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Sulindac Prevents Carcinogen-Induced Intrahepatic Cholangiocarcinoma Formation *In Vivo*

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Background. **Intrahepatic cholangiocarcinoma (ICC) incidence and mortality are increasing in the United States and worldwide. ICC etiologies involve chronic inflammation. We hypothesize that the nonsteroidal anti-inflammatory agent sulindac may prevent ICC by targeting cyclooxygenase-1 and -2 (COX-1, -2) as well as COX-independent pathways.**

Materials and Methods. **ICC was induced with the carcinogen N-nitrosobis(2-oxopropyl)amine (BOP) in Syrian golden hamsters. Cholangiocarcinogenesis was accelerated by a choline-deficient diet and administration of DL-ethionine and L-methionine. Hamsters were gavaged twice daily for 10 wk with vehicle or sulindac 25, 50, or 75 mg/kg/dose. Harvested livers underwent gross and histopathological examinations. Tissues were analyzed by immunostaining, Western blot, and enzyme-linked immunosorbent assay (ELISA).**

Results. **ICC incidence and multiplicity were decreased in sulindac treatment groups *versus* control ($P < 0.05$). In addition, ICC and nontumor lesion sizes decreased in treatment *versus* control animals. Proliferative indices (Ki-67 immunostaining) decreased and apoptosis (ApopTag immunostaining) increased in treatment *versus* control ($P < 0.05$). No changes in COX-1 and -2 protein levels were detected by Western blot. Furthermore, prostaglandin E_2 (PGE_2) levels were unchanged in treatment and control serum and liver tissues ($P > 0.05$), suggesting that the antitumor effects of sulindac are mediated by COX-independent mechanisms. Nuclear p65 (activated NF-κB) immunostaining decreased ($P < 0.05$), and protein levels of the NF-kB inhibitor IκB-α increased in treatment *versus* control groups. p65 ELISA of liver extracts confirmed decreased NF-κB binding activity in sulindac-treated *versus* control animals ($P < 0.05$).**

Conclusion. **Sulindac effectively prevents experimental cholangiocarcinogenesis, in part by inhibiting the NF-κB pathway.**

Key Words: **cholangiocarcinoma; cyclooxygenase; nuclear factor κ-B; sulindac; chemoprevention.**

INTRODUCTION

The incidence and mortality of intrahepatic cholangiocarcinoma (ICC) are increasing in the United States and worldwide [1–3]. Cholangiocarcinoma (CCA) is the second most common primary liver tumor, and ICC comprises one-third of cholangiocarcinoma [1, 4]. Risk factors for ICC include primary sclerosing cholangitis, *Clonorchis sinensis* and *Opisthorchis viverrini* infections, Caroli's disease, choledochal cysts, hepatolithiasis, and Thorotrast exposure [4–6]. Recent reports implicate cirrhosis, inflammatory bowel diseases, and chronic infections with hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) [5, 7]. Prognosis remains poor despite advances in surgical techniques and chemotherapeutics due to the insidious onset and late presentation at diagnosis. Currently, optimal treatment is surgical resection, but 90% of patients are not candidates due to widespread metastases and/or inadequate liver function needed as reserve following partial hepatectomy [8].

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With chronic inflammation as the link between CCA and its risk factors [4], targeting inflammatory pathways involved in cholangiocarcinogenesis may prevent the formation of cholangiocarcinoma in high-risk groups. Cyclooxygenase-2 (COX-2) is an inducible enzyme under conditions of stress and inflammation, while cyclooxygenase-1 (COX-1) is considered a "housekeeping" enzyme, constitutively expressed in nearly all healthy human tissues. Dysregulation of both isoforms has been implicated in tumorigenesis in various cancers [9–13]. Furthermore, both isoforms have been implicated in ICC etiopathology [14–17].

Due to successful chemoprevention with nonspecific COX inhibitors in other cancers, and concerns of cardiovascular complications with prolonged COX-2-specific inhibition in humans, we examined effects of the nonselective COX inhibitor sulindac in a hamster cholangiocarcinogenesis model [18, 19]. *In vitro* and *in vivo* studies support the use of sulindac as an anti-cancer agent in gastrointestinal and inflammatory cancers [20–23]. While known as a non-selective COX inhibitor due to its COX-1 and COX-2 antagonism, sulindac may also target non-COX proteins, notably nuclear factor-kappaB (NF-κB) [22, 24–27]. NF-κB is up-regulated in intrahepatic inflammatory states and hepatopancreatobiliary (HPB) tract carcinomas, and is a potential chemotherapeutic target in HPB tract carcinomas [22, 28–31].

In this study, we employ a hamster cholangiocarcinogenesis model to investigate the effect of sulindac on ICC *in vivo* and provide evidence for a possible mechanistic pathway. We demonstrate that the chemopreventative effects of sulindac are COX-independent and may be mediated by targeting the NF-κB pathway.

MATERIALS AND METHODS

Hamster Homograft Model

To determine appropriate sulindac dosing in hamsters, a homograft model was employed. Syrian golden hamsters (Charles River, Wilmington, MA) were subcutaneously injected with PC-1 tumor cells, generated *de novo* in Syrian golden hamsters exposed to BOP [32]. When tumors reached 1 cm in diameter, a single sulindac dose (0, 12.5, 25, 50, 75, or 100 mg/kg body weight) was administered by orogastric lavage. Animals were sacrificed at 9 or 18 h postinjection and tumors were excised. Intratumoral levels of PGE_2, the endproduct of the reaction catalyzed by COX-1 and COX-2, were determined as described below.

Hamster Carcinogenesis Model

Fifty female Syrian Golden Hamsters (Charles River, Wilmington, MA) were housed and fed in American Association for Accreditation for Laboratory Animal Care (AAALAC)-approved facilities, and animal research and handling were in strict conformance with federal Institutional Animal Care and Use Committee (IACUC) guidelines. Animals were individually caged with alternating 12-h light/dark cycles and fed a diet of Harlan Teklad 4% mouse/rat diet (Harlan, Indianapolis, IN) except where noted. A rapid production/augmentation pressure model [33] was employed with a modification to ethionine administration (see below, days 12–15). On day 1 of the augmentation pressure protocol, 10-wk-old animals weighing approximately 105 g were subcutaneously injected with 70 mg/kg body weight N-nitrosobis(2-oxopropyl)amine (BOP; NCI chemical carcinogen repository, Midwest Research Institute, Kansas City, MO) in PBS. On day 1, animals were randomly divided into treatment groups: group 1 (control (cremophorel/ethanol/water 10%/10%/80%)), group 2 (sulindac [Sigma, St. Louis, MO] 25 mg/kg body weight/dose), group 3 (sulindac 50 mg/kg body weight/dose), and group 4 (sulindac 75 mg/kg body weight/dose). Starting day 1, hamsters received vehicle ± sulindac by orogastric lavage twice daily on weekdays (50, 100, and 150 mg/kg total daily) and once daily on weekends. On days 12–15, hamsters were administered 500 mg/kg ethionine (Lancaster Synthesis, Inc., Windham, NH) suspended in olive oil by daily orogastric lavage while being maintained on a choline-deficient diet (Dyets, Inc., Bethlehem, PA). On day 16, hamsters returned to the basal diet and received a single intraperitoneal injection of 800 mg/kg body weight methionine (Sigma, St. Louis, MO). On day 18, animals received a subcutaneous injection of BOP (20 mg/kg body weight). The augmentation pressure cycle (ethionine/methionine/BOP 20 mg/kg) was repeated on day 26. A final dose of 20 mg/kg BOP was given on day 46. Ten weeks post-initiation, hamsters were sacrificed.

Blood and Tissue Collection and Processing

Upon sacrifice, blood was obtained by cardiac puncture. Approximately 1 mL blood was mixed with 25 μL anticoagulant [EDTA (2g)/NaCl (0.8g) in 100 mL water, pH 7.4] containing 2.4 mg/mL indomethacin (Sigma, St. Louis, MO) and centrifuged (2800 rpm, 15 min, 4°C). Plasma was frozen at –80°C. Representative liver sections from each lobe were frozen in liquid nitrogen and stored at –80°C or fixed in 10% formalin (Sigma, St. Louis, MO) for 48 h. Fixed tissues were transferred to 70% ethanol, paraffin-embedded, and serially cut (5 μm).

Tissue Staining

Slides were stained with hematoxylin and eosin (H and E) according to standard protocols. Immunohistochemistry was performed for: cytokine-7 (CK-7) (pre-diluted, DAKO North America, Carpinteria, CA), Ki-67 (1:50, DAKO), COX-1 (1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), COX-2 (1:500, Cayman Chemical Company, Ann Arbor, MI), and NF-κB/p65 (1:400, Lab Vision Corporation, Fremont, CA). For all immunostains, slides were deparaffinized and hydrated in water. CK-7 slides underwent a 5-min enzymatic digestion with proteinase K (DAKO). Ki-67, COX-2, and NF-κB slides were placed in antigen retrieval citrate buffer, pH 6.0 (DAKO) in a pressure cooker for 15 min and COX-1 slides were placed in antigen retrieval EDTA buffer, pH 8.0. Ki-67 slides also underwent a 10-min avidin/biotin block (DAKO) before all slides were placed in 3% H_2O_2 for 10 min. Ki-67, COX-1, COX-2, and NF-κB slides then were placed in Protein Block (DAKO) for 15 min and then all slides were incubated with appropriate primary and secondary antibodies, and counterstained. Slides were stained for DNA fragmentation using ApopTag peroxidase *in situ* apoptosis detection kit (Millipore, Billerica, MA) per manufacturer's protocol.

Western Blots of Liver Tissue Homogenates

Frozen liver sections were sonicated in radioimmunoprecipitation (RIPA) buffer (PBS, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 1 mmol/L Na_3VO_4). Extracts were centrifuged (10,000 rpm, 10 min, 4°C) and supernatants were obtained. Proteins were resolved on 4% to 20% tris-glycine gels (Invitrogen Corporation, Carlsbad, CA) and transferred to Immobilon-P membranes (Millipore). Blots were