Cyclooxygenase-2 Inhibition Augments the Hepatic Antitumor Effect of Oral Salmonella Typhimurium in a Model of Mouse Metastatic Colon Cancer

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INTRODUCTION: Oral inoculation with a nontoxic, attenuated strain of Salmonella typhimurium reduces tumor burden and improves survival in a mouse model of metastatic colon cancer. These effects are likely mediated by S. typhimurium-induced increases in hepatic natural killer leukocytes. Cyclooxygenase-2 inhibitors may mediate antitumor effects through antiangiogenic, immune, or proapoptotic pathways. We hypothesized that cyclooxygenase-2 inhibitors would act synergistically with S. typhimurium, resulting in additional antitumor effects. METHODS: Four groups of mice were studied: control, S. typhimurium alone, cyclooxygenase-2 inhibitor alone, and S. typhimurium plus cyclooxygenase-2 inhibitor. Mice were given normal drinking water (control, S. typhimurium alone) or water with 1,600 parts per million cyclooxygenase-2 inhibitor (cyclooxygenase-2 inhibitor alone, and S. typhimurium plus cyclooxygenase-2 inhibitor) and orally inoculated with saline (control, cyclooxygenase-2 inhibitor alone) or 10⁷ S. typhimurium (S. typhimurium alone, S. typhimurium plus cyclooxygenase-2 inhibitor) and orally inoculated with saline (control, cyclooxygenase-2 inhibitor alone) or 10⁷ S. typhimurium (S. typhimurium alone, S. typhimurium plus cyclooxygenase-2 inhibitor). Twenty-four hours later, all mice underwent laparotomy, and 5 × 10⁵ MCA38 murine adenocarcinoma cells were injected into the spleen. On Day 14, hepatic tumor number and tumor volume was quantitated and hepatic leukocytes were analyzed by flow cytometry. RESULTS: Compared with control mice orally inoculated with saline, S. typhimurium-treated mice had fewer and smaller tumors; mice treated with cyclooxygenase-2 inhibitor alone had tumor burden similar to control mice, and mice treated with S. typhimurium plus cyclooxygenase-2 inhibitor had fewer and smaller tumors compared with all other groups. Increased liver natural killer cells and decreased CD4⁺ and CD8⁺ T cells were observed in both S. typhimurium-treated groups. No alterations in hepatic leukocyte phenotype were observed in mice receiving cyclooxygenase-2 inhibitor alone. CONCLUSION: Oral cyclooxygenase-2 inhibitor appeared to act synergistically with S. typhimurium to reduce tumor burden. This combination therapy may have clinical application in the treatment or prevention of hepatic metastases associated with colorectal cancer. [Key words: Cancer; Salmonella; Natural killer cells; Cyclooxygenase-2 inhibitors]

Supported by a grant from the ASL Cancer Research Foundation, Minneapolis, Minnesota.

Presented in part at The American Society of Colon and Rectal Surgeons Annual Meeting, San Diego, California, June 2 to 7, 2001. No reprints are available.

Using mice with experimental liver cancer, we have previously reported that oral inoculation with an attenuated S. typhimurium (Δ550) results in no morbidity, is associated with increased hepatic natural killer lymphocytes (NK1.1⁺CD4⁺), and is associated with reduced hepatic tumor burden. Because attenuated S. typhimurium may be suited for use as an adjuvant oral antitumor therapy, we searched for a safe oral agent that may act synergistically with salmonella to reduce tumor burden, and we hypothesized that cyclooxygenase-2 (COX2) inhibition may potentiate the antitumor efficacy of orally administered S. typhimurium.

In mammals, cyclooxygenase-1 (COX1), constitutively expressed on most nucleated cells, enzymatically catalyzes the breakdown of arachidonic acid into eicosanoids including the prostaglandins (PGs), leukotrienes, and thromboxanes. Although COX metabolites generally mediate normal physiologic functions, a growing body of literature implicates COX activity as critical to the process of tumorigenesis of the colon, esophagus, breast, head, and neck. In humans, daily use of drugs that nonspecifically inhibit COX (i.e., nonsteroidal anti-inflammatory drugs) has been associated with a 40 to 50 percent reduction in the relative risk of colorectal cancer over 20 years. COX2 expression is inducible and is elevated in inflamed or dysplastic tissue, including 40 percent of colonic adenomas and 80 percent of colonic adenocarcinoma. Cyclooxygenase-2 specific inhibitors (COX2i) have previously been demonstrated to have
antitumor efficacy in animal models of lung and colon cancer.6,7

Here, we report that in a murine pretreatment model of hepatic metastases of colorectal cancer, oral inoculation with S. typhimurium \( \chi_{4550} \) was associated with reduced hepatic tumor burden. Oral COX2i alone had no effects on tumor burden, but oral COX2i administered in conjunction with oral S. typhimurium resulted in further reduction in tumor when compared with S. typhimurium alone. An increase in liver NK1.1\(^+\) lymphocytes in S. typhimurium-treated animals suggests that these effects may be immune mediated.

**MATERIALS AND METHODS**

**Bacteria**

S. typhimurium \( \chi_{4550} \) is a well-characterized\(^8\) deletion mutant (\( \Delta \text{cyA}, \Delta \text{crp} \)) provided as a gift from Dr. Roy Curtis (Washington University, St. Louis, MO). S. typhimurium \( \chi_{4550} \) has been studied in animals as a vaccine against salmonellosis,\(^9\) and the use of S. typhimurium \( \chi_{4550} \) in mice for the immunotherapy of cancer has been previously reported.\(^1,10\) For use in experiments, overnight tryptic soy broth (Difco Laboratories, Detroit, MI) cultures of S. typhimurium \( \chi_{4550} \) were washed twice and resuspended in sterile saline.

**Mice**

Female 18 to 22 g C57Bl/6 (H-2\(^d\)) mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Mice were housed in biosafety level two containment facilities in cages with filter tops and handled by specially trained personnel. The University of Minnesota Institutional Animal Care and Use Committee approved all protocols.

**Tumor Preparation and Hepatic Metastases Model**

To study hepatic metastases, the model of Lafreniere and Rosenberg\(^11\) was used with minor modifications. MCA-38 is a weakly immunogenic murine colon adenocarcinoma that was originally induced by subcutaneous injection of dimethylhydrazine into C57BL/6 mice.\(^12\) The tumor was maintained in the laboratory by subcutaneous passage in C57BL/6 mice. For use in experiments, single cell suspensions were prepared from subcutaneous tumors. Briefly, tumors were excised, mechanically minced, and digested for 2 hours at room temperature (RT) with a cocktail of 0.01 percent DNase, 0.01 percent hyaluronidase, and 0.1 percent collagenase (Sigma). The preparation was then washed (Hanks balanced salt solution (HBSS) without calcium or magnesium) and resuspended to 200,000 cells/ml in HBSS without calcium or magnesium. Tumor cells were more than 95 percent viable as determined by trypan blue exclusion.

C57BL/6 mice were randomly divided into four groups of 12 mice per group: saline, COX2i alone (celecoxib, Searle, St. Louis, MO), S. typhimurium alone, or S. typhimurium plus COX2i. On Day 0, mice were orally inoculated with saline (control) or 10\(^9\) S. typhimurium (all other groups). No mouse was orally inoculated again for the duration of the experiment, and mice randomized to receive COX2i were maintained on 1,600 ppm in drinking water for the duration of the experiment. This dose has been documented to have antitumor efficacy in previous rodent models of cancer.\(^6\) Twenty-four hours after oral inoculation with saline or S. typhimurium, all mice were anesthetized by intraperitoneal injection of 50\(\mu\)l of 1 part ketamine (100 mg/ml, Sigma) and two parts xylazine (20 mg/ml, Sigma). Through a left subcostal incision, the spleen was injected with 50,000 tumor cells in 250\(\mu\)l of HBSS. Three minutes later, a splenectomy was performed, and the wound was closed. Aseptic technique was followed throughout the procedure. To assess what effect (independent of tumor) administration of COX2i or infection with S. typhimurium may have had on T-cell phenotype, a separate cohort group (termed “No MCA-38” in results) was treated as above except tumor was not introduced. All mice were killed (under anesthesia) 14 days later, and livers were excised. Assessment of hepatic tumor burden was standardized with the following assumptions: surface lesions represent more than 95 percent of tumor burden\(^11\), and the tumor’s visible portion represented the maximal diameter. Hepatic metastases were quantified by counting (3x dissecting scope) and measuring the transverse diameter of each tumor nodule. Individual tumor volume was calculated using the formula for a sphere (\(4/3 \pi r^3\)).

**Cell Preparation**

Liver lymphocytes were prepared as described.\(^13\) Briefly, the liver was mechanically minced, passed through 100-gauge nylon mesh (Sefar America, Inc.,