The ErbB4 Growth Factor Receptor Is Required for Colon Epithelial Cell Survival in the Presence of TNF

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Background & Aims: **The ErbB4 receptor tyrosine kinase regulates cell growth, survival, and differentiation in several tissues, but its role in the gastrointestinal tract has not been reported. We tested the hypothesis that ErbB4 promotes intestinal cell survival and restitution following injury or inflammation.** ***Methods:*** **ErbB4 expression in human inflammatory bowel disease was determined by immunohistochemistry. Mice were subjected to dextran sulfate sodium (DSS, 3%) colitis or injected with tumor necrosis factor (TNF), and ErbB4 expression was quantified by immunohistochemistry and Western blot. Cultured young adult mouse colon (YAMC) cells were exposed to TNF, and ErbB4 messenger RNA, protein, and phosphorylation levels were measured. Cells transfected with ErbB4 small interfering RNA (siRNA), or over expressing ErbB4, were subjected to wound healing and apoptosis assays.** ***Results:*** **ErbB4 levels increased in Crohn's colitis and the colon epithelium of mice with DSS colitis or injected with TNF. In YAMC cells, TNF induced ErbB4 messenger RNA, protein, and phosphorylation; nuclear factor κB activation also stimulated ErbB4 accumulation. ErbB4 siRNA sensitized cells to TNF-stimulated apoptosis, while over expression blocked apoptosis induced by TNF plus cycloheximide. Additionally, ErbB4 siRNA decreased YAMC cell wound healing. ErbB4 knockdown attenuated, while over expression elevated, phosphorylation of Akt in response to TNF. Inhibition of the phosphatidylinositol 3-kinase/Akt signaling cascade reversed the ability of ErbB4 over expression to protect from cytokine-induced apoptosis.** ***Conclusions:*** **ErbB4 expression and signaling are key elements for TNF responses in vivo and in cell culture, protecting intestinal epithelial cells from apoptosis in the inflammatory environment, possibly through Akt activation.**

Regulation of cell proliferation, migration, and apoptosis in the intestinal epithelium by soluble growth factors and their receptors[1,2] is critical for barrier function maintenance and the health of the individual. Signaling through members of the ErbB family of receptor tyrosine kinases, including epidermal growth factor receptor (EGFR), represents a potential therapeutic avenue for gastrointestinal inflammatory disorders, as shown by the efficacy of epidermal growth factor (EGF) in experimental wound healing assays[2] and clinical trials with ulcerative colitis.[3] However, the influence of individual ErbBs on epithelial responses to injury and inflammation is not yet well understood.

ErbB4 is the most recently described ErbB receptor,[4] and compared with the other family members little is known about its biology. Responses to ErbB4 ligands or ErbB4 over expression in cell culture vary widely, ranging from differentiation[5,6] or cell survival[7] to migration,[8] proliferation,[5,9] or growth arrest.[10] These divergent results may be explained by expression of up to 4 receptor isoforms generated by alternative splicing in the regions coding for the juxtamembrane (JM) and cytoplasmic (CYT) domains. The prototypic ErbB4, JM-a/CYT-1, is subject to a sequential 2-step proteolytic cleavage,[10] giving rise to an 80-kilodalton intracellular domain fragment (4ICD). 4ICD is a constitutively active tyrosine kinase[11] that can translocate to the nucleus and function as a transcriptional regulator.[12,13] JM-b forms are resistant to this proteolytic processing and are expressed only as full-length transmembrane receptors.[14] In addition to JM variants, CYT-2 isomerization results in the deletion of a C-terminal phosphatidylinositol 3-kinase (PI3K) binding site.[8] CYT-1 retains this site and can signal through PI3K.

ErbB4 is widely expressed in human fetal and adult tissues, including the digestive tract,[15] but often at low levels, which has made understanding the physiology of the receptor in some tissues difficult. As yet, no function for ErbB4 has been described in the intestinal epithelium,

Abbreviations used in this paper: CYT, cytoplasmic; DSS, dextran sulfate sodium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal–regulated kinase; 4ICD, intracellular domain fragment; GFP, green fluorescent protein; HRG, heregulin; JM, juxtamembrane; NF-κB, nuclear factor κB; PI3K, phosphatidylinositol 3-kinase; RT-qPCR, real-time quantitative polymerase chain reaction; siRNA, small interfering RNA; TACE, tumor necrosis factor–converting enzyme; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; YAMC, young adult mouse colon.

0016-5085/09/$36.00
doi:10.1053/j.gastro.2008.09.023

but the involvement of EGFR and ErbB ligands in recovery from colitis[16,17] suggests potential roles in this response for ErbB4, which can heterodimerize with EGFR and modulate its signaling. In this study, we asked whether ErbB4 is involved in intestinal epithelial responses to injury and inflammation, in particular in response to the proinflammatory cytokine tumor necrosis factor (TNF).

TNF is produced by macrophages and other immune cells as well as epithelial cells and is expressed at high levels in inflammatory bowel diseases (IBD; reviewed by Wang and Fu[18]). Many of the cytotoxic effects of the inflammatory environment are mimicked in cell culture by TNF. Epithelial cells express 2 TNF receptors, the low-affinity TNFR1 and the high-affinity TNFR2, which together trigger an array of cellular outputs. In intestinal epithelial cells, TNF stimulates both cell survival and proapoptotic pathways, with the specific cellular context modulating the balance of signals and determining cell fate.[19] Survival signals induced by TNF include the PI3K/Akt cascade and nuclear factor κB (NF-κB).

Signaling downstream of TNF intersects with EGFR pathways on several levels. TNF stimulates many of the same kinase cascades as EGFR. In contrast, however, TNF can also inhibit phosphorylation and activation of EGFR.[20,21] As yet, little is known about cross talk between TNF signaling and other ErbB family members.

In the current study, we asked whether ErbB4 is regulated by inflammation in intestinal epithelial cells. In both a mouse model of acute colitis and mice injected with TNF, we observe increased ErbB4 expression in the colon epithelium; consistent with these results, we see elevated ErbB4 levels in human IBD. In cultured young adult mouse colon (YAMC) epithelial cells, TNF exposure induced ErbB4 messenger RNA (mRNA), protein, and phosphorylation. Blockade of ErbB4 expression with small interfering RNA (siRNA) inhibited TNF-stimulated Akt phosphorylation and sensitized cells to apoptosis. Conversely, ErbB4 over expression stimulated Akt phosphorylation and protected from cytokine-induced cell death. Taken together, these data position ErbB4 as a key regulator of the response of epithelial cells to injury and inflammation.

Materials and Methods

Apoptosis

Apoptosis was measured by ApopTag terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL; Millipore, Danvers, MA), following the manufacturer's instructions, and by cleaved (active) caspase-3 Western blot analysis. Six-hour TNF exposure (100 ng/mL) combined with either wortmannin (100 nmol/L) or cycloheximide (1 μg/mL) served as positive control.

Immunohistochemistry

Immunostaining for ErbB4 was performed using standard techniques[22] on 5 μm paraffin-embedded sections with anti-ErbB4 or control immunoglobulin G at 2 μg/mL in phosphate-buffered saline for 2 hours. Diaminobenzidine substrate was purchased from Vector Laboratories (Burlingame, CA). Sections were counterstained with methyl green.

Restitution Assays

Restitution assays were performed as previously described.[23] Briefly, fibronectin-plated cultures were subjected to multiple 1.5-mm^2 wounds with a drill-mounted silicone tip. Wounds were photographed over time and measured with ImageJ software (National Institutes of Health, Bethesda, MD).

Constructs

pCDNA3.1-ErbB4 (human JM-a/CYT-1 isoform) expression vector was a gift of Graham Carpenter (Vanderbilt University). Inserts from pCDNA3.1-ErbB4 were polymerase chain reaction amplified (primers: 5′-ATGGCGATCGCATGAAGCCGGCGACAGGACTTTG-3′, *Sgf*I site; 5′-TTGGGCCGGACCGGCCTTACACCACAGTATTCCGGTG-3′, *Sfi*I site) and then cut with *Sfi*I and *Sgf*I and ligated into linearized bicistronic LZRS–green fluorescent protein (GFP) vector (Albert Reynolds, Vanderbilt University). Plasmids were screened by *Sfi*I/*Sgf*I digestion. Phoenix packaging cells (Steve Hanks, Vanderbilt University) were transiently transfected with LZRS-GFP or LZRS-ErbB4-GFP and monitored for GFP expression. YAMC cells were subjected to 5 rounds of infection with filtered viral supernatant supplemented with 4 μg/mL polybrene. GFP-positive cells were sorted at the Vanderbilt Flow Cytometry Core Laboratory using a Becton Dickinson FACSAria (Becton Dickinson, San Jose, CA); the top 20% GFP-expressing cells were maintained as pools.

Mice and Experimental Colitis

C57BL/6 mice were subjected to acute colonic injury and colitis with 3% (wt/vol) dextran sulfate sodium (DSS; mol wt, 36,000–50,000; MP Biochemicals, Solon, OH) in drinking water for 4 days and then fresh water for a 3-day recovery period. On days 0, 4, and 7, colons were Swiss rolled, formalin fixed, and paraffin embedded. Colon epithelium from some mice was isolated for lysates by filleting the colon and scraping the mucosal surface with a glass slide. As an additional model of acute inflammation in vivo, mice were injected intraperitoneally with 10^4 U recombinant TNF. Twenty-four hours later, mice were killed and their colons collected for analysis.

All animal use was approved and monitored by the Vanderbilt Institutional Animal Care and Use Committee.