Saccharomyces boulardii Inhibits EGF Receptor Signaling and Intestinal Tumor Growth in *Apc*^{min} Mice

XINHUA CHEN,* JOHANNES FRUEHAUF,* JEFFREY D. GOLDSMITH,‡ HUA XU,* KIANOOSH K. KATCHAR,* HON–WAI KOON,§ DEZHENG ZHAO,* EFI G. KOKKOTOU,* CHARALABOS POTHOULAKIS,§ and CIARÁN P. KELLY*

*Division of Gastroenterology, Department of Medicine, and ‡Department of Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts; and §Inflammatory Bowel Disease Center, Division of Digestive Diseases, UCLA, Los Angeles, California

BACKGROUND & AIMS: *Saccharomyces boulardii (Sb)* is a probiotic yeast with anti-inflammatory and antimicrobial activities and has been used for decades in the prevention and treatment of a variety of human gastrointestinal disorders. We reported previously that Sb modulates host inflammatory responses through down-regulation of extracellular signal-regulated kinase (Erk)1/2 activities both in vitro and in vivo. The aim of this study was to identify upstream mediators responsible for extracellular signal-regulated kinase (Erk)1/2 inactivation and to examine the effects of *Sb* on tumor development in *Apc*^{Min} mice. **METHODS:** Signaling studies of colon cancer cells were done by western blot. Cell proliferation was measured by MTS and BrdU assay. Apoptosis was examined by flow cytometry, tunel assay and caspase assay. *Apc*^{Min} mice were orally given *Sb* for 9 weeks before sacrifice for tumor analysis. **RESULTS:** We found that the epidermal growth factor receptor (EGFR) was deactivated upon exposure to *Sb*, leading to inactivation of both the EGFR-Erk and EGFR-Akt pathways. In human colonic cancer cells, *Sb* prevented EGF-induced proliferation, reduced cell colony formation, and promoted apoptosis. HER-2, HER-3, and insulin-like growth factor-1 receptor were also found to be inactivated by *Sb*. Oral intake of *Sb* reduced intestinal tumor growth and dysplasia in C57BL/6J Min/+ (*Apc*^{Min}) mice. **CONCLUSIONS: Thus, in addition to its anti-inflammatory effects, *Sb* inhibits EGFR and other receptor tyrosine kinase signaling and thereby may also serve a novel therapeutic or prophylactic role in intestinal neoplasia.**

Saccharomyces boulardii (Sb) is a nonpathogenic yeast that has been used for many years as a probiotic agent to prevent or treat a wide variety of human gastrointestinal disorders of diverse etiologies.[1–3] Preclinical and experimental studies of *Sb* have demonstrated anti-inflammatory, anti-microbial, enzymatic, metabolic and anti-toxin activity.[4–7] *Sb* appears to exert its therapeutic effect by multiple mechanisms and to influence several important facets of intestinal host-pathogen interaction. We and others have reported that *Sb* acts through modulation of host signaling pathways that regulate the intestinal mucosal inflammatory response. Particularly, the extracellular signal-regulated kinase (Erk)1/2 mitogen-activated protein (MAP) kinase pathway is down-regulated by *Sb* both in vitro and in vivo.[8,9]

MAP kinase pathways are located downstream of many growth-factor receptors, including the epidermal growth factor receptor (EGFR), through the Raf/MEK/ERK cascade.[10] Therefore, we hypothesized that *Sb* inhibited Erk activation via an upstream effect on EGFR or other receptor tyrosine kinase signaling. Our data in this study suggested that EGFR signaling is inactivated by *Sb*. It has been well characterized that activation of EGFR results in enhanced cell proliferation, invasion, and tumor metastasis, as well as inhibition of apoptosis.[11] Signaling pathways that emerge from EGFR activation are critical in colon cancer biology.[12,13] EGFR inhibitors have demonstrated clinical benefit for colorectal cancer treatment.[14,15] Because *Sb* has shown beneficial effects in a series of gastrointestinal disorders, we wanted to know whether *Sb* has potential anti-cancer properties by examining whether the effects of *Sb* on EGFR signaling can influence proliferation and apoptosis of colon cancer cells in vitro, as well as intestinal tumor growth in *Apc*^{Min} mice in vivo.

Materials and Methods

Cells and Reagent

HT29, SW480, and HCT-116 cells were obtained from American Type Culture Collection. HT29 and SW480 cells were cultured in Dulbecco's modified Eagle medium (DMEM), whereas HCT-116 were cultured in McCoy's 5A modified media, supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2 mmol/L L-glutamine (GIBCO, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Recombinant human (rh) epidermal growth factor (EGF) and recombinant human Neuregulin (NRG-1) were pur-

Abbreviations used in this paper: EGFR, epidermal growth factor receptor; Erk, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor-1 receptor; MAP, mitogen-activated protein; PCNA, proliferating cell nuclear antigen; PKC, protein kinase C; *Sb*, *Saccharomyces boulardii*; SbS, *Sb* culture supernatant.

0016-5085/09/$36.00
doi:10.1053/j.gastro.2009.05.050

chased from R&D Systems (Minneapolis, MN). Antibodies against Erk, MEK, CamKII kinases, protein kinase C (PKC), EGFR, insulin-like growth factor-1 receptor (IGF-1R), HER-2, HER-3, and Akt, phosphorylated and/or nonphosphorylated forms, were purchased from Cell Signaling Technology (Beverly, MA). DNA constructs myr-Akt-pUSE and pUSE empty vector were gifts from Dr. Grant D Stewart from University of Edinburgh. Preparation of *Sb* culture supernatant (SbS) was done as previously described.[9] Briefly, lyophilized *Sb* (Biocodex Laboratories, Gentilly Cedex, France) was cultured in RPMI 1640 cell culture medium (100 mg/mL) for 24 hours at 37°C. The suspension was then centrifuged at 9000*g* for 15 minutes and the supernatant collected. The supernatant was then passed through a 0.22-mm filter (Fisher Scientific, Pittsburgh, PA) and then a 10-kilodalton cutoff filter (Millipore, Billerica, MA). For SbS used in ligand-induced signaling experiments, ion exchange chromatography was used during preparation.

Western Blot Analysis

HT29, SW480, or HCT-116 cells were incubated with *SbS* at 1:1 dilution for different time periods at different conditions. Treated cells were then lysed in a lysis buffer (62.5 mmol/L Tris-HCl, 10% glycerol, 2% SDS, 0.01% bromphenol blue, and 1% 2-mercaptoethanol). Equal amounts of cell extract were fractionated by 10% SDS-PAGE, and proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) at 300 mA for 3 hours. Membranes were blocked in 5% nonfat dried milk in TBST (50 mmol/L Tris, pH 7.5, 0.15 mol/L NaCl, 0.05% Tween 20) and then incubated with antibodies directed against phospho- or nonphosphorylated forms of ERK1/2, MEK1/2, HER-2, HER-3, IGF-1R (Cell Signaling), and EGFR (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed with TBST and incubated with horseradish peroxidase-labeled secondary antibodies for 1 hour. The peroxidase signal was detected by Supersignal chemiluminescent substrate (Pierce, Rockford, IL), and the image of the signal was recorded by exposure to x-ray film (Fujifilm, Tokyo, Japan).

Colony Formation Assay and Cell Proliferation Assays

HT29 and SW480 cells were seeded at 1000 cells per well in 6-well plates and allowed to attach for 48 hours. SbS was diluted in DMEM at different concentrations and added directly to cell culture wells. Cultures were observed daily for 10–20 days and then were fixed and stained with modified Wright-Giemsa stain (Sigma Chemical Co, St. Louis, MO). Colonies of 30 cells were scored as survivors.[16] Cells were maintained at 37°C in 5% CO_2 in complete humidity. HT29 cells were cultured in DMEM supplemented with 10% (vol/vol) FBS and 2 mmol/L L-glutamine (GIBCO). Cell proliferation assays were carried out using both MTS assay kit (Promega, Madison, WI) and BrdU colorimetric kit (Roche Applied Science, Indianapolis, IN) following manufacturers' instructions.

Cell Transfection

Ninety percent to 100% confluent HT29 cells grown in 12-well dishes or 4 chamber polystyrene vessel glass slides were transfected with pUSE empty vector or pUSE-myr-Akt plasmids using Lipofecatmine 2000 (Invitrogen, Inc., Carlsbad, CA). Twenty-four hours after transfection, cells were treated with SbS for 30 minutes before being lysed for Western blot analysis or treated over night for TUNEL staining.

Apoptosis Assays

Tunel assay was carried out in both HT29 cell culture and intestinal tissues using TUNEL Apoptosis Detection Kit (Upstate Biotechnology Inc., Lake Placid, NY) according to the manufacturer's instructions. For flow cytometry, adherent HT29 cells were released by treatment with 0.25% trypsin. Each sample was fixed overnight with 70% ethanol at 4°C. Cells were rehydrated with phosphate-buffered saline (PBS) and then stained with 10% propidium iodide in 100 U/mL of ribonuclease in PBS for 60 minutes at room temperature. Cells were filtered through 35-μm filters before analysis. The flow cytometer was configured to track the number of events with the FL2 parameter (FACScan; Becton Dickinson, Lincoln Park, NJ). The DNA content was analyzed using a nonlinear least-squares algorithm. Caspase activation was measured with a fluorimetric homogeneous caspase assay kit (Roche Applied Science).

Apc^{Min} *Mice*

C57BL/6J Min/+ (*Apc^{Min}*) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at age 7 weeks. *Sb* was administrated daily in their drinking water, 3×10^8 colony-forming units (CFU) per milliliter, and 3 times each week by oral gavage at a dose of 6×10^8 CFU for 9 weeks. Mice were killed at 16 weeks of age. Intestinal tumor number was counted in the distal 10 cm of the small intestine using Methylene Blue in PBS (0.05%). Tumor sizes were measured by external caliper and taken as diameter to calculate total areas. Proximal sections of the rest of the small intestine (remaining section after usage of 10-cm distal part) were fixed and used for immunohistochemistry. Grade of dysplasia was measured by a blinded pathologist using a simple grading system based on the criteria for adenomatous change in the human colon: high-grade vs low-grade dysplasia, where low-grade dysplasia showed nuclear elongation with a sessile or villous architecture, and high-grade dysplasia showed a cribriform growth pattern with loss of nuclear