Epidermal growth factor receptor signaling modulates apoptosis via p38α MAPK-dependent activation of Bax in intestinal epithelial cells

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Sheng G, Guo J, Warner BW. Epidermal growth factor receptor signaling modulates apoptosis via p38α MAPK-dependent activation of Bax in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 293: G599–G606, 2007. First published July 5, 2007; doi:10.1152/ajpgi.00182.2007.—Previous studies have demonstrated that the proapoptotic protein Bax plays an important role in the elevated enterocyte apoptosis that occurs during the intestinal adaptation response to massive small bowel resection (SBR). Additionally, epidermal growth factor receptor (EGFR) activation prevents SBR-induced enterocyte apoptosis. The present study aims to delineate the relationship between EGFR activity and intestinal epithelial cell apoptosis. Treatment of model intestinal epithelial cells (RIEC-18) with both a selective EGFR inhibitor (ZD1839) and EGFR small interfering RNA knockdown resulted in a dramatic increase in apoptosis, accompanied by rapid phosphorylation of p38α. Concurrently, Bax underwent conformational changes consistent with activation and translocated to mitochondria. In contrast, EGFR stimulation enhanced cell survival by attenuating p38α phosphorylation, Bax conformational change, mitochondrial trafficking, and apoptosis. These results demonstrated that diminished EGFR activity initiates the intrinsic pathway of apoptosis through p38α-dependent Bax activation in intestinal epithelial cells. These findings provide mechanistic insight into the role that EGFR signaling plays in the regulation of enterocyte apoptosis following massive intestinal loss.

intestinal adaptation; short bowel syndrome

FOLLOWING THE RESECTION OF significant intestinal length as required to treat multiple conditions such as midgut volvulus, trauma, or necrotizing enterocolitis, the remaining bowel undergoes a crucial compensatory response termed adaptation. During this process, there is a stimulus for enterocyte proliferation that contributes to the generation of taller villi, deeper crypts, and a greater content of DNA, RNA, and protein per unit length (5, 17). Through these morphological alterations, the remaining intestine attempts to compensate for the loss of mucosal surface area by increasing its capacity for absorption and digestion per unit length.

In addition to increased rates of enterocyte proliferation, our laboratory was the first to demonstrate enhanced enterocyte apoptosis during resection-induced adaptation (13). This observation has been confirmed by other investigators (12, 22–24). Given the highly dynamic nature of the intestinal mucosa, elevated rates of apoptosis probably serve to counter the increased rates of enterocyte proliferation, thus preserving a critical homeostatic balance. Although much attention has focused on understanding the regulation of enterocyte proliferation, few studies have addressed the mechanism for increased apoptosis during this process.

Members of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases serve as critical mediators of the cellular communication network regulating complex biological processes such as growth, differentiation, motility, and death (11, 20). Over the past decade, our laboratory has illuminated a central role for EGFR signaling in the genesis of resection-induced adaptation (26). In addition to affecting rates of enterocyte proliferation, EGFR activity was also found to regulate enterocyte apoptosis after massive small bowel resection (SBR) (14, 18). In wave-2 mice with hypofunctional EGFR activity, apoptosis is markedly enhanced both at baseline and following a massive SBR (14, 15). Conversely, stimulation of the EGFR either by exogenous administration of EGF or in EGF transgenic mice is associated with diminished apoptotic response to SBR (14).

An important class of molecules that regulate apoptosis within complex mammalian cells is the Bcl-2 family of intracellular proteins. By performing intestinal resections in Bax-null mice, we established that Bax was the proapoptotic Bcl-2 family member required for resection-induced enterocyte apoptosis (1, 21). Despite strong evidence for both EGFR signaling and Bax as major regulators of enterocyte apoptosis, a mechanistic link between these two factors is poorly characterized. This is the very basis for the present study.

The p38 mitogen-activated protein kinase (MAPK) is a member of the MAPK pathway and is activated in response to various of physical and chemical stresses, such as oxidative stress, UV irradiation, hypoxia, ischemia, and various cytokines, including interleukin-1 (IL-1) and tumor necrosis factor-α (3). Additionally, it has been shown that p38 phosphorylation plays an important role in enterocyte migration and EGFR degradation following stimulation (6). In preliminary studies from our laboratory, we have revealed that p38 MAPK phosphorylation and activity were significantly enhanced within intestinal crypts following massive SBR. This finding coincided with elevated enterocyte apoptosis in the crypts and suggested a potential role for p38 in resection induced enterocyte apoptosis.

Until recently, the potential interaction between p38 and Bcl-2 family has been poorly understood. A recent study suggested that p38 was required to induce a conformational change in Bax, thereby allowing for its translocation to mitochondria and cytochrome c release following UV damage (25). Our present study attempted to explore the relationship between EGFR modulation of Bax-dependent enterocyte apop-

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tosis via p38, thus providing a novel mechanism for EGFR-regulated enterocyte apoptosis after SBR. Since our in vivo experimental model focuses on physiological processes in normal enterocytes, it is particularly relevant to study the relationship between EGFR signaling and its effect on enterocyte apoptosis by using nontransformed cells. We chose IEC-18 because it is a nontransformed cell line derived from rat terminal ileum and corresponds with the same anatomical location where we observe maximal responses to resection-induced intestinal adaptation.

MATERIALS AND METHODS

Cell culture and materials. IEC-18 cells from ATCC were cultured in DMEM supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. Cells used in experiments were between passage 15 and 20. The EGFR inhibitor ZD1839 was obtained from AstraZeneca (Wilmington, DE). Another EGFR inhibitor, AG1478, and p38 inhibitors SB203580 and SB239063 were purchased from Biosource (Camarillo, CA). Monoclonal anti-Bax 6A7 antibody was purchased from BD Pharmingen (San Diego, CA). Polyclonal anti-EGFR antibody directed against mouse EGFR was generated with a synthetic peptide corresponding to residues 1158–1175 of mouse EGFR as the immunogen. This sequence is unique to EGFR and is identical in rat and mouse, conserved in human and mouse (95% identity). The anti-EGFR serum from rabbit was further affinity purified against the original peptide and specificity tested by Western blot. Polyclonal Bax and phosphorylation-specific p38 antibodies were from Cell Signaling (Beverly, MA).

Western blot and immunoprecipitation. IEC-18 cells were lysed in 1× SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and 5% mercaptoethanol). For immunoprecipitation, the cells were washed with ice-cold PBS and then lysed for 30 min in lysis buffer containing 10 mM HEPES, 150 mM NaCl, and 1% CHAPS plus protease inhibitor tablet (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail I/II (Sigma). The lysates were then centrifuged at 20,000 g for 30 min at 4°C. The supernatant was incubated with protein A plus G beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h (preclearing) at 4°C. Following this, the beads were spun down and discarded, and the supernatants were further incubated with protein A plus G beads and appropriate antibody overnight at 4°C. After being washed in lysis buffer, the absorbed complexes were removed from the beads by heating for 5 min at 100°C in 1× SDS sample buffer and separated on 10% polyacrylamide gels. Proteins resolved on gels were transferred to nitrocellulose membranes and detected with appropriate antibodies by Western blot.

Apoptosis assay. Apoptosis was measured in cells by using an apoptosis ELISA kit (Roche) according to the protocol of the manufacturer. Briefly, IEC-18 cells were induced to undergo apoptosis according to the protocol. Cells were lyzed and fragmented DNA

Fig. 1. A: IEC-18 cells were grown to 80% confluence in 1% FBS in DMEM. To inhibit EGF receptor (EGFR) activity, 2 different concentrations of ZD1839 were used. To enhance EGFR activity, 5 different concentrations of EGF were used to stimulate cells. Cells were harvested 4 h after treatment, and apoptosis was measured by DNA fragmentation ELISA. Results were plotted as fold changes over vehicle (DMSO)-treated cells. Con, control. B: IEC-18 cells were treated with AG1478, a selective EGFR inhibitor, at varied concentrations. Apoptosis was measured 4 h later by DNA fragmentation ELISA and results displayed as fold changes over vehicle (DMSO)-treated cells. C: IEC-18 cells were grown to 50% confluence and transfected with EGFR small interfering RNA (siRNA) at 3 different concentrations for 3 days. Control (scrambled) siRNA were also transfected into a separate group of cells for experimental control. Apoptosis was measured 4 h later by DNA fragmentation ELISA and results were displayed as fold changes over vehicle (DMSO)-treated cells. *P < 0.05 vs. control.
was separated from intact DNA by centrifugation. The amount of fragmented DNA was recognized by a specific antibody against DNA binding proteins and results analyzed by ELISA.

Immunocytochemistry. IEC-18 cells were grown on coverslips in six-well culture plates. Following induction of apoptosis, cells were incubated with 250 nM of MitoTracker Red (Cambrex, Walkersville, MD) for 15 min and then fixed with 4% formalin in PBS and permeabilized with 1% CHAPS. After being blocked with 3% BSA cells were incubated with 1:300 of Bax (6A7) antibody for 1 h in 37°C incubator. After three washes, cells were incubated for 45 min in secondary antibody. Cell nuclei were stained with DAPI for 10 min, slides were mounted, and images were recorded under ×100 and ×400 magnification.

siRNA transfection. Roughly 1 × 10^6 IEC-18 cells were transfected with EGFR, p-38α or Bax small interfering RNA (siRNA; SMARTpool reagent; Lafayette, CO). Control was provided by transfection with a nontargeting RNA (siCONTROL nontargeting siRNA; Dharmacon; Lafayette, CO) at 25 nM concentration with X-tremeGENE (Roche), protocol. Cells were collected 72 h after transfection and used for experiments. For EGFR silencing, EGFR siRNAs were transfected into IEC-18 cells at three different concentrations (10, 15, and 25 nM). To confirm silencing, cells were collected and Western blots were performed to measure target protein expression.

Mitochondrial isolation. For each experiment, 5 × 10^7 cells were collected. Mitochondrial and cytosolic fractions were separated by using a Mitochondrial fractionation kit (Active Motif, Carlsbad, CA). After fractionation, protein concentration in each fraction was measured. Western blot analysis of each fraction was performed.

Statistics. For comparisons of mean values between two groups, Student’s t-test was used. For comparisons of mean values between more than two groups, a one-way analysis of variance was employed. Statistical significance was established at a P value of <0.05.

RESULTS

EGFR activity modulates apoptosis in intestinal epithelial cells. To study the apoptotic response of intestinal epithelial cells with regard to EGFR activity, IEC-18 cells were treated with either EGF or with EGFR inhibitors (ZD1839 or AG1478) at different concentrations. Inhibition of the EGFR was additionally accomplished by siRNA-directed suppression of EGFR expression. Compared with control, EGF stimulation resulted in dose-dependent increases in EGFR phosphorylation and attenuated levels of apoptosis (Fig. 1A). To ensure that EGFR inhibitor-induced apoptosis was not specific to ZD1839, another selective EGFR inhibitor (AG1478) was shown to induce apoptosis in IEC-18 cells in dose-dependent fashion.
Furthermore, knockdown of EGFR protein expression using siRNA resulted in dose-dependent increase in RIEC-18 apoptosis (Fig. 1C).

**EGFR inhibition-induced apoptosis is associated with activation of p38 MAPK and Bax.** Treatment of the cells with the EGFR inhibitor ZD1839 (10 μM) resulted in a significant reduction in baseline EGFR activity as gauged by its phosphorylation status (Fig. 2A). Concurrently, phosphorylation of p38 increased during EGFR inhibition whereas total p38 protein expression remained unchanged. Inhibition of the EGFR was also accompanied by accumulation of the conformationally active form of Bax (6A7) but without change in the expression of native Bax. Cleaved caspase-3 was also elevated and corresponded with the induction of apoptosis. Knockdown of EGFR expression using siRNA similarly resulted in a concentration-dependent suppression of EGFR expression, increased p38 phosphorylation, and activation of Bax (Fig. 2B). Alternatively, stimulation with EGF resulted in dose-dependent phosphorylation of the EGFR, decreased phosphorylation of p38, reduction in Bax activity, as well as diminished cleaved caspase-3 accumulation (Fig. 2C).

**EGFR inhibition induces mitochondrial trafficking of activated Bax.** Bax activation results in initiation of the intrinsic apoptotic pathway by increasing mitochondrial permeability and release of cytochrome c. Following EGFR inhibition, activated Bax became concentrated within the mitochondrial fraction and was reduced in the cytosolic fraction (Fig. 3A). Additionally, cytosolic cytochrome c was increased, consistent with increased release from the mitochondria. The mitochondrial trafficking of activated Bax was confirmed by immunocytochemistry (Fig. 3B). Together, these findings confirm that EGFR inhibition leads to the activation of the intrinsic apoptotic pathway.
EGFR activity induces p38 activation, which in the context of attenuated Bax expression is insufficient to trigger apoptosis.

**EGFR inhibition induces specific p38α subunit phosphorylation.** The p38 MAPK has four distinct isoforms (α, β, δ, and γ). To determine the most important isoform, phosphorylated p38 was immunoprecipitated after EGFR inhibition and specific isoforms were analyzed by Western blotting. As shown in Fig. 5, all four isoforms were detectable in IEC-18 cells; however, only the α-subunit was present within the pulled-down phosphorylated fraction of p38. These findings strongly suggest that the p38α isoform is the most relevant downstream target of EGFR inhibition.

**Inhibition of p38 MAPK results in reduced Bax activity and apoptosis.** To explore a potential link between p38 phosphorylation and Bax activation, IEC-18 cells were pretreated with a selective inhibitor of p38 (SB203580) prior to EGFR inhibition. The results revealed that when p38 phosphorylation was pharmacologically inhibited, EGFR inhibition failed to initiate a significant change in Bax activation (Fig. 6A). Furthermore, the apoptotic response to EGFR inhibition was greatly reduced.

**Pharmacological inhibition of p38 prevents Bax translocation to mitochondria.** As shown earlier, conformationally active Bax translocated to mitochondria following EGFR inhibition. Using immunocytochemistry, we now show that when p38 activity was inhibited pharmacologically, the perturbed EGFR signal to direct mitochondrial translocation of Bax was greatly reduced (Fig. 6B).

**P38α is required for Bax activation and apoptotic response following EGFR inhibition by ZD1839.** Having established that EGFR inhibitor-induced apoptosis in IEC-18 cells involved phosphorylation of p38 as well as activation and translocation of Bax, we next silenced p38α expression using siRNA.

**Bax expression is required for induction of apoptosis by EGFR inhibition.** To verify that Bax is necessary for the induction of apoptosis associated with EGFR inhibition, Bax expression was suppressed in IEC-18 cells by use of siRNA. Significant knockdown of Bax expression was achieved by siRNA when compared with control siRNA-transfected cells (Fig. 4A). The cells with attenuated Bax expression were resistant to apoptosis induced by pharmacological inhibition of the EGFR when compared with cells with normal levels of Bax (Fig. 4B). Nonetheless, p38 phosphorylation in response to EGFR inhibition was not affected in the context of reduced Bax expression (Fig. 4A). These findings support the notion that Bax is required for the apoptotic response to EGFR inhibition. Furthermore, these data suggest that perturbed

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**Fig. 4.** A: Bax expression was suppressed by siRNA transfection for 3 days. After confirmation of gene knockdown by Western blot, cells were treated with ZD1839 (10 µM). Effect of Bax expression knockdown on p38 phosphorylation and Bax activation were evaluated with Western blot and immunoprecipitation, respectively. Initiation of apoptosis was measured by cleaved caspase-3 expression. B: DNA fragmentation ELISA was performed at the same time to evaluate cell death. *P < 0.05 vs. cells not treated with ZD (−ZD).

**Fig. 5.** Following treatment of IEC-18 cells, phosphorylated p38 was immunoprecipitated (IP). Different isoforms of p38 were quantified by Western blot. Western blot analysis of total p38 was used to confirm equal loading.
ENTEROCYTE APOPTOSIS INVOLVES Bax AND p38

**A**

Fold Change in Apoptosis

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<th>Condition</th>
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<td>*ZD1839</td>
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- Active BAX
- Total BAX

**B**

Images showing the effect of ZD1839 on BAX localization:

- ZD1839:
  - Mitochondria
  - Activated Bax
  - Merged Image

- ZD:
  - Mitochondria
  - Activated Bax
  - Merged Image

- SB+ZD:
  - Mitochondria
  - Activated Bax
  - Merged Image

**C**

Western blot images showing the effect of ZD1839 on various proteins:

- P-P38
- P38α
- Active BAX
- BAX
- ERK
- Caspase-3
- Cleaved Caspase-3

**D**

Fold Change in Apoptosis

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* P-value < 0.05
Transfection of IEC-18 cells with p38α siRNA for 3 days resulted in over 90% reduction in its expression with minimal overall toxicity when compared with control siRNA-transfected cells (Fig. 6C). When cells were treated with ZD1839, significant phosphorylation of p38 occurred in the control siRNA-transfected group, whereas in p38α-silenced cells minimal phosphorylation was detected. Although expressions of Bax and total caspase-3 were unchanged by either siRNA transfection or ZD1839 treatment, the EGFR inhibitor-induced Bax activation and caspase-3 cleavage were reduced significantly in the absence of p38 phosphorylation. Additionally, ZD1839-induced increase in intestinal epithelial cell apoptosis was also significantly suppressed in the background of attenuated p38 expression (Fig. 6D).

**DISCUSSION**

Similar to what we have observed using a mouse model of massive intestinal resection in vivo, we have verified in the present study that EGFR signaling plays an important regulatory role for intestinal epithelial cell apoptosis. Suppression of EGFR activity in IEC-18 cells by either siRNA targeting of EGFR expression or pharmacological use of two different inhibitors resulted in significantly enhanced apoptosis and trafficking of activated Bax to the mitochondria. The magnitude of apoptosis directly correlated with EGFR inhibitor concentration and the extent of EGFR inhibition. On the other hand, when EGFR activity was enhanced by ligand-dependent stimulation, apoptosis was decreased to below baseline. We also showed that Bax expression is required for the apoptotic response to EGFR inhibition. Finally, we revealed that, in the context of EGFR inhibition, p38α MAPK is activated and is necessary for the activation and migration of Bax to the mitochondria to induce apoptosis. Collectively, these dataendorse an important role for EGFR signaling to regulate intestinal epithelial cell apoptosis via a pathway of p38α MAPK-dependent activation of Bax.

Although EGFR stimulation generally leads to activation of various downstream signal transduction pathways to include phosphatidylinositol 3-kinase (PI3K), signal transducer and activator of transcription (STAT), and MAPK, EGFR inhibition would be predicted to attenuate downstream signaling pathways. Indeed, we demonstrated EGFR inhibition resulted in impaired phosphorylation of PI3K/Akt, STAT-3, and ERK1/2 (data not shown). However, phosphorylation of p38, but not JNK, was significantly enhanced following EGFR inhibition in our cell line. Conversely, p38 activity was significantly reduced when EGFR was activated, indicating that EGFR activity paradoxically affected the p38 pathway. Although it is generally believed that phosphorylation of p38 is mediated by upstream kinase MAP kinase kinase (MKK) 3/6 in response to variety of stresses (8), we found that MKK3/6 phosphorylation status in response to EGFR modulation remained unchanged (data not shown). Consequently, we believe that phosphorylation of p38 in response to EGFR inhibition was independent of MKK. Interestingly, it has been demonstrated that p38α may undergo autophosphorylation, independent of MKK (9). Another potential link between EGFR inhibition and p38 phosphorylation has been delineated recently in cancer cell lines, suggesting that CARP-1, a novel inducer of apoptosis, played a role in induction of p38 phosphorylation following EGFR inhibition (19). Further studies are needed to understand the precise mechanism by which EGFR activity modulates p38 phosphorylation in intestinal epithelial cells.

With regard to the induction of apoptosis in intestinal epithelial cells, a more established cell survival pathway is mediated by the action of Akt under the regulation of PI3K. In this paradigm, Akt phosphorylates Bax, thus prohibiting its conformational change and mitochondrial translocation to facilitate cell survival (7). Since the PI3K/Akt pathway is directly regulated by the EGFR, we also investigated the role Akt played in the apoptotic response of intestinal epithelial cells in response to EGFR modulation. Although EGFR stimulation resulted in sustained Akt phosphorylation, EGFR inhibition suppressed Akt phosphorylation only briefly (less than 30 min; data not shown). Since significant Bax conformational changes were not detected until 2 h after EGFR inhibition and sustained p38 phosphorylation occurred slightly earlier, it can be inferred that PI3K/Akt pathway probably played a minor role in the induction of apoptosis following EGFR inhibition.

Although p38 MAPK has four isoforms, we illustrated that p38α phosphorylation and expression were required for Bax conformational changes, its mitochondrial translocation, and subsequent induction of apoptosis following EGFR inhibition. The mechanism by which p38α induces Bax activation is presently unknown. Most recently, Bax activation has been demonstrated in hepatoma cell lines via p38-dependent phosphorylation (16). However, it is still unclear whether p38 and Bax can directly interact with each other, because we have failed to coimmunoprecipitate p38 and Bax together. Although other studies have suggested potential links between p38 and Bax activation in response to ionizing radiation (4) or agents that induce the production of reactive oxygen species (10), we believe that this is the first observation that EGFR modulation regulates apoptosis through p38α-dependent activation of Bax in nontransformed intestinal epithelial cells. Although the importance of p38 in resection-induced apoptosis is strongly suggested by its significant phosphorylation after SBR, additional studies are being conducted in p38α-null mice to further characterize its role in vivo. Through these findings, we propose a novel mechanism for small intestinal homeostasis in which EGFR activity plays a crucial role in the balance between survival and death of enterocytes via regulation of p38α phosphorylation and subsequent conformational changes.
in Bax. This mechanism opens the possibility for novel therapeutic options in which pharmacological modulation of EGFR or p38α to attenuate apoptosis could enhance the adaptive response of small intestine. Along these lines, we have already demonstrated that combined therapy to attenuate apoptosis (in Bax-deficient mice) as well as to stimulate enterocyte proliferation and attenuate apoptosis further (by EGF treatment) results in a magnified adaptation response to massive SBR (2). Translation of these data into humans could possibly result in significant clinical improvement by regaining valuable absorptive surface area following the loss of large sum of small intestinal length.

REFERENCES


