Effects of cyclooxygenase 2 inhibitors on biological traits of nasopharyngeal carcinoma cells

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KEY WORDS cyclooxygenase inhibitors; nimesulide; celecoxib; nasopharyngeal neoplasms; apoptosis; cell cycle; angiogenesis inhibitors

ABSTRACT

AIM: To investigate effects of cyclooxygenase 2 (COX-2) inhibitors on nasopharyngeal carcinoma (NPC) cells and on angiogenesis *in vitro*. **METHODS:** Human NPC cell lines (CNE1, CNE2 and SUNE) were treated with nimesulide or celecoxib. MTT assay and colony formation assay were performed to observe antiproliferation activity of COX-2 inhibitors to NPC cell lines. Cell cycle arrest and apoptosis of NPC cell strains were tested by flow cytometry assay, microscopic morphology observation, and DNA fragmentation assay. The effect of COX-2 inhibitor on angiogenesis was tested by chick chorioallantoic membrane (CAM) model. **RESULTS:** Nimesulide (Nim) and celecoxib (Cel) could antiproliferate NPC cell lines with IC_{50} 182 μmol/$L_{Nim\text{-}SUNE}$, 78 μmol/$L_{Nim\text{-}CNE1}$, 175 μmol/$L_{Nim\text{-}CNE2}$, 7.2 μmol/$L_{Cel\text{-}SUNE}$, 8.1 μmol/$L_{Cel\text{-}CNE1}$, and 7.6 μmol/$L_{Cel\text{-}CNE2}$. The antiproliferation presented dose-dependent (Nim 5-400 μmol/L, Cel 0.5-80 μmol/L) and time-dependent manner (Nim IC_{50} 562 μmol/$L_{24\ h}$, 316 μmol/$L_{48\ h}$, 50.1 μmol/$L_{240\ h}$). Nim and Cel arrested SUNE and CNE1 cell cycle at phase G_2/M (cell aggregation rate 28.9 %-45.1 %$_{Nim\ 25\text{-}200\ \mu mol\text{-}12\ h\text{-}SUNE}$, 18.9 %-26.2 %$_{Nim\ 25\text{-}200\ \mu mol\text{-}24\ h\text{-}SUNE}$, 28.8 %-35.6 %$_{Nim\ 25\text{-}200\ \mu mol\text{-}48\ h\text{-}SUNE}$, 30.4 %-16.4 %$_{Cel\ 25\text{-}100\ \mu mol\text{-}12\ h\text{-}SUNE}$, 21.2 %-19.7 %$_{Cel\ 25\text{-}100\ \mu mol\text{-}24\ h\text{-}SUNE}$, 31.1 %-19.9 %$_{Cel\ 25\text{-}100\ \mu mol\text{-}24\ h\text{-}SUNE}$, 20.5 %-34.1 %$_{Nim25\text{-}200\ \mu mol\text{-}12\ h\text{-}CNE1}$, 25.2 %-26.9 %$_{Nim\ 25\text{-}200\ \mu mol\text{-}24\ h\text{-}CNE1}$, 11.5 %-7.1 %$_{Nim\ 25\text{-}200\ \mu mol\text{-}48\ h\text{-}CNE1}$). Apoptosis shape and apoptosis strap displayed in NPC cells after treatment with Nim and Cel. Nim had a feature of anti-angiogenesis on CAM model. **CONCLUSION:** Nim and Cel could suppress proliferation of squamous epithelium NPC cell (SUNE, CNE1 and CNE2) through blocking cell cycle and inducing cell apoptosis. Nim could apparently suppress CAM angiogenesis induced by SUNE cell.

INTRODUCTION

The hypothesis that COX-2 was involved in the pathological process of cancer happening and development was supported by animal studies indicated that tumor genesis was inhibited in COX-2 knockout mice[1,2]. Over expression of COX-2 led to phenotypic changes in intestinal epithelial cells that could enhance their tumorigenic potential[3]. Selective inhibitors of COX-2 have been demonstrated to induce apoptosis in a variety of cancer cells, including those of colon, stomach and prostate and so on. Although COX-2 inhibitors have been reported as effective agents for many tumors, the effect of COX-2 inhibitor on nasopharyngeal carcinoma (NPC) had not been found yet. Our previous work confirmed that synergism or additivity of inhibition of

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Received 2003-08-04 Accepted 2004-02-18

CNE1 and SUNE lines in concomitance of celecoxib with bleomycin, cisplatin, or vincrestine *in vitro*[4]. It seems to be necessary to further evaluate the effects of COX-2 inhibitors on NPC cells, to provide the evidences for using COX-2 inhibitors as chemoprevention agents and therapy agents for NPC, since COX-2 inhibitors are hopeful drugs for chemoprevention against cancer.

MATERIALS AND METHODS

Reagents Nimesulide was provided by Haerbin Pharmaceutical Factory. Indomethacine was from National Institute for the Control of Pharmaceutical and Biological Products. Celecoxib was obtained from Pfizer Pharmaceutical Inc.

Cell culture CNE1 (human nasopharyngeal high differentiated squamous epithelium carcinoma cell), CNE2 and SUNE (human nasopharyngeal low differentiated squamous epithelium carcinomas cell) were obtained from Department of Oncopharmacology, Tumor Institute, Sun Yat-sen University. HT-29 (human colon carcinoma cell) and Hep-G2 (human hepatic carcinoma cell, as a control cell strain) were purchased from Experimental Animal Center of Sun Yat-sen University. The cell strains were maintained in RPMI-1640 supplemented with 10 % calf serum in a 5 % CO_2 atmosphere.

MTT assay To evaluate the antiproliferative activity of COX-2 inhibitors, a MTT assay was performed. Briefly, cells (CNE1, CNE2, SUNE, HT-29 and Hep-G2) were plated at 5000-6000/well in 200 μL volume in 96-well plates and recovered over night. The indicated amounts of tested drugs (nimesulide 5-400 μmol/L, indomethacin 5-400 μmol/L and celecoxib 0.5-80 μmol/L) or negative solvent (Me_2SO 0.25 %) were then added to each well. After incubation for 68 h, 20 μL of MTT (3 g/L) was added, and the cells were incubated at 37 ºC for 4 h. The tetrazolium crystal was solubilized by 100 μL Me_2SO and the absorbance was measured at 570 nm on spectrophotometric plate reader. IC_{50} of antiproliferative activity of COX-2 inhibitors were calculated by interpolation method.

Colony formation assay Colony formation assay was done to evaluate time-dependent effect of COX-2 inhibitors on NPC cell strains. Following digestion by trypsin, CNE1 and SUNE cells were put in 6-well plate at the number of 500 cells in each well. Nimesulide 10-1000 μmol/L and negative fluid were added. Cells were exposed to COX-2 inhibitors for 24 h, 48 h, and 240 h,respectively. At the end of determination time, cells were fixed by 50 % methanol and 5 % acetic acid for 40 min, dyed by Giemsa for 20 min and then washed. The number of colony formation was observed through invert microscope.

Cell cycle analysis by flow cytometry assay Following digestion by 0.25 % trypsin , CNE1 and SUNE cells in logarithmic growth phase were adjusted to a density of 5×10^8-1×10^9/L. A volume of 4 mL cells was placed into a cell culture bottle and incubated over night. Nimesulide 25-200 μmol/L, celecoxib 25-100 μmol/L and negative solvent were added to the bottles. Cells were harvested at several time points, centrifuged, vortexed in 0.3 mL PBS, fixed with 0.7 mL anhydrous alcohol at -20 ºC for 12 h, washed twice with PBS, dyed with 300 μL pyridine iodide (Coulter) at room temperature for 20 min. After incubation cells were analyzed by flow cytometry (Beckman-Coulter, USA) at a wavelength of 488 nm. Data were analyzed by software with the machine.

Apoptosis analysis by morphologic observation and DNA fragmentation assay CNE1 and SUNE cells were incubated at 2×10^4-1×10^5 /well in 6-well plate each well for 24 h. Celecoxib 10-50 μmol/L was added to wells and incubated at 37 ºC for 24 h. Cells were washed with PBS, fixed with 100 % methanol at room temperature for 45 min, dyed with Giemsa for 20 min, then washed and dried. Cells were observed morphologic change under a phase contrast microscope (LiCa, MPS30 Japan). Three sights were observed in each sample and taken a photograph.

After exposed to nimesulide 200 μmol/L or celecoxib 25 μmol/L for 24 h and 48 h, CNE1 and SUNE cells were harvested and re-suspended in 600 μL lysis buffer (Tris-HCl 10 mmol/L, EDTA 10 mmol/L, Triton X-100 2 g/L, pH 7.5) and incubated at 4 ºC for 20 min. Then cells were centrifuged at 1200 r/min for 10 min. Soluble DNAs were recovered from the supernatants by phenol/chloroform (1:1) extraction and isopropanol precipitation. These DNAs were mixed with TE (Tris-HCl 10 mmol/L, EDTA 1.0 mmol/L, pH 7.4) and Rnase A 0.6 g/L, incubated at 37 ºC for 30 min, separated by 1.0 % agarose gel electrophoresis for 1 h. Following dye by ethylingot bromide, the separated samples were analyzed by GDS7500 gel image system.

COX-2 inhibitor suppressed angiogenesis on chick chorioallantoic membrane (CAM) model Sterilized filter paper slip at a diameter 5 mm was placed on 7-day chick chorioallantoic membrane. The slips contained 10 μL supernatant of SUNE cells culture

medium, which was at a concentration of protein 1 g/L with nimesulide 100 μg, or nimesulide 100 μg only, or normal saline 10 μL. CAM method that Wu *et al* established was used to observe effect of COX-2 inhibitors on angiogenesis[5].

RESULTS

Antiproliperative effect of COX-2 inhibitors to NPC cell lines The results showed that nimesulide, celecoxib, and indomethacin could inhibit growth of CNE1, CNE2 and SUNE cell lines in a concentration-dependent manner. IC_{50} of COX-2 inhibitors to NPC cell lines was displayed in Tab 1.

Antiproliperative effect of COX-2 inhibitor on NPC cell lines Colony formation assay showed that incubation of SUNE cells with nimesulide substantially inhibited the ability of cells to form colonies with the IC_{50} value of 562 μmol/L for a 24-h incubation, 316 μmol/L for a 48-h incubation and 50.1 μmol/L for 240-h incubation (Tab 2).

Cell cycle alteration with treatment of COX-2 inhibitors by flow cytometry assay and apoptosis Phase G_2/M block of either SUNE cell or CEN1 cell was dose-dependent (nimesulide 25-200 μmol/L, celecoxib 25-100 μmol/L) and time-dependent at 12-48 h (Tab 3, 4). Aggregation percentage of SUNE cell in phase G_2/M obviously increased with treatment of nimesulide 25, 100, and 200 μmol/L. Celecoxib also could dose-dependently and time-dependently induce SUNE cells gathering in phase G_2/M. The aggregation percentage of SUNE cell in phase G_2/M also increased after treatment with celecoxib 25, 50, and 100 μmol/L. As shown in Tab 4, similar phenomena were also seen in the effects of nimesulide 25, 100, and 200 μmol/L on CNE1 cell line at 12 and 24 h but not at 48 h.

Tab 1. The effect of COX-2 inhibitors on NPC cell survival after incubation for 72 h.

Cell	IC_{50}/μmol·L^{-1}		
	Nimesulide	Celecoxib	Indomethacin
CNE1	175	8.1	208
CNE2	78	7.6	251
SUNE	182	7.2	183
HT-29	134	12.7	152

Tab 2. Survival after incubation of SUNE cell with nimesulide for 24 h, 48 h and 240 h by colony formation assay. *n*=3. Mean±SD.

Time/h	Concentration/μmol·L^{-1}				
	Control	10	100	1000	IC_{50}
24	1.0±0.11	0.98±007	0.94±0.06	0.35±0.12	562
48	1.0±0.05	0.98±0.08	0.85±0.13	0.15±0.08	316
240	1.0±0.04	0.88±0.06	0.34±0.10	0.04±0.05	50.1

Tab 3. Distribution of the cell cycle (%) in SUNE cells following treatment with nimesulide (Nim) or celecoxib (Cel) at 12-48 h. *n*=5. Mean±SD.

Group	12 h			24 h			48 h		
	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
Control	40.3±0.3	44.1±0.7	12.4±0.7	39.5±0.6	42.8±0.4	15.3±0.5	41.6±0.4	43.5±0.4	13.2±0.2
Nim 25 μmol·L^{-1}	35.2±0.4	43.3±0.5	28.9±0.6	39.9±0.6	36.6±0.5	18.9±0.3	43.2±0.9	29.4±0.7	28.8±0.8
Nim 100 μmol·L^{-1}	23.3±0.5	43.1±0.3	35.5±0.2	42.6±0.7	32.8±0.4	24.8±0.4	48.5±0.7	21.5±0.6	30.5±0.8
Nim 200 μmol·L^{-1}	16.6±0.3	38.3±0.2	45.1±0.6	44.9±0.9	30.3±0.3	26.2±0.8	49.4±0.5	19.7±0.3	35.6±0.3
Cel 25 μmol·L^{-1}	32.6±0.4	43.9±0.6	30.4±0.3	28.9±0.6	33.1±0.2	21.2±0.7	46.6±0.8	27.1±0.5	31.1±0.2
Cel 50 μmol·L^{-1}	19.9±0.6	40.4±0.8	38.9±0.8	43.3±0.7	26.8±0.5	28.7±0.3	49.2±0.2	18.9±0.4	33.0±0.4
Cel 100 μmol·L^{-1}	24.2±0.3	35.9±0.7	16.4±0.2	51.1±0.4	16.4±0.7	19.7±0.2	44.6±0.9	17.5±0.8	19.9±0.5

Tab 4. Distribution of the cell cycle (%) in CNE1 cells following treatment with nimesulide (Nim) at 12-48 h. *n*=5. Mean±SD.

Group	12 h			24 h			48 h		
	G_1	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
Control	61.1±0.2	26.0±0.4	14.2±0.8	58.5±0.7	24.7±0.5	16.2±0.3	62.4±0.7	25.8±0.1	13.3±0.3
Nim 25 μmol·L^{-1}	48.0±0.2	28.6±0.6	20.5±0.7	48.5±0.5	26.6±0.9	25.2±0.5	68.2±0.7	20.3±0.4	11.5±0.2
Nim 100 μmol·L^{-1}	43.6±0.2	32.9±0.3	25.1±0.8	42.9±0.5	31.2±0.4	28.5±0.4	75.3±0.7	18.2±0.6	9.1±0.4
Nim 200 μmol·L^{-1}	37.2±0.2	28.8±0.5	34.1±0.3	44.2±0.3	29.1±0.5	26.9±0.7	72.2±0.1	12.0±0.2	7.1±0.9

The apoptosis rate of SUSE and CNE1 after treatment with nimesulide and celecoxib were shown in Tab 5. Celecoxib could induce SUNE cells gathering in phase G_2/M, but a plenty of cells became apoptosis within 24 h after treatment with celecoxib 100 μmol/L.

Morphological change of NPC cells Apoptosis characteristics with nucleus condense, shrinkage could be observed in NPC cell incubated with COX-2 inhibitors under invert microscope (Fig 1, 2). Analysis of DNA trapezoid strap revealed that an apoptosis strap feature obviously appeared in NPC cells after treatment with celecoxib 25 μmol/L and nimesulide 200 μmol/L for 48 h (Fig 3).

COX-2 inhibitor suppressed angiogenesis on chick chorioallantoic membrane model (CAM) As shown in Fig 4, blood vessels in CAM manifested ner-

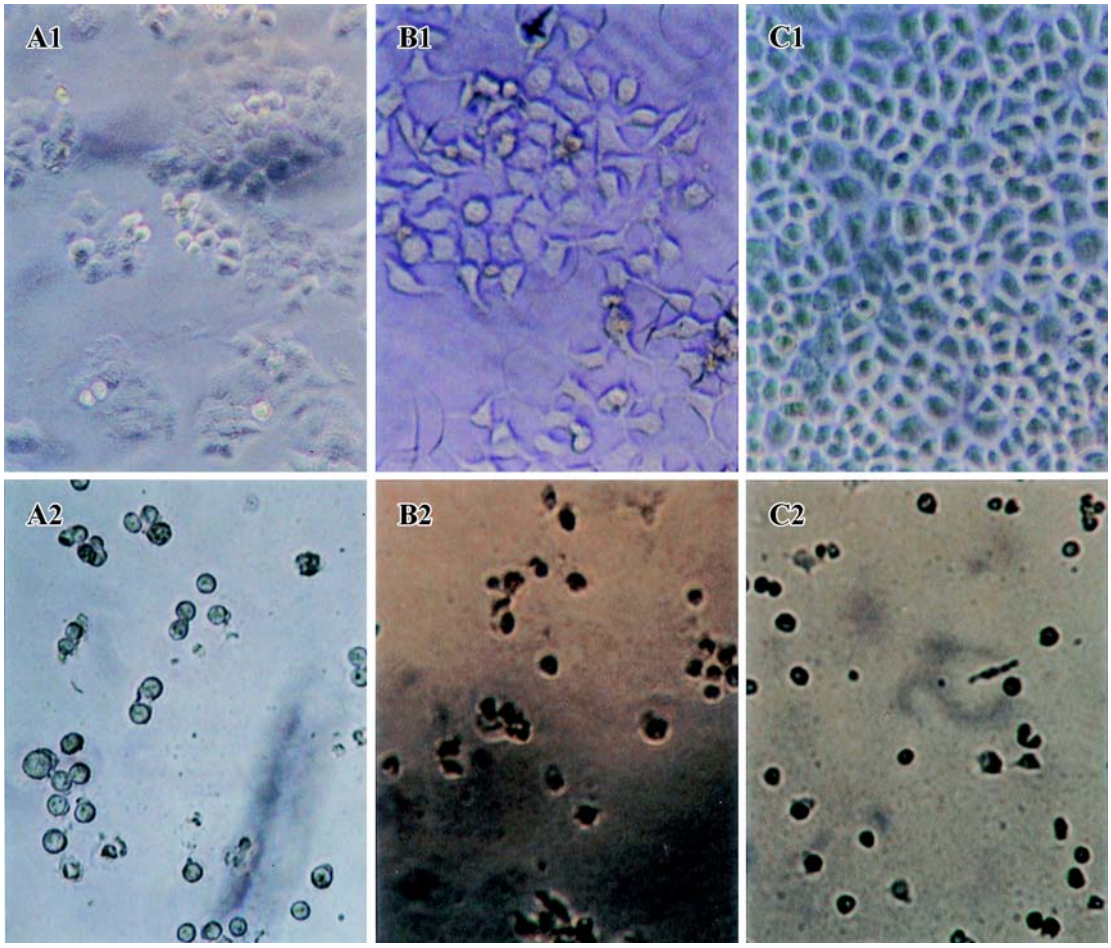

Fig 1. Effect of nimesulide (Nim) on SUNE, CEN1, and CNE2 cells observed with phase-contrast microscope (×200). A1: SUNE, A2: SUNE with Nim 100 μmol/L; B1: CNE1, B2: CNE1 with Nim 100 μmol/L; C1: CNE2, C2: CNE2 with Nim 100 μmol/L.

Tab 5. Apoptosis rate (%) in SUNE and CNE1 cells following treatment with nimesulide (Nim) and celecoxib (Cel) at 12-48 h. *n*=5. Mean±SD.

Group	SUNE			CNE1		
	12 h	24 h	48 h	12 h	24 h	48 h
Nim 25 μmol·L^{-1}	3.6±0.2	4.3±0.3	5.9±0.4	4.1±0.3	6.6±0.2	10.6±0.7
Nim 100 μmol·L^{-1}	2.5±0.1	7.0±0.5	11.2±0.6	5.7±0.2	8.9±0.4	24.0±0.5
Nim 200 μmol·L^{-1}	1.7±0.2	3.8±0.6	4.0±0.7	2.9±0.3	4.6±0.5	6.4±0.2
Cel 25 μmol·L^{-1}	3.8±0.3	5.2±0.3	7.1±0.5	–	–	–
Cel 50 μmol·L^{-1}	4.4±0.2	5.1±0.6	6.7±0.4	–	–	–
Cel 100 μmol·L^{-1}	51.0±0.6	69.4±0.7	9.2±0.9	–	–	–

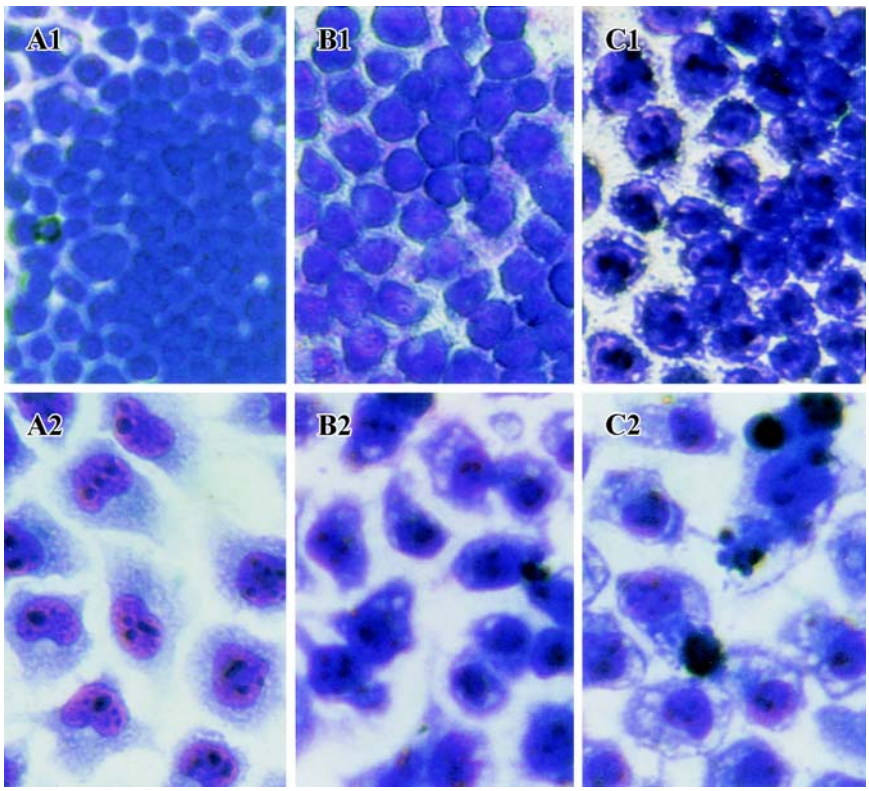

Fig 2. Celecoxib (Cel)-induced morphological characteristics of apoptosis in SUNE and CNE1 cells. Photo was taken under phase-contrast microscope (×200). A1: SUNE; B1: SUNE with Cel 10 μmol/L; C1: SUNE with Cel 50 μmol/L. A2: CNE1; B2: CNE1 with Cel 10 μmol/L; C2: CNE1 with Cel 50 μmol/L.

vure shape in natural condition. Blood vessels formed as radiation with paper slip as the center, some vessels ran through area of paper slip, and vessels far from slip attracted each other after normal saline was added. Vessel growth of CAM was mildly suppressed under the slip with nimesulide 100 μmol/L and area around the slip compared with blank slip. Diameter of free vessel area was about 4-6 mm as a center of slip. Normal nervure shape vessels were seen in free drug area. Counting number of vessels showed that nimesulide (66.7±12.5, *n*=7, *P*<0.05) could mildly depress blood vessel growth compared with that of control (90.0±18.7, *n*=4). A great deal vessels with radiation in CAM formed outward with the position of SUNE cell culture liquid as the center. Diameter of the outward vessels was more than 3 cm. Density of the vessel increased. Vessel number intersected circle was 142±45 (*n*=4). CAM angiogenesis was clearly depressed after supernatant of SUNE cul-

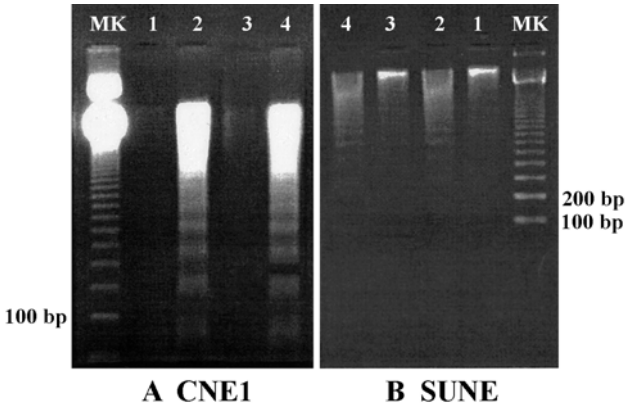

Fig 3. Agarose gel electrophoresis of DNA fragmentation analysis on CEN1 and SUNE cells treated with nimesulide (Nim) 200 μmol/L and celecoxib (Cel) 25 μmol/L for 24 and 48 h. A: 1) Nim 24 h; 2) Nim 48 h; 3) Cel 24 h; 4) Cel 48 h. B: 1) Nim 24 h; 2) Nim 48 h; 3) Cel 24 h; 4) Cel 48 h.

ture solution and nimesulide 100 μmol/L were placed to slip. Number of vessels was 70±14 (n=5).

DISCUSSION

Both nimesulide and celecoxib are COX-2 inhibitors. The former is a benzenesulfonyl structure NSAID to be considered for predominant inhibiting COX-2 (IC_{50} 7.1 μmol/L) and light COX-1 inhibitor (IC_{50} 985 μmol/L), while the later is a selective COX-2 inhibitor (IC_{50} 0.04 μmol/L, for COX-1 IC_{50} 15 μmol/L) with a structure of tricycle.

SUNE and CNE2 are human low differentiated NPC squamous epithelium cell. SUNE was certified as positive LMP1 (latent membrane protein 1). CNE1 is a human high differentiated NPC squamous epithelium cell. HT-29 is a human colon carcinoma strain with positive COX-2, and often used to study effect of COX-2 inhibiter on cancer of colon. HT-29 was a positive line of sensitive COX-2 inhibitor in our experiment.

NPC cells proliferation was evidently suppressed by nimesulide, celecoxib, and indomethacin from MTT assay. IC_{50} (7-8 μmol/L) of celecoxib to depress NPC cells approached IC_{50} of clinical concentration (3 μmol/L). IC_{50} of celecoxib and nimsulide to depress NPC was much more than that to inhibit COX-2, but less than that to inhibit COX-1. Compared with positive control HT-29 ($IC_{50\ nimesulide}$ 134 μmol/L, $IC_{50\ indomethacin}$ 152 μmol/L) and Hep-G2 ($IC_{50\ celecoxib}$ 12.7 μmol/L), it was valuable for nimesulide, celecoxib and indomethacin to present antiproliferation to NPC cells. Because the three drugs did not balance in the fields of depressing NPC cells and inhibiting of COX-2, other pathways to be function might exist except for depression of NPC through inhibiting COX-2.

Results of colony formation analysis clearly displayed that antiproliferation of COX-2 inhibitors to SUNE was time- and dose-dependent. The longer time and the more dose, the more evident antiproliferating action was (n=3, P<0.05).

COX-2 inhibitor inhibited NPC cells mainly due to cell cycle arrest and apoptosis demonstrated by flow cytometry assay, cell morphological change and DNA fragmentation agarose gel electrophoresis. There was a temporary block phase G_2/M for about 24 h in nimesulide and celecoxib acting to NPC cell. The cell number in phase G_0/G_1 relatively increased as drug action time prolongation and the cell number in phase G_2/M decreased. NPC cell eventually gathered in phase G_0/G1 and apoptosis started to display. NPC cell in phase G_2/M increasingly gathered with early time in apoptosis and apoptosis rate increasing when COX-2 inhibitor concentration was higher. Our experimental results differed from that NSAID influenced cell cycle chiefly by blocking G_0/G_1 in references[6,7]. Causes of

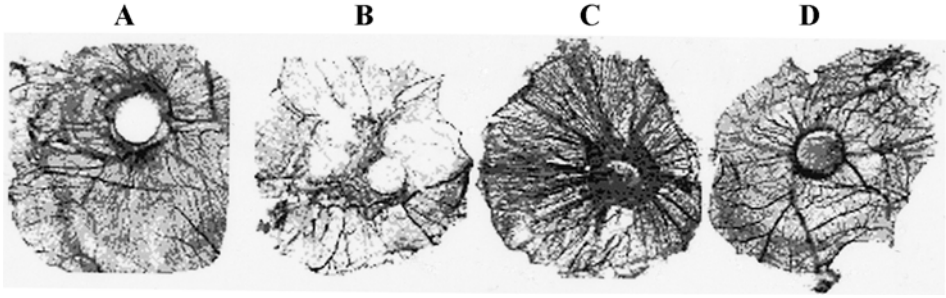

Fig 4. Effect of nimesulide (Nim) 100 μmol/L on angiogenesis of chick chorioallantoic membrane. A: Normal saline, B: Nim, C: Supernatant of SUNE culture solution, D: Supernatant of SUNE culture solution and Nim.

the difference probably were as a result of different cell strain with different biological characteristics in experiment and different observation time. The observed time reported in references was often more than 24 h that was the beginning of G_2/M decrease and G_0/G_1 increase.

Tumogenesis, on the one hand, is considered to be out of control of cell proliferation and abnormal differentiation by gene regulation and control. On the other hand, cell apoptosis process might be inhibited. Tumor cell apoptosis and alteration of cell cycle play an important role in the treatment of tumor. Our experimental results displayed that nimesulide, celecoxib and indomethacin could all inhibit NPC cell proliferation, induce apoptosis and change cell cycle in cell culture *in vitro*. These suggested that COX-2 inhibitors could probably play a certain part in caner therapy. Because expression of COX-2 gene in tumor cell was much more than that in normal cell, COX-2 inhibitors could selectively act on cancer cell, but relatively little effect on normal cell function[8]. It was inferred that COX-2 inhibitors might potentiate the action of anticancer agents[4].

Filter paper slip could induce blood vessel formation as an exogenous irritant in our experiments. It seems to be more suitable for a carrier of drug to depress anginogenesis than cellothyl. Supernatant from SUNE cell culture solution strongly induced CAM angiogenesis. Nimesulide 100 μmol/L, however, could not only depress CAM angiogenesis, but also depress angiogensis that induced by treatment with supernatant of SUNE cell culture solution (P=0.01). The results implied that COX-2 inhibitors could possibly inhibit angiogenesis-induced materials synthesized by SUNS cell. SUNE used in our experiment was a NPC cell with LMP1 gene. Murono *et al* found that LMP1 gene expression in NPC cell might induce the expression of COX-2 gene, whereas activity increase of COX-2 would further induce the expression of vascular endothelial growth factor (VEGF)[9]. The study results suggested that SUNE cell might induce angiogenesis through a pathway of LMP1-COX-2-VEGF. If COX-2 inhibitors will become anti-angiogenesis agents is supposed to be further investigated.

In conclusion, COX-2 inhibitors (nimesulide and celecoxib) could suppress proliferation of squamous epithelium NPC cell (SUNE, CNE1 and CNE2) through cell cycle block and inducing cell apoptosis. The antiproliferaion presented dose- and time-dependent features. And nimesulide could apparently suppress CAM angiogenesis induced by SUNE cell, although did not obviously suppress normal angiogenesis in CAM.

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