Tumor necrosis factor inhibits ligand-stimulated EGF receptor activation through a TNF receptor 1-dependent mechanism

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McElroy SJ, Frey MR, Yan F, Edelblum KL, Goettel JA, John S, Polk DB. Tumor necrosis factor inhibits ligand-stimulated EGF receptor activation through a TNF receptor 1-dependent mechanism. Am J Physiol Gastrointest Liver Physiol 295: 285–293, 2008. First published May 8, 2008; doi:10.1152/ajpgi.00425.2007.—Tumor necrosis factor (TNF) and epidermal growth factor (EGF) are key regulators in the intricate balance maintaining intestinal homeostasis. Previous work from our laboratory shows that TNF attenuates ligand-driven EGF receptor (EGFR) phosphorylation in intestinal epithelial cells. To identify the mechanisms underlying this effect, we examined EGFR phosphorylation in cells lacking individual TNF receptors. TNF attenuated EGF-stimulated EGFR phosphorylation in wild-type and TNFR2−/−, but not TNFR1−/−, mouse colon epithelial (MCE) cells. Reexpression of wild-type TNFR1 in TNFR1−/− MCE cells rescued TNF-induced EGFR inhibition, but expression of TNFR1 deletion mutant constructs lacking the death domain (DD) of TNFR1 did not, implicating this domain in EGFR downregulation. Blockade of p38 MAPK, but not MEK, activation of ERK rescued EGF-stimulated phosphorylation in the presence of TNF, consistent with the ability of TNFR1 to stimulate p38 phosphorylation. TNF promoted p38-dependent EGFR internalization in MCE cells, suggesting that desensitization is achieved by reducing receptor accessible to ligand. Taken together, these data indicate that TNF activates TNFR1 by DD- and p38-dependent mechanisms to promote EGFR internalization, with potential impact on EGF-induced proliferation and migration key processes that promote healing in inflammatory intestinal diseases.

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The single layer of epithelial cells lining the gut forms an essential barrier to a wide range of noxious substances found in the intestinal lumen. Integrity of this barrier is regulated by a number of soluble growth factors and cytokines (35, 47). Imbalances in the expression of molecules such as tumor necrosis factor (TNF) or epidermal growth factor (EGF) have been observed both clinically and in experimental models of inflammatory bowel disease (IBD) and necrotizing enterocolitis (NEC) (11, 19, 39, 42); such dysregulated signal transduction may contribute to defects in mucosal repair and potentiate disease states.

TNF, a potent proinflammatory cytokine, is produced as a 26-kDa homotrimeric transmembrane precursor that is cleaved by metalloproteinase (MMP) activity into a soluble 17-kDa polypeptide (6, 7, 33) to bind two distinct transmembrane receptors, the 55-kDa low-affinity TNF receptor (TNFR) 1, and the 75-kDa high-affinity TNFR2 (26, 28, 31). These two receptors mediate distinct effects in intestinal epithelial cells, with TNFR1 promoting growth arrest and cytokine-mediated inflammation and TNFR2 promoting cellular proliferation and migration (14, 28, 29). These diverging affinities and responses suggest preferential binding to TNFR2 at physiological concentrations and increased relative TNFR1 activation during states of heightened TNF levels such as inflammation (44, 49).

TNFR1 has two well-characterized cytoplasmic domains, the Death Domain (DD) and the neutral sphingomyelinase domain (NSD) (1). The DD is an 80-amino acid span localized to the COOH-terminal portion of the receptor. It is thought to be critical for generation of cytotoxic death signals, anti-viral responses, and acid sphingomyelinase activation, which promotes inflammation and apoptosis through induction of NF-κB and other pathways (5, 43, 45). The NSD is a nine-amino acid motif immediately adjacent to the DD (1). Activation of the NSD causes activation of inflammatory signals including MAPK pathways (1, 2, 46).

To maintain both absorptive and barrier functions in the face of inflammation or other damage, intestinal epithelial tissues express a number of molecules that drive restitution and repair. EGF receptor (EGFR, aka ErbB-1), a well-characterized promoter of intestinal cell growth and response to damage, is a 170-kDa transmembrane protein containing an intrinsic cytoplasmic tyrosine kinase domain and docking sites for various signaling effector molecules (4, 27). Following binding to EGF or other ligands, the tyrosine phosphorylation on the cytoplasmic tail increases and stimulates a variety of downstream cascades. Signal termination is accomplished through receptor internalization, ubiquitinylation and proteolytic degradation, and/or inactivation by tyrosine phosphatases. EGFR is widely expressed in mammalian epithelial tissues and initiates signals for cellular growth and survival, cellular migration, and wound healing (8, 20, 24, 41, 60). During intestinal inflammation, EGF has been used effectively in the treatment of rodent models of NEC, as well as in clinical trials for ulcerative colitis (19, 42), suggesting that this signaling axis is an important target for IBD and NEC therapy.

In addition to their individual effects on cell growth, proliferation, and apoptosis, TNF and EGF regulate intestinal homeostasis via complex signaling cross-talk mechanisms. TNF, for example, promotes MMP activity, leading to release of EGF family members and promotion of EGFR activation (32, 37, 53). In contrast, previous findings in our laboratory have shown that TNF decreases EGF-stimulated EGFR phosphorylation (28). Similarly, a recent report by Yarden and
colleagues (59) demonstrated p38-dependent TNF-induced internalization of EGFR in two cancer cell lines. Here we define TNF inhibition of EGF-stimulated EGFR phosphorylation as a TNFR1 DD-dependent process. Furthermore, both TNF-induced EGFR inhibition and internalization require p38 MAPK activity. These findings have important implications for understanding injury repair mechanisms in a high TNF environment, such as the gastrointestinal mucosa in a number of inflammatory disorders.

MATERIALS AND METHODS

Cells. Conditionally immortalized mouse colon epithelial (MCE) cells were established from the colonic epithelium of H-2Kb-tsA58 Immortomice by Robert Whitehead at the Vanderbilt University Digestive Disease Research Center novel cell line core (54). These cells express a heat-labile SV40 large T antigen under the control of an interferon-γ-inducible promoter. Young adult mouse colon (YAMC), TNFR1−/− MCE, TNFR2−/− MCE, and EGFR−/− MCE knockout cells were generated from TNFR1, TNFR2, or EGFR-null mice crossed with Immortomice as previously described (14). Receptor deletion was confirmed by PCR and immunofluorescence analysis. Intestinal epithelial cell (IEC)-6 cells are a well-known epithelial cell line derived from rat small intestine (American Type Culture Collection, Manassas, VA).

Cell culture. YAMC and EGFR−/− MCE cells were maintained on rat tail collagen-coated plates (Mediatech, Herndon, VA). All cells were maintained as a monolayer in RPMI 1640 medium with 5% fetal bovine serum, 5 U/ml mouse interferon-γ (Intergen, Norcross, GA), 100 U/ml penicillin and streptomycin, 5 μg/ml transferrin, and 5 μg/ml selenous acid (BD Biosciences, San Jose, CA) at 33°C (permissive conditions) under 5% CO2. Before experiments, cells were transferred to RPMI 1640 medium with 0.5% fetal bovine serum and 100 U/ml penicillin and streptomycin at 37°C (nonpermissive conditions) for 16–18 h.

Treatment protocol. After incubation in nonpermissive conditions, cells were pretreated with TNF (100 ng/ml) for 45 min and then treated with EGF (10 ng/ml) for 1 min, unless otherwise noted. Inhibitors (SB220025 or U0126, 10 μM each) were added to the media 30 min before TNF treatment. TNFR1 agonist antibody was used at 2 μg/ml and was added to media 45 min before treatment with EGF. In all experiments, cells remained in nonpermissive conditions during treatments.

Antibodies, inhibitors, cytokines, and growth hormones. Murine TNF was purchased from PeproTech (Rocky Hill, NJ). Mouse EGF was a gift from Stanley Cohen (Vanderbilt University, Nashville, TN). EGF phosho-specific antibodies P-Y845, P-Y1045, P-Y1068, and P-Y1173, anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies, phospho-Akt, and phospho-focal adhesion kinase (FAK) antibodies were purchased from Cell Signaling Technology (Beverly, MA). We have previously characterized the EGFR phosho-specific antibodies for specificity (23). Anti-EGFR antibody was purchased from Upstate Biotechnology (Charlottesville, VA). Anti-active ERK polyclonal antibody and the MEK inhibitor U0126 were purchased from Promega (Madison, WI). Horseradish peroxidase-conjugated mouse anti-phosphotyrosine (PY20) was purchased from BD Transduction Laboratories (Franklin Lakes, NJ). Anti-FAK, anti-TNFR1, and anti-TNFFR2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human TNFR1 agonist antibody was purchased from R&D Systems (Minneapolis, MN). The p38 inhibitor SB220025 was purchased from Calbiochem (La Jolla, CA). The proteasome inhibitor MG132 was purchased from Sigma Aldrich (St. Louis, MO).

Cell lysates and Western blotting. Cell monolayers were washed twice with ice cold PBS and scraped on ice cold lysis buffer (1% Triton, 10% 200 μM HEPES, pH 7.4). Cellular lysates were cleared and boiled in Laemmli sample buffer (30). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h in Tris-buffered saline (50 mM Tris, 150 mM NaCl, and 25 mM KCl, pH 8.0) with 0.05% Tween 20 (TBST) and 5% nonfat dry milk, incubated with primary antibody overnight at 4°C, and incubated with secondary antibody (except for PY20) for 45 min. Horseradish peroxidase was detected with the Western Lightning enhanced chemiluminescence kit (PerkinElmer Life Sciences, Wellesley, MA).

Immunoprecipitation. Cellular lysates were precleared by incubating with protein AG agarose beads (Santa Cruz Biotechnology) for 30 min followed by centrifugation. Supernatants were incubated with 2 μg anti-EGFR antibody for 1 h at 4°C, and then beads were added for 1 h at 4°C. Immunocomplexes were collected by centrifugation, washed three times in lysis buffer, and boiled in Laemmli sample buffer (3, 30) for SDS-PAGE Western blot analysis.

Constructions. TNFR1 was cloned by RT-PCR with mRNA isolated from YAMC cells to generate full-length wildtype (WT) mouse TNFR1. SNP constructs were generated using the site directed mutagenesis kit TaqMan (Applied Biosystems, Branchburg, NJ). The ΔDD construct was terminated at aa-348, and the ΔCT construct was terminated at aa-228, where CT is cytoplasmic tail. Constructs were cloned into the LZRS retroviral vector and amplified with the use of Phoenix ecotropic packaging cells before infection of TNFR1−/− MCE cells. Infected cells were sorted on the basis of green fluorescent protein expression.

EGFR internalization and fluorescence microscopy. YAMC cells were plated on four-well chamber slides (Nalge Nunc International, Rochester, NY) and treated with SB220025 (10 μM), TNF (100 ng/ml), or SB220025 and TNF together for 45 min. EGF (10 ng/ml, 30 min) served as the positive control for EGFR internalization. Cells were fixed with methanol at −20°C for 5 min, solubilized with PBS + 0.2% Triton X-100, and blocked with 10% goat serum. Slides were stained with anti-EGFR and Cy3-labeled rabbit secondary antibody (Jackson Laboratories, Bar Harbor, ME). EGFR localization was detected by ApoOptome optical sectioning with the use of an Axiovert 200 microscope (Zeiss, Thornwood, NY). TNFR1 and TNFR2 identification by fluorescence microscopy. YAMC, TNFR1−/− MCE, and TNFR2−/− MCE cells were cultured on chamber slides. Cells were washed with PBS and incubated in 10% normal donkey serum (Zymed Laboratories, San Francisco, CA) for 1 h to decrease nonspecific antibody binding. Cells were stained with anti-TNFR1 or TNFR2 (1:1,000) in PBS with 5% donkey serum overnight at 4°C, then with donkey anti-rabbit IgG-FITC (1:2,000, Zymed Laboratories).

Replicates and statistical analysis. All data are representative of at least three independent experiments. Statistical significance of differences between mean values was assessed with a Student’s t-test analysis. Minimum level of significance was set at 0.05.

RESULTS

TNF inhibits EGF-stimulated EGFR phosphorylation. Our laboratory (28) and others (59) have reported that TNF decreases EGF-stimulated EGFR activation. To determine the optimal conditions for TNF inhibition of EGFR phosphorylation, YAMC cells were incubated with TNF for various times (Fig. 1A) and then exposed to EGF for 1 min in this and subsequent experiments, except where otherwise indicated. Whole cell lysates and EGFR immunoprecipitates were prepared and subjected to Western blot analysis for tyrosine-phosphorylated EGFR. TNF substantially attenuated EGFR phosphorylation in response to EGF, with maximal effect seen at 45 min (Fig. 1A). Decreased EGF-induced phosphorylation was observed both in whole cell lysate and immunoprecipitated EGFR (Fig. 1B). To determine whether this response is
phosphosite specific, we performed Western blot analysis using phosphospecific EGFR antibodies that we have previously characterized (Y845, Y1045, Y1068, and Y1173) (23). A marked decrease in phosphorylation was detected when cells were pretreated with TNF before EGF exposure (Fig. 1C). Similar downregulation of EGF-dependent EGFR activation was seen in IEC-6 cells pretreated with TNF and EGF as above and subjected to Western blot analysis for PY-1068. Densitometry shows averaged results from 3 or more experiments. WB, Western blot; IP, immunoprecipitation. *P < 0.04 vs. EGF treatment. EGFR and actin blots shown as loading controls.

Fig. 1. Tumor necrosis factor (TNF) inhibits epidermal growth factor (EGF)-stimulated EGFR receptor (EGFR) phosphorylation. A: young adult mouse colon (YAMC) cells were exposed to 100 ng/ml TNF for the indicated times before 1-min EGF stimulation, and lysates were subjected to Western blot analysis for phosphotyrosine (PY). B and C: lysates or EGFR immunoprecipitates (as indicated) from YAMC cells pretreated with 100 ng/ml TNF for 45 min before EGF exposure were subjected to Western blot analysis with antibodies specific for PY (B), EGFR, or indicated EGFR phosphorylation sites (C). D: intestinal epithelial cell (IEC)-6 cells were treated with TNF and EGF as above and subjected to Western blot analysis for PY-1068. Densitometry shows averaged results from 3 or more experiments. WB, Western blot; IP, immunoprecipitation. *P < 0.04 vs. EGF treatment. EGFR and actin blots shown as loading controls.

Blockade of EGFR phosphorylation by TNF requires TNFR1. Most studies suggest that distinct cellular responses are regulated by TNFR1 and TNFR2. Thus we tested the roles of these receptors in attenuation of EGF-stimulated EGFR activation, using TNFR1−/−/MCE or TNFR2−/−/MCE, which lack their respective TNFRs but express EGFR at levels equal to or higher than in YAMC cells (Fig. 3, A and B). As in

Fig. 2. TNF inhibits a subset of EGFR-stimulated signaling. YAMC cells were pretreated with TNF for 45 min and then exposed to EGF as in Fig. 1. Whole cell lysates were analyzed by Western blot analysis using the indicated phosphospecific antibodies. Focal adhesion kinase (FAK) is shown as loading control. Densitometry shows averaged results from 3 or more experiments. *P < 0.006 vs. EGF treatment.
YAMC cells, which express both TNFR1 and TNFR2, TNF blocked ligand-stimulated EGFR phosphorylation in TNFR2<sup>−/−</sup> MCE cells. In contrast, TNF had no effect on EGFR phosphorylation in cells lacking TNFR1 (Fig. 3C). Furthermore, a TNFR1-specific agonist antibody inhibited EGFR activation as efficiently as TNF in YAMC cells (Fig. 3D).

**TNFR1 mediates EGFR blockade through the DD.** TNFR1 contains two well-characterized signal transduction regulatory domains, the DD and the NSD. We examined their role in TNF-induced EGFR inhibition by infecting mutant human TNFR1 viral constructs lacking the DD (ΔDD) or both the DD and NSD (ΔCT) into TNFR1<sup>−/−</sup> MCE cells. Cells were exposed to EGF and TNF as above, and lysates were subjected to Western blot analysis for EGFR phosphorylation. As shown in Fig. 4, TNF attenuated EGFR activation only in cells expressing TNFR1 containing the DD.

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**Fig. 3.** Tumor necrosis factor receptor 1 (TNFR1) is required for TNF-induced EGFR inhibition. YAMC cells, TNFR1<sup>−/−</sup> mouse colon epithelial (MCE), or TNFR2<sup>−/−</sup> MCE cells were subjected to immunofluorescence analysis for TNF receptor expression (A) and Western blot analysis for EGFR expression (B). C: cells were treated as above, and EGFR phosphorylation was determined. D: YAMC cells were incubated with a TNFR1 agonist antibody for 45 min before EGF stimulation. Densitometry shows averaged results from 3 or more experiments. *P < 0.02 vs. EGF treatment. Actin blot is shown as a loading control.

**Fig. 4.** TNF-induced EGFR inhibition requires the TNFR1 death domain (DD). A: TNFR1<sup>−/−</sup> cells were stably infected with pRK7 vector, wild-type TNFR1, ΔDD TNFR1, or ΔC, TNFR1 constructs, where C<sub>t</sub> is the cycle threshold, as described in MATERIALS AND METHODS, and then expression was validated by RT-PCR. B: mutant TNFR1 cell lines were exposed to TNF and EGF as above. Lysates were subjected to Western blot analysis for EGFR phosphorylation. Densitometry shows averaged results from 3 or more experiments. *P < 0.05 vs. EGF treatment. Actin is shown as a loading control.
Fig. 5. TNFR1-mediated inhibition of EGFR phosphorylation requires p38 MAPK activity. A: YAMC cells were pretreated with p38 inhibitor (SB220025) (SB) 30 min before TNF and EGF as above. Cells were lysed and probed by Western blot analysis for EGFR phosphorylation. B: YAMC cells were pretreated with MEK inhibitor (U0126) and exposed to TNF before EGF. Cells were lysed, and Western blot analysis for EGFR phosphorylation was performed. C: TNFR1Δ-DD cells were transiently transfected with vector, wild-type TNFR1, ΔDD TNFR1, or ΔC, TNFR1. Cells were treated with TNF, lysed, and subjected to Western Blot analysis using antibody specific for phospho (active)-p38. Right: TNFR1Δ-DD MCE and TNFR2Δ-DD MCE cells were treated with TNF for the times indicated, and cellular lysates were analyzed by Western blot analysis for P-p38 and total actin. D: EGFR internalization in YAMC cells was assessed by immunofluorescence localization analysis following 45-min TNF or EGF treatment, with or without SB220025. Densitometry shows averaged results from 3 or more experiments. *P < 0.03 vs. EGF treatment. Actin, total EGFR, and total p38 are included as loading controls. Con, control.
TNF inhibition of EGFR activation requires p38 MAPK activity. A recent report by Zwang and colleagues (59) describes p38-dependent transient EGFR internalization in response to UV irradiation or TNF in HeLa and SW480 cells. To test whether this mechanism explains our findings, we pretreated YAMC cells with a pharmacological p38 inhibitor (SB220025) 30 min before treatment with TNF and EGF as above. TNF exposure attenuated ligand-stimulated EGFR phosphorylation in vehicle-treated but not SB220025-treated cells, implicating a p38 MAPK-dependent process (Fig. 5A). In contrast, blockade of MEK-ERK1/2 signaling with the MEK inhibitor U0126 had no effect on TNF-induced EGFR inhibition (Fig. 5B), suggesting that p38 is acting independently of other MAPKs. Similar results were obtained with another MEK inhibitor (PD98059, data not shown).

As both TNFR1 DD signaling and p38 are required for TNF-induced EGFR desensitization, we tested the requirement for TNFR1 and its DD in p38 activation in colon epithelial cells. TNFR1−/− and TNFR2−/− MCE cells were treated with TNF for 0–120 min, and p38 phosphorylation in whole cell lysates was assessed by Western blot analysis. Only cells expressing TNFR1 displayed increased p38 phosphorylation above baseline in response to TNF treatment (Fig. 5C). Furthermore, TNF promoted p38 activation in TNFR1−/− MCE cells reconstituted with WT, but not ΔDD or ΔC, TNFR1 (Fig. 5C).

To determine whether TNF-induced p38 activation causes EGFR internalization as previously described (59), YAMC cells were treated for 45 min with TNF, SB220025, or both. Cells were fixed and subjected to immunofluorescence analysis for EGFR subcellular localization. Thirty-minute EGF exposure served as a positive control for internalization. Both EGF and TNF treatment promoted EGFR internalization. However, p38 blockade with SB220025 prevented TNF-induced EGFR internalization (Fig. 5D), suggesting that TNFR1-induced EGFR inhibition may be via this mechanism. In contrast, EGF-driven receptor internalization was not blocked by p38 inhibitor.

TNF-stimulated EGFR inhibition occurs independent of proteasome inhibition. EGFR can be internalized either constitutively or by a ligand-binding-induced mechanism (55). Once internalized, the receptor is sorted by incompletely understood mechanisms through multivesicular endosomes (12, 18, 25) and designated for either destruction or recycling to the plasma membrane (10, 16). Our data show that TNF inhibits EGFR via p38-dependent EGFR internalization. To test whether this mechanism impacts receptor degradation as well as internalization, we pretreated YAMC cells with a proteasome inhibitor (MG132, 10 μM) 30 min before treatment with TNF and EGF as above. MG132 attenuated EGFR-induced EGFR degradation but did not affect the ability of TNF to inhibit EGFR phosphorylation (Fig. 6). Thus altered receptor degradation is not the primary mechanism for TNF-induced EGFR desensitization. This result is consistent with the observation that TNF does not alter total EGFR levels in YAMC cells (Figs. 1, A–D, and 5C).

DISCUSSION

In this study we provide evidence that signaling through TNFR1, but not TNFR2, inhibits EGFR-stimulated EGFR phosphorylation in murine colon epithelial cells by a mechanism requiring the TNFR1 DD and p38 MAPK. We show that TNF activation of p38 through TNFR1 promotes internalization of EGFR in murine colon epithelial cells. Internalization under these conditions was concomitant with EGFR inhibition but independent of proteasome activity.

TNF-induced EGFR blockade may be an important signaling mechanism in the acute injury initiated by the innate immune response with implications for both acute and chronic IBDs. TNF is a key proinflammatory cytokine expressed at high levels in IBD and NEC (11, 39); conversely, EGF has been shown effective in treatment of both experimental NEC and clinical ulcerative colitis (19, 42). The data presented here indicate that activation of TNFR1, the receptor likely to be preferentially activated in the presence of high TNF levels during active disease, induces EGFR desensitization via p38-driven internalization. Taken in the context of our recent work showing that p38, which is elevated in several IBD models (51), is also required for ligand-stimulated ubiquitinylation/degradation of EGFR (14), these results suggest that signaling through this MAPK regulates EGFR at multiple levels.

P38 is involved in cellular stress responses and apoptosis (15, 57), as well as wound healing in epithelial cells with impact on migration and proliferation (17, 23, 40). Our data showing that TNFR1-stimulated EGFR internalization in colon epithelial cells requires p38 are in agreement with a recent study (59) describing EGFR internalization following cell stressors such as UV irradiation and TNF exposure. In that paper, p38 phosphorylation of the cytoplasmic tail of the receptor was
linked to transient receptor endocytosis (59). Similarly, Vergarajauregui and colleagues (50) have demonstrated EGFR internalization as a result of forced p38 activation with anisomycin. Thus transient receptor endocytosis may be an important mechanism by which TNFR1 attenuates responses to EGF in IECs.

TNFR1 has two well-characterized cytoplasmic domains, the NSD and the DD (1). Our data show that the DD is necessary for both p38 activation and EGFR inhibition by pathological TNF concentrations (Figs. 4 and 5). The 80-amino acid cytoplasmic DD region of TNFR1 is thought to be critical for generation of cytotoxic death signals, anti-viral responses, and activation of acid sphingomyelinase, which promotes inflammation and apoptosis through induction of NF-κB and other pathways (5, 43, 45). Previous studies (9, 13) in mouse fibrosarcoma cells have shown that the DD was competent to promote p38 signaling; our data extend this by showing conclusively that, in the absence of the TNFR1 DD, p38 phosphorylation does not occur, despite pathological TNF concentrations. Because p38 is a key regulator of EGFR signaling through both TNF-induced internalization (Fig. 5 and (59)) and EGFR processing (23), our data further define the important role of the TNFR1 DD in colon epithelial cell physiology.

EGFR is found predominantly on the cell surface but constantly undergoes shuttling and recycling between the plasma membrane and the endosomal compartment. Following ligand binding, EGFR is quickly internalized through a coated pit pathway to the endosomal compartment of the cell and then sorted through incompletely understood mechanisms through multivesicular endosomes (12, 18, 25). Once internalized, the receptor is sorted for either destruction or recycling to the plasma membrane (10, 16). Because EGFR is internalized shortly after ligand binding, it is thought that significant EGFR signaling can occur within the endosomal compartment (55).

Although active EGFR is able to signal when internalized, EGFR internalized while still inactive is presumably unable to bind ligands present at the cell surface. In addition, receptor trafficking is slower for nonligand bound EGFR by 5–10-fold (52, 56), maintaining the receptor in a ligand inaccessible compartment for longer periods of time. Our laboratory and others (50, 59) have seen that p38 activation can induce EGFR internalization (Fig. 6). Additionally, a recent report from our laboratory showed that EGFR-stimulated p38 activation is required for EGF-induced cobalamin (Cbl) activation and subsequent receptor ubiquitinylation and degradation (23). However, TNF-induced p38 activation in the present study was not associated with changes in EGFR levels (Fig. 1) or Cbl phosphorylation (data not shown). Thus this cytokine-inducible pathway appears to be independent of lysosomal EGFR degradation and may involve p38 activation with different kinetics or in a different subcellular compartment than that required for EGF-driven receptor downregulation.

EGFR signaling is integral to the IEC response to damage and inflammation. In vitro, EGF induces epithelial migration (24) and intestinal epithelial proliferation (23). In animal models of IBD and NEC, EGFR-dependent responses reduce disease severity and promote recovery. Mice lacking TGF-α show a significantly increased susceptibility to dextran sodium sulfate (DSS)-induced colitis (22), whereas, in contrast, mice treated with exogenous EGF or overexpressing TGF-α are protected from DSS-induced colitis (21, 38). Neonatal rat pups pretreated with EGF show a reduced incidence of experimental NEC compared with controls (19), and 8-wk-old rabbits with jejunal resections treated with EGF exhibit an increase in glucose absorption (34). Thus there is a key role for EGFR in both the healing process from and prevention of intestinal epithelial injury.

A developing literature suggests that there is complex and tightly controlled cross-talk between TNFRs and EGFR. One example of this interaction is through TNF-α-converting enzyme (TACE). TACE is a membrane-bound protein containing MMP and disintegrin domains involved in TNF-induced cleavage of TGF-α (37). TNF can transactivate EGFR by promoting MMP-dependent cleavage of TGF-α and other EGFR ligands into their active forms (32, 37, 53). Mice that are deficient in active TACE have epithelial defects, including in the intestinal tract, which are similar to those reported in mice lacking EGFR (37). A second avenue for TNF-induced regulation of EGFR is the stimulation of protein tyrosine phosphatase activity. ME-180 cells treated with TNF showed an increase in protein tyrosine phosphatase (PTP)1B, a 37-kDa protein with tyrosine-specific protein phosphatase activity in cervical cancer cells (36, 48). Because of this association, we examined two common nonreceptor PTPs, Src-homology 2 domain containing phosphatase (SHP)-1 and SHP-2, but found no increase in either PTP with TNF treatment (data not shown). Our findings expand the understanding of the relationship between TNF and EGFR by identifying a third mechanism by which TNF signals through the TNFR1 DD to activate p38 and stimulate internalization of EGFR in colon epithelial cells.

In summary, we have shown that TNF negatively regulates EGF-dependent EGFR tyrosine phosphorylation and activity in a p38-dependent manner. This effect is a specific response to stimulation of TNFR1 and requires the presence of the DD-containing cytoplasmic tail region of the receptor. These data provide evidence of negative regulation of EGFR signal transduction by the proinflammatory cytokine TNF in murine IEC, with implications for impaired injury response mechanisms in a number of gastrointestinal disorders such as IBD and NEC. Understanding these interactions may lead to important new approaches to pharmacological prevention and/or treatment of IBDs.

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