Nuclear factor-κB activation promotes restitution of wounded intestinal epithelial monolayers

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Egan, Laurence J., Ana de Lecea, Evan D. Lehrman, Gennett M. Myhre, Lars Eckmann, and Martin F. Kagnoff. Nuclear factor-κB activation promotes restitution of wounded intestinal epithelial monolayers. Am J Physiol Cell Physiol 285: C1028–C1035, 2003.—Epithelial restitution, the movement of wound-edge cells into an area of epithelial cell denudation, is an important early step in the ulcer healing process. Growth factors regulate epithelial restitution, yet little is known about the transcriptional pathways that mediate their effects on cell migration. The transcription factor nuclear factor (NF)-κB is a master regulator of the host inflammatory response that is activated in the epithelium in intestinal inflammation, which often accompanies epithelial injury. We hypothesized that NF-κB may be an important transcriptional regulator of epithelial restitution. In an in vitro model of scrape-wounded monolayers of nontransformed rat intestinal epithelial (RIE-1) cells, NF-κB was activated in epithelial cells at the wound edge. Blocking of NF-κB activation by either pharmacological or genetic approaches inhibited intestinal epithelial restitution. Moreover, scrape wounded activated the epidermal growth factor receptor (EGFR) in cells at the wound edge, and, importantly, inhibiting EGFR tyrosine kinase activity decreased scrape wound-induced NF-κB activation and cell migration. These results indicate a novel role of NF-κB in a signaling pathway important for restitution and healing of intestinal epithelia. To the extent NF-κB may have parallel functions in vivo, they also suggest a need for caution in the proposed use of NF-κB inhibitors for the treatment of conditions associated with inflammation and injury of intestinal and other mucosal surfaces.

METHODS

Cell lines. Rat intestinal epithelial (RIE-1) cells (5) provided by K. Brown, Babraham Institute, Cambridge, UK were maintained in Dulbecco’s modified Eagle’s medium containing 2 mM l-glutamine and 5% fetal bovine serum at 37°C in 95% air-5% CO2 and were used between passages 22 and 12–14, 35). These migration-promoting factors affect cellular functions by activating specific signaling cascades (48). For example, activation of the epidermal growth factor receptor (EGFR) by its cognate ligands initiates intracellular signaling cascades, which involve phospholipase C, protein kinase C, phosphatidylinositol-3 kinase, and mitogen-activated protein kinases, that are important for the migration of intestinal epithelial cell lines (35, 48).

The transcription factor NF-κB is important for the initiation of the inflammatory response and protection of cells from proapototic stimuli (28). NF-κB is activated in intestinal epithelial cells in vitro in response to environmental stresses such as infection with enteric bacterial pathogens and stimulation with proinflammatory cytokines (16–18, 23, 27) and in vivo in inflammatory bowel disease and diverticulitis (36, 40). This suggests that NF-κB has an important role in regulating epithelial cell functions under conditions of inflammation and injury.

Given that epithelial ulceration is typically accompanied by mucosal inflammation and activation of NF-κB (36, 40), we hypothesized that NF-κB also may be linked to pathways that signal intestinal epithelial restitution. Using an in vitro model of scrape-wounded intestinal epithelial cell monolayers, we have demonstrated that wound injury activates NF-κB in intestinal epithelial cells at the wound edge and that blocking NF-κB activation decreases epithelial restitution. Moreover, this injury rapidly activates the EGFR, and blocking EGFR activation abrogates the downstream activation of NF-κB and epithelial restitution. Thus a pathway linking the EGFR to NF-κB is an important, previously unrecognized motogenic cascade in intestinal epithelial cells.
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30. REV and RSR cell lines were generated for this study by transfecting RIE-1 cells with pRCβ-actin (REV-3, REV-11) or pRCβ actin (IcBeA4) (RSR-1, RSR-6) (9), respectively, using Lipofectamine Plus (Invitrogen, Carlsbad, CA). G418 (0.4 mg/ml) was used to select and maintain stable transfectants. Cell viability was assessed by trypan blue exclusion.

Scraper wounding of RIE-1 cell monolayers. To measure the rate of epithelial restitution, cells were grown to confluence in six-well tissue culture plates. Monolayers were wounded by scraping cells off with a razor blade, as previously described (8, 37). Three wounds were made in each well. Before experiments, cells were washed with prewarmed Hanks’ balanced salt solution and recultured in media containing 5% fetal bovine serum. The razor blade imprinted a line on the surface of the plate visible by phase-contrast microscopy that indicated the start of the wound. At various time points after wounding, the number of cells crossing 1 mm of the wound was counted using a 1-cm reticle mounted in the eyepiece of an inverted phase-contrast microscope, using a 10× objective lens.

To prepare whole cell or nuclear protein extracts, cells were grown to confluence in 10-cm tissue culture dishes. The monolayer was cross-scraped 20 times in each of 4 directions (80 total scrapes) with a 1-ml pipette tip to maximize the length of the wound edge, after which the monolayer was washed as described above.

For immunofluorescence experiments, cells were grown to confluence on sterile 12-mm glass coverslips placed in 24-well tissue culture plates. Two perpendicular scrapes were made through the monolayer with a 0.2-ml pipette tip, after which the monolayer was washed as described above.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays (EMSA) were done as previously described (4, 17).

RelA/p65 immunofluorescence. Cells grown on glass coverslips were fixed with 4% paraformaldehyde. Cells were permeabilized with 0.5% Triton X-100 and blocked with PBS containing 1% bovine serum albumin and 5% goat serum, after which polyclonal rabbit anti-p65 IgG (sc-372; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer was added and cells were further incubated at 4°C overnight. After washing, Cy3-conjugated goat anti-rabbit Ig (Jackson ImmunoResearch Labs, West Grove, PA) in blocking buffer was added. Cells in which the nuclear fluorescence intensity was the same as or greater than the cytoplasmic intensity were scored as positive for nuclear RelA/p65.

EGFR immunofluorescence. Subcellular localization of the EGFR was evaluated using laser scanning confocal microscopy of RIE-1 monolayers grown on glass coverslips. The monolayers were stained as described above, using anti-EGFR antibody (06-847; Upstate Biotechnology, Lake Placid, NY).

Immunoprecipitation and Western blotting. Cells were scraped into lysis buffer containing 50 mM Tris·HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1:200 protease inhibitor cocktail set III (Calbiochem, San Diego, CA), 40 mM β-glycerophosphate, 1 mM orthovanadate, and 1 mM sodium fluoride. Lysate containing 500 μg of protein was incubated with anti-EGFR antibody at 4°C overnight on a rocking platform. Protein A/G Plus agarose (Santa Cruz Biotechnology) was added for 1 h, after which washed immunoprecipitates were separated by SDS-PAGE on 7.5% acrylamide/Tris·HCl gels. Proteins were transferred to nitrocellulose and blotted with anti-phosphotyrosine antibody (clone 4G10; Upstate Biotechnology) and then stripped and reblotted with anti-EGFR antibody. Bound primary antibodies were detected by using peroxidase-conju-
gated secondary antibodies and ECL reagents (Amersham Biosciences, Piscataway, NJ).

Statistical analysis. Differences between means were compared using t-tests or ANOVA with a Tukey post test for significance. P values <0.05 were considered significant. All experiments were repeated at least three times and representative results are shown.

RESULTS

NF-κB is activated in wound-edge RIE-1 cells. To study the cellular signaling pathways involved in reepithelialization of the intestine after epithelial cell denudation, we used the nontransformed rat intestinal epithelial cell line RIE-1 (5, 6) in an in vitro model of epithelial wounding and restitution (8, 37). Given that NF-κB can be activated in response to direct activation or transactivation of the EGFR (25, 26) and that NF-κB is activated in intestinal epithelial cells of the inflamed and ulcerated intestinal mucosa (36, 40), we hypothesized that NF-κB may have a role in regulating intestinal epithelial restitution. As a first step toward testing this hypothesis, we evaluated NF-κB activation in scrape-wounded intestinal epithelial cells. Nuclear proteins from scrape-wounded RIE-1 cells were analyzed by EMSA, using a labeled NF-κB oligonucleotide. Whereas only low levels of constitutive NF-κB binding activity were observed in unwounded monolayers, scrape wounding led to a marked increase in NF-κB activation within 30 min, which subsequently decreased to baseline (Fig. 1A). To determine whether NF-κB activation occurred throughout the monolayer.

Fig. 1. Wound-edge rat intestinal epithelial (RIE-1) cells activate NF-κB. A: confluent monolayers of RIE-1 cells were scrape wounded. At the indicated times, NF-κB activity was determined by EMSA. Specificity of binding was established by incubation of extracts with 100-fold excess unlabeled NF-κB (κB) oligonucleotide or 100-fold excess of an unrelated (mut) oligonucleotide. B: confluent monolayers of RIE-1 cells grown on glass coverslips were scrape wounded. At the indicated times after wounding, monolayers were fixed and the cellular localization of RelA/p65 was determined by indirect immunofluorescence. Photographs show the indicated areas of the monolayer.
after injury or was confined to cells at the wound edge, we analyzed the subcellular localization of the NF-κB RelA/p65 protein in scrape-wounded and control RIE-1 monolayers, because this is a predominant NF-κB subunit that is activated in intestinal epithelial cells in response to proinflammatory stimuli (4, 17). Nuclear fluorescence characteristic of activated and translocated RelA/p65 was confined to cells at the monolayer wound edge and was not seen in cells more than 5–10 cell diameters from the wound edge (Fig. 1B).

Activation of the EGFR in scrape-wounded RIE-1 monolayers. Signaling through the EGFR is known to promote epithelial restitution in murine intestinal epithelial cells (35). Consistent with this, scrape wounding of RIE-1 monolayers induced a marked increase in EGFR tyrosine phosphorylation within 2.5 min, which returned to near baseline levels by 60 min (Fig. 2A). After activation, the EGFR is internalized in endocytic vesicles before hydrolysis and degradation (48). In scrape-wounded RIE-1 monolayers, analysis of the subcellular localization of the EGFR using confocal microscopy revealed internalization of the EGFR into

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**Fig. 2.** Wound edge RIE-1 cells activate the epidermal growth factor receptor (EGFR). A: confluent monolayers of RIE-1 cells were incubated in the absence or presence of AG 1478 (200 nM) for 30 min, after which the monolayers were either scrape wounded or stimulated with EGF (5 ng/ml). Cell lysates were prepared at the indicated times after wounding or 15 min after EGF treatment. The EGFR was immunoprecipitated (IP), and Western blotting (WB) was performed using anti-phosphotyrosine (P-Tyr) antibodies (top). The blot was stripped and reprobed using an anti-EGFR antibody (bottom). B: confluent monolayers of RIE-1 cells were incubated in the absence or presence of AG 1478 (200 nM) for 30 min, after which the monolayers were scrape wounded. After 15 min, the cells were fixed and the subcellular localization of the EGFR was determined by indirect immunofluorescence and laser scanning confocal microscopy.

**Fig. 3.** NF-κB activation downstream of EGFR in scrape-wounded RIE-1 monolayers. A: confluent RIE-1 cells were treated with vehicle (DMSO) or AG 1478 (200 nM), and 30 min later the monolayers were scrape wounded. At the indicated times after wounding, NF-κB activation was assessed by EMSA. B: confluent RIE-1 cells on glass coverslips were left untreated or were treated with the indicated concentrations of AG 1478, and 30 min later the monolayers were scrape wounded. After 2 h, cells were fixed and the subcellular localization of RelA/p65 was determined by indirect immunofluorescence (top). The percentage of wound-edge cells with nuclear RelA/p65 staining was counted (bottom). Data are means ± SD; n = 3 monolayers. *P < 0.05 compared with vehicle-treated monolayers (post test).

**C:** confluent RIE-1 cells on glass coverslips were left untreated (open bars) or treated with AG 1478 (200 nM) (filled bars). After 30 min, the monolayers were scrape wounded and the subcellular localization of RelA/p65 in wound-edge cells was determined at the indicated times after wounding. Values are means ± SD; n = 3 monolayers. *P < 0.05 compared with vehicle-treated monolayers.
dense aggregates only in cells at the wound edge, suggesting that cells only at this location activated this receptor (Fig. 2B). Moreover, pretreatment with AG 1478 inhibited EGFR internalization (Fig. 2B), indicating that receptor internalization depended on auto-phosphorylation.

**Activation of the EGFR results in downstream NF-κB activation.** We next asked whether EGFR activation plays a role in the activation of NF-κB in scrape-wounded RIE-1 cells. For these studies, NF-κB activation was assessed after inhibition of EGFR tyrosine kinase activity with AG 1478. AG 1478 dose-dependently inhibited the activation of NF-κB in scrape-wounded monolayers, as determined by EMSA and RelA/p65 immunofluorescence (Fig. 3, A and B). Maximal inhibition was observed 1–2 h after scrape wounding (Fig. 3C), which was also the time of greatest NF-κB activation in untreated cells (Fig. 1A). Nonetheless, AG 1478 did not completely block NF-κB activation after scrape wounding, even at the highest concentrations. Thus scrape-wound injury of intestinal epithelial cells appears to activate NF-κB via EGFR-dependent and -independent signaling pathways.

**Inhibitors of NF-κB and the EGFR decrease RIE-1 cell migration.** After scrape wounding, RIE-1 cells migrated across the wound edge and filled in the area that was denuded of epithelium by the scrape (Fig. 4A). The number of RIE-1 cells that crossed the wound edge increased linearly over the first 24 h after the injury. As noted in a similar model (8, 37), the rate of cell proliferation, determined by bromodeoxyuridine incorporation, did not differ between migrating and stationary RIE-1 cells over the initial 24-h period (data not shown), indicating that early epithelial restitution in this model is proliferation independent. To determine whether NF-κB activation in scrape-wounded RIE-1 monolayers has functional consequences for cell migration, we first treated scrape-wounded monolayers with two pharmacological inhibitors of NF-κB, the calpain inhibitor ALLN (38) and the antioxidant pyrrolidine dithiocarbamate (39). Both agents dose-dependently inhibited the migration of RIE-1 cells by up to 50% (Fig. 4B) without affecting viability of the RIE-1 cells throughout the monolayers or at the wound edge. This suggests that NF-κB activation regulates targets that promote restitution of the wounded intestinal epithelium. Similarly, AG 1478 decreased intestinal epithelial restitution in scrape-wounded RIE-1 monolayers (Fig. 4C), consistent with prior reports in murine intestinal epithelial cells (35).
To determine whether NF-κB/H9260, the EGFR was expressed and shown in Fig. 6 the restitution of REV-3 and RSR-6 monolayers. As restitution, we examined the effect of AG 1478 on transcriptional mediator of EGFR-dependent epithelial restitution resulted in rapid activation of NF-κB from scrape-wounded REV-3 and RSR-6 cells. Scrape NF-κB after this stimulus (Fig. 5 REV-3 cells, whereas RSR-6 cells did not activate NF-κB kinase complex, rendering it resistant to degradation. Cells expressing IkB-DD are defective in their capacity to activate NF-κB in response to most stimuli (9, 18, 27). Consistent with this, RelA/p65 remained cytoplasmic in G418-resistant clones transfected with pRCβactIkB-DD after stimulation with tumor necrosis factor-α (Fig. 5A). These cell lines were designated RSR (RIE-1 cells with the NF-κB superrepressor). In contrast, parental RIE-1 cells (data not shown) and all clones transfected with pRCβact, designated REV (RIE-1 cells with empty vector) responded to tumor necrosis factor-α by translocating RelA/p65 to the nucleus. The population doubling times of RIE-1, RSR, and REV cell lines were not significantly different.

To determine whether NF-κB activation due to scrape wounding was inhibited by expression of IkB-DD, we performed EMSA with nuclear extracts from scrape-wounded REV-3 and RSR-6 cells. Scrape wounding resulted in rapid activation of NF-κB in REV-3 cells, whereas RSR-6 cells did not activate NF-κB after this stimulus (Fig. 5B). Comparable results were obtained when NF-κB activation in scrape-wounded REV-3 and RSR-6 cells was assessed by the nuclear/cytoplasmic distribution of RelA/p65 immunofluorescence (data not shown).

To determine whether blocking NF-κB activation by stable expression of IkB-DD inhibited epithelial restitution, we scrape wounded confluent monolayers of REV and RSR cells. Migration of RSR cells was significantly less than that of REV cells (Fig. 5C), confirming that NF-κB activation has an important role in intestinal epithelial cell migration in this model of epithelial restitution.

NF-κB-independent morphogenic functions of EGFR. To determine whether NF-κB activation is the sole transcriptional mediator of EGFR-dependent epithelial restitution, we examined the effect of AG 1478 on the restitution of REV-3 and RSR-6 monolayers. As shown in Fig. 6A, the EGFR was expressed and tyrosine phosphorylated to the same extent in REV-3 and RSR-6 cells after either scrape wounding or EGF treatment, indicating that the lack of NF-κB induction had no effect on EGFR activation. Furthermore, AG 1478 inhibited the migration of REV-3 cells by an average of 40% after 24 h (Fig. 6B), which was comparable to that seen in the wild-type parental RIE-1 cells. In contrast, AG 1478 inhibited the migration of RSR-6 cells, on average, by 14%, which was significantly different from the magnitude of inhibition in REV-3 cells but nonetheless constituted a statistically significant level of inhibition compared with untreated cells. Together, these findings suggest that the EGFR can exert its morphogenic functions through at least two pathways, which differ in their dependence on NF-κB.

DISCUSSION

We report the novel finding that the transcription factor NF-κB has an important role in intestinal epithelial restitution. NF-κB in intestinal epithelial cells regulates the transcription of genes whose products can activate and regulate host innate immune and
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inflammatory responses (16, 18, 23, 27). NF-κB also regulates the transcription of genes whose products have survival effects when cells are exposed to proapoptotic stimuli (28). The novel finding herein that NF-κB promotes the restitution of wounded intestinal epithelial cell monolayers in a model of epithelial injury and repair suggests that NF-κB activation may also promote ulcer restitution and healing, which was a previously unrecognized function for NF-κB in the intestine.

The activation of NF-κB and its effects on the motogenic response occurred downstream of EGFR activation. Given that EGFR ligands were not added to wounded monolayers, activation of the EGFR following scrape wounding could involve the release of EGFR ligands from wounded cells, possibly by ectodomain shedding of EGFR ligands (3, 32, 42). Alternatively, the EGFR may be transactivated in the absence of exogenous ligands by intracellular signaling; for example, from integrins or in response to increases in intracellular Ca²⁺ (29, 46). Downstream of the EGFR, activation of phospholipase C-γ1, protein kinase C (35), and mitogen-activated protein kinases have been reported to play a role in intestinal epithelial restitution of a murine intestinal epithelial cell line (10, 19, 24). Consistent with a recently described pathway (25, 26), these or other signaling intermediates may be involved in the transduction of motogenic signals from the EGFR to NF-κB. Whereas blocking EGFR phosphorylation with the tyrosine kinase inhibitor AG 1487 markedly decreased epithelial restitution in wounded monolayers, some RIE-1 cells nonetheless continued to migrate across the wound edge under those conditions, and NF-κB activation was still noted in a small fraction (15–20%) of wound-edge cells. Whether this comprises a sufficient motogenic signal or, alternatively, activation of the EGFR and NF-κB are not absolute requisites for epithelial cell migration in wounded monolayers is not known. However, in this regard we note that wild-type intestinal trefoil factor, which is produced by intestinal epithelia and can activate the EGFR, as well as intestinal trefoil factor containing mutations rendering the molecule defective in activating the EGFR, promotes epithelial restitution (11, 30).

A role for NF-κB in epithelial wound healing has important therapeutic implications for patients with diseases in which NF-κB is activated in intestinal epithelial cells (e.g., inflammatory bowel disease and diverticulitis) (36, 40). Agents such as glucocorticoids (2) and aminosalicylates (17, 43) that are used for treating intestinal inflammation are known inhibitors of NF-κB activation in vitro. Nonetheless, it is not known whether the relevant mechanism of action of these drugs on disease activity in vivo is mediated through NF-κB inhibition or whether their use promotes the healing of epithelial ulcers. Notably, aspirin, a highly ulcerogenic drug, blocks NF-κB activation though the inactivation of the inhibitory κB kinase complex (49), suggesting that it may facilitate ulceration by inhibiting NF-κB required for epithelial restitution.

Nitric oxide and prostaglandins, whose production is regulated, in part, by the expression of the NF-κB target genes NOS2 (inducible nitric oxide synthase) (22) and COX2 (cyclooxygenase-2) (47), respectively, were reported to promote migration of other nontransformed epithelial cells (15, 34). Thus NOS2 and COX2 are candidate mediators of the motogenic effects of NF-κB in scrape-wounded RIE-1 monolayers. NF-κB was noted recently to promote the migration of vascular smooth muscle cells (44, 45) and invasive breast cancer cells, the latter being due to the NF-κB-dependent secretion of plasminogen activator (41). Thus it will be important in future studies to define the relevant target genes responsible for the novel motogenic properties of NF-κB.

In summary, NF-κB activation is shown to be an important downstream regulator of intestinal epithelial cell migration in response to epithelial wounding. This highlights a possible role for intestinal epithelial NF-κB as a key transcription factor in the healing process of inflammatory diseases of the intestinal tract that are associated with mucosal ulceration. Importantly, to the extent that NF-κB may have parallel functions in vivo, our data further suggest that therapies designed to inhibit NF-κB as a strategy to attenuate the inflammatory response (33) potentially could have adverse effects on epithelial wound healing.

Fig. 6. Effect of AG 1478 on migration of REV-3 and RSR-6 cells. A: confluent monolayers of REV-3 or RSR-6 cells were left untreated (0), scrape wounded, or stimulated with EGF (5 ng/ml). After 2.5 min, the EGFR was immunoprecipitated, and Western blotting was performed using P-Tyr antibodies (top). The blot was stripped and reprobed using an anti-EGFR antibody (bottom). B: confluent monolayers of REV-3 or RSR-6 cells were incubated in medium containing DMSO (open bars) or AG 1478 (200 nM; filled bars) for 30 min, after which the monolayers were scrape wounded. The numbers of cells that had crossed the wound edge were counted after 24 h. For each cell line, values are shown as the percentage of migration that occurred in vehicle-treated controls. Values are means ± SD; n = 3 wounds. *P < 0.05 for effect of AG 1478 on REV-3 and RSR-6 migration and for comparison of the percentage inhibition of migration by AG 1478 between REV-3 and RSR-6 cell lines (t-test).
REFERENCES


