carlsbad, CA), supplemented with 6% fetal bovine serum (FBS), 5 ng/mL EGF, 5 μ g/mL insulin, and 40 μ g/mL gentamicin (supplemented D/F12).³¹ Cells were grown for 1 to 2 days and kept subconfluent in 5% CO₂, 95% ambient air, at 37°C. To optimize cell responsiveness to EGF, before experimentation cells were starved by incubation in medium devoid of serum and EGF for 24 hours.

Scratch Wound Healing Assay

Cells were grown to subconfluence in 35-mm culture plate wells in serum-supplemented D/F12, starved for 24 hours, and scratch-wounded using the sharp edge of a sterile cell scraper. Floating cells were removed, and culture was refed with fresh medium in the presence or absence of either EGF (10 ng/mL) or RvE1 (0.001–0.1 μ M). The selective EGFR inhibitor AG1478 (1 μ M) blocked EGFR activation. LY294002 (1 μ M) or wortmannin (0.1 μ M) inhibited PI3-K activation.³² BIRB796 (0.5 μ M) suppressed p38 MAPK activation.³³ All inhibitors were added to the medium 30 minutes before exposure to RvE1 and remained throughout the experiment. The cell cycle inhibitor hydroxyurea (2.5 mM) was also added to the medium to reduce proliferation during the experiment. This concentration inhibited cell proliferation by 95% and had a minimal effect on cell viability (data not shown). The wound closure rate was monitored from photographs taken with an inverted-stage microscope (Diaphot; Nikon Inc., Morton Grove, IL). Wound areas were measured with an image analysis program (Sigma Scan Pro 5; Systat Software, Inc., San Jose, CA). Wound area closure for each condition was calculated as a percentage by first obtaining the difference between the initial wound area and the remaining wound area at 24 hours and dividing this difference by the initial wound area. The quotient was then normalized in each case to the remaining wound area in the untreated control cells. Four fields from each dish were measured. Each experimental condition was repeated at least three times.

Western Blot Analyses

Western blot experiments were performed as previously described.³⁴ In brief, cells were gently washed twice in cold phosphate-buffered saline (PBS) and harvested in 0.5 mL cell lysis buffer. Cell lysates were centrifuged at 13,000g for 15 minutes. Supernatants were collected, and equal amounts of protein from the cell lysates were applied to 7.5% or 10% polyacrylamide sodium dodecyl sulfate (SDS) minigels. The gel-separated proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% nonfat skim milk in PBS containing 0.1% Tween-20 (PBST) for 1 hour and were incubated overnight at 4°C with antibodies for detecting the phosphorylated forms of EGFR (Tyr1068), Akt, p38, GSK-3 α / β (Ser21/9), or paxillin (Tyr118) (1:300–1:1000 dilutions; Cell Signaling Technology, Inc., Beverly, MA). The membranes were washed three times with PBST and incubated with appropriate horseradish peroxidase-conjugated secondary antibody-labeled IgG (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. Immunoreactive bands were detected with an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences Inc., Piscata-

way, NJ). Resolved bands were quantified with ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Equivalent protein loading was confirmed by probing with either anti-Akt antibody or IgG. Images shown in each case are representative of three independent experiments. Protein concentrations were measured with a bicinchoninic acid assay protein assay kit (Pierce Biotechnology, Chicago, IL).

Immunocytochemical Localization

Cells were seeded onto chamber slides (Laboratory-Tek; Nunc, Naperville, IL) and were allowed to reach confluence. Cell layers were then washed twice with PBS, fixed in ice-cold PBS/4% paraformaldehyde for 30 minutes, washed three times with PBS, rendered permeable by a 10-minute incubation in 0.1% Triton-PBS solution, and blocked by 15-minute incubation in 3% bovine serum albumin (BSA)-PBS. These processed slides were incubated for 16 hours at 4°C with mouse anti-paxillin antibody (1:400; Millipore, Billerica, MA) made in 1.5% BSA-PBS. After the cells were washed, they were incubated for 30 minutes in a mix of AlexaFluor568-phalloidin (1:50) and AlexaFluor488-goat anti-mouse IgG (1:500; Invitrogen) at room temperature. Finally, sections were washed three times with PBS and mounted with antifade mounting medium containing 1.5 μ g/mL DAPI (Santa Cruz Biotechnology). Fluorescence was visualized using a fluorescence microscope with a 60 $\times$ oil objective lens. Images were processed using confocal laser scanning microscope software (LSM; Zeiss, Thornwood, NY) and image editing software (Photoshop 6.0; Adobe Systems Incorporated, San Francisco, CA).

Materials

D/F12, FBS, and PBS were from Invitrogen. Wortmannin, LY294002, GM6001, CRM197, heparin-bound EGF-like growth factor (HB-EGF), EGF, bovine insulin, gentamicin, and 0.05% trypsin-EDTA solution were purchased from Sigma RBI (St. Louis, MO). BIRB796 and RvE1 (RX-10001) were provided by Resolvix Pharmaceuticals, Inc. (Bedford, MA).

Statistical Analysis

Experiments were repeated at least three to six times. Statistical significance was determined by Student's unpaired *t*-test, and *P* < 0.05 was taken to be significant.

RESULTS

Resolvin-Stimulated Migration

To determine whether RvE1 stimulates cell migration, its dose-dependent effect on wound closure rates were investigated. Figure 1A shows a typical photomicrograph comparing the migration response by RvE1 at 0.1 μ M with that of 10 ng/mL EGF after 24 hours of drug exposure. Figure 1B confirms dose-dependent effects of RvE1. At a concentration of 0.1 μ M, the rates normalized to those of untreated controls reached 2.24 ± 0.08 (*n* = 11) and were of the same magnitude as those obtained with EGF (2.22 ± 0.19 ; *n* = 11).

Resolvin-Mediated Increases in Akt, p38 MAPK, and GSK Activation

To investigate whether RvE1 stimulates wound closure through activation of pathways previously described as essential for HCEC migration, its effects on PI3-K/Akt and p38 MAPK pathway activation were determined. RvE1 (0.1 μ M) induced time-dependent increases in Akt, p38, and GSK-3 α / β phosphorylation that reached maximal value at 5 to 15 minutes but remained evident for up to 60 minutes. Figures 2A–C show typical Western blot analyses and summaries of the time courses of these responses.

FIGURE 1. RvE1 enhances cell migration. Scratch-wound assay was performed on HCECs in DMEM without serum. (A) Representative photomicrographs show enhanced wound closure in the presence of EGF (10 ng/mL) or RvE1 (0.1 μ M). (B) Concentration-dependent increases in HCEC migratory rates by RvE1 ($n = 11$).

Resolvin-Induced Increases in Cell Migration Elicited by PI3-K and p38 MAPK Pathway Signaling

Cells were preexposed to a PI3-K inhibitor (LY294002 [1 μ M] or wortmannin [0.1 μ M]) for 30 minutes, followed by the addition of either 10 ng/mL EGF or 0.1 μ M RvE1 for another 24 hours (Fig. 3). As shown in Figure 1, EGF and RvE1 increased wound closure rates to twice those seen in unstimulated controls. These increases were blocked by either LY294002 or wortmannin, confirming a role for the PI3-K pathway. Wortmannin was efficacious and even suppressed cell migration rates to values less than those of the controls. This difference did not occur because suboptimal concentrations of LY294002 were used; rather, for each inhibitor, preliminary dose-titration experiments were performed to select an optimal dose (data not shown). The p38 inhibitor BIRB796 (0.5 μ M) also inhibited RvE1-stimulated migration. The data suggest that the inhibition of p38 phosphorylation at an optimal concentration of BIRB796 had less of an inhibitory effect on migration than did the inhibition of PI3-K. The combined results show that re-

FIGURE 2. RvE1 induces PI3-K and p38 MAPK activation. Western blot analysis was performed on HCEC lysates. (A) HCECs were serum starved for 24 hours and stimulated with RvE1 (0.1 μ M) for the time indicated. Akt activation was assessed by Western blot analysis based on changes in Akt phosphorylation. Equal loading of cell lysis proteins was confirmed by reprobating the same blot with total Akt antibody. (B) RvE1 time dependently induced changes in p38 phosphorylation. (C) RvE1 inhibited GSK-3 α/β by stimulating its phosphorylation in a time-dependent manner. Typical results are shown from experiments performed in triplicate. Summarized graphs are shown at the bottom of each panel. Data represent the mean $\pm$ SEM ($n = 3$).

FIGURE 3. RvE1 mediates increases in cell migration through EGFR-linked pathways. After scratch-wound, HCE cells were exposed to 10 ng/mL EGF or RvE1 (0.1 μ M) for 24 hours. Thirty minutes of preexposure to 1 μ M LY294002, 0.1 μ M wortmannin, or 0.5 μ M BIRB796 inhibited RvE1-stimulated migration ($n = 3$).

solvin-enhanced cell migration can be suppressed through the inhibition of either PI3-K or p38 MAPK signaling pathways.

Resolvin-Induced EGFR Transactivation and PI3-K and p38 MAPK Signaling

To determine whether EGFR transactivation could play a role in resolvin-mediated activation of PI3-K/Akt and p38 MAPK pathways, we evaluated its effects on Akt, p38 MAPK, and GSK-3 α/β phosphorylation in the presence of the EGFR tyrosine kinase inhibitor AG1478. Figure 4 shows that after 5 minutes, either EGF (10 ng/mL) or RvE1 (0.1 μ M) induced Akt, p38 MAPK, and GSK-3 α/β phosphorylation, respectively. Preexposure to AG1478 (1 μ M) suppressed these increases, causing them to remain near their baseline levels, indicating a dependence on EGFR transactivation for the observed activation of the PI3-K and p38 MAPK pathways.

Resolvin-Stimulated Cell Migration through EGFR Transactivation

The inhibition of resolvin-induced phosphorylation of PI3-K and p38 MAPK kinase by the EGFR tyrosine kinase inhibitor AG1478 strongly suggested that a migration response to RvE1 is dependent on EGFR transactivation. Using Tyr1068 phosphorylation as an indicator of EGFR activation, we confirmed that exposure to RvE1 (0.1 μ M) for up to 60 minutes increased EGFR phosphorylation, which was prevented by preexposure to AG1478 (1 μ M) for 30 minutes (Figs. 5A, 5B). AG1478 also completely blocked the cell migration responses to RvE1 (Fig. 5C). The observation that AG1478 reduced the responses below those of controls suggested that there may be an autocrine release of EGF in response to the wounding itself or possibly a release of EGF during the serum starvation period.

Dependence of Resolvin-Induced EGFR Transactivation on HB-EGF Release

A mechanism described for EGFR transactivation occurs through shedding of HB-EGF, which commonly occurs in re-

sponse to protease upregulation. To determine whether RvE1 induces EGFR transactivation through HB-EGF shedding, cells were pretreated with GM6001, a matrix metalloproteinase (MMP) inhibitor, or CRM197, a heparin-binding EGF-like growth factor inhibitor. GM6001 (50 μ M) and CRM197 (10 μ g/mL) not only decreased RvE1 (0.1 μ M)-stimulated EGFR and Akt phosphorylation, they also abolished its stimulation of migration (Figs. 6A, 6B). CRM197 reduced the wound closure rate to a value even lower than that of the control, again suggesting that wounding itself may induce protease upregulation and autocrine stimulation of the EGFR. HB-EGF (50 ng/mL) supplementation reversed CRM197-mediated inhibition of increases in migration elicited by RvE1.

Resolvin-Stimulated Paxillin Phosphorylation

Paxillin is a scaffolding protein whose phosphorylation status affects focal adhesion kinase (FAK) activation, which then leads to increases in cell migration. As RvE1 increases migration through EGFR transactivation and stimulation of these pathways, we determined whether it elicits this response through paxillin phosphorylation. Another indication for making such an assessment is that in HCECs, EGF induces increases in cell migration through complexation between paxillin and

FIGURE 4. RvE1 stimulates PI3-K and p38 MAPK through EGFR. (A) Akt phosphorylation was induced by exposure to EGF (10 ng/mL) or RvE1 (0.1 μ M) for 5 minutes. EGFR inhibition with AG1478 (1 μ M) for 30 minutes suppressed Akt activation. (B) p38 phosphorylation was induced by exposure to EGF or RvE1 for 5 minutes. Preexposure to AG1478 (1 μ M) for 30 minutes suppressed p38 activation. (C) EGF or RvE1-stimulated GSK-3 α/β phosphorylation was blocked by AG1478. Blotting images show representative results of three independent experiments.

FIGURE 5. RvE1 increased cell migration through EGFR phosphorylation. (A) Western blot analysis showed EGFR phosphorylation to be induced by exposure to RvE1 (0.1 μ M) up to 60 minutes. Inhibition of EGFR activation by preexposure to AG1478 (1 μ M) suppressed EGFR activation by RvE1. Typical results are shown from experiments performed in triplicate. (B) RvE1 stimulated EGFR phosphorylation in a time-dependent manner. Data are expressed as mean $\pm$ SEM ($n = 3$). (C) After scratch wound, HCECs were exposed to 10 ng/mL EGF or RvE1 (0.1 μ M) for 24 hours with and without exposure to AG1478 (1 μ M; $n = 3$).

FAK.^{35–37} Figure 7A shows that RvE1 (0.1 μ M) time dependently stimulated paxillin Tyr118 phosphorylation.

Immunocytochemistry staining shows that RvE1 induces remodeling of the actin cytoskeleton and relocalization of the scaffolding protein paxillin. Figure 7B is a negative control in which the cells exhibit thick cortical actin staining but no paxillin staining because the anti-paxillin antibody was omitted. In addition, increases in membrane protrusion induced by EGF (10 ng/mL) are evident relative to cells not exposed to this growth factor. After wounding, at their leading edges, cells have bundles of thick actin fibers parallel to the wound edge, with well-defined lamellipodia extending into the denuded area. In RvE1 (0.1 μ M)-treated cells, the cortical actin ring is reduced, whereas there are more prominent long protrusions at the wound edge. Paxillin is punctate in the cytoplasm and

more localized at the cell periphery, with the leading edge forming spikes and puncta-like structures (arrow). On the other hand, CRM197 (10 μ g/mL) completely abolished RvE1-stimulated cytoskeleton reorganization and peripheral membrane protrusions into the wound (Fig. 7C). Actin staining displayed thickened cortical staining, whereas paxillin remained diffuse throughout the cytoplasm. However, after HB-EGF (50 ng/mL) supplementation, actin-positive protrusions at the wound edge reappeared along with paxillin staining. Thus, resolvin-induced increases in paxillin phosphorylation shown in Figure 7A appear to precede its redistribution toward the wound edges shown in Figure 7C.

FIGURE 6. Dependence of RvE1-induced EGFR transactivation on shedding of HB-EGF. (A) Growth factor-starved cells were exposed to 0.1 μ M RvE1 for 5 minutes with and without preexposure to GM6001 (50 μ M) or CRM197 (10 μ g/mL) for 30 minutes. Cells were lysed at the end of stimulation, and equal amounts of cell lysates were immunoblotted by antibodies against phosphorylated Akt (pAkt) or phosphorylated EGFR (pEGFR; Tyr1068). The same cell lysates were subjected to Western blot analysis with anti-Akt or IgG to assess protein loading equivalence. (B) Scratch-wound healing kinetics were assessed under the same conditions. Changes in the extent of wound closure are shown as ratios compared with untreated control. HB-EGF (50 ng/mL) reversed the inhibitory effect of CRM197 on increases in cell migration induced by RvE1 ($n = 3$).

FIGURE 7. RvE1 stimulates paxillin phosphorylation and cytoskeleton reorganization. (A) Cells were exposed to RvE1 (0.1 μ M) for the time indicated. Western blot analysis was used to evaluate paxillin phosphorylation status. (B, C) Confluent cells were wounded with a 200- μ L pipette tip and were further incubated with or without EGF (10 ng/mL) or RvE1 (0.1 μ M). Wound edge was photographed 2 hours after wounding. Immunocytochemistry staining detected actin filament organization with AlexaFluor568-phalloidin (red). The localization of paxillin was visualized by incubating cells with mouse anti-paxillin antibody, followed by AlexaFluor488 goat anti-mouse IgG (green). Cell nuclei are evident based on DAPI staining (blue). Asterisks: wound edge. Arrows: paxillin-positive cell protrusions. (B) Lack of staining with isotype antibody validates selectivity of anti-paxillin antibody (negative control). EGF induces distinct punctate paxillin staining localized to protrusions at the wound edge. (C) RvE1 induces distinct punctate paxillin staining, which was lost when HCECs were pretreated with an HB-EGF inhibitor, CRM197 (10 μ g/mL), before wounding. HB-EGF (50 ng/mL) added in the presence of CRM197 rescued cytoskeleton reorganization and peripheral membrane protrusions into the wound. Images shown are representative of three independent experiments. Scale bar, 100 μ m.

DISCUSSION

Resolvins are highly potent endogenous anti-inflammatory mediators derived from the essential omega-3 polyunsaturated fatty acid eicosapentaenoic acid.^{22,38} In both acute and chronic

models of inflammation, the endogenous RvE1 was shown to accelerate the resolution of inflammation.^{22,28,39} Although it would be expected that controlled resolution of an inflammatory process would more rapidly allow tissue homeostatic processes to normalize tissue function, nothing was known about a direct effect of resolvins on tissue repair.

We now show for the first time that RvE1 directly stimulates cell migration and hastens wound closure. The effects of RvE1 were concentration dependent and, at the highest concentration investigated, induced increases in cell migration to a level similar to that maximally induced by EGF. Its stimulation of cell migration was dependent on EGF release from its binding to heparin, followed by EGFR transactivation and subsequent stimulation of p38 MAPK and PI3-K signaling. There is ample evidence that a number of different receptor types elicit control of responses through EGFR transactivation in the corneal epithelium,^{14–19,21,40} and in numerous other tissues as part of a normal regulation of tissue function.^{41–49} G protein-coupled, receptor-mediated Erk1/2 and PI3-K activation often occur through the transactivation of receptor tyrosine kinases (RTKs), which leads to sequential activation of linked cell-signaling pathways.^{41,50} Many corneal epithelial studies indicate a pivotal role for EGFR, including receptor transactivation, in mediating responses to wounding and other pathophysiological challenges.^{10–21} Initial evidence from our laboratory, including the inhibition by pertussis toxin, indicates that the GTP-binding protein G_i mediates resolvins-induced EGFR transactivation and stimulation of cell migration (data not shown).

Specifically in our study, blocking EGFR activation with AG1478, a selective tyrosine receptor kinase inhibitor, not only

FIGURE 8. Cell-signaling pathways mediating RvE1-induced increases in migration. RvE1 stimulates cell migration through EGFR transactivation. This response occurs subsequent to MMP-mediated ectodomain shedding of HB-EGF. EGFR activation by released EGF elicits concomitant stimulation of the linked PI3-K, p38, and ERK/MAPK signaling pathways. PI3-K-induced Akt activation suppresses constitutively active GSK-3 α/β by phosphorylation on Ser21/9. Such inhibition decreases the phosphorylation status of the phosphatase MKP-1, which results in its destabilization. Declines in MKP-1 levels suppress a negative feedback effect on EGF-induced p38 and ERK/MAPK phosphorylation and prolong their activation. Such prolongation enhances EGFR-induced increases in migration through increased phosphorylation of the scaffolding protein paxillin. Akt stimulation selectively phosphorylated paxillin at Tyr118, whereas Erk1/2 activation induces Ser126 phosphorylation.³⁶ EGF also induces paxillin-FAK complex formation and increases in cell migration.³⁷

abolished the resolvin-induced migration responses, it also blocked the phosphorylation of both Akt and p38. Furthermore, inhibitors that blocked the phosphorylation of either Akt or p38, such as the PI3-K-specific inhibitor wortmannin or LY294002 or the specific p38 inhibitor BIRB796, also inhibited the migration responses to RvE1. EGFR transactivation commonly occurs in response to sheddase-dependent release of HB-EGF.²¹ Adding either an MMP inhibitor (GM6001) or a direct HB-EGF inhibitor (CRM197) to the scratch-wound assay also blocked resolvin-mediated EGFR phosphorylation and inhibited the cell migratory responses, further confirming a dependence on EGFR transactivation. The inhibitory effects of CRM197 on resolvin-induced increases in migration were circumvented by supplementing the medium with HB-EGF. Similar response rescue results were described in another study using the same cell line.⁵¹

EGFR transactivation by RvE1 induced persistent increases in GSK-3 α/β and p38 MAPK phosphorylation. The time dependence of these changes was different from that induced by EGF.³⁶ With EGF, the induced changes were more transient than those elicited by RvE1. Nevertheless, the increases in cell migration induced by RvE1 and EGF in the present study are indistinguishable from one another. The fact that the same increase in cell migration can be induced through differences in cell signaling activation patterns reflects that there are other aspects of this process that require further elucidation. Some insight into this question stems from comparing the specific residues on paxillin that are phosphorylated by different EGFR-linked signaling pathways. We found that RvE1 induces Tyr118 phosphorylation on paxillin, whereas Erk1/2 and JNK/SAPK pathways induced Ser126 and Ser178 phosphorylation, respectively.^{36,52} In each case, these effects led to increases in cell migration, even though different paxillin residues undergo phosphorylation. These differences suggest that cell migration can be stimulated by alternative phosphorylation of different residues on paxillin. Furthermore, they indicate that EGFR activation is both tissue and ligand specific. This means that in some cases an increase in migration can be elicited through different patterns of cell signaling activation, leading to phosphorylation of a variety of residues on one of the mediators of this response. In HCECs, such redundant control of paxillin phosphorylation affects focal-adhesion dynamics and cell migration. A recent study³⁷ shows that paxillin phosphorylation by ERK pathway stimulation enhances its interaction with FAK, thus promoting cell migration through focal adhesion disassembly. Therefore, different patterns of cell signaling activation can mediate the phosphorylation of paxillin on various residues, and phosphorylation of one or more of these residues may be sufficient to trigger an increase in cell migration.

Activation of the PI3-K/Akt pathway in HCECs suggested that resolvin may also activate pro-survival pathways, but this was not further studied. However, in a recent report⁵³ of myocardial ischemia/reperfusion injury in the rat, RvE1 administered at the time of reperfusion reduced the infarct size by up to 70%. The same study in isolated cardiomyocytes exposed to hypoxia/reoxygenation in vitro showed RvE1 to activate survival pathways, including PI3-K and Erk1/2, that were dependent on HB-EGF shedding and EGFR transactivation. The demonstration of profound cellular protective and repair pathway activation in two tissues as diverse as epithelial cells and myocardial cells may indicate that EGFR transactivation is a major mechanism for the tissue-homeostatic effects attributed to RvE1. These observations extend the importance of the resolvin biology beyond the effects linked to a resolution of inflammation, particularly in chronic models, including those of colitis,²⁵ lung inflammation,^{28,54} and periodontitis.^{26,27}

Our current understanding of the mechanisms underlying resolvin-induced stimulation of HCEC migration is summarized

in Figure 8. In the corneal epithelium, increases in paxillin amino acid residue phosphorylation status is a complex response that is controlled by changes in the activation status of either the Akt, JNK/SAPK, or ERK/MAPK signaling pathway. Each of these can increase the phosphorylation status of paxillin at different residues on paxillin. In addition, Akt-induced GSK-3 α/β inhibition (i.e., phosphorylation) prolongs ERK and p38 activation. This control by GSK-3 α/β of ERK and p38 phosphorylation entails a negative feedback effect wherein GSK-3 α/β activation is inversely related to the phosphorylation status of ERK and p38. Such control is elicited through GSK-3 α/β regulation of the expression level of MAPK phosphatase 1 (MKP-1). When GSK-3 α/β is active (i.e., dephosphorylated), GSK-3 α/β phosphorylates MKP-1 and stabilizes its level of expression.⁵⁵ On the other hand, Akt-mediated GSK-3 α/β phosphorylation inhibits GSK-3 α/β , causing losses in MKP-1 levels. Declines in MKP-1 levels, in turn, prevent the dephosphorylation of these two MAPK intermediates, which prolongs their phosphorylation activity. Such prolongation, resulting from Akt phosphorylation, leads to increases in cell migration that are dependent on the duration of GSK-3 α/β inactivation.^{20,36,52,56}

Finally, the present study used a corneal cell line generated by immortalization with the SV40-large T-antigen.³¹ Although this cell line is frequently applied to study signal transduction events underlying the control of corneal epithelial migration, it is possible that immortalization altered signaling events, accounting for resolvin-induced stimulation of cell migration. Hence, notwithstanding the practical difficulties associated with working in vitro with rapidly differentiating primary corneal epithelial cells, future selective reconfirmation of our findings with the SV40 cell line are warranted. Some recent evidence mitigates concern regarding the physiological relevance of using the SV40 cell line for characterizing the signaling mechanisms controlling cell migration. Our comparison of microarray-based global gene expression in stratified HCECs cultured at an air-liquid interphase with whole fresh primary epithelium shows that though differentiation in the HCECs is markedly perturbed, the genes encoding signal transduction events investigated in the current report undergo only moderate changes (Wolosin M, unpublished observations, 2010).^{57,58}

In summary, RvE1 induces increases in cell migration through EGFR transactivation which is dependent on the shedding of HB-EGF. EGFR stimulation, in turn, activates PI3-K/Akt/GSK-3 α/β , p38 (MAPK) signaling, leading to selective Tyr118 paxillin phosphorylation. This change on paxillin promotes lamellipodia extension into the wound edges. RvE1 may provide new strategies for promoting corneal barrier integrity as an added therapeutic feature to their well-established anti-inflammatory properties. The now demonstrated stimulation of epithelial wound healing could be of potential therapeutic benefit in hastening the restoration of epithelial barrier integrity.

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