• LIVER CANCER •

Effect of Nimesulide on proliferation and apoptosis of human hepatoma SMMC-7721 cells

Geng Tian, Jie-Ping Yu, He-Sheng Luo, Bao-Ping Yu, Hui Yue, Jian-Ying Li, Qiao Mei

Geng Tian, Jie-Ping Yu, He-Sheng Luo, Bao-Ping Yu, Hui Yue, Jian-Ying Li, Qiao Mei, Gastroenterology department, Renmin hospital of Wuhan university, Wuhan 430060, Hubei Province, China
Correspondence to: Jie-Ping Yu, Gastroenterology department. Renmin hospital of Wuhan university, 238Jie-fang Road,Wuhan 430060,Hubei Province,China. tg3030330@sina.com
Telephone: +86-27-88077184
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Abstract

AIM: **Cyclooxygenase-2 (COX-2) has been suggested to be associated with carcinogenesis. We sought to investigate the effect of the selective COX-2 inhibitor, Nimesulide on proliferation and apoptosis of SMMC-7721 human hepatoma cells.**

METHODS: **This study was carried out on the culture of hepatic carcinoma SMMC-7721 cell line. Various concentrations of Nimesulide (0,200µmol/L,300µmol/L, 400µmol/L) were added and incubated. Cell proliferation was detected with MTT colorimetric assay, cell apoptosis by electron microscopy, flow cytometry and TUNEL.**

RESULTS: **Nimesulide could significantly inhibit SMMC-7721 cells proliferation dose-dependent and in a dependent manner compared with that of the control group. The duration lowest inhibition rate produced by Nimesulide in SMMC-7721 cells was 19.06%, the highest inhibition rate was 58.49%. After incubation with Nimesulide for 72h, the most highest apoptosis rate and apoptosis index of SMMC-7721 cells comparing with those of the control were 21.20%±1.62% *vs* 2.24%±0.26% and 21.23±1.78 *vs* 2.01±0.23 (*P*<0.05).**

CONCLUSION: **The selective COX-2 inhibitor, Nimesulide can inhibit the proliferation of SMMC-7721 cells and increase apoptosis rate and apoptosis index of SMMC-7721 cells. The apoptosis rate and the apoptosis index are dose-dependent. Under electron microscope SMMC-7721 cells incubated with 300µmol and 400µmol Nimesulide show apoptotic characteristics. With the clarification of the mechanism of selective COX-2 inhibitors, These COX-2 selective inhibitors can become the choice of prevention and treatment of cancers.**

INTRODUCTION

Hepatic carcinoma was one of most common malignant tumors in China. Its death rate was the third among all cancers, second to gastric carcinoma and lung carcinoma. Although there is a progress in diagnosis and treatment of hepatic carcinoma, its prognosis is still poor. Investigating its pathogenesis and finding new diagnostic and treatment methods is important. Recent epidemiological studies indicate an inverse relationship between the risk of colorectal cancer and intake of NSAIDs. NSAIDs could reduce the incidence of gastric carcinoma and pancreatic carcinoma. It could inhibit tumor cells proliferation and induce apoptosis[1-41]. Cyclooxygenases (COXS) are key enzymes in the conversion of arachidonic acid to prostaglandins and other eicosanoids. Recently two isoforms of the enzyme have been identified. COX-1 is constitutively expressed in a number of cell types, whereas the isoform designated COX-2 is inducible by a variety of factors, as cytokines, growth factors, and tumor promoters. Some studies have suggested that COX-2, but not COX-1, was involved in colon carcinogensis and might thus be the target of chemopreventive effect by the COX inhibor, nonsteroidal anti-inflammatory drugs. The effects of COX-2 on inflammation, procancarous conditions and cancers have been delineated[42-47]. To date the effects of Nimesulide on the growth and apoptosis of human hepatoma cell line SMMC-7721 in vitro have not been analyzed, and that is the aim of this study.

MATERIALS AND METHODS

RPMI 1640 medium is a product of CIBCO; Nimesulide and MTT were from Sigma; In situ cell death detection kit was from Boehringer Mannheim, Germany; 96-well plates were from Costar.

Cell lines and culture

Human hepatoma SMMC-7721 cells were obtained from the Wuhan University Center for type culture collection. The cells were grown as monolayers in RPMI1640 medium supplemented with 10% fetal calf serum (FCS, Gibco) and incubated at 37℃ in the humidified incubator with 5% CO_2 in air.

Assay of cell proliferation

The SMMC-7721 cells were seeded at $5×10^4$/ml density in 96-well plates 200µl cell suspension per well. Each group had four wells with a non-treated group as control. When the cells anchored to the plates, various concentrations (0,200µmol/L,300µmol/L,400µmol/L) of Nimesulide were added and the slides were incubated at 37℃, 5% CO_2 for 5 days. In order to maintain Nimesulide concentrations, we changed the culture medium (included various concentrations of Nimesulide) every day. When the cells described above were cultured for 48h, 72h, 96h, 120h, 0.5% MTT 20µl was added to each well and cultured for another 4h. The supernatant was discarded and dimethyl sulfoxide (DMSO) 200µl added. When the crystals were dissolved, the optical density(OD) value of the slides was read on an enzyme-labeled Minireader II at 492nm. Cellular proliferation inhibition rate (CPIR) was calculated using the following equation: CPIR=(1-average OD value of experimental group/average OD value of control group)×100%

Electron microscopic observation

The SMMC-7721 cells were seeded in culture flasks. Four culture bottles were divided into normal group and control group. When the cells were anchored to the plates, various concentrations (0,200µmol/L, 300µmol/L,400µmol/L) of Nimesulide were added and the cells incubated at 37℃, 5% CO_2 for 3 days. Then hepatoma cells were

digested by 0.25% trypsinase and collected. After rinsing with PBS, the cells were fixed with 2.5% glutaraldehyde for 30min and washed with PBS. After routine embedding and sectioning, the cells were observed by Hitachi H-600 electronic microscope.

Flow cytometric analysis

The SMMC-7721 cells were seeded in culture flasks. The culture bottles were divided into normal and three control groups. Each group had three culture bottles. When the cells were anchored to the plates, various concentrations (0,200µmol/L,300µmol/L,400µmol/L) of Nimesulide were added and the cells incubated at 37℃, 5% CO_2 for 3 days. Then each group of cells were washed with PBS, trypsinized and fixed with 70% ethanol at -20℃ for 30 minutes. Fixed cells were incubated with IP/Rnase solution for 15 minutes and 10^6 cells of each culture bottle were harvested and analyzed with FACScan Becton Dickeuson Flow Cytometer.

In situ apoptotic cell death detection by TUNEL

A TUNEL kit (Boehringer Mannhein, IN) was used to detect DNA fragmentation, the characteristic of apoptotic cell death. The SMMC-7721 cells were seeded in culture flasks. Culture bottles were divided into normal and three control groups. Each group had three culture bottles. When the cells were anchored to the plates, various concentrations (0,200µmol/L,300µmol/L,400µmol/L) of Nimesulide were added and the cells incubated at 37℃, 5% CO_2 for 3 days. In order to maintain Nimesulide concentrations, we changed the culture medium (including various concentrations of Nimesulide) every day. After having been cultured for 3 days, each culture bottle cells were scraped and centrifuged 800r/min for 5 minutes. Then the deposited cells were smeared and air-dried. Following the manufacturer's directions, smears were incubated with the TUNEL reaction mixture for 60min at 37℃ and then with converter-POD for 30min. The DAB-substrate solution was added to the smears and kept at room temperature until positive signal appeared. Then they were dried and analyzed under light microscope.

Under light microscope, the TUNEL positive nuclei were stained brown. Selecting 5 fields randomly (the number of cells in each field >1000).

Apoptosis index (AI)=(number of apoptotic cells/ the number of cells in each field) ×100%.

Statistical analysis

Statistical analysis was performed using the student's t test and analysis of variance. $P<0.05$ was considered significant.

RESULTS

Effect of Nimesulide in various concentrations on the growth of SMMC-7721

We analyzed the effects of Nimesulide on cell proliferation in cultured human hepatoma cell line SMMC-7721 after 5 days of treatment. Nimesulide, a selective COX inhibitor, produced a dose-dependent inhibition of cells growth (Table 1 and Figure 1). The lowest inhibition rate produced by Nimesulide in SMMC-7721 cells was 19.06%, the highest being 58.49%.

Morphology observation

Under the electron microscope, SMMC-7721 cells exhibited characteristics of apoptosis including plasma membrane blebbing, cytoplasmic condensation, pyknotic nuclei, condensed chromatin and apoptotic bodies. Compared with control groups, 300µmol/L and 400µmol/L groups cells had many more cells with apoptotic characteristics (Figure 2).

Table 1 Inhibition effect of Nimesulide on proliferation and growth in hepatic carcinoma cell line SMMC-7721

Nimesulide Concentrations (µmol/L)	OD Value			
	the 2nd day	the 3rd day	the 4th day	the 5th day
0	1.039±0.066	1.516±0.117	2.142±0.072	2.467±0.080
200	0.841±0.027[a]	1.109±0.231[a]	1.416±0.080[a]	1.341±0.021[a]
300	0.796±0.019[a]	1.002±0.274[a]	1.101±0.028[a]	1.243±0.168[a]
400	0.581±0.164[a]	0.825±0.016[a]	0.943±0.032[a]	1.024±0.026[a]

[a]$P<0.05$ *vs* control group
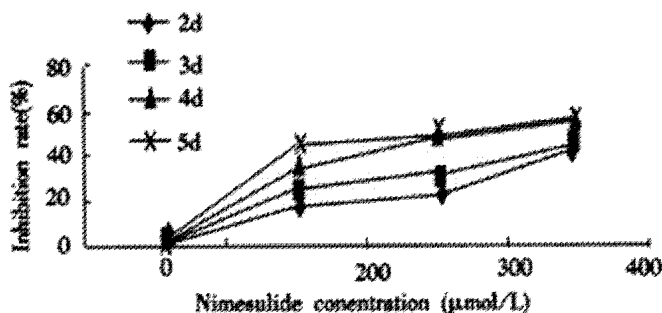
Figure 1 Inhibition rate of Nimesulide on proliferation of SMMC-7721 cells. Cells were incubated with 200µmol/L,300µmol/L,400µmol/L Nimesulide for 2d,3d,4d,5d respectively.

Figure 2 Transmission electron micrograph of SMMC-7721 cells treated with Nimesulide at the concentration of 300µmol/L for 72h.The picture showed early change of apoptosis, the nuclear chromatin condensation.

Flow-cytometry analysis of cell apoptosis

The peak value appearing before the G_1 peak is called apoptotic peak. As shown in Figure 3 and Table 2, the apoptotic peak and rate increased with increasing concentrations of Nimesulide. Furthermore, Nimesulide induced cells apoptosis in a dose and time-dependent manner ($P<0.01$).

Table 2 Apoptosis rate of SMMC-7721 cells induced by Nimesulide

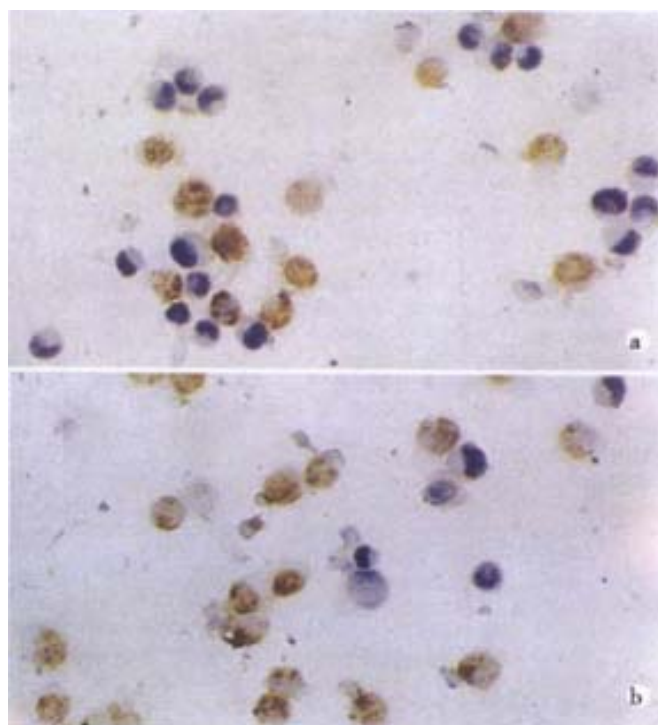| Nimesulide concentration (µmol/L)	Apoptosis rate (%)
0	2.24±0.26
200	7.42±0.43[b]
300	9.84±1.54[b]
400	21.20±1.62[b]

[b]$P<0.01$ *vs* control group

Analysis of apoptosis by TUNEL

As shown in Figure 4 and Table 3, the apoptotic index increased with increase of Nimesulide concentrations which what appeared to be dose-dependent relationship in the alone groups ($P<0.05$).
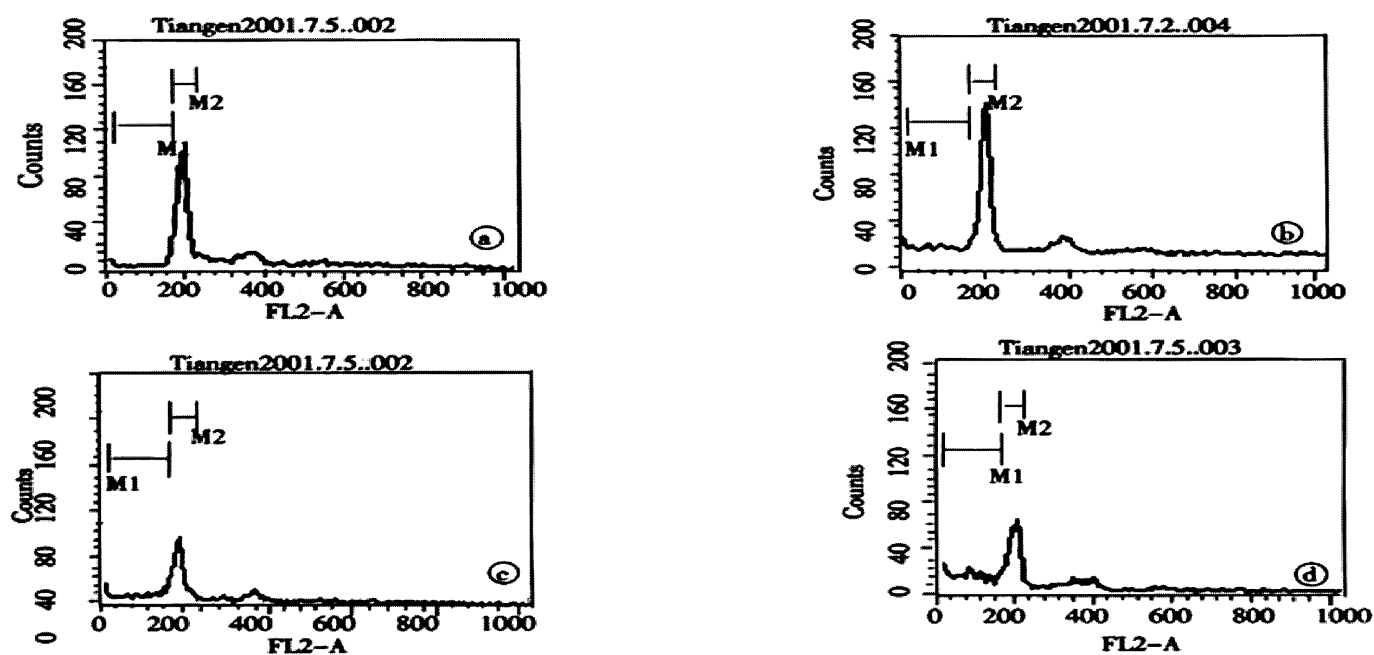
Figure 3 Cell apoptosis was determined by flow-cytometry,SMMC-7721 cells were treated with Nimesulide at various concentrations (0,200,300,400μmol/L respectively A to D).

Figure 4 TUNEL stain showed SMMC-7721 cells apoptosis. SMMC-7721 cells were treated with Nimesulide at various concentrations (300,400μmol/L A and B).

Table 3 Aapoptosis index of SMMC-7721 cells induced by Nimesulide

Nimesulide concentration (μmol/L)	Apoptosis index (%)
0	2.016±0.23
200	7.64±0.34[a]
300	10.14±1.42[a]
400	21.23±1.78[a]

[a]$P<0.05$ *vs* control group

DISCUSSION

It had been shown that selective COX-2 inhibitors inhibited tumor cells proliferation and induced tumor cells apoptosis, in colon and prostate carcinoma cell lines[48,49]. To date, their effects on human hepatoma SMMC-7721 cell lines have not yet been studied. The aim of this study was to investigate the effect of Nimesulide, a selective COX inhibitor, on the proliferation and apoptosis of SMMC-7721 cell lines. The results indicated that various concentrations of Nimesulide could change the morphology of SMMC-7721 cells and inhibit SMMC-7721 cells proliferation obviously in a dose and time-dependent manner. Nimesulide could induce SMMC-7721 cells apoptosis and cause death in a dose-dependent manner. The precise mechanism by which selective COX-2 inhibitors inhibit tumor cells growth and induce tumor cells apoptosis was not been clearfield. The available data supported the two hypotheses.

Some studies indicate that COX-2 is a key enzyme in the conversion of arachidonic acid to prostaglandins. Selective COX-2 inhibitors can decrease prostaglandins biosynthsis, and prostaglandins can inhibit cell-mediated immunity, which enables the tumor cells escaping the host-immunity[50,51]; PG_S also can conjugate with PPARá and activate cell proliferation passage of signal conduction, promote cells proliferation[52]; PG_S can also inhibit cells apoptosis and cause cells division uncontrollable, thus accelerating tumor genesis[53,54]; the effects of COX-2 inhibitors might involve prostaglandin biosynthesis. Some studies indicated that the effects of COX-2 were not related to COX-2 expression and PG_S. Hanif *et al*[55] verified that $NASID_S$ (nonselective COX inhibitors) could induce apoptosis of colon carcinoma cell line HCT-15. HCT-15 cells have no COX gene transcription, and does not produce PG_S. When adding exogenous PG_S to the HCT-15 cells, it could not reverse the induction of HCT-15 cells apoptosis by $NASID_S$.

On the whole, Nimesulide, a selective COX-2 inhibitor, can inhibit the growth of hepatoma cells and induce tumor cells apoptosis. With the clarification of the mechanism of selective COX-2 inhibitors, These COX-2 selective inhibitors can become the choice of prevention and treatment of cancers.

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