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Inhibitors of cyclooxygenase-2 (COX-2) suppressed the proliferation and differentiation of human leukaemia cell lines

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Abstract

Prostaglandins (PG) are known to play important roles in the proliferation and differentiation of leukaemia cells. The effect of the inhibitors of cyclooxygenase-2 (COX-2), a rate-limiting enzyme for the synthesis of PG, on the proliferation and differentiation of leukaemia cell lines was investigated. COX-2 inhibitors, NS-398 and nabumetone, suppressed the proliferation of U-937 and ML-1 cells by inducing a G0/G1 cell-cycle arrest. Cell-cycle arrest induced by these COX-2 inhibitors was not associated with an upregulation of the cyclin-dependent kinase inhibitors. COX-2 inhibitors also inhibited the differentiation of these cells induced by interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and retinoic acid (RA). Treatment with NS-398 did not suppress the levels of PGs produced by these cells. Although COX-2 antisense oligonucleotide showed a similar inhibitory effect on these cells, its inhibitory effect was smaller than that of NS-398. These results suggest that COX-2 inhibitors may suppress the proliferation and differentiation of leukaemia cells both via COX-2-dependent and -independent pathways.

Keywords: U-937; ML-1; COX-2 inhibitor; NS-398; Nabumetone; Proliferation; Differentiation; G0/G1 arrest

1. Introduction

Arachidonic acid metabolites, collectively referred to as eicosanoids, such as hydroxyeicosatetraenoic acids, prostaglandins (PGs), thromboxanes (TXs), and leucotrienes (LTs), affect a number of signal transduction pathways that modulate the growth and differentiation of many types of cancer including leukaemia cells [1–4]. PGs are produced by the action of cyclooxygenase (COX) enzymes on the free arachidonic acid liberated from membrane phospholipids by phospholipases. Prostaglandin endoperoxidase (also referred to as (COX)) is the rate-limiting enzyme for the production of PGs and TXs from free arachidonic acid [5]. Two forms of COX have now been described: a constitutive enzyme (COX-1) present in most cells and tissues; and an inducible isoenzyme (COX-2) expressed in response to cytokines, growth factors, and other stimuli [6–10]. These COX isoforms have both overlapping, as well as distinct physiological and pathological functions [11,12]. While COX-1 is involved in the homeostasis of various physiological functions, COX-2 is responsible for many pathological processes such as inflammation and cancer. *COX-2* mRNA levels, as well as COX-2 protein levels, are markedly increased in human colorectal adenocarcinomas relative to normal colonic mucosa, and overexpression of COX-2 has been identified as an early central event in colon carcinogenesis [13–17]. A previous study has demonstrated that constitutive expression of COX-2 in human colon cancer cells promotes tumour invasion and the metastatic potential of these cells [18]. As well as colon cancer, increased COX-2 expression is found in cancers of various organs including breast, lung and the mucous membrane of the head and neck [19–22]. Thus, it is recognised that COX-2 inhibitors may be effective chemopreventive agents against various types of cancer.

In this study, we examined the effects of COX-2 inhibitors on the proliferation and differentiation of leukaemia cells. We also examined the effects of COX-2 on the expression of cell-cycle regulating molecules.

2. Materials and methods

2.1. COX-2 inhibitors

COX-2 inhibitors, NS-398 and nabumetone, were purchased from Calbiochem (La Jolla, CA, USA) and

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the Sigma Chemical Company (St Louis, MO, USA), respectively. According to the manufacturer's information, NS-398 at 75 μM selectively inhibits COX-2 activity without inhibiting COX-1 activity. It has also been reported that nabumetone at concentrations exceeding 1 mM can selectively inhibit COX-2 [23]. Therefore, NS-398 and nabumetone at 75 and 35 μM, respectively, were employed in this study. The chemical structure of NS-398 is shown in Fig. 1.

2.2. Cell lines and cell culture

A human monocytoid leukaemia cell line, U-937, and a human myeloblastic leukaemia cell line, ML-1, were maintained as suspension cultures in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin (complete medium).

2.3. Proliferation assay

Cells were cultured in a 24-well microplate by adding 1 ml of complete medium containing 1×10^5 cells in the absence or presence of NS-398 (75 μM) or nabumetone (35 μM). The effects of NS-398 and nabumetone on cell growth and cell viability were assayed on a portion of the cell suspension. The cell number was determined with a haemocytometer, and viability was estimated by trypan blue dye exclusion.

2.4. Differentiation assays

Cell differentiation was monitored by determining the appearance of various cellular markers usually associated with the maturation of monocytic elements.

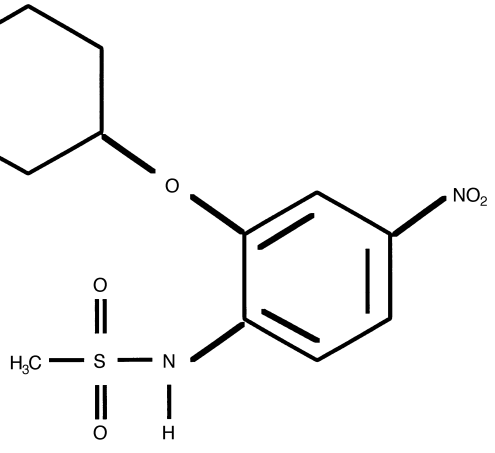

Fig. 1. The chemical structure of NS-398.

2.4.1. Nitroblue tetrazolium (NBT)-reducing activity

NBT-reducing activity was assayed microscopically as previously described [24]. Briefly, 6×10^4 cells were suspended in a flat-bottomed 96-well microplate with 0.2 ml of complete medium containing test materials at the determined concentration, and incubated at 37°C. Three days after incubation, 1% of NBT (Sigma, St Louis, MO, USA) dye and 30 ng of 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma, St Louis, MO, USA) were added to each well and incubated at 37°C for 60 min. After incubation, the percentage of cells containing blue-black formazan deposits was determined by counting at least 200 cells under a microscope.

2.4.2. Fc receptor expression

U-937 cells were cultured in 15 ml of complete medium containing 3×10^6 cells and test materials at the desired concentrations. The expressions of Fcγ receptor (FcγR) type II and type III were then analysed as follows. U-937 cells were incubated for 2 days for FcγR type II analysis and 3 days for FcγR type III analysis. The cells were treated with mouse anti-human FcγR type II and FcγR type III monoclonal antibodies (Immunotech, France) and incubated at room temperature for 15 min for FcγR type II and for 30 min for FcγR type III analysis. After washing with phosphate-buffered solution (PBS), the cells were treated with goat anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated antibody (Becton Dickinson, San Jose, CA, USA) and incubated at 4°C for 10 min. After washing with PBS, the percentage of labelled cells was analysed by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA, USA).

2.4.3. Morphological differentiation

U-937 cells were incubated with interferon (IFN)-γ (10^3 U/ml) and NS-398 (75 μM), either alone or in combination, for 4 days. The appearance of morphological differentiation was assessed on day 5 in stained slide preparations. The slides were prepared by centrifugation in a Cytospin (Shandon Southern Products Ltd, UK), followed by staining with May–Gruenwald's solution for 2 min and diluted Giemsa solution for 30 min. The cells were viewed on a microscope, and photographed with Kodak DYNA EX-100 films.

2.5. Competitive reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

U-937 cells were cultured in 10 ml of complete medium containing 7×10^6 cells in the presence of IFN-γ (10^3 U/ml) and incubated at 37°C. Total RNAs were extracted at 0, 48 and 72 h after incubation, and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was carried out using internal RNA standards. The RNA standard, heterologous competitors, was

made using a Competitive RNA transcription Kit (Takara Biochemicals, Tokyo, Japan), and contained the same primer sites, but was made approximately 50 bp longer than the cellular RNA being amplified. One μg of cellular RNA samples were mixed with between 0.005 and 20 pg of internal standard RNA, and competitive RT-PCR analysis was performed using a TaKaRa RNA PCR Kit Ver. 2.1 (Takara Biochemicals, Tokyo, Japan). The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Thermocycling was performed at 94°C for 30 s, 60°C for 30 s and 72°C for 90 s, repeated 35 times, and followed by a final extension at 72°C for 10 min (Programmed Temperature Control System, Astec, Fukuoka, Japan). The specific primers used in this study were as follows: *COX-2*: sense, 5′-TCCAGATCACATTTGATTGACA-3′, antisense, 5′-TCTTTGACTGTGGGAGGATACA-3′.

2.6. Cell cycle analysis

U-937 cells were cultured in 10 ml of complete medium containing 7×10^6 cells in the presence of test materials at the desired concentrations and incubated at 37°C. At 24 and 48 h after incubation, cell-cycle analysis was performed on these cells using DNA staining and flow cytometry. The cells were washed twice with PBS, treated with 0.2% of TritonX-100 and 0.5% of RNase, and then stained with 50 μg/ml of propidium iodide (PI). The stained cells were analysed by fluorescent activated cell sorter (FACS) Calibur. The relative DNA content per cell was obtained by measuring the fluorescence of PI that bound stoichiometrically to DNA.

2.7. Western blot analysis

U-937 cells were cultured in 10 ml of complete medium containing 7×10^6 cells in the presence of test materials at the desired concentrations and incubated at 37°C. At 0, 12, 24 and 48 after incubation, the cells were lysed with lysis buffer [20 mM Tris–HCl (PH 7.5), 0.1% sodium dodecyl sulphate (SDS), 1% TritonX-100, 1% sodium deoxycholate, 1 mM pheny methyl sulphonyl fluoride (PMSF), 10 μg/ml leupeptin, 20 μg/ml aprotinin] at 4°C. Insoluble material was removed by centrifugation at 12 000*g* for 20 min at 4°C. Proteins from cell lysates (20 μg) were separated on acrylamide-bisacrylamide-SDS gels and electrophoretically transferred to Immobilon-NC Pure membranes (Nihon Millipore, Tokyo, Japan) in 25 mM Tris–HCl–0.19 M glycine–10% methanol–0.05% Triton X100. The membranes were blocked in blocking solution [5% bovine serum albumin (BSA) in Tris-buffered saline Tween (TBS-T)] for 1 h at room temperature. After being washed in TBS-T, the blots were then incubated with the first antibodies in blocking solution for 1 h at room temperature. After being washed in TBS-T, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Pharmacia Biotech, UK, 1:500) in TBS-T for 1 h and exposed to X-ray film at room temperature. The first antibodies (Abs) used in this study were as follows: anti-p21 polyclonal Ab [rabbit (r) Immunoglobulin (Ig) G, C-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA], anti-p27 polyclonal Ab (rIgG, N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p16 polyclonal Ab (rIgG, N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.8. Measurement of eicosanoid levels

U-937 cells (4×10^4 cells in 200 μl of complete medium) were seeded into 96-well microplates and allowed to adhere overnight. The cells were then incubated in the presence or absence of NS-398 (75 μM) for 24 h. Aliquots of culture medium were harvested and PGE_2, $PGF_{2\alpha}$, and TXB_2 levels in the culture medium were determined using EIA kits, according to the manufacturer's instruction.

2.9. COX-2 *phosphorothioate oligonucleotide studies*

COX-2 phosphorothioate antisense oligonucleotide (5′-CAGTTCAGTCGAA CGTTCTTTTAGTAGTAC-3′), and its counterpart were synthesised by Hokkaido System Science (Sapporo, Japan). U-937 cells were incubated in the presence of 0.05 μM antisense oligonucleotides or sense oligonucleotides. Four hours after incubation, the proliferation of U-937 cells was determined as described above.

3. Results

3.1. Effects of COX-2 inhibitors on the proliferation and viability of U-937 and ML-1 cells

To determine the effects of COX-2 inhibitors on the proliferation of leukaemia cells, U-937 and ML-1 cells were cultured in the presence of NS-398 or nabumetone and the cell number and viability were examined. Both NS-398 and nabumetone remarkably inhibited the proliferation of U-937 cells (Fig. 2a). The growth inhibitory effect of COX-2 inhibitors could be seen on day 2, and became obvious on days 3 to 4. Similar growth inhibition by COX-2 inhibitors was obtained in the ML-1 cells (Fig. 2b). NS-398 exhibited almost the same growth inhibitory effect as nabumetone against both cell lines. Neither COX-2 inhibitor was cytotoxic against U-937 or ML-1 cells, since the viability of these cell lines exposed to COX-2 inhibitors for 4 days was approximately 99% (data not shown). Aspirin, a predominantly

COX-1 inhibitor, showed only a slight growth inhibitory effect both on U-937 and ML-1 cells (data not shown).

3.2. Effects of COX-2 inhibitors on the induction of NBT-reducing activity of U-937 and ML-1 cells

U-937 and ML-1 cells can be induced to differentiate into the monocyte/macrophage pathway by protein inducers called differentiation-inducing factors (DIFs) such as IFN-γ, tumour necrosis factor-α (TNF-α), and retinoic acid (RA). We examined the effects of COX-2 inhibitors on the differentiation-inducing activity of these DIFs. U-937 and ML-1 cells were incubated in the presence of DIFs and/or COX-2 inhibitors, and the maturation processes of these cell lines were monitored by observing various differentiation-associated characteristics. One of these indicators, the ability to reduce NBT dye, was measured on day 3. TNF-α (1 ng/ml), IFN-γ (10^3 U/ml) or RA (10^{-8} M) induced NBT-reducing activity of U-937 cells (Fig. 3a). NBT-reducing activity induced by these DIFs was suppressed by treatment with NS-398 or nabumetone. There was no significant difference between the inhibitory effect of NS-398 and that of nabumetone on the NBT-reducing activity of U-937 cells. TNF-α exerted cytotoxic activity against U-937 cells at concentrations exceeding 10 ng/ml. Accordingly, we employed a lower concentration of TNF-α (1 ng/ml). At this concentration, TNF-α, alone or in combination with NS-398 or nabumetone, was not cytotoxic against U-937 cells. The viability of U-937 cells incubated with TNF-α and/or NS-398 was approximately 99% (data not shown). TNF-α was less cytotoxic against ML-1 cells, and therefore we employed 10 ng/ml of TNF-α to induce NBT-reducing activity in the ML-1 cells, and similar results were obtained with ML-1 cells (Fig. 3b).

3.3. Effect of NS-398 on FcγR expression on U-937 cells

The effects of NS-398 and nabumetone on the proliferation and induction of NBT-reducing activity were almost the same. Therefore, we employed NS-398 for subsequent experiments, since NS-398 is highly selective against COX-2 (the ratio of inhibitory concentration (IC_{50}) against COX-2/COX-1 is 26.3) compared with nabumetone. The effect of NS-398 on the maturation of the cells was then monitored by FcγR expression induced by IFN-γ, which is also a typical marker associated with cell differentiation into the monocyte/macrophage lineage. U-937 cells were incubated with IFN-γ (10^3 U/ml) and/or NS-398 (75 μM), and the expressions of FcγR type II (FcγR II) and FcγR III on U-937 cells were analysed on days 2 and 3, respectively, by flow cytometry. Treatment with IFN-γ upregulated the expression levels of both FcγR II and FcγR III on the U-937 cells, and NS-398 suppressed IFN-γ-induced FcγR II and FcγR III expression (Fig. 4a and b). NS-398 alone did not affect the expression levels of either receptor on the U-937 cells (data not shown).

3.4. Effect of NS-398 on morphological differentiation of U-937 cells induced by IFN-γ

U-937 cells were incubated with IFN-γ (10^3 U/ml) and NS-398 (75 μM), either alone or in combination. The appearance of morphological differentiation was assessed on day 5 in stained slide preparations. Compared with untreated U-937 cells, U-937 cells treated with IFN-γ exhibited the characteristics of intermediate stages of macrophage-like cells such as increased cytoplasm, abundant granules, and eccentrically placed oblate nucleis with loosely stranded nuclear chromatin. In addition to the other cellular differentiation markers,

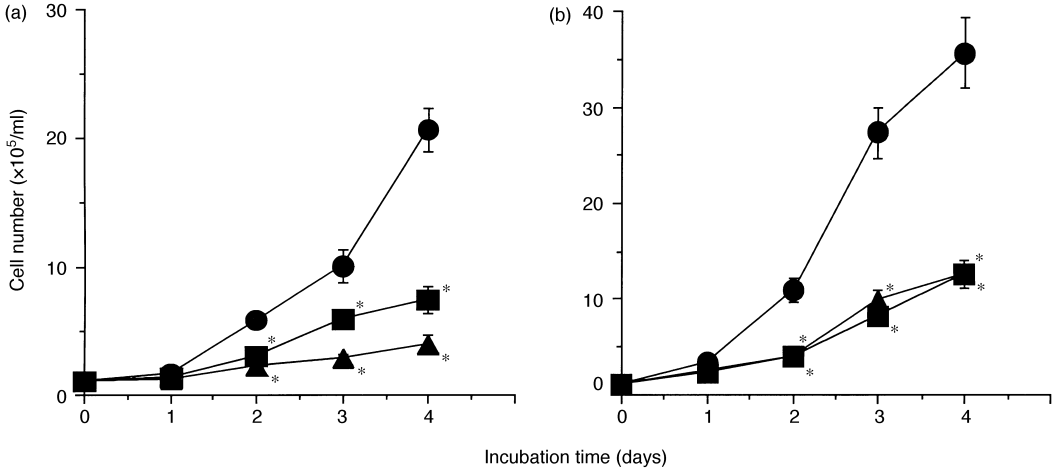

Fig. 2. Effects of cyclooxygenase-2 (COX-2) inhibitors on the proliferation of U-937 (a) and ML-1 (b) cells. Control (closed circle); NS-398 (75 μM, closed triangle); nabumetone (35 μM, closed rectangular). Values are means ± standard deviation (S.D) of triplicate determinations. *$P < 0.05$ versus control.

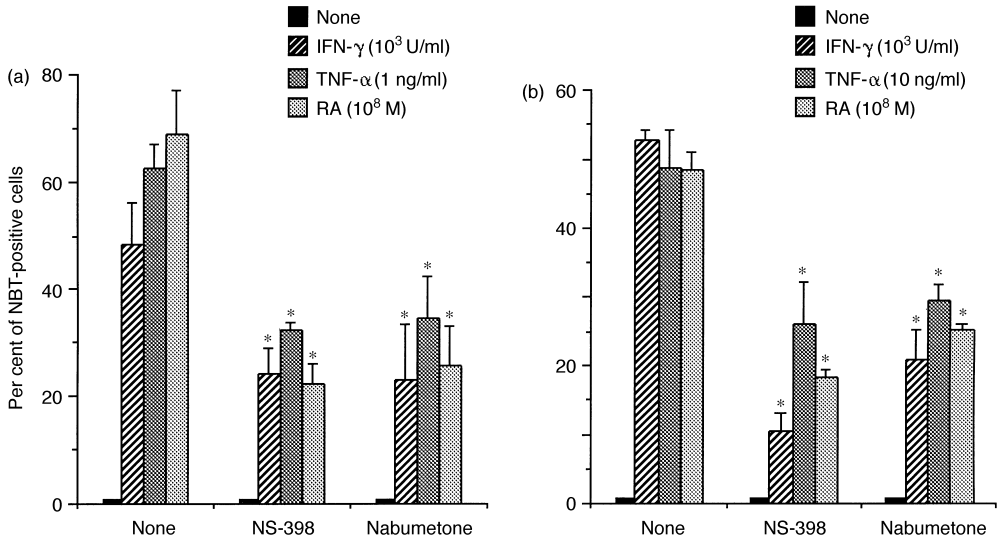

Fig. 3. Effects of COX-2 inhibitors on Nitroblue tetrazolium (NBT)-reducing activity of U-937 (a) and ML-1 (b) cells. Values are means±S.D. of triplicate determinations. *$P<0.05$ versus non-treatment with a COX-2 inhibitor. IFN-γ, interferon-γ; TNF-α, tumour necrosis factor-α; RA, retinoic acid.

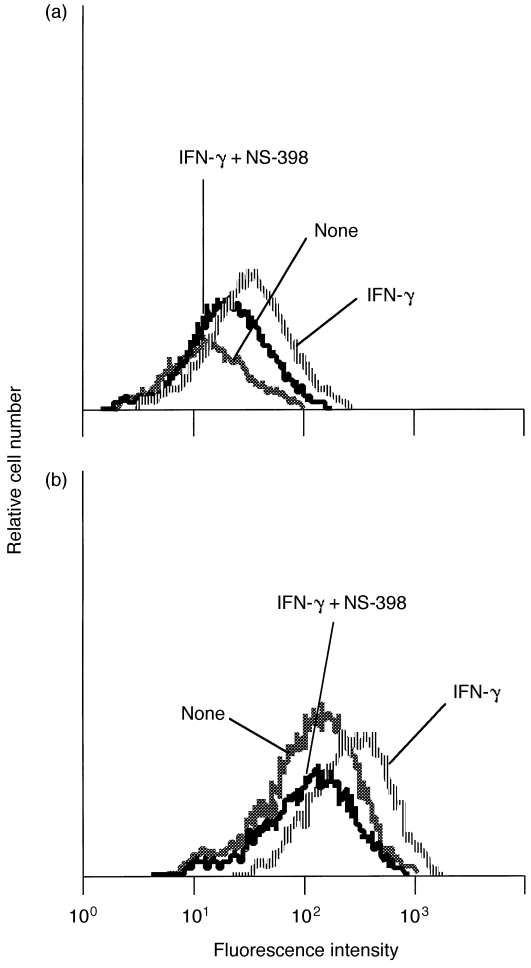

Fig. 4. Effect of NS-398 on FcγR type II (FcγR II) (a) and FcγR III (b) on U-937 cells induced by IFN-γ. Results from a representative analysis are shown.

treatment with NS-398 reduced the appearance of macrophage-like changes induced by IFN-γ. However, the suppression by NS-398 was partial, since U-937 cells treated with IFN-γ and NS-398 still retained characteristics of the intermediate stages of macrophage-like cells to some extent. Treatment with NS-398 alone induced no morphological changes in the U-937 cells (data not shown).

3.5. Competitive RT-PCR analysis of the expression of COX-2 *mRNA in U-937 cells treated with IFN-γ*

Since the differentiation-inducing activity of the DIFs against U-937 cells was inhibited by COX-2 inhibitors, we next examined the expression of *COX-2* mRNA in both undifferentiated and differentiated U-937 cells. U-937 cells were incubated in the presence or absence of IFN-γ (10^3 U/ml), and total RNA was extracted at 48 and 72 h after incubation. According to the Northern blot analysis, *COX-2* mRNA was barely detectable in the RNA samples extracted from untreated and IFN-γ-treated U-937 cells (data not shown). Therefore, we employed semiquantitative competitive RT-PCR methods for the analysis of *COX-2* mRNA expression in the U-937 cells.

Competitive RT-PCR revealed that *COX-2* mRNA was constitutively expressed in the untreated U-937 cells, and treatment with IFN-γ for up to 72 h did not alter the level of *COX-2* mRNA expressed in the U-937 cells (Fig. 5).

3.6. Cell cycle analysis of U-937 cells treated with NS-398

As we had observed that NS-398 could inhibit the proliferation of U-937 cells without inducing cytotoxicity, we

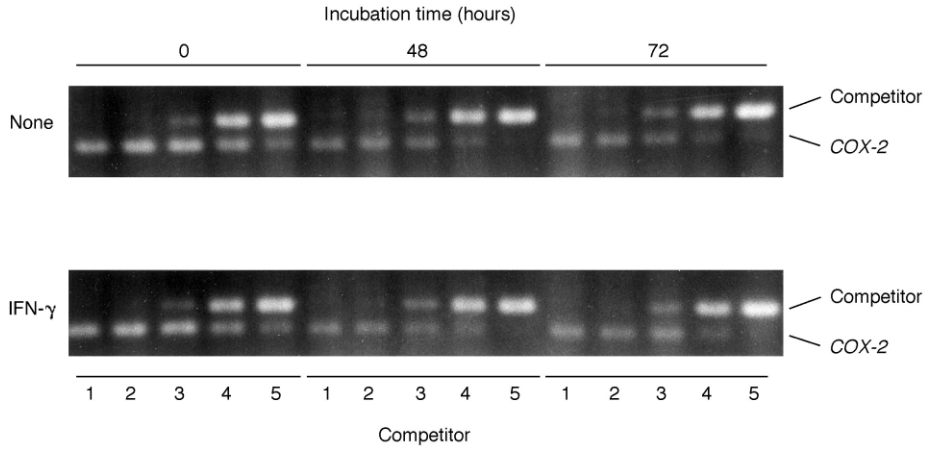

Fig. 5. Competitive RT-PCR analysis of *COX-2* mRNA expression in U-937 cells treated with IFN-γ. The amounts of competitors are as follows. Lane 1, 10^2 copies; lane 2, 3×10^2 copies; lane 3, 10^3 copies; lane 4, 3×10^3 copies; and lane 5, 10^4 copies. Results from a representative analysis are shown.

intended to determine if this reflected changes in the cell, cycle distribution. U-937 cells were treated with NS-398, and harvested over 24 and 48 h periods for flow cytometric analysis. As shown in Table 1, 48 h treatment with NS-398 resulted in altered distributions of cells in specific phases of the cell cycle compared with untreated U-937 cells. U-937 cells showed a tendency to accumulate in G0/G1 when exposed to NS-398 for 48 h. In parallel, a decrease in the number of cells in G2-M (in particular) and S was observed on treatment with NS-398. In contrast, the overall distribution of cells was not altered significantly upon 24 h treatment with NS-398 (data not shown). The data indicate that treatment with NS-398 resulted in an accumulation of U-937 cells in G0/G1.

3.7. Effect of NS-398 on CKI expression in U-937 cells

The cell-cycle progression of leukaemia cells is negatively regulated by a series of proteins called cyclin-dependent kinase inhibitors (CKIs). Therefore, the effect of NS-398 on the expression of CKIs in the U-937 cells was investigated. U-937 cells were incubated in the presence or absence of NS-398. At 0, 12, 24 and 48 h after incubation, cells were lysed and CKI protein levels were determined by Western blot analysis. p21 was slightly upregulated in the untreated U-937 cells over the first 24 h (Fig. 6). However, the expression level of p21 was not upregulated by treatment with NS-398 for up to 48 h. In addition to p21, p16 and p27 were expressed in the untreated U-937 cells at 0 h, while the expression levels of p16, and p27 were not modified by NS-398 treatment.

Table 1
Effect of NS-398 on the cell cycle distribution of U-937 cells

	% of cell number	
Phase in cell cycle	None	NS-398
G0/G1	48.06	62.99
G2/M	5.68	5.49
S	46.26	31.52

U-937 cells were incubated in the presence of NS-398 (79.5 μM) for 48 h. Values are means±standard deviation (S.D.) of triplicate determinations.

3.8. Effect of NS-398 on the production of eicosanoids by U-937 cells

U-937 cells were seeded into 96-well microplates and incubated for 24 h in the presence or absence of NS-398. The levels of PGE_2, PGF_{2a} and TXB_2 in the culture medium were determined by EIA kits. PGE_2 and PGF_{2a} were detectable in culture medium, and the production of these eicosanoids was not suppressed by NS-398. TXB_2 was undetectable in the culture medium of U-937 cells (data not shown).

3.9. COX-2 phosphorothioate oligonucleotide studies

To elucidate the functional role of COX-2 in the regulation of cell proliferation, sense and antisense oligonucleotides of *COX-2* were added to U-937 cells. The *COX-2* antisense oligonucleotide inhibited the proliferation of U-937 cells on day 4 (Fig. 7). The inhibitory effect of the *COX-2* antisense oligonucleotide was smaller than that of NS-398.

4. Discussion

Recent reports have shown that COX-2, rather than COX-1, is a key enzyme in cellular adhesion, growth, differentiation and apoptosis [1,15,25]. To investigate the role of COX-2 in cell growth and differentiation of leukaemia cells, we employed selective COX-2 inhibitors,

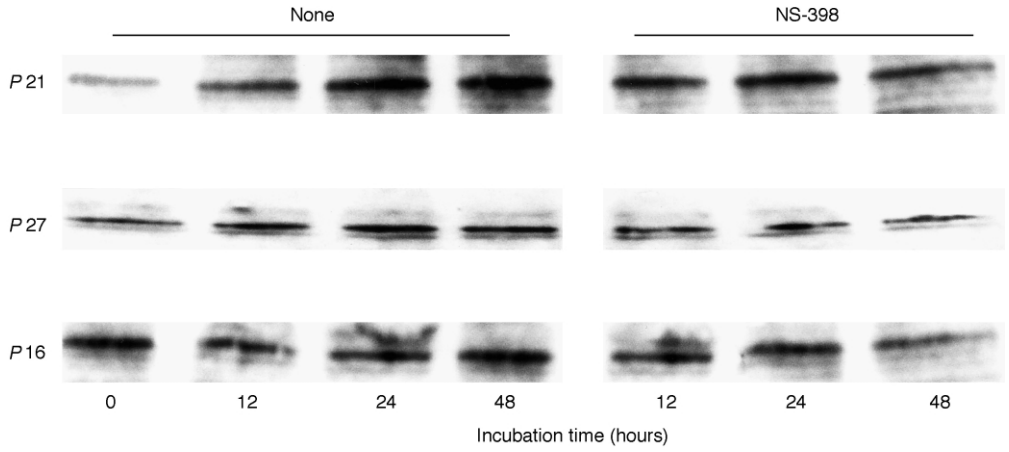

Fig. 6. Western blot analysis of the effect of NS-398 on cyclin-dependent kinase inhibitor (CKI) expression in U-937 cells. Results from a representative analysis are shown.

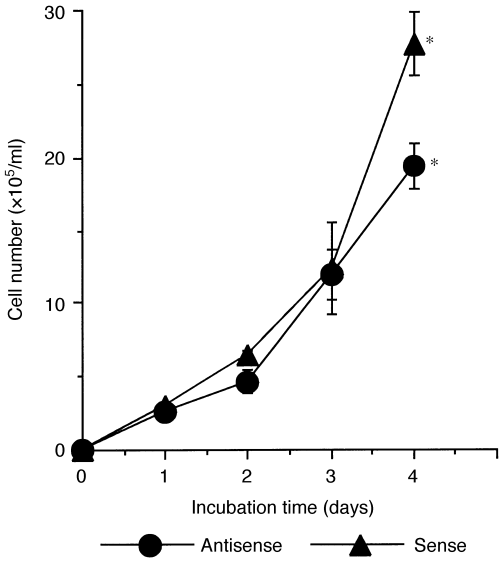

Fig. 7. Effects of antisense (closed cycle) and sense (closed triangle) oligonucleotides of *COX-2* on the proliferation of U-937 cells. The antisense or sense oligonucleotides (0.05 μM) were added to U-937 cells. Results from a representative analysis are shown. $*P<0.05$.

NS-398 and nabumetone. We have shown here that both inhibitors suppressed proliferation of two leukaemic cell lines, U-937 and ML-1. Growth suppression by COX-2 inhibitors was accompanied by the inhibition of monocytic differentiation of these cells induced by various DIFs such as IFN-γ, TNF-α, and RA, suggesting that COX-2 may share at least partially, the same signal transduction pathway which is necessary for both proliferation and differentiation.

In this study, we employed NS-398 to investigate the role of COX-2 in leukaemia cells, because NS-398 has a highly selective inhibitory activity for COX-2: a half maximal inhibitory concentration (IC_{50}) for COX-2 is 0.05 μM and an IC_{50} for COX-1 is more than 100 μM [26]. The selectivity of this inhibitor depends on subtle differences in the active site of the two COX isoforms, and it may react with COX-1 or other target molecules as well as COX-2. If COX-2 is a relevant target of NS-398 in U-937 cells, then another class of COX-2 inhibitors should also be effective at inhibiting the growth and differentiation of U-937 cells. To test this hypothesis we used nabumetone, a COX-2 inhibitor, in both the proliferation and differentiation assays. The proliferation, as well as differentiation induced by DIFs, of both U-937 and ML-1 cells was suppressed by the treatment with nabumetone. It would also be interesting to confirm these data with the COX-2 inhibitor, celecoxib that unfortunately was not available in Japan at the start of this study. However, these findings are consistent with the hypothesis that COX-2 is involved in the regulation of proliferation and differentiation of leukaemia cells.

Induced terminal differentiation of transformed haematopoietic cells involves both the cessation of DNA synthesis and the expression of genes crucial for the differentiated phenotype. The decision as to whether a cell continues to divide or withdraws from the cell cycle and differentiates occurs during the G1-phase. Induction of growth arrest of various cell types in the G1-phase of the cycle by several factors (e.g. TGF-β_1, phorbol esters) is linked to the upregulation of CKIs, such as p21, p27, p15 and p16, all of which block cyclin-dependent kinase (cdk)-cyclin complex activity in G1 [27–29]. Cell-cycle analysis revealed that treatment with NS-398 resulted in a G0/G1 arrest in U-937 cells. However, upregulation of p21, p27 and p16 by NS-398 was not observed in this study. Furthermore, p15 was undetectable in the U-937 cells as determined by Western blot analysis, and RT-PCR analysis revealed that there was no upregulation of *p15* mRNA by NS-398 in the U-937 cells (data not shown). These results suggest that although NS-398 induces a G0/G1 arrest, subsequent inhibition of proliferation and differentiation in U-937 may not be due to an upregulation of the CKIs.

Thus, the precise mechanisms by which COX-2 inhibitors suppressed proliferation and differentiation of leukaemia cells remains to be clarified. U-937 cells spontaneously produced certain levels of eicosanoids such as PGE_2, PGF_{2a}, TXB_2 and NS-398 did not reduce the ability of U-937 cells to produce these eicosanoids, as determined by specific EIA. Recent studies have suggested that COX-1 and COX-2 utilise different pools of arachidonic acid for synthesising prostanoids. It has been demonstrated that in murine fibroblasts and macrophages, ligand-induced prostaglandin production occurs via expression of COX-2, while the COX-1 present in these cells cannot metabolise ligand-released arachidonic acid [30]. Although the K_m value of COX-1 to arachidonic acid was almost the same as that of COX-2, low concentrations of arachidonic acid existing intracellularly are preferentially utilised by COX-2 [31]. These differences between the isoforms in the metabolism of arachidonic acid are due to alternative requirements for the initiator hydroperoxide. Thus, it may be hypothesised that, in differentiating U-937 cells, there is a increased peroxide level and a change of localisation of arachidonic acid, and these changes make COX-2 able to synthesise intracellular PGs in U-937 cells. Further analysis of the peroxide level and localisation of arachidonic acid in differentiating U-937 cells should determine how the COX-2 inhibitor works without changing the total eicosanoid levels produced by U-937 cells.

It is generally accepted that COX-2 inhibitors exert their action via blocking PG synthesis by direct COX inhibition. However, whether NS-398 blocks proliferation of cancer cells solely by blocking PG synthesis is a matter for discussion. Several studies have shown that COX-2 inhibitors can act through mechanisms that are independent of their ability to inhibit COX-2. For example, celecoxib, a selective COX-2 inhibitor, induces apoptosis of a prostate cancer cell line through a target other than COX-2 [32]. COX-2 inhibits cell-cycle progression in a variety of cell types by a novel mechanism that does not require the synthesis of PG [33]. Therefore, it is apparent that selective COX-2 inhibitors also possess COX-2-independent pathways which are responsible for their function. When U-937 were treated with COX-2 antisense oligonucleotide, U-937 cells accumulated in G0/G1 (data not shown). However, the degree of suppression of proliferation with NS-398 was higher than that obtained with the COX-2 antisense oligonucleotide. This result indicates that accumulation in G0/G1 by NS-398 might be independent of decreased PG biosynthesis resulting from the downregulation of COX-2 expression.

Treatment of amnion-derived WISH cells with TNF-α caused a dose-dependent increase in COX-2 expression and a subsequent biosynthesis of PGE_2 that persisted for at least 48 h [3]. Accordingly, the alternative explanation for the inhibitory effect of NS-398 on differentiation of U-937 cells may be that DIFs induce COX-2 expression which effectively utilises intracellular arachidonic acid, and that this COX-2 induced by DIFs is inhibited by NS-398. However, COX-2 was not induced by the treatment with IFN-γ, indicating that COX-2 is not induced in the differentiation process of U-937 cells.

COX-2 inhibitors suppressed both proliferation and DIF-induced differentiation of leukaemia cells. Therefore, our present results may lead to new possibilities in chemotherapy for patients with leukaemia and the chemoprevention of haematopoietic malignancies by COX-2 inhibitors. We are currently investigating the effects of COX-2 inhibitors on telomerase activity in these cells to further understand the mechanism of the inhibitory activity.

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