Sulindac and Exisulind Exhibit a Significant Antiproliferative Effect and Induce Apoptosis in Human Hepatocellular Carcinoma Cell Lines

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Abstract

This is the first report enumerating a superb antiproliferative effect of both sulindac and exisulind on hepatocellular cancer cell lines. The growth inhibition and cytotoxicity of sulindac in human hepatocellular carcinoma cell lines HepG2, Huh-7, and KYN-2 were investigated by studying cell growth, cell cycle distribution, and induction of apoptosis. In the presence of sulindac, there was a marked time- and dose-dependent decrease in cell proliferation and viability. Also, exisulind exhibited a similar growth-inhibitory effect on the KYN-2 cell line. The findings of this study suggest that sulindac exhibits a growth-inhibitory effect on human hepatocellular carcinoma cell lines; therefore, these drugs might serve as an effective tool for hepatocellular carcinoma chemoprevention.

Introduction

Accumulating evidence indicates that the NSAIDs possess an antiproliferative effect and induce apoptosis and tumor regression (1, 2). Initially, the research regarding the chemopreventive effect of NSAIDs was largely confined in colorectal-originated tumor cell lines; however, rapid development in this field urged researchers to extend this finding to other carcinomas, including breast, prostate, and pancreatic carcinoma cell lines (2-4). HCC is one of the most lethal malignancies and ranks worldwide as the seventh most common cancer (5). Both colorectal cancer and HCC have a unique similarity in their natural history of development, often characterized by multistage tumor development with distinct morphological and biological phases. Extensive work regarding the chemopreventive effect of a new generation of NSAIDs, sulindac, in colorectal lesions and cell lines has been accomplished (6, 7); however, thus far, there has been no report in HCC. Hence, the present study was undertaken to analyze the effect of sulindac and its irreversible oxidized derivative, sulindac sulfone (exisulind), in three human HCC cell lines. Also, the rationale for elucidation of the effect of sulindac in HCC cell lines was proposed by a report indicating that well-differentiated HCCs express COX-2 more frequently and strongly than less-differentiated ones (8).

Materials and Methods

Cell Lines and Cell Culture. Three HCC-derived cell lines designated KYN-2, HepG2, and Huh-7 were used. The KYN-2 cell line was kindly provided by the First Department of Pathology, Kurume University School of Medicine (Kurume, Japan). HepG2 and Huh-7 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM supplemented with 5% fetal bovine serum (Life Technologies, Gaithersburg, MD), penicillin (100 units/ml), and streptomycin (100 μg/ml) in an atmosphere of 5% CO₂.

Growth Inhibition and MTT Assays. Approximately 5 × 10⁴ cells were plated in 60-mm-diameter Petri dishes in triplicate. Cells were allowed to grow, and after 24 h sulindac (Sigma Chemical Co., St. Louis, MO) and exisulind (a generous gift from Merck, Rahway, NJ) at various concentrations (25, 50, and 100 μM) dissolved in DMSO were added to the treatment dishes. The final concentration of DMSO was <0.1%. Cells were harvested at definite time intervals by trypsinization, and aliquots were counted using a hematocytometer. On day 3, only KYN-2 cells were harvested by trypsinization and were used for MTT assay (Boehringer Mannheim, Mannheim, Germany). This assay relies on the ability of viable cells to reduce the tetrazolium salt MTT metabolically to a purple formazan product, which can be quantified colorimetrically. One hundred-microliter aliquots of cells were transferred to triplicate wells of a flat-bottomed 96-well microtiter plate and treated with 10 μl of MTT for 4 h at 37°C. After that, 100 μl of solubilization solution were added, and the mixture was incubated at 37°C for overnight. The solubilized formazan product was spectrophotometrically quantified using a microtiter plate reader (EAR 400; FW Sh-Lab Instruments, Groedig, Austria) at 550-nm wavelength. The morphology of the KYN-2 cells both in the sulindac and control group was checked by a phase-contrast light microscope.

Cell Cycle Analysis. The proportion of cells in G₀-G₁, S, and G₂-M phases was determined by flow cytometric analysis of DNA content (EPICS Elite ESP flow cytometer; Coulter Electronics, Miami, FL). Cell cycle distribution in KYN2 cells was measured after 24 h of treatment with 100 μM sulindac. In brief, cell suspension was prepared by trypsinization, and ~2 × 10⁶ cells/ml were washed twice with PBS. The cells were resuspended with 10 ml of 70% ethanol (~20°C), incubated at 4°C for 4 h, washed twice in PBS, incubated with RNase (Sigma) at a concentration of 0.25 mg/ml at 37°C for 15 min, followed by treatment with propidium iodide (50 μg/ml), and incubated for 30 min at 4°C in the dark. DNA histograms were analyzed using Multicycle AV software (Phoenix, San Diego, CA) to evaluate cell cycle compartments.

Detection of Apoptosis. Apoptosis was detected by an annexin V-FITC kit (Immunotech, Marseille, France) according to the manufacturer’s instructions. Briefly, KYN-2 cells were collected by trypsinization after a 72-h treatment, and the number of cells was adjusted to ~1 × 10⁶ cells/ml. Cells were washed with ice-cold DMEM and were centrifuged to collect the cell pellet. Cell pellet was resuspended in ice-cold binding buffer. After that, annexin V-FITC (10 μl/ml) and PI (10 μl/ml) solutions were added to the cell suspension and mixed gently. The tube was then incubated for 10 min in the dark before being analyzed by the flow cytometer. All steps were carried out on ice. Aliquots of stained cells were smeared onto glass slides, and morphological changes were examined under a fluorescence microscope.

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2 The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; HCC, hepatocellular carcinoma; COX-2, cyclooxygenase-2; RT, reverse transcription; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI, propidium iodide.

Immunostaining and RT-PCR for COX-2 Expression. Expression of COX-2 was checked at the protein and mRNA levels by immunohistochemistry and RT-PCR, respectively, in all three cell lines used for this study. Immunohistochemistry was done by the avidin-biotin-peroxidase complex method as described elsewhere (4). Briefly, cell smears were fixed in 4% paraformaldehyde, endogenous peroxidase activity was quenched with 3% H₂O₂, and nonspecific binding was blocked by normal rabbit serum. Slides were treated with mouse anti-human COX-2 antibody (Cayman Chemical, Ann Arbor, MI) at a 1:500 dilution overnight at 4°C. All other steps were done using a Histofine SAB-PO® (Nichirei Corp., Tokyo, Japan) kit according to the manufacturer’s instructions. The reaction product was visualized by 3-amin-o-ethylcarbazole (Histofine, Tokyo, Japan). Counterstaining was done using hematoxylin.

For RT-PCR, total RNA was extracted using the TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer’s instructions. RT-PCR was performed as described previously (4).

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**Statistical Analysis.** A minimum of three experiments measuring the growth inhibition was performed, and the mean of the inhibitory effect was expressed as percent inhibition calculated as (number of treated cells/number of control cells × 100). Pairwise group comparisons between different groups were done using the unpaired Student’s t test. Significant differences were assumed when the chance of differences arising from a sampling error was <1 in 20 (i.e., P < 0.05).

**Results**

**Growth Inhibition.** All three cell lines used in this study, KYN-2, HepG2, and Huh-7, expressed COX-2 strongly, and also the expression of COX-2 at the mRNA level was confirmed by RT-PCR (Fig. 1). Sulindac exhibited a statistically significant dose-dependent growth-inhibitory effect on all HCC-derived cell lines evaluated in this study (Fig. 2a). Supplementation of KYN-2 cell culture medium for 72 h with 25, 50, and 100 μM sulindac produced almost 10, 48, and 70% reduced cell growth, respectively, when compared with cells in medium supplemented with DMSO only (Fig. 2b). As shown in Fig. 2b, at comparable concentrations, exisulind had a comparatively greater dose-dependent inhibitory effect than that of the sulindac at 72 h of treatment; however, there was no statistical difference between the two groups. Moreover, the growth-inhibitory effect of sulindac on KYN-2 cells was found to be time dependent, because the inhibitory effect became gradually stronger with the passage of time after treatment, with the most significant effect observed at 72 h. (Fig. 2c).

As shown in Fig. 2d, the results of manual counting of cell numbers conformed well with those performed with the MTT assay. Sulindac- and exisulind-induced morphological changes were evident by 72 h of treatment in all cell lines. Cells in dishes supplemented with sulindac or exisulind became sparse, rounded, and detached from the dishes (Fig. 2f).

**Cell Cycle Effect.** DNA histograms prepared from cells cultured in medium supplemented with sulindac showed accumulation of cells in G0-G1, and a corresponding reduction in percentages of cells in S and G2-M (Fig. 3). In contrast to the apoptotic effect, arrest of the cell cycle was not time dependent. Sulindac produced cell quiescence during the first 24 h of cell culture in the preconfluent state, and after that the cell cycle progressed to S and G2-M as noted in the control cells at 48 h after sulindac treatment (data not shown).

**Apoptosis and Secondary Necrosis.** To evaluate whether the growth-inhibitory effect of sulindac was associated with apoptosis or necrosis, a double-staining method using FITC-conjugated annexin V and PI was done. Annexin V, a calcium- and phospholipid-binding protein, binds preferentially to a negatively charged inner membrane phospholipid, phosphatidylserine, on exposure to the cell surface and detects early cellular apoptotic changes, whereas the normally impermeable vital dye PI detects cells undergoing necrotic changes. As shown in Fig. 4a, 100 μM sulindac induced cellular apoptosis without producing cellular necrosis. This effect was time dependent, because

Fig. 1. Immunostaining and mRNA expression of COX-2 in KYN-2, Huh-7, and HepG2 cell lines. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.
the percentages of cells undergoing apoptosis increased from 16.6% at 12 h to 66.5% at 48 h (Figure 4, a–d). Furthermore, annexinV- and PI-stained slides were inspected for morphological changes associated with apoptosis. A representative slide at 48 h after sulindac treatment shows nuclear condensation and fragmentation as the hallmark of cellular apoptosis (Fig. 4e).

Discussion

The potential for sulindac as an antineoplastic agent has been unanimously established in colorectal carcinoma cell lines. Subsequently, similar results have been reported in several other carcinomas (2–4); however, the effect has yet to be elucidated in HCC-originated cell lines. Sulindac, a new generation of NSAID, inhibits both isoforms of COX (COX-1 and COX-2), key enzymes that catalyze the formation of prostaglandins from arachidonic acid. This inhibitory effect of sulindac is attributable to its antineoplastic effect, although much controversy remains around this plausible hypothesis. Much of this controversy looms around a similar antineoplastic effect exerted by exisulind, which has virtually no inhibitory effect on either of the COX enzymes (3). As established previously in colorectal carcinoma cell lines, similarly both sulindac and exisulind had a uniform growth-inhibitory effect on all HCC-originated cell lines used for this study.

Fig. 2. Growth-inhibitory effect of sulindac and sulindac sulfone. A concentration-dependent growth inhibitory effect of sulindac on all HCC cell lines was noticed (a), and a similar effect was also noted for sulindac sulfone in KYN-2 cells (b). A time-dependent effect of sulindac on KYN-2 cells was evident at a 100 μM dose (c). The manually counted data of cell number were reproducible by the MTT assay (d). e and f, Morphological characteristics of KYN-2 cells before (e) and after (f) sulindac treatment.

Fig. 3. DNA histograms of KYN-2 cells cultured in medium supplemented with DMSO (a) and sulindac (b).
All of the three cell lines used in this study had different differentiation statuses, with HepG2 and Huh-7 being well differentiated (9) and KYN-2 being less differentiated (10). Koga et al. (8) reported that COX-2 is involved in early stages of HCC carcinogenesis and is expressed frequently in well-differentiated carcinomas. However, in this study, irrespective of their differentiation status, all three cell lines expressed COX-2 both at the protein and mRNA levels. Supplementation of culture medium with both sulindac and its irreversible oxidative form exisulind at comparable concentrations produced a concentration-dependent growth-inhibitory effect. A growing body of evidence suggests that exisulind induces apoptosis in various tumor cell lines and inhibits chemically induced colonic (11) and mammary carcinogenesis (2). In these reports the authors concluded, and we concur with them, that the antineoplastic effect of sulindac may be irrespective of its inhibitory effect on COX enzymes and prostaglandin biosynthesis. It is worth mentioning that a comparable growth-inhibitory effect of sulindac could be achievable at a much lower concentration (100 μM) in HCC cell lines than in other cell lines, because sulindac had a significant inhibitory effect at 100 μM in HCC cell lines, whereas in pancreatic and colon cancer cell lines it was 200 and 400 μM, respectively (4, 7). Sulindac is a prodrug and is metabolized to sulindac sulfide by the gut flora and in the liver before it exerts its effect on COX enzymes (12). It might be possible that HCC cell lines efficiently converted sulindac to its sulfide form for effectiveness at a relatively lower concentration.

Although the exact mechanism for the antineoplastic effect of NSAIDs has yet to be delineated, the antiproliferative effect and/or induction of apoptosis have been attributed to this antineoplastic effect. The antiproliferative effect of NSAIDs by quiescence of the cell cycle has been questioned by several authors, but most of them agree regarding the proapoptotic effect of NSAIDs. In a recent report, Elder et al. (13) demonstrated that NS-398, a new generation of NSAID had no effect on cell cycle distribution in a colon carcinoma cell line, whereas Piazza et al. (14) showed that sulindac produced G1...

Fig. 4. Percentages of apoptotic cells after sulindac treatment in KYN-2 cells at 0 h (a), 12 h (b), 24 h (c), and 48 h (d). Quadrants 2–4, Secondary necrotic cells, viable cells, and apoptotic cells, respectively. The percentages of apoptotic cells at 0, 12, 24, and 48 h after sulindac treatment were 0, 16.2, 42.9, and 66.5%, respectively. e, Morphological changes as evidence of apoptosis in sulindac-treated KYN-2 cells.
arrest in proliferating colon carcinoma cell lines. In this study, we found that both cell cycle arrest and apoptosis contributed to the growth-inhibitory effect of sulindac in HCC cell lines. In the preconfluent state, sulindac produced significant cell cycle arrest in G_{1}/G_{2}, as early evidence of an antineoplastic effect; however, this was not time dependent, because the cells emerged from the arrest and entered into S-phase. In contrast, the rate of apoptosis increased uniformly in a time-dependent manner and reached a maximum of 66.5% at 48 h of sulindac treatment, which was followed by a maximum growth inhibition at 72 h of treatment (Fig. 4).

Several mechanisms have been proposed affecting the molecular pathways regulating the cellular proliferation and apoptosis by the NSAIDs, although the key mechanism still remains a dilemma. Only a few of these mechanisms are related to their capability to inhibit COX, and most of them are COX independent, including modulation of ras signal transduction (15), mitogen-activated protein kinase activation (16), nuclear factor κB activation (17), cyclin expression (7), activation of the sphingomyelin-ceramide pathway (18), and p53 expression (14). In this study, we used two well-differentiated HCC cell lines of differentially mutated p53 and ras genes with an N-ras mutation at codon 61 position 2 in the HepG2 cell line, and a p53 point mutation resulted in the amino acid changes of cysteine for tyrosine at codon 220 in the Huh-7 cell line (9, 19). Sulindac was equally effective in both of these cell lines and produced similar growth inhibition, indicating that neither of these pathways is crucial for the antineoplastic effect of sulindac.

HCC is the leading cancer in men in Taiwan and is one of the most common causes of malignancy-related death in Africa and Asia (20). Despite an enthusiastic effort to diagnose the disease at an early stage, the prognosis is still dismal, thus implying that emphasis should be given on preventive measures. Unfortunately, to date, there is no effective preventive measure in this highly malignant disease. Both viral infection and chemical carcinogens are thought to be responsible for the development of HCC, with distinct preceding steps of premalignant lesions in the vicinity of cirrhotic liver. Epidemiological studies have shown a 40–50% reduction in mortality rates from colon cancer in patients receiving NSAID therapy (21). It is worth mentioning that the results of this study might create a new avenue for the chemoprevention of HCC by the new generations of NSAID therapy.

In the late 1970s, Hial et al. (22) demonstrated that COX-dependent NSAIDs exerted an antiinflammatory effect on a rat hepatoma cell line. For long-term chemoprevention, the COX-dependent NSAIDs are not suitable because of their deleterious effect on gastrointestinal functions. Therefore, the new generation of COX-independent NSAIDs such as exisulind and COX-2-specific inhibitors might be suitable for HCC chemoprevention. However, before recommending these drugs as routine prophylaxis, further studies regarding the safety profile of these drugs and identification of cohorts at high risk for subsequent development of HCC should be conducted.

References
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