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• ORIGINAL RESEARCH •

Growth inhibition and apoptosis induction of Sulindac on Human gastric cancer cells

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Abstract

AIM: **To evaluate the effects of sulindac in inducing growth inhibition and apoptosis of human gastric cancer cells in comparison with human hepatocellular carcinoma (HCC) cells.**

METHODS: **The human gastric cancer cell lines MKN45 and MKN28 and human hepatocellular carcinoma cell lines HepG$_2$ and SMMC7721 were used for the study. Anti-prolife rative effect was measured by MTT assay, and apoptosis was determined by Hoechst-33258 staining, electronography and DNA fragmentation. The protein of cyclooxygen ase-2 (COX-2) and Bcl-2 were detected by Western dot blotting.**

RESULTS: **Sulindac could initiate growth inhibition and apoptosis of MKN45, MKN28, HepG$_2$ and SMMC7721 cells in a dose-and time-depen dent manner. Growth inhibitory activity and apoptosis were more sensitive in HepG$_2$ cells than in SMMC7721 cells, MKN45 and MKN28 cells. After 24 hours incubation with sulindac at 2 mmol·L^{-1} and 4 mmol·L^{-1}, the level of COX-2 and Bcl-2 prote in were lowered in MKN45, SMMC7721 and HepG$_2$ cells but not in MKN28 cells.**

CONCLUSION: **Sulindac could inhibit the growth of gastric cancer cells and HCC cells effectively *in vitro* by apoptosis induction, which was associated with regression of COX-2 and Bcl-2 expression. The growth inhibition and apoptosis of HCC cells were greater than that of human gastric cancer cells. The different effects of apoptosis in gastric cancer cells may be related to the differentiation of the cells.**

Subject headings sulindac; apoptosis; gastric cancer; HCC;COX-2

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin were widely used for the treatment of inflammatory joint and muscle pain. The stuidies of past two decades indicated that NSAID could prevent colorectal cancer[1-3]. As a member of NSAID, sulindac has been shown to induce regression of adenomas in familial adenomatous polyposis (FAP) patients, prevent recurrence of adenomas[4-5] and reduce the risk of colon cancer[6]. Rahman *et al*[7] demonstrated that sulindac and its irreversible oxidized derivative, sulindac sulfone exhibited a growth inhibitory effect on human hepatocellular carcinoma (HCC) cell lines, indicating that sulindac has chemopreventive effect on colon cancer as well as on other types of cancer in GI tract. Gastric cancer is one of the most common causes of malignancy-related death in China[8-11]. It is imperative to find effective chemopreventive methods to reduce the morbidity and mortality of gastric cancer. Lots of chemical agents have been proved having the chara cter of inducing apoptosis in human gastric cancer cells[12-13]. Recent studies gave the concept that overexpression of cyclooxygenase-2 (COX-2) was the early event in carcinogenesis of gastric cancer[13] and made it the target of chem oprevention against gastric cancer[14]. The present study was undertaken to analyze the effect of sulindac in two gastric cancer cell lines as compared with two HCC cell lines.

MATERIALS AND METHODS

Cell lines and culture

The huamn gastric cancer cell lines MKN45 and MKN28 were obtained from the Japanese Cancer Res earch Resources Bank (Tokyo, Japan). HepG$_2$ and SMMC7721 cells were available commercially from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. Cells were grown in RP M I 1640 (Gibco) supplemented with 100 mL·L^{-1} fetal bovine serum (Sijiqing, HangZhou , China), penicilin (100 mg·L^{-1}) and streptomycin (100 mg·L^{-1}) in a humidified atmosphere of 50 mL·L^{-1} CO_2 at 37℃. Sulindac purchased from Sigma Chemical Co. (St Louis, MO, USA) was freshly prepared in DMSO (less than 5 g·L^{-1} in w hole medium) before use. Vehicle controls of DMSO was included in the studies.

MTT assay

Antiproliferative effects were measured by MTT assay. About 10 000 cells per well were plated in 96-well microtitre plates and incubated overnight in 100 μL of culture media. Then cells were treated with various concentrations of sulindac for 24 h and 48 h (0, 0.25, 0.5, 1, 2 and 4 mmol·L^{-1} for MKN45, MKN28 and SMMC77 21 cells, 0, 25, 50, 100, 200 and 400 μmol·L^{-1} for HepG$_2$ cells); 100 μL MTT (1 g·L^{-1}) was then added to each well, and the cells were further incubated for 4 h. The supernatant was removed and 100 μLDMSO was added to each well, and then incu bated for 30 min. The absorbance at wavelength of 570 nm was measured by a micro - ELISA reader. The negative control well has no cells and used as zero point of a bsorbance. Each assay was performed three times in triplicate.

Morphological measurement of apoptosis

The morphological change of apoptosis was assayed under fluorescence microscope following staining with Hoechst33258. The cells were fixed in 200 mL·L^{-1} ethanol/PBS, stained with 5 mg·L^{-1} Hoechst33258 (CNI) for 30 min at 37℃, then visualized under UV fluorescence microscope. Apoptotis cells were de fined as cells

showing nuclear and cytoplasmic shrinkage, chromatin condensation and apoptosis body. At least 300 cells were counted and the percentage of apoptotic cells (Apoptotic Index) was calculated.

DNA fragmentation analysis

Following sulindac treatment, cells were washed by PBS, and fixed in ice-c old 700 mL·L^{-1} ethanol for 24 h. After the ethanol was removed, cells were rinsed in 40 μL 0.2 mol·L^{-1} Na_2HPO_4/0.1 mol·L^{-1} citric acid (192:8, pH7.8) for 60 min at room temperature. After being centrifuged 5 min (1000G), the supernatant was c ollected in Eppendorf tubes, 4 μL 2.5 g ·L^{-1} NP-40 and 4 μL 10 g ·L^{-1} RNase A were added at 37℃ for 30 min, then 4 μL Protein K (1 mg/mL, Promega) were added at 50 ℃ for 30 min. Equal amount of DNA (15 μL) was electrophoresed in 10 g·L^{-1} a garose gels impregnated with ethidium bromide (5 mL·L^{-1}) for 2 h at 80 V. DNA fragments were visualized by ultraviolet transillumination.

Western Dot blot analysis of COX-2 and Bcl-2

Cells incubated with various concentrations of sulindac for 24 h (0, 2 and 4 mmol·L^{-1} for MKN28, MKN45 and SMMC7721 cells, 0, 400 and 800 μmol ·L^{-1} for $HepG_2$ cells). These cells were washed by PBS and dissolve d in 100iL extraction buffer (50 mmol·L^{-1} Tris-HCL (pH 8.0), 150 mmol·L^{-1} NaCl, 10 g·L^{-1} Triton-X-100 and 1mg·L^{-1} Aprotinine). Equal amounts of cell lysates (100 μg) were dotted on nitrocellulose membrane (Amersham). The membrane was incubated first with a primary antibody overnight at 4℃ and then with peroxidase- conjugated anti-rabbit or anti-mouse IgG antibody for 2 h. Protein was detected by chemilu minescence (ECL) system. The membranes were washed and added by equal amounts of luminal reagent A and B (Santa Cruz) for 2 min and exposed to film. Rabbit anti-h u man polyclonal antibody COX-2(H-62, sc-7951), goat anti-mouse IgG conjugate d with HRP(Cat#sc-2005) , Goat anti-rabbit IgG conjugated with HRP(Cat#sc-2004) we re purchased from Santa Cruz Co. Mouse anti-human bcl-2 monoclonal antibody (Cat.No.M-0025) was purchased from Antibody Diagnostica Inc(USA).

Statistical analysis

Student's *t* test was used for results comparison among different groups. The presented data were mean values of at least three different experiments and expressed as $\bar{x}\pm s$. A *P* value of less than 0.05 is considered statistically significant.

RESULTS

Effects of sulindac on cell growth

Various concentrations of sulindac were incubated with cells for 24 h and 48 h. Cell growth was determined by MTT assay. As shown in Figure 1, sulindac could inhibit the growth of gastric cancer cells and HCC cells in a dose-and time-dependent manner. Sulindac showed a more potent effect in reducing $HepG_2$ cells' growth as compared with SMM C77 21, MKN45 and MKN28 cells. The cell death rate was more obvious in MKN45 cells than in MKN28 cells (Figure 1).

Apoptosis of cells induced by sulindac

To evaluate the apoptosis of cells, Hoechst-33258 staining and agarouse gel electrophoresis of genomic DNA were used. The Hoechst-33258 staining showed apoptosis in all four types of cells, which was characterized by cytoplasmic and nuclear shrinkage, chromatin condensation and apoptosis body (Figure 2). The apoptosis was mo re evident in $HepG_2$ cells than in SMMC7721 and gastric cancer cells and the AI of MKN45 cells were higher than that of MKN28 cells (Figure 3). DNA fragmentation was shown as a ladder pattern on agarose gel.

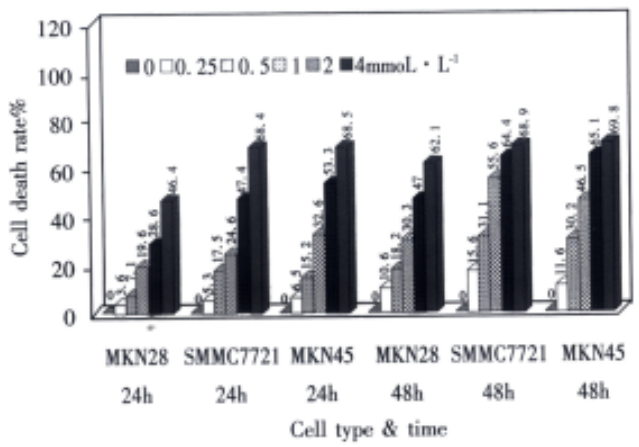

Figure 1A Dose-response of sulindac on growth of cell lines by MTT assay. (N = 3)

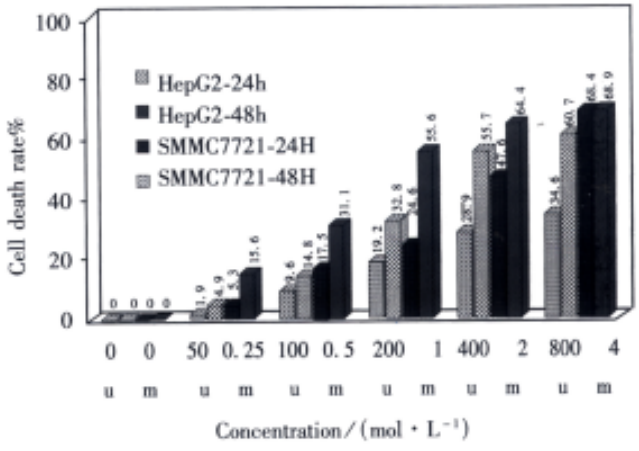

Figure 1B Dose-response of sulindac on growth of HCC cell line s by MTT assay. (N = 3)

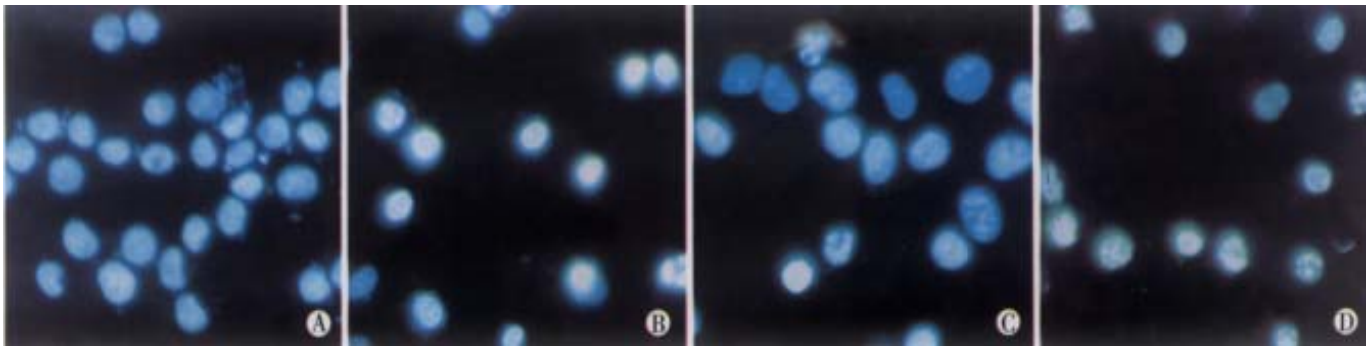

Figure 2 Morphological changes of MKN45 and $HepG_2$. Cells stained with Hoechst33258×400. A: MKN45 cells; B:MKN45 cells treated with 2 mmol·L^{-1} sulindac for 24 h; C: $HepG_2$ cells; D: $HepG_2$ cells treated with 400 μmol·L^{-1} sulindac for 24 h.

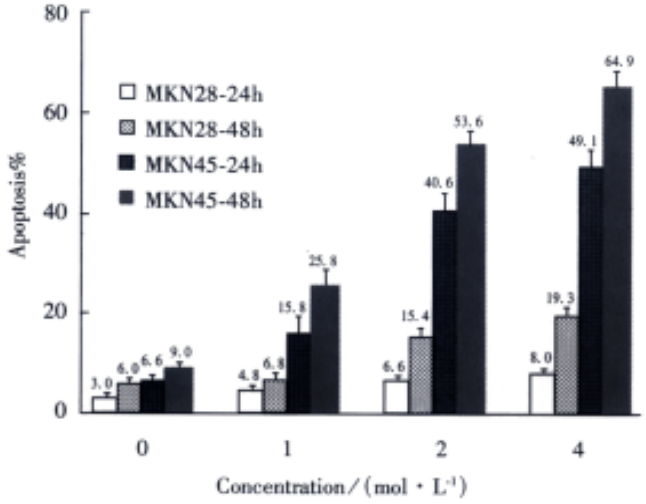

Figure 3A The apoptosis of gastric cancer cells induced by sulindac by Hoechst 33258 staining. (N = 3)

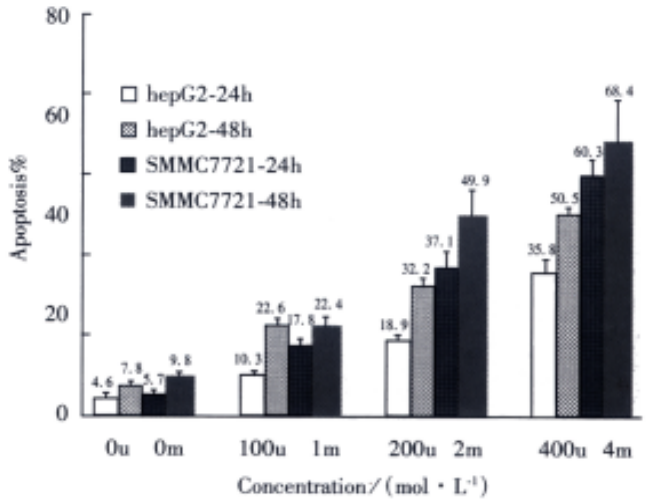

Figure 3B The apoptosis of 2 HCC cells induced by sulindac by Hoechst 33258 staining. (N = 3)

Differential expression of COX-2 and Bcl-2 protein in sulindac-treated cells

The protein levels of COX-2 and Bcl-2 were determined by Western dot blotting. After treatment with 2 mmoL ·L^{-1} and 4 mmoL·L^{-1} of sulindac for 24 h, the protein level of COX-2 and Bcl-2 showed marked decrease in MKN45, HepG$_2$ and SMMC7721 cells, whereas the protein level remained unchanged in MKN28 cells (Figure 4).

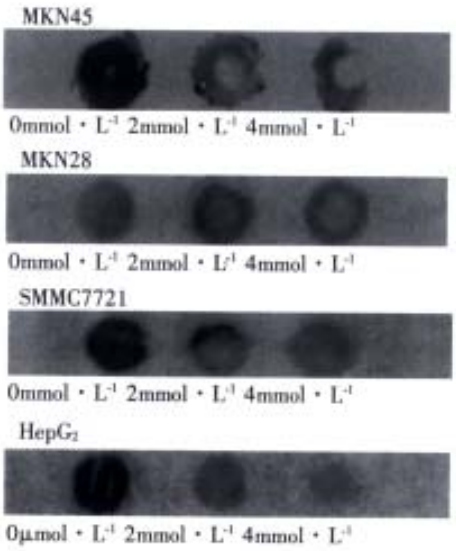

Figure 4A COX-2 protein levels in human gastric cancer and HCC cells with sulindac for 24 h.

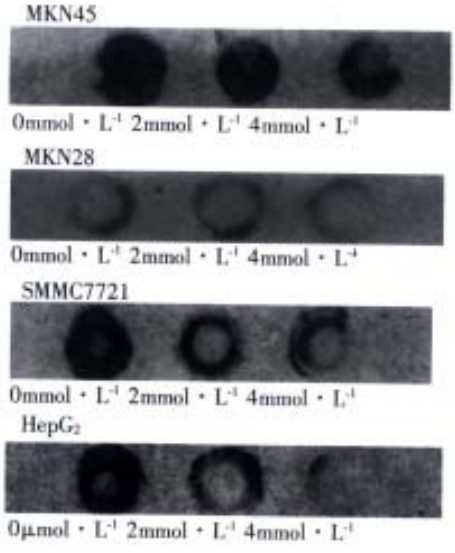

Figure 4B Bcl-2 protein levels in human gastric cancer cells and HCC cells with sulindac for 24 h.

DISCUSSION

Since Adolphie *et al* reported that certain NSAIDs were capable of inhibiting proliferation of Hela cells in 1972, the chemopreventive effect of NSAIDs has been widely studied *in vivo* and *in vitro* in recent years[15-16]. Most results indicated that the mechanism related to this capability was by the inhibition of cyclooxygenase-2 (COX-2) which was not found in most normal tissues and could be induced by cytokines and growth factors[17-19]. Elevated level of COX-2 suggested the existance of inflammation or carcinoma[20-25]. Lim *et al* found that all 104 gastric cancer tissues showed positive expression of COX-2 but not in normol gastric mucosa. Ratnasinghe *et al*[26] found that gastric cancer tissues could produce more prostagland in than normal gastric mucosa. COX-2 was also found to be related to tumor angiogenesis[27-28] and metastasis[29-31]. In this study, we confirmed the results of other studies that COX-2 was positive in MKN45, MKN28 and HepG$_2$ cell lines as well as in SMMC7721 cells and the level of COX-2 protein was much lower in MKN 2 8 cells. The results also confirmed that COX-2 was correlative with carcinogenesis in GI tract.

One of the strongest evidence that NSAID has the capability of chemoprevention and treatment of colorectal cancer was the obvious effect of sulindac in treatment of FAP[32-33]. Pasricha *et al* reported that the number and size of polyps were reduced in 24 FAP patients after treatment with sulindac and induction of apoptosis was regarded

as the main mechanism. However, there we re no studies about growth inhibition and apoptosis induction of sulindac in gastric cancer. In this study, two gastric cancer cell lines with different status of differentiation were used. The growth inhibition and apoptosis of $HepG_2$ cells were more obvious as compared with MKN45 and MKN28 cells. Since sulindac is a pro-drug and is metabolized to sulindac sulfide and sulfone by the gut flora and in the liver[34], HCC cells might be able to convert sulindac to its metabolic derivative and increase its capability of growth inhibition and apoptosis inducti on. We suggested that the effects of growth inhibition of sulindac on gastric cancer might be increased *in vivo* than *in vitro*. We also concluded that sulindac could induce apoptosis in gastric cancer cells and HCC cells, which may account for its growth inhibitory effects.

Bcl-2 is one of the most important factors in apoptosis process[35]. The elevated expression of COX-2 could increase the level of Bcl-2 in the epithelial cells of rat colon and decrease the apoptotic rate of colonic cells[36]. Prostaglandin E_2, mediated by COX-2 from a rachidonic acid, could inhibit apoptosis of human colon cancer cells *in vitro* and increase expression of Bcl-2 in cancer cells[37]. Liu *et al*[38] also confirmed the relationship between COX-2 and Bcl-2 in prostate cancer. We found that the levels o f COX-2 in MKN45, SMMC7721 and HepG 2 cells as well as Bcl-2 were decreased after treatment with sulindac, whereas both COX-2 and Bcl-2 were unchanged in MKN28 cells. Apoptosis was also more evident in MKN45, SMMC7721 and $HepG_2$ than in MKN28 cells. It was suggested that COX-2 and Bcl-2 were involved in apoptosis of gastric cancer cells and HCC cells induced by sulindac.

Several mechanisms have been proposed affecting the pathways regulating cellular proliferation and apoptosis by NSAIDs. Although parts of the mechanisms were related to COX-2 inhibition, most of them were COX independent[39-44]. Sulindac and its derivatives have different mechanism in inducing apoptosis of cancer cells. Sulfone neither inhibits COX nor has anti-inflammatory properties, but can produce chemopreventive effect similar to that of sulindac[45-46]. The death rate of MKN28 cells was higher than its apoptotic rate in our study, which might be caused by the COX-2 in dependent way.

As COX-2 specific inhibitors, Celecoxib and Rofecoxib have been used clinically with few side effects[47]. The chemopreventive eff ect of COX-2 specific inhibitor has not yet been well studied as compared with COX-2 nonsp ecific inhibitors[48-49]. As one of COX-2 nonspecific inhibitors, sulindac had little effect in renal prostanoid synthesis and provides addtional advantage for its use in clinical trials[50]. The concentration and time course of sulindac in inhibiting growth and inducing apoptosis of gastric cancer cells *in vivo* need further investigations. Other possible mechanisms of action of sulindac need to be further studied.

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