Experimental study on the antitumor effect of a selective cyclooxygenase-2 inhibitor combined with 5-fluorouracil in a mouse model of colon cancer

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Summary

Selective cyclooxygenase (COX)-2 inhibitors have been reported to be promising means to prevent and treat colon cancer. We studied the effects of the selective COX-2 inhibitor nimesulide (NIM) alone or in combination with 5-fluorouracil (5-FU) upon in vitro growth of mouse colon cancer cells (colon 26 cells), and evaluated the antitumor effect and influence on survival of NIM with or without 5-FU in a mouse model of colon cancer prepared by subcutaneous implantation of colon 26 cells. NIM inhibited colon 26 cell growth concentration-dependently, showing correlation with apoptosis induction. The combination of NIM and 5-FU (NIM + 5-FU) enhanced the colon 26 cell growth inhibition by 5-FU in an additive manner. In mice with subcutaneously implanted colon cancer, NIM alone did not show antitumor effect but promoted growth of cancer cells. Although NIM + 5-FU failed to enhance the antitumor effect of 5-FU, it decreased toxicity-related deaths following administration of 5-FU 40 mg/kg and prolonged survival significantly as compared with 5-FU alone. Animals receiving NIM alone, 5-FU (20 mg/kg) alone or NIM + 5-FU had a significantly prolonged survival over the solvent treated control animals. These results demonstrate that NIM inhibited colon 26 cell growth in vitro, the inhibition being additively enhanced by the concomitant use of 5-FU. NIM showed no antitumor effect in the mouse model of colon cancer prepared by subcutaneous implantation of colon 26 cells, while it significantly prolonged survival of these cancer bearing mice and its combination with 5-FU resulted in prolonged survival and in reduced toxicity of 5-FU.

Combination chemotherapy with NIM and 5-FU was considered to be a promising treatment for colon cancer, especially useful for the improvement of the patient’s quality of life.

Introduction

Colon cancer mortality has shown a 6- to 7-fold increase over a little less than 50 years in Japan, and prevention and treatment methods urgently need to be established.

Recent epidemiologic studies reported that long-term use of nonsteroidal antiinflammatory drugs reduced the risk for colon cancer by 40 to 50%. The mechanism by which nonsteroidal antiinflammatory drugs inhibit the onset of colon cancer has not been fully elucidated, except that cyclooxygenase (COX), an action target of these drugs, might be involved. COX is a key enzyme that transforms arachidonic acid to various prostaglan-
dins and its two isozymes have been identified (COX-1 and COX-2). COX-1 is constitutively expressed in a variety of organs including the gastrointestinal tract and plays a role in maintaining organ functions, while the expression of COX-2 is induced in response to excitation by cytokines, tumor promoters and growth factors8-13. The observation that COX-2 is expressed at high levels in 80 to 90% of colon cancer patients14-16 suggests the possibility of a selective COX-2 inhibitor being an effective measure not only for prevention but also for the treatment of colon cancer.

According to previous studies, selective COX-2 inhibitors do not reduce tumor size but delay tumor growth17-24 and this seems to indicate that COX-2 inhibitors should be combined with cytotoxics with different mechanisms of action for clinical application25.

In this study, nimesulide (NIM), a selective COX-2 inhibitor, and 5-fluorouracil (5-FU) were evaluated for their in vitro effects on the growth of colon 26 cells derived from mouse colon cancer when used alone or in combination. We also evaluated the antitumor effect of the drugs in combination as well as their contribution to survival in a colon cancer model prepared by subcutaneous implantation of colon 26 cells into the mouse abdomen.

Materials and methods

Drugs and reagents

NIM (N-[4-nitro-2-phenoxypyphenyl]-methanesulfonamide) was purchased from Sigma Chemical Co., St. Louis, MO, USA, 5-FU from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan, and methylcellulose (MC) and dimethylsulfoxide (DMSO) from Wako Pure Chemical Industries, Ltd., Asaka, Japan.

Cell strain

Colon 26 cell strain, a mouse colon cancer strain, was a gracious gift from Tsukuba ARL (Tsukuba, Ibaraki, Japan). Animals

CDF1 mice at six weeks of age were purchased from Charles River, Atsugi, Japan and subjected to one-week acclimation. They were housed in a controlled environment (room temperature : 24±1°C ; humidity : 50±10%).

In vitro experiment

To examine whether simultaneous treatment with 5-FU plus NIM increased apoptosis, we carried out FACS Caliber flow cytometry (Becton Dickinson Co., Ltd., Franklin Lakes, NJ, USA) analysis using Annexin V apoptosis kit (BioVision Research Product, Mountain View, CA, USA). NIM and 5-FU were dissolved in DMSO. Colon 26 carcinoma cells were exposed to NIM, 5-FU and NIM plus 5-FU at final concentrations of 200 μM, 5 μg/ml and 200 μM plus 5 μg/ml, respectively. DMSO was present in all experiments at a final concentration of 0.05%. Colon 26 cells cultured with RPMI 1640 medium containing 10% fetal bovine serum (FBS) were trypsinized and gently washed three times with phosphate buffered saline (PBS) solution supplemented with 2% FBS. After 1×10⁶ cells were incubated for 48 h with the same medium, the culture media were decanted and cells were harvested with trypsin. The cells collected were gently washed three times with the above PBS solution and 1×10⁶ cells were resuspended in 500 μl of Annexin V binding buffer and 5 μl of Annexin V-FITC. After incubation at room temperature for 5 min, the apoptosis of colon 26 cells was analyzed with a flow cytometer.

Animal experiments

1. Drug administration

NIM suspended and 5-FU dissolved in 0.5% MC were diluted to the desired concentration and administered by oral gavage (0.1 ml/10 g body weight) once daily at a specified time (10:00 a.m. for NIM and 4:00 p.m. for 5-FU). Animals were assigned to a total of 6 groups of 10 mice each: Group A : solvent (used as a control); Group B : 5-FU 20 mg/kg alone; Group C : 5-FU 40 mg/kg alone; Group D : NIM 110 mg/kg alone; Group E : NIM 110 mg/kg plus 5-FU 20 mg/kg; Group F : NIM 110 mg/kg plus 5-FU 40 mg/kg.

2. Experimental design

Mice were subjected to subcutaneous implantation of 1×10⁶ colon 26 cells into the flank. 5-FU was administered orally for 5 days starting from the day when the tumor grew to around 10 mm in diameter (Day 0) and was withheld for 2 days (constituting one cycle) followed by another 5-day oral administration. NIM was started from the day when 5-FU was started and was continued independently of whether 5-FU was given or withheld. In Experiment 1, survival and tumor volume were estimated during two cycles of 5-FU treatment and until all animals died. In Experiment 2 to determine tumor enzyme activity, 5-FU was administered up to the 10th day (Day 10), when mice were sacrificed by cervical dislocation 3 hours after the last dose of 5-FU to isolate tumors for biochemical analysis.


Tumor volume and body weight were measured in surviving animals every other day from the day of initial dosing (Day 0) and mean values of each group were obtained for intergroup comparison. Tumor volume and T/C ratio (%) were calculated from the expressions:

\[
\text{Tumor volume} = \frac{\text{Major dimension (mm) × (Minor dimension)^2}}{2}
\]
where Vo represented the tumor volume measured just before initial dosing and V the tumor volume measured at a designated time. A reduction in tumor volume of 25% or more was considered to indicate that the regimen was antitumoral.

4. Measurement of biochemical parameters
1) 5-FU concentrations
5-FU was quantified at room temperature by high performance liquid chromatography (HPLC) according to the method described by Masuike et al.26 The HPLC column used was Develosil 60-3 (14.6×100 mm, Nomura Chemical Co., Ltd., Seto, Aichi, Japan) and the guard column was Nova-Pak Silica (Waters Corp., Milford, MA, USA).

2) Enzyme activity
Among the enzymes involved in the metabolism of 5-FU, thymidylate synthase (TS) and orotate phosphoribosyl transferase (OPRT) were quantified. The method reported by Spears et al.27 was applied to quantification of TS. That is, 50 µmol/L phosphate buffered solution (pH 7.4) was added to tissue samples, homogenized and centrifuged. The supernatant was collected and divided into two portions: one was assayed for [3H]-5-fluorodeoxyuridine monophosphate (FdUMP) bindable to the total TS composed of complex TS and free TS (TS\textsubscript{total}) and the other for [3H]-FdUMP bindable to free TS (TS\textsubscript{free}). In the former assay, to the supernatant was added an equal volume of 50 mmol/L Tris buffer (pH 8.0), then [3H]-FdUMP and tetrahydro-folic acid (mTHF) were added and incubated at 25°C, the resulting complex was isolated and its radioactivity determined. In the latter, to the supernatant was added an equal volume of 50 mmol/L Tris buffer (pH 8.0) followed by [3H]-FdUMP and mTHF, the resulting complex was isolated and its radioactivity determined as in the assay for TS\textsubscript{total}. TS inhibition was calculated from TS\textsubscript{total} and TS\textsubscript{free} values following the expression:

\[
\text{TS inhibition} \text{ (%) } = \left[ 1 - \frac{\text{TS}_{\text{free}}}{\text{TS}_{\text{total}}} \right] \times 100
\]

OPRT activity was determined by the paper disk method according to Laskin et al.28. Tris-HCl at 50 mmol/L (pH 7.5) containing 1.5 mmol/L MgCl\textsubscript{2} and 2 mmol/L diethyldithiocarbamate was added to tissue samples, homogenized and centrifuged (105,000×g, 1 hr, 4°C). The supernatant was collected, and [3H]-5-FU was added as a substrate and incubated at 37°C. The reaction solution was sampled at 5, 10 and 15 minutes and immediately placed in a hot-water bath at a temperature of 100°C to stop reaction. After centrifugation, the supernatant was passed through a DEAE-cellulose ion exchange filter paper and washed repeatedly to remove nonreacting [3H]-5-FU. The filter paper was placed in a scintillation vial, to which was added a liquid scintillation cocktail, and the radioactivity of the resulting [3H]-5-fluorouridine monophosphate (FUMP) was determined to obtain FUMP concentrations. The reaction rate was estimated from the relationship between reaction time and product concentration. This reaction rate together with the separately determined protein concentration was used to calculate OPRT activity (nmol/min/mg protein).

COX-2 activity was determined as follows. Cooled 0.1 mol/L Tris-HCl at pH 7.8 (buffer for extraction) containing 1 mmol/L EDTA, 0.3 mmol/L diethyldithiocarbamic acid and 250 mmol/L mannitol was added to tumor tissue samples and homogenized using a Polytron homogenizer under ice-cooling. The resulting homogenate was centrifuged at 4°C, 10,000×g, for 10 minutes and the supernatant was subjected to 30-minute ultracentrifugation at 4°C, 100,000×g. To the precipitate, the extraction buffer was added, re-homogenized as described above, and ultracentrifuged. The resulting precipitate (microsome fraction) was assayed for COX-2 activity using a cyclooxygenase activity kit (COX Activity Assay, Cayman Chemical Company, Ann Arbor, MI, USA).

3) COX-2 messenger RNA (mRNA) (determined by real-time PCR)
COX-2 mRNA expression in tumor cells was determined. For total RNA isolation, Trizol was added to tissue samples (1 ml/100 mg tissue) and homogenized. The homogenate was extracted by adding 200 µl of chloroform, precipitated with ethanol and re-dissolved in diethylpyrocarbonate-treated water (30 µl). PCR proceeded as follows. For cDNA synthesis and fragmentation, 3'-terminal cDNA was synthesized from total RNA in the usual way. Two primer sets were designed and synthesized to optimize PCR conditions for each gene. The primer used had the upper sequence 5'-CCCAAACACAGTGCACCTACA-3' from position 333 at the 5'-end and the lower sequence 3'-ACCCAGGTCTCGCTATTAGAT-5' from position 740 at the 3'-end, based on GenBank accession No. PCR conditions were those for the Light Cycler (Roche, Mannheim, Germany) with syber green I staining. For data processing, fluorescence resulting from PCR was measured with the Light Cycler and a calibration curve was constructed through simultaneous assay of the reference standard. The relative copy number of the target transcription product was read from the calibration curve.

Statistical analysis
Body weight and biochemical data were analyzed by Dunnett's multiple comparison test, incidence of apoptosis by Student's t-test and survival data by the log-rank test.
rank test.

Results

1. In vitro experiment

Induction of apoptotic cells was significant in the experimental groups treated with 5-FU, NIM or NIM+5-FU over the solvent-treated control group (p<0.05). The combination of NIM+5-FU showed an additive effect on apoptosis induction when compared with each component used alone (Fig. 1).

2. Antitumor effect

Figure 2 gives the time course of mean tumor volume. Antitumor effect was noted on Day 10 in the groups given 5-FU 40 mg/kg alone (T/C=0.42) or NIM 110 mg/kg+5-FU 40 mg/kg (T/C=0.58). NIM was devoid of antitumor effect on any day of determination; it showed a tendency to increase tumor volume on Day 10 as compared with the control group (T/C=1.94).

3. Body weight

Figure 3 gives the time course of mean individual body weight. Animals on NIM alone weighed significantly more than control animals on Day 12. On Day 16, a significant increase in body weight was observed in animals on NIM alone and on NIM 110 mg/kg+5-FU 20 mg/kg as compared with solvent-treated controls (p<0.05 for each of the experimental groups).

4. Survival

Survival was significantly prolonged in the experimental groups given 5-FU 20 mg/kg alone, NIM alone or NIM 110 mg/kg+5-FU 20 mg/kg over the control group (Fig. 4, p<0.05 for each of the experimental groups). The combination of NIM 110 mg/kg+5-FU 40 mg/kg resulted in longer survival than the same dosage level of 5-FU alone, the difference being significant (Fig. 5, p<0.05). However, this NIM 110 mg/kg+5-FU 40 mg/kg group was not significantly different from the control group in terms of survival (Fig. 4).

5. 5-FU concentrations

Tumor 5-FU concentrations exceeded 100 ng/g tissue in the groups given 5-FU. There was no significant difference in tumor 5-FU concentrations whether the drug had been used alone (235.5±102.3 ng/g, mean±
Fig. 3 Time course of mean individual body weight. Animals on NIM alone weighed significantly more than control animals on Day 12. On Day 16, a significant increase in body weight was observed in animals on NIM alone and on 5-FU 20 mg/kg + NIM 110 mg/kg as compared with solvent-treated controls (p<0.05 for each of the experimental groups).

Fig. 4 Time course of survival. Survival data were analyzed by the log rank test. Survival was significantly prolonged in the experimental groups given 5-FU 20 mg/kg alone, NIM alone or 5-FU 20 mg/kg + NIM 110 mg/kg (p<0.05).

Fig. 5 Time course of survival. When compared with 5-FU alone (40 mg/kg), the combination 5-FU 40 mg/kg + NIM 110 mg/kg prolonged survival significantly (p<0.05).

standard deviation) or in combination with NIM (Fig. 6).

6. Inhibition of TS enzyme
Inhibition of TS enzyme was significantly and dose-dependently enhanced by 5-FU as compared with the solvent (14.4±3.5%, mean±standard deviation) (p<0.01) since inhibition was estimated to be 68.1±9.1% for 5-FU 20 mg/kg alone and 88.1±7.0% for 5-FU 40 mg/kg alone. No significant difference was observed between the NIM group and the solvent group. A comparison between NIM+5-FU and the same dosage of 5-FU alone failed to reveal any influence of NIM on the enzyme inhibition enhanced by 5-FU (Fig. 7).

7. COX-2 activity
Measured COX-2 activities expressed in nmol/min/mg protein were 3.25±0.73 (mean±standard deviation) in the control group, 3.50±1.29 in the 5-FU 20 mg/kg group, 2.97±1.37 in the NIM group and 3.66±2.01 in the NIM 110 mg/kg+5-FU 20 mg/kg group. Thus, there were no significant intergroup differences (Fig. 8).

8. Expression of COX-2 mRNA
None of the experimental groups was significantly different from the control group in regard to COX-2 mRNA expression (Fig. 9).
Fig. 6  Mean 5-FU concentrations in tumor tissue. There was no significant difference between 5-FU alone and 5-FU combined with NIM.

Fig. 7  Inhibition of TS enzyme. A significant difference was noted between 5-FU treated groups and the solvent control group (p<0.01), but the group given NIM alone was not different from the control.

Fig. 8  Mean COX-2 activity. There was no significant difference between drug treated and solvent treated groups.

9. OPRT activity
OPRT activity was significantly higher in the 5-FU 20 mg/kg group and NIM group than in the control group, as the values obtained in nmol/min/mg protein were $1.482 \pm 0.245$ (mean ± standard deviation), $1.662 \pm 0.145$ and $1.059 \pm 2.000$, respectively (p<0.05 and p<0.01,
Discussion

It was expected from our experiments using colon 26 cells that a selective COX-2 inhibitor, when used alone, might exert antitumor effect causing apoptotic death in tumor cells and that this antitumor effect might be additively enhanced by combination with a metabolic antagonist. In the therapeutic experiment in a mouse colon cancer model with subcutaneously implanted colon 26 cells, however, NIM neither showed antitumor effect when used alone nor enhanced antitumor effect of 5-FU when used in combination. Although the lack of in vivo antitumor effect of celecoxib, another selective COX-2 inhibitor, against colon 26 cells\(^{29}\) does not rule out the possibility of low in vivo sensitivity of colon 26 cells to COX-2 inhibitors, the present authors and other investigators reported evident antitumor effect with celecoxib\(^{25,30}\), SC58125\(^{31}\) and meloxicam\(^{25}\) in nude mice implanted with human colon cancer cells and this suggests that the intensity of NIM was not sufficient to be effective against colon 26 cells in our study. The similarity of the two parameters determined in parallel with antitumor effect: COX-2 activity and COX-2 mRNA expression in colon 26 cells between the NIM- and solvent-treated groups also forms a support for the deduction that the dose of NIM used in this study was not large enough to inhibit COX-2 activity in tumor cells or to inhibit tumor cell growth. However, the tendency for colon 26 cell growth to increase in the animals given NIM alone seems to indicate that the dose of NIM used corresponds to the level at which the release of tumor necrosis factor-\(\alpha\) is inhibited\(^{25,34}\) and cancer cachexia-related cytokines affected.

In this study the daily dose of NIM selected (110 mg/kg) was that at which colon 26 cancer bearing mice...
receiving NIM alone had tended to live longer in the preliminary dose finding study, with the expectation that NIM might enhance the antitumor effect of 5-FU when used in combination. This expectation was not met, however. Prolonged survival was observed in animals on NIM alone, on NIM + 5-FU (20 mg/kg) and on 5-FU 20 mg/kg alone over solvent-treated controls. Body weight on the 16th day of treatment was significantly increased in the groups given NIM alone or NIM + 5-FU 20 mg/kg as compared with the solvent group; thus, NIM might affect cancer cachexia-related cytokines by inhibiting release of tumor necrosis factor-α by reducing synthesis of interleukin-6, for example. The absence of significant survival prolongation in the NIM + 5-FU 40 mg/kg group as compared with the solvent control group can be explained by the toxicity of 5-FU causing such major signs as convulsion and weakness occurring from the 9th day of treatment, which had been observed from the 6th day in the group on 40 mg/kg 5-FU alone. The occurrence of death attributable to 5-FU was apparently delayed in the combination treatment groups in comparison with the groups on 5-FU alone, and the significantly prolonged survival attained by the combination regimens suggests reduction of 5-FU toxicity by NIM.

Among biochemical parameters determined in parallel with evaluation of antitumor effect, OPRT activity was significantly enhanced by NIM alone. This finding is of interest in pondering clinical applications of the combination of NIM with 5-FU. It also indicates the possibility that NIM, when administered prior to 5-FU, may enhance the activity of the latter, for phosphorylation of 5-FU by OPRT is essential as the first step toward activation of this drug against cancer cells.

This experimental study demonstrated that NIM combined with 5-FU prolonged survival by reducing the toxicity of 5-FU, although it failed to enhance the antitumor effect of 5-FU. Various attempts have been made to establish a standard chemotherapy for colon cancer combining 5-FU with cytotoxics. Regimens so far proposed are found very toxic, and the combination of NIM and 5-FU, through careful dose finding studies, can be a useful treatment for colon cancer with due quality-of-life consideration.

Conclusion

NIM when used alone was not antitumoral in a mouse colon cancer model prepared by subcutaneous implantation of colon 26 cells. NIM when used in combination with 5-FU did not increase the antitumor effect of 5-FU. However, NIM proved to reduce the toxicity of 5-FU, since the survival of cancer bearing mice was prolonged by NIM combined with a low dose of 5-FU and was significantly prolonged by NIM combined with a high dose of 5-FU as compared with the same doses of 5-FU alone.

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COX-2 阻害剤及び 5-fluorouracil の併用投与による
マウス大腸癌モデルにおける抗腫瘍効果に関する影響の検討

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【要旨】 Cyclooxygenase (COX)-2 選択阻害剤は、大腸癌の予防および治療薬として有望である可能性を報告されてきた。我々は COX-2 選択的阻害剤の nimesulide (NIM)、NIM および 5-fluorouracil (5-FU) の併用処理による、マウス大腸癌 colon-26 細胞の in vitro での増殖に与える影響、および colon 26 細胞を皮下移植したマウス大腸癌モデルにおける抗腫瘍効果ならびに生存期間に与える影響を検討した。NIM は colon26 細胞の増殖を濃度依存的に抑制し、その抑制は、アポトーシスの誘導と関連した。NIM および 5-FU の併用 (NIM + 5-FU) 処理は、5-FU による同細胞の増殖抑制効果を相加的に増強した。マウスの大腸癌皮下移植モデルにおける NIM 単剤投与では、抗腫瘍効果を示さず、むしろ癌細胞の増殖を促進した。NIM + 5-FU 投与においては、5-FU の抗腫瘍効果を増強しなかったが、5-FU 40 mg/kg 投与による毒性死を減少させ、5-FU 単剤投与群に比較して生存期間の有意な延長が観察された。また、NIM 単剤、5-FU (20 mg/kg) 単剤および NIM + 5-FU 群を各々宿主対照群と比較したとき、有意な生存期間の延長が認められた。これらの結果から、NIM は、in vitro で colon26 細胞の増殖を抑制し、5-FU との併用により相加的に同細胞の増殖を抑制した。一方、同細胞を皮下移植したマウス大腸癌モデルにおいては、抗腫瘍効果を示さなかったが、NIM 単剤投与により、胆癌マウスの生存期間の有意な延長をもたらし、また 5-FU との併用により 5-FU の毒性軽減を伴う生存期間の延長をもたらすことが明らかとなった。

大腸癌の治療法として NIM と 5-FU の併用による化学治療法は、有望であり、とくに患者の quality of life を考慮した治療法として、有用なものになる可能性があると考えられた。

〈Key words〉 Antitumor effect, cyclooxygenase-2 inhibitor, 5-fluorouracil, mouse colon cancer model, nimesulide