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Celecoxib inhibits Ewing sarcoma cell migration via actin modulation

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ABSTRACT

Background: Ewing sarcoma (ES) is an aggressive childhood solid tumor in which 30% of cases are metastatic at presentation, and subsequently carry a poor prognosis. We have previously shown that treatment with celecoxib significantly reduces invasion and metastasis of ES cells in a cyclooxygenase-2-independent fashion. Celecoxib is known to downregulate β-catenin independently of cyclooxygenase-2. Additionally, the actin cytoskeleton is known to play an important role in tumor micrometastasis. We hypothesized that celecoxib's antimetastatic effect in ES acts via modulation of one of these two targets.

Methods: ES cells were treated with celecoxib, and the levels of β-catenin and total actin were examined by Western blot and quantitative polymerase chain reaction. Cells were transfected with small interfering RNA targeting β-catenin, and invasion assays were performed. Immunofluorescence staining for β-catenin and F-actin was performed on treated and untreated cells. Additionally, cells were subjected to a wound healing assay to assess migration.

Results: Celecoxib had no effect on the messenger RNA or protein levels of β-catenin but did significantly decrease the amount of total actin within ES cells. Reduction of β-catenin by small interfering RNA had no effect on invasion, and celecoxib treatment of the β-catenin depleted cells continued to inhibit invasion. Immunofluorescence staining demonstrated no change in β-catenin with treatment but did show a significant reduction in the amount of F-actin, as well as morphologic changes of the cells. Wound healing assays demonstrated that celecoxib significantly inhibited migration.

Conclusions: Celecoxib does not exert its antimetastatic effects in ES through alteration of β-catenin but does significantly modulate the actin cytoskeleton.

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1. Introduction

Ewing sarcoma (ES) and the ES family of tumors are a group of malignant tumors of the bone and soft tissue. They are characterized as small, round blue cell tumors, which typically affect children between the ages of 5–15 and are often quite aggressive [1]. Although the overall 5-y survival for ES is between 60 and 70%, approximately 25% of patients present with metastatic disease, which carries a poor prognosis [2–4]. Fewer than 40% of patients with distant metastasis on initial diagnosis survive past 5 y [5,6]. Strategies to treat and prevent metastasis continue to be the focus of significant research.

We have previously shown that the selective cyclooxygenase-2 (COX-2) inhibitor celecoxib prevents lung metastasis in a murine model of ES, with minimal effect on the primary tumor [7,8]. Furthermore, celecoxib inhibits invasion by ES cells in a COX-2-independent fashion [9]. Recent clinical studies have shown that the addition of celecoxib and vinblastine to standard treatment protocols (alternating cycles of vincristine/doxorubicin/cyclophosphamide and ifosfamide/etoposide) in ES doubled the 2-y event-free survival for patients with lung metastases, from 36%–71% [10]. The mechanism by which celecoxib prevents malignant progression of ES has yet to be elucidated.

Celecoxib exhibits many of its antitumor effects via a decrease in COX-2 activity, particularly in colon cancer [11–15]. Celecoxib inhibition of COX-2 also plays a role in treating various other malignancies including breast, endometrial, and pancreatic cancers [16–18]. However, myriad off-target effects of celecoxib treatment are being evaluated for their antitumor properties. A 2006 review by Grösch *et al.* [19] identified over 40 different targets for the COX-2-independent effects of celecoxib on apoptosis, cell cycle regulation, and angiogenesis and/or metastasis. In particular, it alters the Wnt signaling pathway through modulation of β-catenin. Studies in colorectal cancer models have demonstrated that celecoxib works in part by inducing the rapid translocation of predominantly membrane-bound β-catenin to the cytoplasm and nucleus, followed by its degradation [20–23]. Other cancers, such as the lung, liver, and glioblastoma, have likewise shown a β-catenin response to celecoxib treatment [24–27]. Furthermore, β-catenin has been shown to specifically influence the metastatic potential of ES [28,29].

Another potential mechanism through which celecoxib may exert its antitumor effects is through modification of the actin cytoskeleton. Actin dynamics are known to be vital for cell motility, migration, shape, and cell–cell junctions and interactions [30,31]. β-actin is overexpressed in various cancers [32,33] and is especially overexpressed in samples taken from metastases [32–34]. Relative invasiveness of cancer cells has been associated with increases in β-actin, as well as increases in the proportion of polymerized F-actin to monomeric G-actin, resulting in the reorganization of actin within the cell [35–38]. Although some have suggested the importance of the actin cytoskeleton in ES [39–42], little is known about celecoxib's influence on this integral cellular protein, which may have particular importance in tumor micrometastasis.

2. Materials and methods

2.1. Cell lines

SK-NEP1 (ATCC, Manassas, VA, HTB-48) cells, a well-described ES cell line, were maintained in McCoy's media (Gibco [Invitrogen], Grand Island, NY) supplemented with 15% fetal clone II serum (Hyclone; Thermo Scientific, Waltham, MA) and 1% penicillin-streptomycin, and incubated at 37°C and 5% CO_2. Additionally, a portion of the cells were transfected with small interfering RNA (siRNA) oligomers targeting β-catenin or with nontargeting control siRNA (Dharmacon, Lafayette, CO). Transfection was accomplished using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA) transfection reagent.

Caco-2 (ATCC, HTB-37) cells, a well-described colon cancer cell line, were maintained in Delbecco Modified Eagle medium (Hyclone; Thermo Scientific) supplemented with 10% fetal clone II serum (Hyclone; Thermo Scientific) and 1% penicillin-streptomycin, and incubated at 37°C and 5% CO_2.

2.2. Reagents

Celecoxib (TSZ Scientific, Framingham, MA) was dissolved in 100% dimethyl sulfoxide and stored at 4°C. Cultrex (Trevigen Inc, Gathersburg, MD) basement membrane extract (BME) was aliquoted and stored at −20°C. Antibodies used included anti–β-catenin (Cell Signaling, Boston, MA), anti–actin C-2 (Santa Cruz Biotechnology, Dallas, TX), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) FL-335 (Santa Cruz Biotechnology). Fluorescent antibodies for use with the LiCor Odyssey imaging system were obtained from LiCor (Lincoln, NE). Rhodamine phalloidin (Invitrogen) dissolved in methanol and DAPI (4',6-diamidino-2-phenylindole) (Invitrogen) were used for immunofluorescence.

2.3. Western blotting

SK-NEP1 cells were treated for 72 h with 5 μM of celecoxib for β-catenin analysis and with 5, 15, or 20 μM of celecoxib for total actin analysis. Caco-2 cells were treated for 20 h with 5 and 50 μM of celecoxib. Cells were then lysed using TNE lysis buffer (dH_2O, 1 M of Tris [pH 8.0], 5 M of NaCl, 0.6 M of NaF, NP-40, protease inhibitor, and sodium orthovanadate), and protein contents were measured using the Pierce bicinchoninic acid protein assay kit (Thermo Scientific). Equal amounts of protein were loaded into Mini-PROTEAN TGX Gels (Bio-Rad, Hercules, CA) and subjected to electrophoresis. Proteins were then transferred to polyvinylidene difluoride transfer membranes (Thermo Scientific). Membranes were incubated with primary antibodies diluted in Odyssey Blocking Buffer (LiCor) at concentrations of 1:1000 (anti–β-catenin), 1:5000 (anti–β-actin), or 1:500 (anti–actin C-2 and anti–GAPDH FL-335) overnight at 4°C, washed with phosphate-buffered saline (PBS)-Tween-20 thrice and incubated with their corresponding secondary antibodies (LiCor) for 1 h at room temperature. Membranes were imaged using the LiCor Odyssey System (LiCor).

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