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Erlotinib promotes endoplasmic reticulum stress-mediated injury in the intestinal epithelium

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ABSTRACT

Erlotinib, a popular drug for treating non-small cell lung cancer (NSCLC), causes diarrhea in approximately 55% of patients receiving this drug. In the present study, we found that erlotinib induced barrier dysfunction in rat small intestine epithelial cells (IEC-6) by increasing epithelial permeability and down-regulating E-cadherin. The mRNA levels of various pro-inflammatory cytokines (*Il-6*, *Il-25* and *Il-17f*) were increased after erlotinib treatment in IEC-6 cells. Erlotinib concentration- and time-dependently induced apoptosis and endoplasmic reticulum (ER) stress in both IEC-6 and human colon epithelial cells (CCD 841 CoN). Intestinal epithelial injury was also observed in male C57BL/6J mice administrated with erlotinib. Knockdown of C/EBP homologous protein (CHOP) with small interference RNA partially reversed erlotinib-induced apoptosis, production of IL-6 and down-regulation of E-cadherin in cultured intestinal epithelial cells. In conclusion, erlotinib caused ER stress-mediated injury in the intestinal epithelium, contributing to its side effects of diarrhea in patients.

Introduction

Erlotinib is an orally administered, low molecular weight, quinazoline-based agent (chemical structure of erlotinib was shown in Supplementary Fig. 1A) which selectively and reversibly inhibits epidermal growth factor receptor (EGFR) kinase activity (Hidalgo et al., 2001). Erlotinib is currently approved for the treatment of advanced or metastatic resistant non-small cell lung cancer (NSCLC) and for use in combination with gemcitabine to treat advanced, unresectable, or metastatic pancreatic cancer (Bareschino et al., 2007). However, side effects including skin rash, diarrhea, and pulmonary toxicity often limit the application of erlotinib, particularly for long-term treatment. Diarrhea is the second most common side effect of erlotinib. The National Cancer Institute of Canada Clinical Trials Group Study BR.21 documented that 55% of patients treated with erlotinib had diarrhea, compared to 19% of patients on placebo. In the Iressa Pan-Asia Study (IPASS), 47% of patients treated with erlotinib developed diarrhea (Nguyen and Neal, 2012). It has been reported that severe diarrhea caused by erlotinib can result in fluid and electrolyte losses, which may lead to dehydration, electrolyte imbalances and renal insufficiency (Melosky, 2012). Epidermal growth factor (EGF) has been shown to inhibit Ca^{2+}-dependent Cl^- transport in T84 human colonic epithelial cells. EGFR inhibitors may cause diarrhea by blocking this inhibitory loop and causing chloride secretion (Uribe et al., 1996). Still, the mechanisms by which erlotinib destroys the intestinal epithelium remain unclear.

The intestinal epithelium forms a protective barrier between the luminal contents and the external environment. Breakdown of the gut barrier is associated with increased intestinal permeability and gut inflammation (Krzyzaniak et al., 2011). Epithelial cells interconnect by an array of intercellular adhesion complexes including adherens junctions (AJs), tight junctions (TJs) and desmosomes (Perez-Moreno et al., 2003). E-cadherin is a major component of AJs, impaired expression of which in the small intestine and colon has been linked to disturbed intestinal homeostasis and barrier function (Schneider et al., 2010).

The endoplasmic reticulum (ER) is a principal site for protein synthesis and folding, calcium storage and signaling, and it is highly sensitive to alterations in calcium homeostasis and environmental perturbations (Lin et al., 2008). Physiologic or drug-induced disruption of protein folding causes misfolded, aggregated, or unassembled proteins to accumulate in the ER lumen, triggering a response called ER stress (Kaufman, 1999). The ability of cells to respond to perturbations in ER function, or 'ER stress', is critical for cell survival, but chronic or unresolved ER stress can lead to apoptosis (Tabas and Ron, 2011). ER stress has been reported to contribute to gastrointestinal inflammation in epithelial dysfunction (Hotamisligil, 2010b; Kaser and Blumberg, 2009, 2010). Our previous study has shown that human immunodeficiency

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virus protease inhibitor disrupts epithelial barrier integrity through activation of ER stress and unfolded protein response in intestinal epithelial cells (Wu et al., 2010). However, no reports concern ER stress and erlotinib-induced diarrhea.

In the present study, we found for the first time that erlotinib induced injury and dysfunction in the intestinal epithelium through ER stress.

Materials and methods

Materials. Antibodies against ATF4 (#11815), phospho-eIF2α (#3398) and eIF2α (#5324) were purchased from Cell Signaling Technology (Beverly, MA). Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit was purchased from BD Biosciences (San Jose, CA). ELISA kit for interleukin-6 (IL-6) was purchased from R&D Systems (Minneapolis, MN). Antibodies against E-cadherin (sc-8426), ZO-1 (sc-10804), ATF6 (sc-22799), XBP-1 (sc-7160), CHOP (sc-575) and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC–dextran, dimethyl sulfoxide (DMSO) and insulin were obtained from Sigma Chemical Co. (St. Louis, MO). Protease inhibitor cocktail was obtained from Roche Technology (Basel, Switzerland). Erlotinib was obtained from Genentech Inc. (South San Francisco, CA).

Cell culture and erlotinib treatment. Rat small intestine epithelial cells (IEC-6) were maintained in DMEM (Life Technologies, Carlsbad, CA) supplemented with 100 mU/ml of insulin and 10% fetal bovine serum (Life Technologies, Carlsbad, CA) under a humidified 5% (v/v) CO_2 atmosphere at 37 °C. CCD 841 CoN human colon epithelial cells were incubated in MEM (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA) in a 5% CO_2 atmosphere at 37 °C. Erlotinib was dissolved in DMSO to a concentration of 20 mM (stock solution) and stored at −20 °C.

Permeability measurement. Cells were seeded into 12-well transwell plates (0.4-μm pore diameter, Corning-Costar Corp., Cambridge, MA) and grown to 100% confluency. The cells were incubated with 0.1% DMSO and 5, 10 or 20 μM erlotinib for 24 h, respectively. FITC–dextran (MW, 4.4 kDa) was dissolved in culture medium and used at a final concentration of 2.2 mg/ml in the apical cell compartment. After 4 h of incubation, 100 μl aliquots were obtained from the medium of basal chamber. Fluorescence was measured with a fluorescence spectrometer (excitation 490 nm; emission 520 nm).

Cytotoxicity assay. The cytotoxic activity of erlotinib was examined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and Lactate Dehydrogenase (LDH) assay. Cells were treated with erlotinib for indicated time periods in 96-well plates. For MTT assay, MTT solution (4 mg/ml in phosphate buffered saline [PBS]) was added (20 μl/well) into each well and incubated for 4 h at 37 °C. Then the supernatant was discarded and the purple formazan crystals were dissolved in 200 μl of DMSO for 5 min. The plates were read on an automated microplate spectrophotometer (Sunrise, Tecan, Austria) at 570 nm. For LDH assay, the supernatant was harvested and the released LDH was detected. Total LDH was detected after cells were lysed. Cytotoxicity was evaluated by the released LDH/total cell LDH.

Isolation and culture of primary rat intestinal cells. Primary rat intestinal cells were isolated from male rats and cultured as described (Campbell, 2010).

Reverse transcription and real-time quantitative PCR (Q-PCR). RNA samples were treated by DNase and subjected to real-time quantitative PCR. First-strand cDNAs were generated by reverse transcription using oligo(dT) (Hidalgo et al., 2001). Quantitative PCR was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR Green I dye (Biotium, Inc., Hayward, CA), and threshold cycle numbers were obtained using ABI Prism 7000 SDS software version 1.0. The cDNA amplification was performed for 30 cycles using the primers listed in Table 1 with the following settings: 94 °C for 5 min, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and 72 °C for 10 min.

Flow cytometry. Annexin V–FITC/PI double staining assay was performed to detect apoptosis. Cells were harvested and resuspended in Annexin-V binding buffer. The suspension was incubated with 2.5 μl of Annexin V–FITC and 2 μl of PI for 10 min at room temperature in the dark, followed by cytometric analysis (EPICS XL, Beckman Coulter, Fullerton, CA) within 30 min of staining. Samples were analyzed using a FACSCalibur flow cytometer.

ELISA. Cytokine (IL-6) was measured with ELISA kit (R6000B, M6000B) from R&D systems (Minneapolis, MN) according to the manufacturer's protocol.

Western blot. Proteins were extracted in lysis buffer (30 mmol/l Tris, pH 7.5, 150 mmol/l sodium chloride, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and protease inhibitor cocktail). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies overnight at 4 °C, and then incubated with a horseradish peroxidase-coupled secondary antibody for 1 h at room temperature. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

Small interfering RNA (siRNA) transfection. C/EBP homologous protein (CHOP) siRNA sequence used in CCD 841 CON cells was: 5′-UUCAUC UGAAGACAGGACCUCUUGC-3′. Luciferase siRNA was used as described before (Wang et al., 2011). CHOP siRNA and nonspecific siRNA used in IEC-6 cells were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells were cultured to 60% confluency in a 6-well plate and transfected with luciferase siRNA or CHOP siRNA using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) for 30 h. Then cells were

Table 1
Primers used for amplification.

Gene	Primer sequence
Cdh1 (rat)	5′-ATCTAAAGCTTCACAAGCTGGA-3′
	5′-TGATCTGTGACTGTGACCACTA-3′
Tjp1 (rat)	5′-CCATCTTTGGACCGATTGCTG-3′
	5′-TAATGCCCGAGCTCCGATG-3′
Itjb1 (rat)	5′-GACCTGCCTTGGTGTCTGTGC-3′
	5′-AGCAACCACACCAGCTACAAT-3′
Il-6 (rat)	5′-CCGGAGAGGAGACTTCACAG-3′
	5′-CAGAATTGCCATTGCACAAC-3′
Socs3 (rat)	5′-CCCGCTTTGACTGTGTACT-3′
	5′-TGAGTACCAGCGGGATCTTCTC-3′
Il-25 (rat)	5′-CTTACCCAGATGCTGTCCC-3′
	5′-GATTCAAGTCCCTGTCCAAC-3′
Gapdh (rat)	5′-GGCATTGCTCTCAATGACAA-3′
	5′-AGGGCCTCTCTCTTGCTCTC-3′
Il-6 (mouse)	5′-CTGCAAGAGACTTCCATCCAG-3′
	5′-AGTGGTATAGACAGGTCTGTTGG-3′
Il-25 (mouse)	5′-ACAGGGACTTGAATCGGGTC-3′
	5′-TGGTAAAGTGGGACGGAGTTG-3′
Il-17f (mouse)	5′-TGCTACTGTTGATGTTGGGAC-3′
	5′-CAGAAATGCCCTGGTTTTGGT-3′
Gapdh (mouse)	5′-AATGGATTTGGACGCATTGGT-3′
	5′-TTTGCACTGGTACGTGTTGAT-3′
CDH1 (human)	5′-CGAGAGCTACACGTTCACGG-3′
	5′-GGGTGTCGAGGGAAAAATAGG-3′
IL-6 (human)	5′-ACTCACCTCTTCAGAACGAATTG-3′
	5′-CCATCTTTGGAAGGTTCAGGTTG-3′
Gapdh (human)	5′-TGTGGGCATCAATGGATTTGG-3′
	5′-ACACCATGTATTCCGGGTCAAT-3′