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Apoptosis Induced by NS-398, a Selective Cyclooxygenase-2 Inhibitor, in Human Colorectal Cancer Cell Lines

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Recent studies have suggested that apoptosis is a key phenomenon in the chemopreventive action of nonsteroidal antiinflammatory drugs (NSAIDs), which exhibit cancer-preventive and tumor-regressive effects in the human colon. The effect of NS-398, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide, which is a selective inhibitor of cyclooxygenase-2 (COX-2), on the induction of apoptosis in two human colorectal cancer cell lines (Colo320 and THRC) was determined. The apoptotic ratios (-fold vs. control value) of Colo320 in the presence of 100 μM indomethacin and NS-398 were 3.3±1.5 and 9.0±0.94, and those of THRC were 2.3±0.46 and 7.4±0.87, respectively. The ability of NS-398 to induce apoptosis is greater than that of indomethacin. Both indomethacin and NS-398 reduced the cell proliferation in a concentration-dependent manner. The IC_{50} values of NS-398 (54.8±3.6 and 77.2±4.9 μM) were significantly lower than those of indomethacin (206.3±43.0 and 180.3±22.6 μM) at $P<0.01$ in Colo320 and THRC cell lines, respectively. These findings suggest that NS-398, a selective inhibitor of COX-2, is a possible candidate for a chemopreventive agent with a potent apoptosis-inducing effect and low ulcerogenic activity.

Key words: Apoptosis — COX-2 inhibitor — NS-398 — Colorectal cancer — DNA fragmentation

Epidemiological studies of regular use of aspirin in humans[1,2] and clinical trials of sulindac to reduce the risk of colorectal cancer in familial adenomatous polyposis have indicated the effectiveness of nonsteroidal antiinflammatory drugs (NSAIDs) in chemoprevention of colorectal cancers.[2,3] Such chemopreventive effects of NSAIDs have been demonstrated in animal models of colorectal carcinogenesis.[4,5]

Prostaglandin (PG) G/H synthase, also known as cyclooxygenase (COX), is a key enzyme in the biosynthesis of PGs and, as such, is the target of NSAIDs. Recently two genetically different isoforms have been described; a constitutive (COX-1) enzyme present in a large number of cell types and tissues, and an inducible (COX-2) isoenzyme generated in many cells in response to inflammatory mediators. NSAIDs currently available for clinical use inhibit both isoforms, but a selective COX-2 inhibitor would eliminate gastric ulceration, which is the main undesirable side-effect of common NSAIDs.[6,7]

Sulindac, which has been examined in clinical trials for the ability to reduce the risk of colorectal cancer in familial adenomatous polyposis, is metabolized to a pharmacologically active sulfide derivate that inhibits PG synthesis. The sulfone derivate of sulindac, which essentially lacks anti-PG synthetase activity, also reduces tumor occurrence in an azoxymethane-induced rodent colon carcinogenesis model,[8] suggesting that the antineoplastic activity of NSAIDs is based not on the reduction of PG levels, but on other biological mechanisms. Recent studies have suggested that cell death caused by apoptosis is responsible for the chemopreventive effects of NSAIDs, such as sulindac derivates,[9] salicylate,[10] naproxen, indomethacin and piroxicam,[11] on growth inhibition of colorectal tumor cell lines.

The specific aim of this study was to investigate the effects of NS-398, a COX-2-specific inhibitor, on the proliferation and apoptosis of cultured colorectal cancer cells to see whether it might have potential for chemoprevention of colorectal cancers with low risk of gastric lesions.

MATERIALS AND METHODS

Cell culture The human colorectal carcinoma cell lines, Colo320 and THRC 1, were used in this study. Colo320 was kindly provided by Colorado University and THRC 1 was provided by Dr. M. Yamauchi (Tokai Central Hospital, Gifu). Both cell lines were cultured at 37°C in an atmosphere of 5% CO_2 and 95% air in RPMI 1640 medium supplemented with 10% fetal calf serum, 300 μg/ml L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Treatment with NSAIDs NS-398,[7] *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide, was generously provided by Dr. N. Futaki (Research Center of Taisho Pharmaceutical Company, Saitama). Indomethacin, ibuprofen and piroxicam were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of NS-398, indomethacin, ibuprofen and piroxicam were made at 100× concentrations in dimethylsulfoxide (DMSO) and added to the culture medium. The final concentration of DMSO for all treatments including the negative control was maintained at 1%. All compound solutions were

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prepared within 30 min before testing. Exponentially growing cells were trypsinized and seeded in 24-well multiwell culture plates (Becton Dickinson Co., Oxnard, CA) at densities of 1×10^4 cells/ml for Colo320 and 5×10^4 cells/ml for THRC 1. The culture plates were used for testing at 3 days after seeding. Cells were treated in triplicate with NS-398 and other NSAIDs for 4 days, and then the attached cells (those still adhering to the tissue culture plates) and floating cells (those having detached from the tissue culture plates) were counted separately.

Measurement of apoptosis Hague *et al.*[12] and Heerdt *et al.*[13] have demonstrated that the majority of cells floating in the media of colonic tumor cells grown on culture plates are morphologically apoptotic and exhibit characteristic ladders of cleaved DNA. According to the method described by Elder *et al.*,[10] the degree of apoptosis in Colo320 and THRC 1 cells was determined by measuring the level of floating cells as a proportion of the total cells (both floating and attached cells). After treatment with NS-398 and other NSAIDs for 96 h, the proportion of the floating cells to the total cell number was determined at each concentration of the drugs.

Morphological analysis Following the treatment with NS-398 and other NSAIDs at the concentration of 300 μM for 96 h, the attached cells and floating cells were gathered separately and spun down onto slide glasses by a Cytocentrifuzer CF-12SB (Sakurai-Seiki, Tokyo). The slides were fixed in 90% ethanol, immersed in 0.5% Triton X-100/10 mM EDTA/10 mM Tris-HCl (pH 7.4), and stained with 0.1 μg/ml 4′-diamidino-2-phenylindole solution (Boehringer Mannheim GmbH, Mannheim, Germany) for fluorescence microscopic evaluation. Apoptotic cells were identified by their characteristic features, such as condensed chromatin and budding nuclei.

Demonstration of DNA laddering DNA fragmentation was visualized by the method of Sellins and Cohen[14] with some modification. Briefly, after treatment with NS-398 and indomethacin at the concentration of 300 μM for 0, 2, 4, 12, 36 and 96 h, the total cells (attached and floating) were collected by centrifugation at 600g for 5 min. The pellet was lysed with 100 μl of hypotonic buffer (10 mM Tris 10 mM EDTA, pH 7.5) containing 0.5% Triton X-100, and the lysates were centrifuged at 20,000g for 20 min. The supernatant, containing fragmented DNA, was removed and the fragments were precipitated overnight with 0.25 M NaCl and 50% isopropyl alcohol. DNA samples of 4×10^5 cells at each concentration of drugs were run on a 1% (w/v) agarose gel containing 0.1 μg/ml ethidium bromide at 100 V for 30 min.

RESULTS

Effects of NS-398 and indomethacin on apoptosis and attached cell yield The effects of NS-398 and indometh-

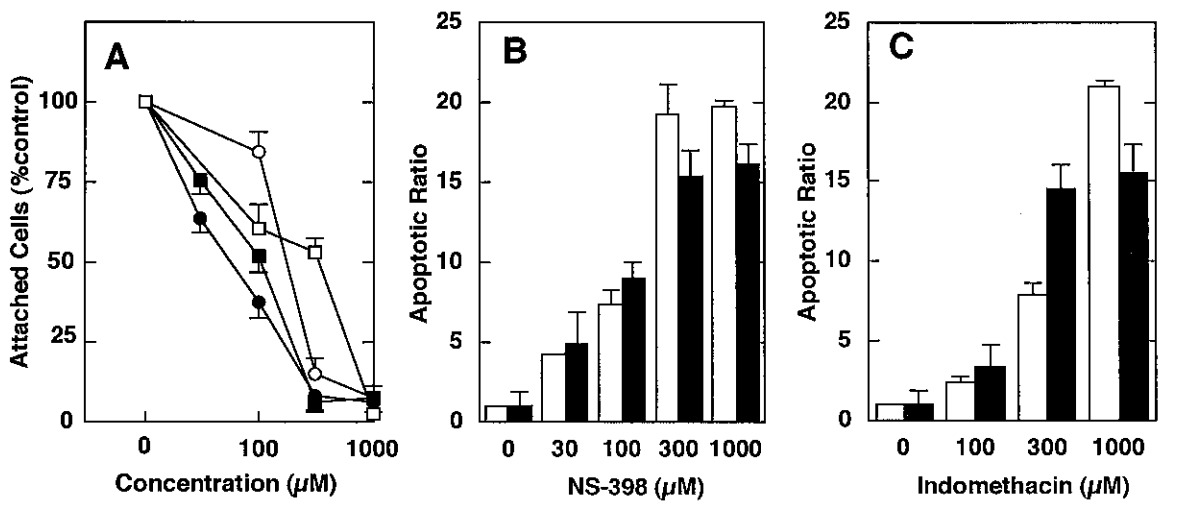

Fig. 1. Dose-dependent growth inhibition (A) and dose-dependent induction of apoptosis (B, C) of colorectal carcinoma cell lines following 96-h treatment with NS-398 and indomethacin. In A, the attached cell yields (number of cells remaining attached to the tissue culture plate) following 96-h treatment with NS-398 and indomethacin are expressed as a percentage of control values. Colo320 cells (○, ●) and THRC 1 cells (□, ■) were treated with indomethacin (○, □) or NS-398 (●, ■). In B and C, apoptotic ratios indicate the level of floating cells, as a proportion of the total cell number (both floating and attached), following 96-h treatment with NS-398 (B) and indomethacin (C) at each concentration in the relation to the control level (assigned as 1). When not shown, the SD is smaller than the symbol. Bars, SD of 3 experiments. □, THRC 1; ■, Colo320.

acin on the induction of apoptosis and the proliferation of attached cells in each cell line were determined after 96 h of treatment at various concentrations. Both drugs induced an intense concentration-dependent reduction in the attached cell yield of Colo320 and THRC 1. The growth inhibition of NS-398 was stronger than that of indomethacin at 100, 300 and 1000 μM concentration in both cell lines. NS-398 showed complete cell growth suppression at the concentration of both 300 and 1000 μM in each cell line. NS-398 even at the concentration of 30 μM, at which no significant growth inhibition was seen with the other NSAIDs (indomethacin, ibuprofen and piroxicam), profoundly inhibited cell growth in both cell lines. The IC_{50} values for NS-398 and indomethacin were calculated by the probit method using the dose-response curves for each cell line. The IC_{50} values for NS-398 in Colo320 and THRC 1 were 54.8 ± 3.6 and 77.2 ± 4.9 μM, respectively. Those for indomethacin in Colo320 and THRC 1 were 206.3 ± 43.0 and 180.3 ± 22.6 μM, respectively. The statistical significance of differences was determined by using the two-tailed unpaired Student's t test. The IC_{50} value of NS-398 was significantly lower than that of indomethacin ($P<0.01$) in each cell line.

Fig. 1 illustrates the effects of NS-398 and indomethacin on the induction of apoptosis in Colo320 and THRC 1. Both drugs were found to increase, in a concentration-dependent manner, the proportion of apoptotic cells in both cell lines. NS-398 showed maximum induction of apoptosis at the concentration of 300 μM in each cell line. NS-398 even at the concentration of 30 μM, at which no induction of apoptosis was noted with other NSAIDs (indomethacin, ibuprofen and piroxicam), induced apoptosis amounting to 4.9 ± 1.9 and 4.2 ± 0.1 times the control value in Colo320 and THRC 1, respectively.

Relative levels of apoptosis induction of NS-398 and other NSAIDs The mean relative levels of apoptosis induction of NS-398, indomethacin, ibuprofen and piroxicam (indomethacin was assigned as 100) at 300 μM are summarized in Table I. The effects of NS-398 on the apoptosis induction in both cell lines were stronger than those of the other three NSAIDs.

Confirmation of apoptosis induction by NS-398 and indomethacin The floating cells in both cell lines after treatment with 300 μM NS-398 and indomethacin exhibited typical features of apoptosis, whereas the attached cells, stained after trypsinization, contained few apoptotic cells (less than 5%). Representative photomicrographs of the floating and attached cell populations of NS-398-treated Colo320 cell line are shown in Fig. 2.

DNA ladders were recognized in both cell lines following treatment with NS-398 and indomethacin at the concentration of 300 μM. The most intense DNA fragmentation was recognized after 36-h treatment with each

Table I. Relative Levels of Apoptosis Induction of NS-398 and Other NSAIDs

	Colo320	THRC 1
NS-398	105[a)]	243
Indomethacin[b)]	100	100
Ibuprofen	15	51
Piroxicam	19	41

a) The figures indicate the relative levels of the mean apoptotic ratios for triplicate experiments with each drug at the concentration of 300 μM.

b) The apoptotic ratio of indomethacin, determined as the proportion of the floating cells to the total cell number, was assigned as 100.

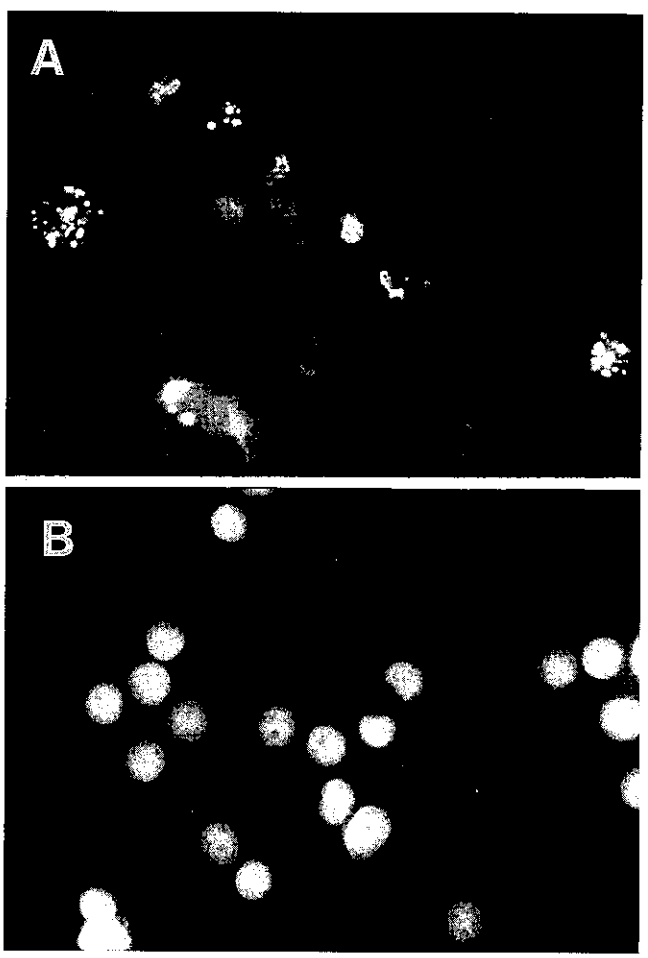

Fig. 2. Fluorescence photomicrographs of the floating cells (A) and the attached cells (B) of Colo320 after 96-h treatment with 300 μM NS-398. The floating cells show typical feature of apoptosis, whereas the attached cells, stained after trypsinization, contain few apoptotic cells.

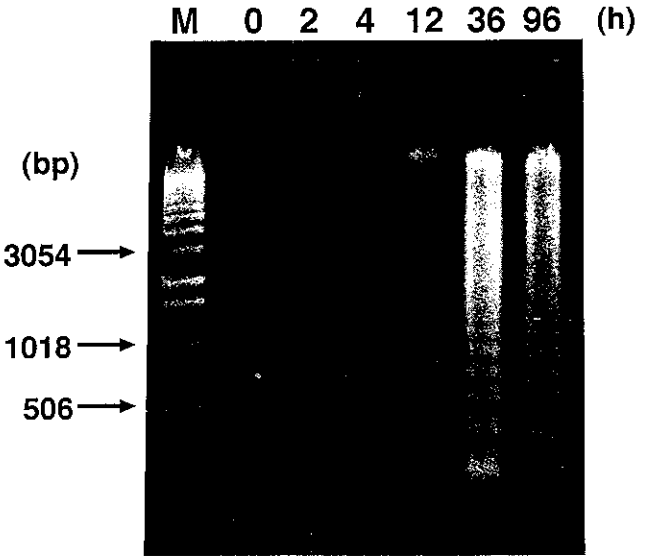

Fig. 3. DNA ladders produced by Colo320 cell line after different times of treatment (0, 2, 4, 12, 36 and 96 h) with NS-398 at the concentration of 300 μM. The DNA in each lane corresponds to 4×10^5 cells. DNA was loaded into an ethidium bromide-containing 1.0% agarose gel. After electrophoresis, DNA was visualized under UV light. The most intense DNA fragmentation was seen after the 36-h treatment with NS-398. M, DNA size markers.

drug. DNA ladders produced by Colo320 cells after different times of treatment with NS-398 at the concentration of 300 μM are shown in Fig. 3.

DISCUSSION

NSAIDs are known to have cancer-preventive and tumor-regressive effects in the human colon[15] and to inhibit chemical carcinogenesis in rodent models.[16] However, the mechanisms underlying these phenomena are still unclear. Human colorectal cancers and experimental colonic tumors in rodents contain increased amounts of PGE_2, and therefore, PGE_2 is thought to play an important role in both human and experimental colon carcinogenesis. The suppressive effects of NSAIDs on colon carcinogenesis are considered to derive from their inhibition of COX, resulting in the reduction of PG levels. However, a recent study revealed that the sulfone derivate of sulindac, which essentially lacks anti-PG synthetase activity, also reduces tumor occurrence in azoxymethane-induced rodent colon carcinogenesis.[8] Furthermore, Maxwell *et al.*[17] reported that the primary source of PGE_2 may be inflammatory cells such as tissue-fixed macrophages rather than tumor cells in human colon cancers. This does not explain the direct inhibitory effect of NSAIDs on the tumor cells *in vitro*. These findings suggest that the antineoplastic activity of NSAIDs is based not on the reduction of PG levels, but on other biological mechanisms.

Recent studies have revealed that growth inhibition of colorectal cancer cells with non-selective NSAIDs, which inhibit both COX-1 and COX-2, involves the induction of apoptosis. Our results demonstrated that NS-398, a COX-2-specific inhibitor, also induced apoptosis, producing typical changes in nuclear morphology and DNA electrophoresis profile. Furthermore, dose-dependent inhibitory effects of NS-398 on the proliferation of colorectal carcinoma cell lines were much stronger than those of indomethacin. Our findings that indomethacin, among the non-selective NSAIDs, has the most potent inhibitory efficacy for proliferation of the colorectal carcinoma cells are in agreement with a previous study.[11] Therefore, NS-398 may be a superior agent to other non-selective NSAIDs in terms of the growth inhibition of colorectal cancer cells. The biochemical mechanism responsible for the induction of apoptosis by NS-398 is still unclear. It is likely that the induction of apoptosis by NS-398 is based on a mechanism other than inhibition of PG synthesis. Further studies are warranted to elucidate the intracellular pathway through which apoptosis is induced after NS-398 treatment of colorectal carcinoma cells.

Non-selective NSAIDs, which inhibit both COX-1 and COX-2, block gastric PG, and consequently, produce gastric lesions. Indomethacin[18] and other NSAIDs[19] inhibit the growth of cultured cells at concentrations significantly in excess of those which are required to inhibit PG synthesis. Thus, non-selective NSAIDs are likely to cause gastrointestinal side effects, such as gastritis, gastric ulcer and gastrointestinal bleeding at concentrations which may inhibit the growth of colorectal carcinomas.[20] In contrast, a selective COX-2 inhibitor, which does not block gastric PG, may not produce gastric lesions. In fact, NS-398, a COX-2-specific inhibitor, in a single dose of up to 1,000 mg/kg, p.o. did not cause significant gastric ulceration, while other nonsteroidal anti-inflammatory drugs, such as loxoprofen, indomethacin, diclofenac and ibuprofen, produced distinct gastric lesions.[6]

In conclusion, the selective COX-2 inhibitor NS-398 possesses two major advantages compared with other non-selective NSAIDs. One is the stronger level of growth inhibition caused by the induction of apoptosis in colorectal carcinoma cell lines, as compared with non-selective NSAIDs. The other is the low risk of gastrointestinal side-effects. Thus, specificity of NS-398 for COX-2 inhibition should be highly advantageous, and a clinical trial for chemoprevention of colorectal cancers seems to be justified.

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