Cyclooxygenase-2 inhibitor NS-398 suppresses cell growth and constitutive production of granulocyte-colony stimulating factor and granulocyte macrophage-colony stimulating factor in lung cancer cells

Hideshi Nakata, Yoshiki Uemura,[1] Makoto Kobayashi, Ryoji Harada and Hirokuni Taguchi

Department of Internal Medicine, Kochi Medical School, Kohasu, Okocho, Nankoku, Kochi 783-8505

(Received October 10, 2002/Revised December 9, 2002/Accepted December 13, 2002)

We previously established two lung cancer cell lines, OKa-C-1 and MI-4, which constitutively produce abundant granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF). Inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β stimulated the expression of G-CSF, GM-CSF, and cyclooxygenase (COX)-2 in the two cell lines. It is known that increased COX-2 activity promotes tumor growth and induces G-CSF and GM-CSF expression in non-malignant cells, and that selective COX-2 inhibitors inhibit the growth of some types of malignant cells. Therefore, we hypothesized that inhibition of COX-2 activity might suppress constitutive production of G-CSF or GM-CSF in addition to reducing the growth of malignant cells. We confirmed that the selective COX-2 inhibitor, NS-398 suppressed the constitutive production of G-CSF and GM-CSF, and the cell growth in both OKa-C-1 and MI-4 cell lines. Prostaglandin E_2 (PGE_2) reversed the inhibitions of G-CSF and GM-CSF expression, as well as cell growth, by NS-398. This result confirms that the effects of NS-398 are based on the inhibition of COX activity. Some studies have indicated that nuclear factor kappa B (NF-κB) or MAPK (mitogen-activated protein kinase) activation is related to upregulation of G-CSF, GM-CSF or COX-2 expression in some types of cells. Therefore, we examined if the actions of NS-398 might be mediated by the MAP kinase pathway or NF-κB activity in OKa-C-1 and MI-4 cells. We found that NS-398 inhibits G-CSF and GM-CSF production and cell growth through an extracellular signal-regulated kinase kinase (MEK) signaling pathway in these cell lines. The prognosis of non-small cell lung cancer showing *G-CSF* gene expression is significantly worse. G-CSF overproduction by tumor cells is observed at an advanced clinical stage. Our findings imply that a COX-2 inhibitor might improve the prognosis of patients with lung cancer through the reduction of G-CSF or GM-CSF. (Cancer Sci 2003; 94: 173–180)

Occasionally, leukocytosis is observed in patients with several cell types of carcinoma with an aggressive clinical course.[1–8] The overproduction of hematopoietic growth factor granulocyte-colony stimulating factor (G-CSF) or granulocyte macrophage-colony stimulating factor (GM-CSF) by tumor cells is thought to be responsible for this paraneoplastic syndrome.[9, 10] Several reports have suggested that these cytokines from tumor cells could have a potential role in stimulating tumor growth and invasion.[11–13] Therefore, it is possible that suppression of these constitutive cytokines might lead to an improvement in the prognosis of patients with cancer in an advanced stage.[14]

Recently, a new class of nonsteroidal anti-inflammatory agents (NSAIDs) that selectively inhibits the cyclooxygenase (COX)-2 has been developed. Traditional NSAIDs such as aspirin and indomethacin can inhibit both COX-1 and COX-2. Selective COX-2 inhibitors provide therapeutic benefit with less toxicity than traditional NSAIDs because the inhibition of COX-1 has serious side effects such as peptic ulcer formation and renal dysfunction.[15, 16] In addition to inhibiting inflammatory reactions, selective COX-2 inhibitors have been shown to reduce proliferation and to increase apoptosis in some carcinoma cell types.[17, 18] COX-2 activity also potentiates G-CSF release by human vascular cells.[19] These data raise the possibility that COX-2 activation contributes to constitutive production of G-CSF or GM-CSF by tumor cells, in addition to stimulating tumor growth.

The signaling pathway of the constitutive G-CSF and GM-CSF induction remains obscure in tumor cells. We have demonstrated that tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) induced constitutive G-CSF or GM-CSF production in a dose-dependent manner using two lung cancer cell lines, MI-4 and OKa-C-1. It has been shown that TNF-α and IL-1β activate nuclear factor-kappa B (NF-κB)[20] and mitogen-activated protein kinase (MAPK)[21] in some cell types, and activated NF-κB and MAPK upregulate G-CSF and GM-CSF production.[22, 23] The suppression of cellular proliferation by NS-398 is mediated by the inhibition of a MAPK signaling pathway in a gastric cancer cell line,[24] and the expression of COX-2 is largely regulated by NF-κB in several cells,[25, 26] suggesting that constitutive production of G-CSF or GM-CSF and the cell growth might be mediated through a NF-κB and MAP kinase signaling pathway in OKa-C-1 and MI-4 cells as well.

The aim of this study was to examine whether the selective COX-2 inhibitor NS-398 could suppress constitutive G-CSF or GM-CSF expression in addition to cell growth, and to assess the signaling pathways affecting the inhibition by NS-398 in our two lung carcinoma cell lines OKa-C-1 and MI-4.

Materials and Methods

Reagents and supplies. Recombinant human TNF-α, IL-1β, G-CSF, GM-CSF and prostaglandin E_2 (PGE_2) were obtained from Sigma (St. Louis, MO) and N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS-398) from Alexis Biochemicals (San Diego, CA). Pyrrolidinedithiocarbamate (PDTC), PD98059, and SB203580 were purchased from Calbiochem (San Diego, CA). NS-398, PD98059 and SB203580 were prepared with dimethyl sulfoxide (DMSO) as 1000× stock and were handled in subdued light conditions. Antihuman COX-2 monoclonal antibody was purchased from BD Biosciences (Franklin Lakes, NJ). Enzyme-linked immunosorbent assay (ELISA) kits for measuring the contents of G-CSF and GM-CSF in culture medium were from TECHNE Corporation (Minneapolis, MN). "Cell Titer 96" AQueous one solution cell pro-

[1]To whom correspondence and reprint requests should be addressed.
E-mail: uemuray@kochi-ms.ac.jp

liferation assay kit and "Cyto Tox 96" non-radioactive cytotoxicity assay kit were purchased from Promega (Madison, WI). NF-κB p65, Trans-AM kit for measuring the NF-κB activation was from Active Motif (Carlsbad, CA). Rabbit anti-phospho-p38 MAPK and rabbit anti-phospho-p44/42 MAPK antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Cell lines. In our laboratory, we have established two lung cancer cell lines, OKa-C-1 and MI-4.[6, 27] The OKa-C-1 and MI-4 lines were derived from patients with squamous cell carcinoma and large cell carcinoma, respectively. The two lung cancer cell lines have been maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS) at 37°C in an incubator, under a 100% humidified 5% CO_2 atmosphere. Subcultures were carried out twice weekly by removing the cells from 75-cm^2 tissue flasks with 0.025% trypsin in 0.02% EDTA and splitting them 1:3. For experiments, all cultures were grown to confluency in 24-well tissue culture plates in the presence of 10% FCS. Then, the medium was changed to a fresh one containing 1% FCS. After incubation for 24 h, the cells were treated with either NS-398, TNF-α, or IL-1β, or one of the combinations under the conditions indicated in the figure legends. Each conditioned medium contained DMSO at the same final concentration as the control. After 24 h, the cells were harvested with a cell scraper (Becton Dickinson, Lincoln Park, NJ) for RNA and protein analysis. The culture medium was collected after 48 h for measurement of the G-CSF and GM-CSF contents by ELISA, centrifuged at 1000*g* for 10 min to remove cell debris and stored at −80°C. Each experiment was performed in triplicate.

Expression of COX-2 protein following treatment with TNF-α and IL-1β. The constitutive enzyme, COX-1, is present in most cells and tissues, while the inducible enzyme, COX-2, is expressed in response to a variety of stimuli.[28, 29] We examined whether inflammatory cytokines, TNF-α and IL-1β could induce COX-2 expression in OKa-C-1 and MI-4 cells. OKa-C-1 and MI-4 cells were plated in 100-mm^3 dishes and grown to 80% confluence. The medium was changed to a fresh one containing 1% FCS and incubated for 24 h. Then, the cells were treated with 10 ng/ml of TNF-α or IL-1β. After incubation for 24 h, cells were collected, and lysates were prepared as described below.

Western blot analysis. The medium was removed, then the cell monolayers were washed three times with phosphate-buffered saline and lysed in ice-cold buffer [50 m*M* Tris-buffered saline (pH 8.0), 150 m*M* NaCl, 0.002% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, 200 m*M* sodium orthovanadate, 1% (octylphenoxy)polyethoxyethanol, and 0.5% sodium deoxycholate] for 20 min on ice followed by centrifugation at 4°C for 5 min to sediment the particulate material. The protein concentration of the supernatant was measured with a Bradford assay (Bio-Rad, Hercules, CA). Ten micrograms of protein was separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad). Each membrane was blocked overnight at 4°C using I-block (Tropix, Bedford, MA) and incubated with 1:500 dilution of mouse monoclonal antihuman COX-2 antibody (BD Biosciences). Ovine COX-2 protein standards (BD Biosciences) served as a positive control. Chemiluminescence was determined using the Western-Star kit (Tropix) according to the manufacturer's instructions.

Quantitative RT-PCR. Total RNA was extracted from the cultured cells exposed to a range of concentrations of NS-398 (0–100 μ*M*) after 24 h by the guanidine thiocyanate method using ISOGEN solution (Nippon Gene Co., Tokyo). One microgram of the RNA was converted to cDNA with Molony murine leukemia virus reverse transcriptase in 20 μl of reaction mixture. For quantification, 1 μl aliquots of cDNA samples were subjected to PCR in 50 μl of reaction solution containing 0.5 μ*M* of each specific primer, 10 m*M* Tris-HCl, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP, and 1 unit of *Taq* polymerase. Primers used for G-CSF were 5′-TAGAGCAAGTGAGGAAGATC-CAGG-3′ for sense and 5′-AGTTCTTCCATCTGCTGCCA-GATG-3′ for antisense, giving a 328 bp fragment. Primers used for GM-CSF were 5′-ATGTGGCTGCAGAGCCTGCTGC-3′ for sense and 5′-CTGGCTCCCAGCAGTCAAAGGG-3′ for antisense, giving a 424 bp fragment. Primers used for COX-2 were 5′-CAGCACTTCACGCATCAGTT-3′ for sense and 5′-TCTGGTCAATGGAAGCCTGT-3′ for antisense, giving a 756 bp fragment. Primers used for β-actin were 5′-ACCTTCAA-CACCCCAGCCATG-3′ for sense and 5′-GGCCATCTCT-TGCTCGAAGTC-3′ for antisense, giving a 309 bp fragment. Reaction was performed for 20 cycles for β-actin and 30 cycles for G-CSF in a DNA thermal cycler (TaKaRa, Ohtsu). The PCR steps included denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and polymerization at 72°C for 1 min. Amplification cycle numbers were optimized for each sequence using the serial dilution method to achieve a dose-dependent amplification.[30] One microliter of PCR product was electrophoresed on 1% NuSieve GTG agarose gel and stained with ethidium bromide. The intensity of the bands was evaluated using a UV-light box imaging system (Atto, Tokyo).

Cell proliferation assay for NS-398. For the evaluation of sensitivity to NS-398, cell proliferation assay was performed using the "Cell Titer 96" AQueous One Solution Cell Proliferation Assay kit (Promega). Briefly, 2×10^5 cells were plated in 96-well plates and exposed continuously for 48 h to a range of concentrations of NS-398. The absorbance of the solution was measured at 490 nm in a spectrophotometer. Three independent experiments were carried out in quadruplicate.

Detection of G-CSF and GM-CSF. The concentration of G-CSF or GM-CSF in the culture medium was determined using an ELISA kit according to the supplier's instructions. The sensitivities of these kits are 0.4 pg/ml and 3.0 pg/ml for G-CSF and GM-CSF, respectively. The value of G-CSF or GM-CSF in the conditioned medium was normalized for the relative number of cells using values obtained in the cell proliferation assay. G-CSF or GM-CSF level in the fresh medium was below the determination limit of these kits.

G-CSF or GM-CSF expression and cell growth following treatment with NS-398. To evaluate the effect of a specific COX-2 inhibitor on the G-CSF or GM-CSF expression and the cell growth, cells were treated with various concentrations of NS-398 (0–100 μ*M*). G-CSF or GM-CSF content and mRNA expression, and cell growth were assayed by the methods described above. PGE_2 is an enzymatic product of COX. We tested whether PGE_2 would reverse the inhibitory effect of NS-398 on G-CSF or GM-CSF expression and cell growth. Cells were pretreated with NS-398 (10 μ*M*) for 8 h then were incubated with PGE_2 (10 μ*M*). In addition, we investigated whether exogenous G-CSF or GM-CSF could reverse the inhibition of cell growth imposed by the treatment with NS-398. Cells were pretreated with NS-398 (10 μ*M*) for 8 h and then were incubated with various concentrations of G-CSF or GM-CSF. G-CSF or GM-CSF mRNA expression and cell growth were assayed after 24 h and 48 h, respectively, by the methods described above.

Effect of specific NF-κB or MAPK inhibitor on G-CSF and GM-CSF production, and cell growth. To investigate the involvement of NF-κB and the MAPK pathway in constitutive G-CSF or GM-CSF production and cell growth, we treated the cells with a specific NF-κB inhibitor, PDTC (10 μ*M*), an extracellular signal-regulated kinase kinase (MEK) inhibitor, PD98059 (10 μ*M*), or a p38 MAPK inhibitor, SB203580 (10 μ*M*). The G-CSF or GM-CSF content, and the cell growth were assayed by the methods described above.

Microwell colorimetric NF-κB assay. To evaluate the effect of NS-398 on the NF-κB activation, cells were treated with various

concentrations of NS-398 (0–100 μM) for 24 h. The NF-κB activation in the cell extract was quantified using a TransAM NF-κB p65 kit according to the supplier's instructions. The absorbance of the solution was measured at 450 nm in a spectrophotometer. We checked the specificity of the assay in a competition experiment using the wild-type NF-κB-consensus binding sequence.

Effect of NS-398 on phosphorylation of p38 and p44/42 MAPK. To evaluate the effect of NS-398 on the phosphorylation of two major types of MAPKs, ERK and p38 MAPK in MI-4 and OKa-C-1 cells, the cells were exposed to various concentrations of NS-398 (0–100 μM) for 3 h. The cells were lysed, and the lysate was subjected to SDS-polyacrylamide gels electrophoresis and transferred onto nitrocellulose membranes as described above. Membranes were incubated with 1:2000 dilution of rabbit anti-phospho-p44/42 MAPK antibody or rabbit anti-phospho-p38 MAPK antibody. Chemiluminescence was determined using the Western-Star kit (Tropix) according to the manufacturer's instructions.

Cytotoxic effect of NS-398, PDTC, PD98059 and SB203580 on OKa-C-1 and MI-4 cells. To evaluate the cytotoxic effect of NS-398, MAPK inhibitors and NF-κB inhibitor, the cells were cultured with 10 μM PDTC, PD98059 or SB203580 for 24 h. Released lactate dehydrogenase (LDH) in culture supernatants was quantitatively measured using a "Cyto Tox 96" cytotoxicity assay kit. The absorbance of the solution was measured at 490 nm in a spectrophotometer.

Statistics. Values are presented as mean±SE. The unpaired t test was used to determine the statistical significance of differences. For all analyses, a value of $P<0.05$ was considered significant.

Results

TNF-α or IL-1β induces G-CSF and GM-CSF production. TNF-α or IL-1β dose-dependently stimulates the release of G-CSF and GM-CSF into the medium and induces mRNA expression (data not shown) in OKa-C-1 and MI-4 cells (Fig. 1).

TNF-α or IL-1β induces COX-2 expression in OKa-C-1 and MI-4 cells. To determine the COX-2 expression, OKa-C-1 and MI-4 cells were stimulated with TNF-α (10 ng/ml) or IL-1β (10 ng/ml) for 24 h, and western blot analysis was performed with anti-COX-2 monoclonal antibody. As shown in Fig. 2a, TNF-α or IL-1β significantly induced COX-2 expression in OKa-C-1 and MI-4 cells. The induction of COX-2 appeared to be stronger in OKa-C-1 cells than in MI-4. Quantitative RT-PCR also showed that TNF-α or IL-1β induced COX-2 expression in these cell lines. In addition, the COX-2 inhibitor NS-398 (10 μM) suppressed this inducible COX-2 expression (Fig. 2b).

NS-398 inhibits G-CSF or GM-CSF expression and cell growth. OKa-C-1 and MI-4 cells were treated with vehicle or NS-398 at concentrations ranging from 0.1–100 μM. The effects on G-CSF or GM-CSF production and mRNA expression were determined after 48 h and 24 h of treatment, respectively. We found that NS-398 induced a dose-dependent inhibition of G-CSF or GM-CSF release and mRNA expression in OKa-C-1 and MI-4 (Fig. 3, a, b). The mRNA expression and the release of these cytokines were inhibited by as little as 1.0 μM NS-398 in OKa-C-1 and MI-4 cells. The effects on cell growth were determined after 48 h of treatment. NS-398 dose-dependently inhibited cell growth of OKa-C-1 and MI-4 (Fig. 3c). Growth inhibition was induced even with 1.0 μM NS-398 in MI-4, while 1–10 μM was required in OKa-C-1. Concentrations of 1–10 μM NS-398 had no cytotoxic effect in these cell lines (Fig. 3d).

PGE_2 reverses the effect of NS-398 on G-CSF or GM-CSF expression and cell growth. To confirm that the effects of NS-398 on G-CSF or GM-CSF expression and cell growth were caused by the suppression of COX-2 activity, PGE_2 (10 μM) was added after treatment with NS-398 (10 μM). PGE_2 reversed G-CSF or GM-CSF mRNA expression (Fig. 4a) and cell growth (Fig. 4b).

Exogenous G-CSF or GM-CSF reverses the inhibition of cell growth imposed by treatment with NS-398 in OKa-C-1 and MI-4 cells. We

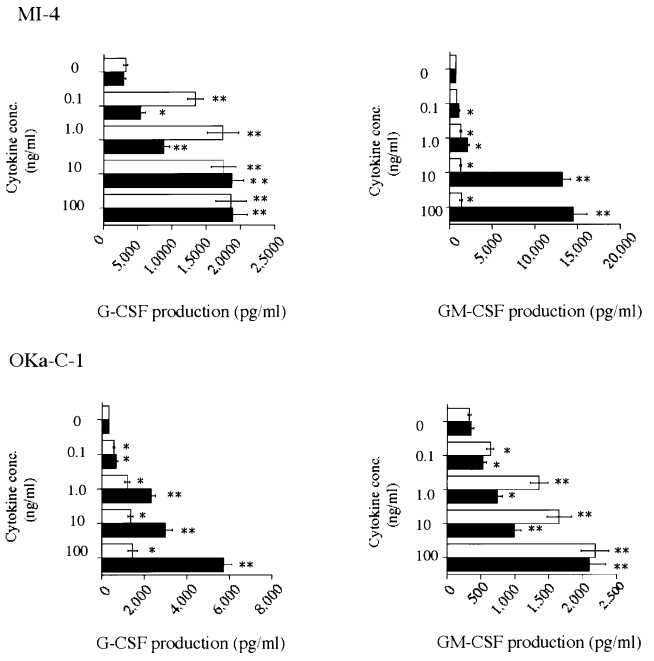

Fig. 1. IL-1β and TNF-α enhance G-CSF and GM-CSF production in OKa-C-1 and MI-4 cells. These cells were treated with various concentrations (0–100 ng/ml) of TNF-α and IL-1β for 24 h. The G-CSF or GM-CSF content in the culture medium was determined using an ELISA kit. The data represent the mean±SE of 4 wells from three independent experiments. * $P<0.01$ vs. control. ** $P<0.001$ vs. control. □ IL-1β, ■ TNF-α.

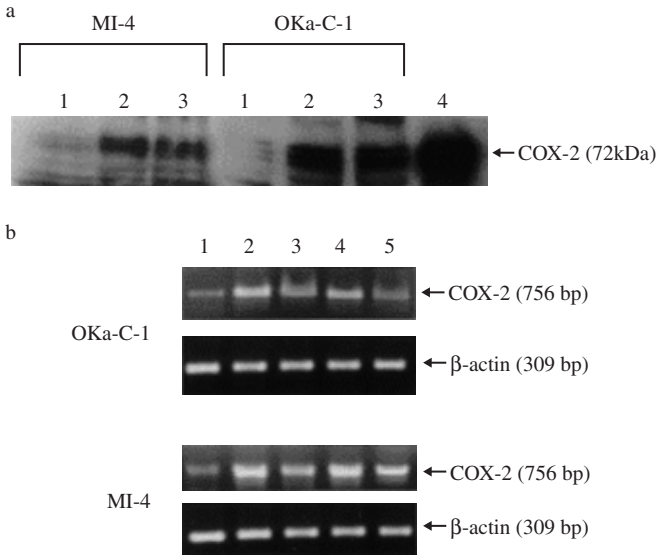

Fig. 2. IL-1β and TNF-α induce COX-2 expression in OKa-C-1 and MI-4 cells. These cells were treated with 10 ng/ml of TNF-α or IL-1β for 24 h. (a) COX-2 protein levels were examined by western blot analysis. Lane 1, vehicle; lane 2, TNF-α (10 ng/ml); lane 3, IL-1β (10 ng/ml); lane 4, positive control (ovine COX-2 protein standards). (b) COX-2 mRNA levels were also examined by RT-PCR. Lane 1, vehicle; lane 2, TNF-α (10 ng/ml); lane 3, TNF-α (10 ng/ml) + NS-398 (10 μM); lane 4, IL-1β (10 ng/ml); lane 5, IL-1β (10 ng/ml) + NS-398 (10 μM). Results are representative of three independent experiments.

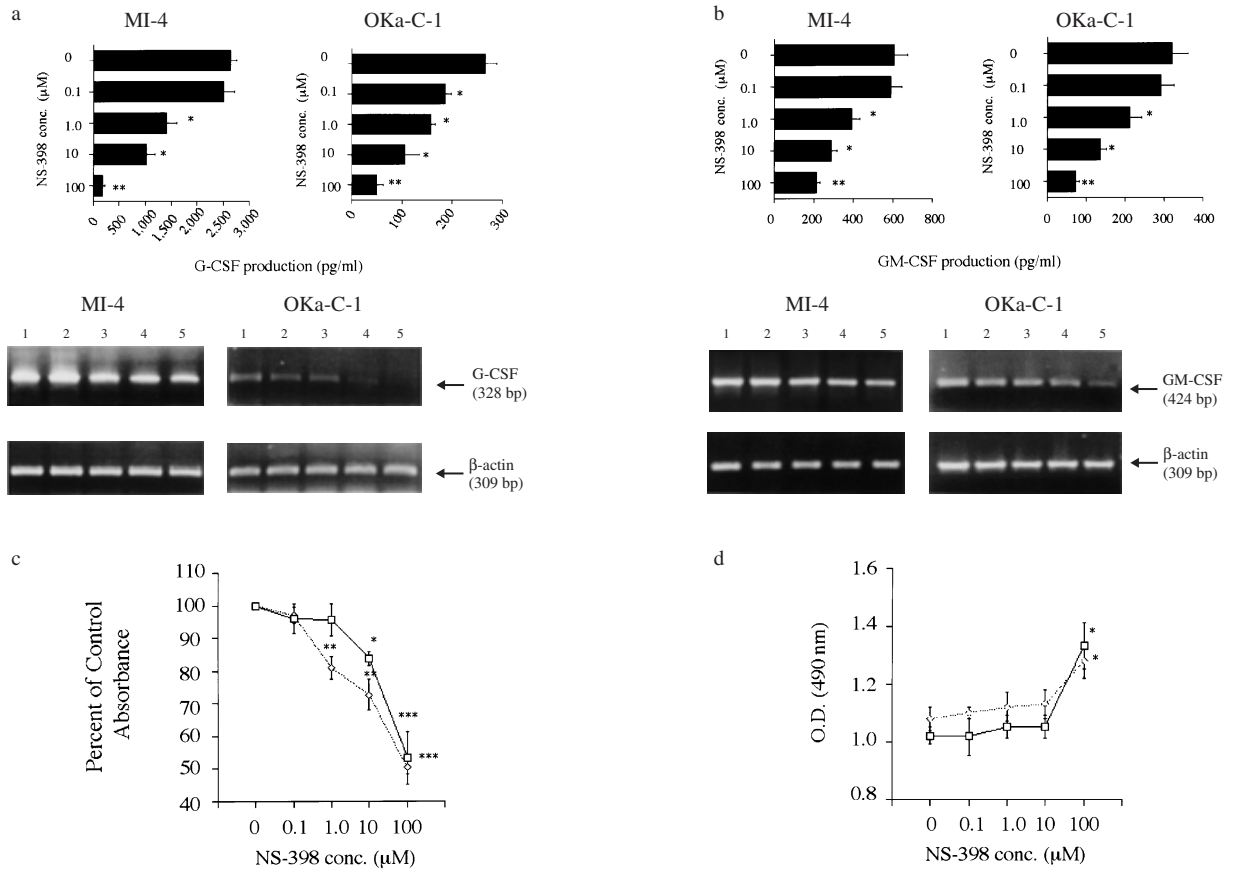

Fig. 3. NS-398 inhibits G-CSF and GM-CSF expression in OKa-C-1 and MI-4 cells in a dose-dependent manner. These cells were cultured in 1% FCS medium containing vehicle or various concentrations (0–100 μM) of NS-398. The G-CSF (a) or GM-CSF (b) in the culture medium was determined after 24 h using ELISA kit. The data represent the mean±SE of 4 wells from three independent experiments. * $P<0.01$ vs. control. ** $P<0.001$ vs. control. Total RNA was isolated from the cells after 24 h as well, then G-CSF (a), GM-CSF (b) and β-actin mRNAs expressions were examined by RT-PCR analysis. Results are representative of three independent experiments. Lane 1, vehicle; lane 2, NS-398 (0.1 μM); lane 3, NS-398 (1.0 μM); lane 4, NS-398 (10 μM); lane 5, NS-398 (100 μM). (c) Growth-inhibitory effects of NS-398 were examined after 48 h by a cell proliferation assay kit. The data represent the mean±SE of 4 wells from three independent experiments. * $P<0.05$ vs. control. ** $P<0.01$ vs. control. *** $P<0.001$ vs. control. □ OKa-C-1, ◇ MI-4. (d) Cytotoxic effects of NS-398 were examined after 24 h by a cytotoxicity assay kit. The data represent the mean±SE of 4 wells from three independent experiments. * $P<0.05$ vs. control. □ OKa-C-1, ◇ MI-4.

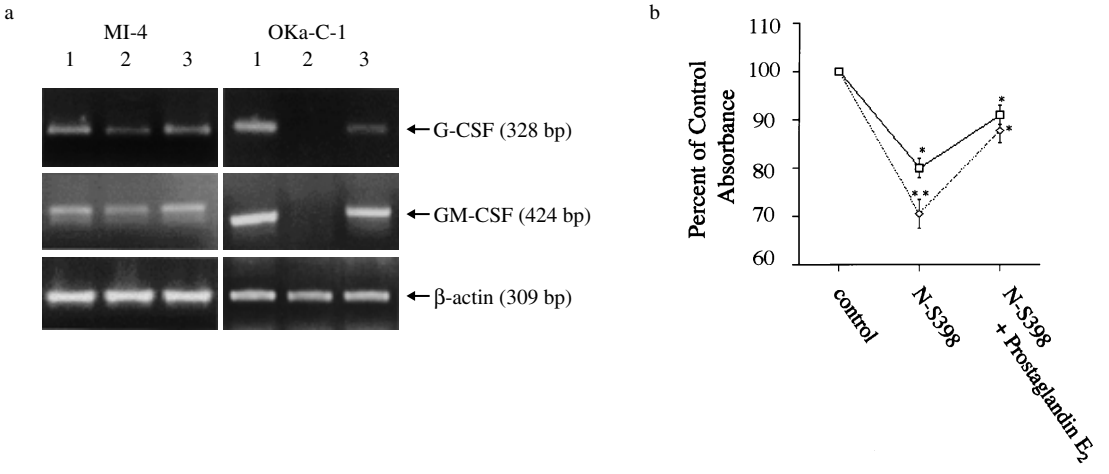

Fig. 4. PGE_2 reverses the inhibitions of G-CSF and GM-CSF expression, and cell growth in OKa-C-1 and MI-4 cells. Cells were pretreated with NS-398 (10 μM) for 8 h, then incubated with PGE_2 (10 μM) for 24 h. (a) PGE_2 reverses the inhibition of G-CSF and GM-CSF expression by NS-398 in OKa-C-1 and MI-4 cells. Results are representative of three independent experiments. Lane 1, vehicle; lane 2, NS-398 (10 μM); lane 3, NS-398 (10 μM) + PGE_2 (10 μM). (b) PGE_2 reverses the inhibition of cell growth by NS-398 in OKa-C-1 and MI-4 cells. The data represent the mean±SE of 4 wells from three independent experiments. * $P<0.05$ vs. control. ** $P<0.01$ vs. control. □ OKa-C-1, ◇ MI-4.

examined whether exogenous G-CSF or GM-CSF could reverse the inhibition of cell growth imposed by NS-398. G-CSF or GM-CSF sufficiently reversed cell growth at a low concentration in both cell lines (Fig. 5).

NF-κB and MAPK inhibitors inhibit G-CSF or GM-CSF expression. To investigate whether NF-κB activation and the MAPK pathway are associated with G-CSF or GM-CSF production in OKa-C-1 or MI-4 cells, cells were treated with 10 μM PDTC, PD98059 or SB203580. After 48 h, the G-CSF and GM-CSF in the medium were measured with ELISA kits. In MI-4 cells, PD98059 and SB203580 reduced the G-CSF production by ~30% in protein level, and PD98059 suppressed the GM-CSF production by ~7%, while PDTC had no effect on G-CSF and GM-CSF production. In OKa-C-1 cells, all three inhibitors reduced G-CSF production by ~20%. SB203580, PD98059 and PDTC reduced GM-CSF production by ~20%, ~50% and ~60%, respectively (Fig. 6a). The expression of G-CSF and GM-CSF mRNAs was examined after 24 h by RT-PCR. Changes of G-CSF or GM-CSF mRNA expression were similar to those of protein in OKa-C-1 and MI-4 cells (Fig. 6b). Growth inhibition by SB203580, PD98059 or PDTC was examined after 48 h with a "Cell Titer 96" cell proliferation assay kit. SB203580 and PD98059 inhibited cell growth dose-dependently, while PDTC was ineffective at 0–10 μM (Fig. 6c). SB203580, PD98059 and PDTC at 10 μM had scarcely any cytotoxic effect on these cell lines (Fig. 6d).

Involvement of p44/42 MAPK (Erk1/2) pathway in NS-398 inhibition signaling in MI-4 and OKa-C-1 cells. To examine the effects of NS-398 on NF-κB activation in MI-4 and OKa-C-1 cells, we mea-

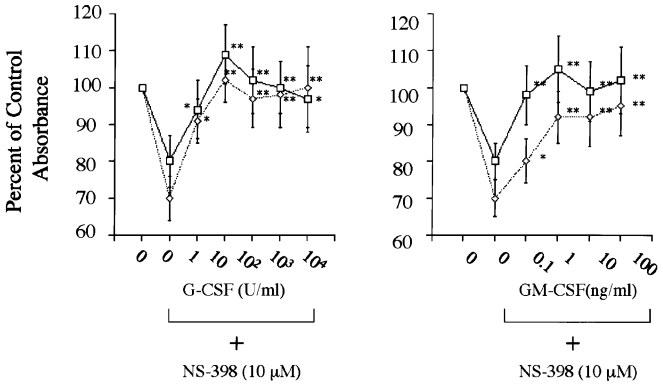

Fig. 5. Exogenous G-CSF or GM-CSF reverses inhibition of cell growth imposed by treatment with NS-398 in OKa-C-1 and MI-4 cells. Cells were pretreated with NS-398 (10 μM) for 8 h, then incubated with G-CSF (1–10^4 U/ml) or GM-CSF (0.1–100 ng/ml) for 48 h. The data represent the mean±SE of 4 wells from three independent experiments. * $P<0.05$ vs. treatment with NS-398 (10 μM) alone. ** $P<0.01$ vs. treatment with NS-398 (10 μM) alone. □ OKa-C-1, ◇ MI-4.

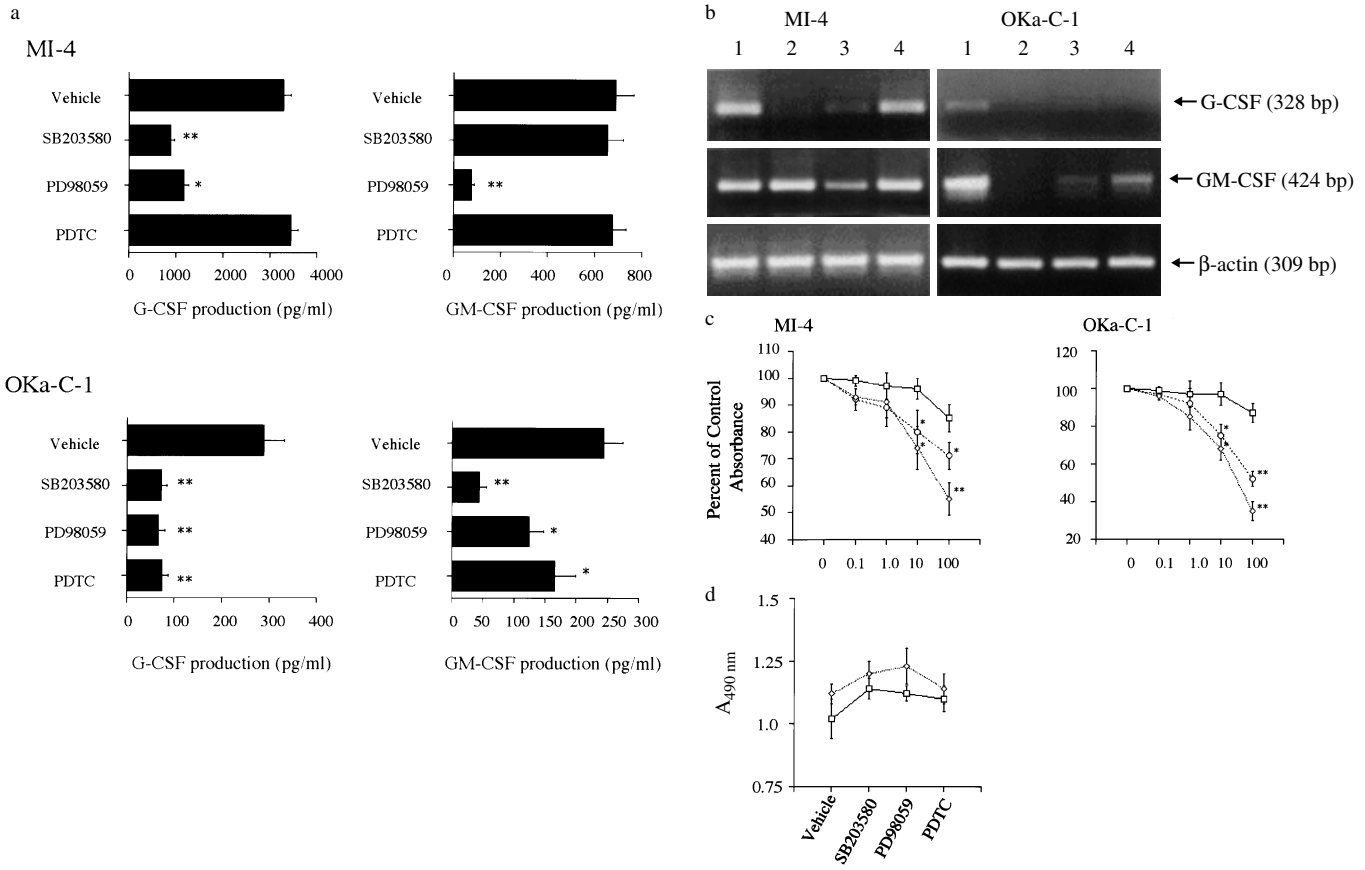

Fig. 6. NF-κB and MAPK inhibitors suppress G-CSF and GM-CSF production, and cell growth in OKa-C-1 and MI-4 cells. (a) These cells were treated with vehicle, SB203580 (10 μM), PD98059 (10 μM) and PDTC (10 μM). G-CSF and GM-CSF in the medium were measured after 48 h by ELISA kits. The data represent the mean±SE of 4 wells from three independent experiments. * $P<0.01$ vs. control. ** $P<0.001$ vs. control. (b) The expression of G-CSF and GM-CSF mRNAs was examined after 24 h by RT-PCR. Lane 1, vehicle; lane 2, SB203580 (10 μM); lane 3, PD98059 (10 μM); lane 4, PDTC (10 μM). Results are representative of three independent experiments. (c) The effects of SB203580, PD98059 or PDTC on cell growth were examined after 48 h with a cell proliferation assay kit. The data represent the mean±SE of 4 wells from three independent experiments. * $P<0.05$ vs. control. ** $P<0.01$ vs. control. □ PDTC, ○ PD98059, ◇ SB203580. (d) Cytotoxic effects of SB203580, PD98059 and PDTC were examined after 24 h with a cytotoxicity assay kit. The data represent the mean±SE of 4 wells from three independent experiments. □ OKa-C-1, ◇ MI-4.

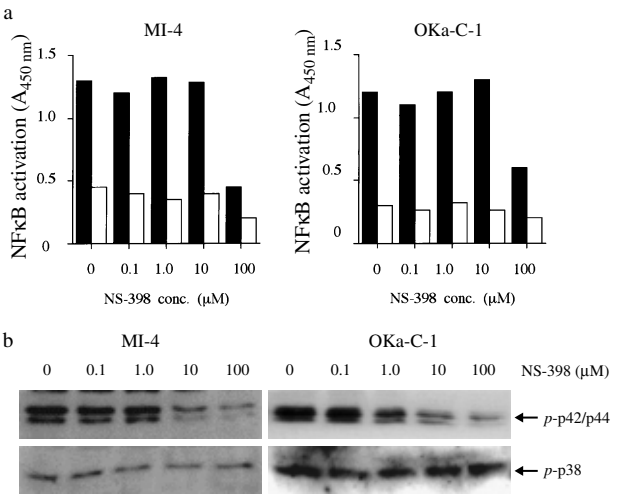

Fig. 7. NS-398 does not inhibit NF-κB activation in OKa-C-1 and MI-4 cells. NS-398 decreases the phosphorylation of p44/42 MAPK in OKa-C-1 and MI-4 cells. These cells were treated with various concentrations (0–100 *μM*) of NS-398. (a) The NF-κB activation in the cell extract was quantified after 24 h using a TransAM NF-κB p65 kit. The absorbance of the solution was measured at 450 nm in a spectrophotometer. We checked the specificity of the assay in a competition experiment using the wild-type NF-κB consensus binding sequence. □ oligonucleotide (+), ■ oligonucleotide (–). (b) The effects of NS-398 on the phosphorylation of p38 MAPK and p44/42 MAPK were examined after 3 h by western blot analysis. Results are representative of three independent experiments.

sured the NF-κB activation by NF-κB p65, using a Trans-AM kit. NS-398 at 0–10 *μM* did not decrease the NF-κB activation in the cell lines (Fig. 7b). Time course studies revealed that 10 *μM* NS-398 did not decrease NF-κB activation within 24 h in these cell lines (data not shown). We analyzed the effects of NS-398 on the phosphorylation of two major types of MAP kinases, ERK and p38 MAPK, by western blotting in MI-4 and OKa-C-1 cells. The phosphorylation of p44/42 MAPK was suppressed by NS-398 in a dose-dependent manner in the two cell lines, while that of p38 MAPK was not (Fig. 7b). Time course studies revealed that decreases in phosphorylation of p44/42 MAPK by NS-398 (10 *μM*) were detectable at 2–4 h in the cell lines, while no change of p38 MAP kinase was seen within 24 h (data not shown).

Discussion

We previously established two lung cancer cell lines, a squamous cell carcinoma cell line OKa-C-1 and a large cell carcinoma cell line MI-4, from patients with marked leukocytosis. Both cell lines constitutively produce high levels of G-CSF and GM-CSF. However, the biological properties associated with the overproduction of G-CSF and GM-CSF by tumor cells are not well known. Several reports have demonstrated the presence of an autocrine growth loop for G-CSF[31, 32] and GM-CSF[13] in non-hematopoietic tumor cells. However, our cell lines did not have an autocrine growth loop for G-CSF,[27] and they were not investigated for GM-CSF. To our knowledge, there have been no reports about autocrine growth control by G-CSF in lung cancer. Nevertheless, the prognosis is significantly worse in non-small cell lung cancer showing G-CSF production.[14] In addition, G-CSF production by tumor cells significantly increases at an advanced clinical stage. These results suggest that G-CSF overproduction by lung carcinoma cells might lead the lung tumor to grow via a non-autocrine mechanism, so control of G-CSF and GM-CSF production by lung tumor cells might improve the prognosis of patients.

In addition to inflammatory reactions, COX-2 has been implicated in the development of colon cancer and may play a role in promoting invasion, metastasis, and angiogenesis in established tumors.[16–18] Previous reports have shown that a significantly increased expression of COX-2 is frequently seen in a specific type of lung cancer (i.e., adenocarcinoma), in contrast to the scattered weak reactivity seen in normal peripheral airway epithelial cells, and that such an increase in COX-2 expression may be a clinically significant prognostic factor for patients undergoing surgical resection of early-stage adenocarcinomas.[33, 34] Selective COX-2 inhibitors reduce proliferation and increase apoptosis in some cell types of carcinoma.[17, 18] These results indicate that increased COX-2 activity also might have a growth advantage for lung cancer. A previous report has shown that human vascular smooth muscle cells are capable of releasing G-CSF and GM-CSF as well as large amounts of prostaglandins following induction of COX-2, when stimulated with cytokines.[19] Thus, COX-2 activity appears to be closely associated with inducible G-CSF and GM-CSF production in nonmalignant cells. However, it is unclear whether increased COX-2 activity can also promote G-CSF and GM-CSF production in tumor cells. In this study, we examined whether constitutive G-CSF or GM-CSF production by tumor cells, and the cell growth could be inhibited by the COX-2 inhibitor NS-398 in the two lung cancer cell lines, OKa-C-1 and MI-4. We found that NS-398 dose-dependently reduced the G-CSF or GM-CSF expression and the cell growth. The growth-inhibitory effect of NS-398 on lung cancer cell lines has been reported previously by Chang and Weng.[35] They found that some COX-2 inhibitors potently induce apoptosis, while other COX-2 inhibitors primarily induce growth inhibition. Thus, COX-2 inhibitors have variable effects on cancer cells. They concluded that the level of COX-2 expression correlates with the extent of apoptosis induced by COX-2 inhibitors. In our study, less than 10 *μM* NS-398 had scarcely any cytotoxic effect on OKa-C-1 or MI-4 cells, suggesting that NS-398 might primarily induce growth inhibition rather than apoptosis in the cell lines under the present culture conditions.

In OKa-C-1 and MI-4 cells, even less than 10 *μM* NS-398 decreased G-CSF and GM-CSF expression at both mRNA and protein levels. Inhibition of cell growth was also induced at a low dose of NS-398. NS-398 has been shown to lose its selectivity for COX-2 at high concentrations.[36] The use of a high concentration of NS-398 might affect the nuclear peroxisome proliferator activated receptor (*PPAR*) or other genes involved in proliferation,[37] suggesting that the possibility of an independent action mechanism of COX-2 inhibitor other than COX-2 inhibition cannot be excluded at this time. Therefore, we investigated whether an enzymatic product of COX, PGE_2, might reverse the inhibitory effect of NS-398 in OKa-C-1 and MI-4 cells. We found that 10 *μM* PGE_2 reversed the inhibitions of cell growth and G-CSF or GM-CSF mRNA expression caused by 10 *μM* NS-398 in these cell lines. These results indicate that NS-398 specifically inhibits COX activity in our cell lines. We examined whether exogenous G-CSF or GM-CSF could reverse the inhibition of cell growth imposed by NS-398 (10 *μM*) in order to clarify whether the reduction of G-CSF or GM-CSF is biologically significant in the COX-2 inhibitor-mediated growth inhibition. We found that G-CSF and GM-CSF significantly reversed the cell growth even at low concentrations. These results suggest that NS-398 inhibits anchorage-independent growth and invasiveness through the reduction of G-CSF or GM-CSF.

COX-2 is induced by cytokines and tumor promoters in inflammatory cells and human tumors.[38–40] We showed that the inflammatory cytokines, TNF-α and IL-1β induced COX-2 expression in addition to G-CSF or GM-CSF in OKa-C-1 and MI-4 cells.[27, 41] Some studies have reported that NF-κB activa-

tion is required to upregulate G-CSF, GM-CSF, or COX-2 expression,[42–49] and that inactivation of the NF-κB also induces rapid pro-apoptotic action.[45] COX-2 activation was also shown to follow the activation of MAPK in another cell type.[46] Besides p38, NF-κB and MEK, the Akt/PKB pathway plays an important role in COX-2 mRNA and protein induction and in tumorigenesis.[47] We examined whether the inhibition of G-CSF or GM-CSF production and the cell growth by NS-398 was mediated through NF-κB or the MAPK signaling pathway in OKa-C-1 and MI-4 cells. In MI-4 cells, the inhibition of p38 MAPK or MEK suppressed G-CSF production, and MEK GM-CSF. In OKa-C-1 cells, the p38 MAPK, MEK, and NF-κB inhibitiors all suppressed both G-CSF and GM-CSF production. SB203580, PD98059 and PDTC (10 μM), which were used to suppress G-CSF or GM-CSF expression, had no cytotoxicity in these cell lines. The p38 MAPK inhibitor and the MEK inhibitor suppressed cell growth dose-dependently, but PDTC had scarcely any influence. NS-398 decreased the phosphorylation of Erk1/2 but not p38, and had no effect on NF-κB activation. Overall, NS-398 appeared to inhibit constitutive G-CSF or GM-CSF production through the MEK signaling pathway in the cell lines. However, in this study, reversal of the inhibition of G-CSF or GM-CSF expression and cell growth by PGE_2 was partial, not complete, suggesting that constitutive G-CSF or GM-CSF production by tumor cells and cell growth might not necessarily be regulated by COX-2 activity alone. The promoter region of the human *COX-2* gene contains two NF-κB consensus sites,[48] the activating transcription factor (ATF)/cAMP-response element (CRE) site[49] and the NF/IL6 site,[50] though the extent to which each site contributes to COX-2 induction is unknown. Some reports suggest that the *COX-2* gene is regulated at the posttranscriptional level, though this represents a minor element in COX-2 mRNA induction.[51] Thus, further studies are required to elucidate the mechanism of constitutive G-CSF and GM-CSF production by tumor cells.

To our knowledge, this is the first report to show that a selective COX-2 inhibitor suppresses the constitutive G-CSF and GM-CSF expression in lung tumor cells. These results provide an experimental basis for clinical studies designed to determine whether COX-2 inhibitors will be useful in the chemoprevention or treatment of G-CSF or GM-CSF-producing lung cancer with a progressive clinical course.

1. Lilly MB, Devlin PE, Devlin JJ, Rado TA. Production of granulocyte colony-stimulating factor by a human melanoma cell line. *Exp Hematol* 1987; **15**: 966–9.
2. Ito N, Matsuda T, Kakehi Y, Takeuchi E, Takahashi T, Yoshida O. Bladder cancer producing granulocyte colony-stimulating factor. *N Engl J Med* 1990; **323**: 1709–10.
3. Takeda T, Ichiyanagi A, Sano K, Yoshida J, Tsutsumi Y, Miyaji T. A case of gallbladder cancer producing granulocyte-colony-stimulating factor. *Gastroenterol Jpn* 1990; **25**: 762–7.
4. Shijubo N, Inoue Y, Hirasawa M, Igarashi T, Mori M, Matsuura A, Uede T, Suzuki A. Granulocyte colony-stimulating factor-producing large cell undifferentiated carcinoma of the lung. *Intern Med* 1992; **31**: 277–80.
5. Teramachi M, Miyamoto N, Yamamoto Y, Sasaka T, Nakamura T, Kitamura F. A case of large cell carcinoma of the lung which is suspected of producing granulocyte colony-stimulating factor. *J Jpn Respir Soc* (in Japanese) 1992; **30**: 1327–32.
6. Asahi Y, Kubonishi I, Imamura J, Kamioka M, Matsushita H, Furihata M, Ohtsuki Y, Miyoshi I. Establishment of a clonal cell line producing granulocyte colony-stimulating factor and parathyroid hormone-related protein from a lung cancer patient with leukocytosis and hypercalcemia. *Jpn J Cancer Res* 1996; **87**: 451–8.
7. Watanabe M, Ono K, Ozeki Y, Tanaka S, Aida S, Okuno Y. Production of granulocyte-macrophage colony-stimulating factor in a patient with metastatic chest wall large cell carcinoma. *Jpn J Clin Oncol* 1998; **28**: 559–62.
8. Higaki I, Hirohashi K, Fukushima S, Wanibuchi H, Seike N, Yamane T, Kubo S, Tanaka H, Shuto T, Yamamoto T, Kinoshita H. Renal pelvic carcinoma producing granulocyte colony-stimulating factor: report of a case. *Surg Today* 2001; **31**: 266–8.
9. Baba M, Hasegawa H, Nakayabu M, Shimizu N, Suzuki S, Kamada N, Tani K. Establishment and characteristics of a gastric cancer cell line (HuGC-OOHIRA) producing high levels of G-CSF, GM-CSF, and IL-6: the presence of autocrine growth control by G-CSF. *Am J Hematol* 1995; **49**: 207–15.
10. Sharkey LC, Rosol TJ, Grone A, Ward H, Steinmeyer C. Production of granulocyte colony-stimulating factor and granulocyte-macrophage colony stimulating factor by carcinomas in a dog and a cat with paraneoplastic leukocytosis. *J Vet Intern Med* 1996; **6**: 405–8.
11. Berdel WE, Danhauser-Riedl S, Steinhauser G, Winton EF. Various human hematopoietic growth factors (interleukin-3, GM-CSF, G-CSF) stimulate clonal growth of nonhematopoietic tumor cells. *Blood* 1989; **73**: 80–3.
12. Young MR, Wright MA, Lozano Y, Prechel MM, Benefield J, Leonetti JP, Collins SL, Petruzzelli GJ. Increased recurrence and metastasis in patients whose primary head and neck squamous cell carcinomas secreted granulocyte-macrophage colony-stimulating factor and contained CD34+ natural suppressor cells. *Int J Cancer* 1997; **74**: 69–74.
13. Oshika Y, Nakamura M, Abe Y, Fukuchi Y, Yoshimura M, Itoh M, Ohnishi Y, Tokunaga T, Fukushima Y, Hatanaka H, Kijima H, Yamazaki H, Tamaoki N, Ueyama Y. Growth stimulation of non-small cell lung cancer xenografts by granulocyte-macrophage colony-stimulating factor (GM-CSF). *Eur J Cancer* 1998; **34**: 1958–61.
14. Kasuga I, Makino S, Kiyokawa H, Katoh H, Ebihara Y, Ohyashiki K. Tumor-related leukocytosis is linked with poor prognosis in patients with lung carcinoma. *Cancer* 2001; **92**: 2399–405.
15. Piazza GA, Alberts DS, Hixson LJ, Paranka NS, Li H, Finn T, Bogert C, Guillen JM, Brendel K, Gross PH, Sperl G, Ritchie J, Burt RW, Ellsworth L, Ahnen DJ, Pamukcu R. Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Res* 1997; **57**: 2909–15.
16. Thompson HJ, Jiang C, Lu J, Mehta RG, Piazza GA, Paranka NS, Pamukcu R, Ahnen DJ. Sulfone metabolite of sulindac inhibits mammary carcinogenesis. *Cancer Res* 1997; **57**: 267–71.
17. Souza RF, Shewmake K, Beer DG, Cryer B, Spechler SJ. Selective inhibition of cyclooxygenase-2 suppresses growth and induces apoptosis in human esophageal adenocarcinoma cells. *Cancer Res* 2000; **60**: 5767–72.
18. Masferrer JL, Leahy KM, Koki AT, Zweifel BS, Settle SL, Woerner BM, Edwards DA, Flickinger AG, Moore RJ, Seibert K. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. *Cancer Res* 2000; **60**: 1306–11.
19. Stanford SJ, Pepper JR, Mitchell JA. Release of GM-CSF and G-CSF by human arterial and venous smooth muscle cells: differential regulation by COX-2. *Br J Pharmacol* 2000; **129**: 835–8.
20. Krasnow SW, Zhang LQ, Leung KY, Osborn L, Kunkel S, Nabel GJ. Tumor necrosis factor-alpha, interleukin 1, and phorbol myristate acetate are independent activators of NF-kappa B which differentially activate T cells. *Cytokine* 1991; **3**: 372–9.
21. Andersen NA, Larsen CM, Mandrup-Poulsen T. TNFalpha and IFNgamma potentiate IL-1beta induced mitogen activated protein kinase activity in rat pancreatic islets of Langerhans. *Diabetologia* 2000; **43**: 1389–96.
22. Munoz C, Pascual-Salcedo D, Castellanos MC, Alfranca A, Aragones J, Vara A, Redondo MJ, de Landazuri MO. Pyrrolidine dithiocarbamate inhibits the production of interleukin-6, interleukin-8, and granulocyte-macrophage colony-stimulating factor by human endothelial cells in response to inflammatory mediators: modulation of NF-kappa B and AP-1 transcription factors activity. *Blood* 1996; **88**: 3482–90.
23. Griego SD, Weston CB, Adams JL, Tal-Singer R, Dillon SB. Role of p38 mitogen-activated protein kinase in rhinovirus-induced cytokine production by bronchial epithelial cells. *J Immunol* 2000; **165**: 5211–20.
24. Husain SS, Szabo LI, Pai R, Soreghan B, Jones KM, Tarnawski SA. MAPK (ERK2) kinase-a key target for NSAIDs—induced inhibition of gastric cancer cell proliferation and growth. *Life Sci* 2001; **69**: 3045–54.
25. Hla T, Neilson K. Human cyclooxygenase-2 cDNA. *Proc Natl Acad Sci USA* 1992; **89**: 7384–8.
26. Yamamoto K, Arakawa T, Ueda N, Yamamoto S. Transcriptional roles of nuclear factor kappa B and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *J Biol Chem* 1995; **270**: 31315–20.
27. Harada R, Uemura Y, Kobayashi M, Zamecnikova A, Nakata H, Taguchi T, Furihata M, Otsuki Y, Taguchi H. Establishment and characterization of a new lung cancer cell line (MI-4) producing high levels of granulocyte colony stimulating factor. *Jpn J Cancer Res* 2002; **93**: 667-76.
28. Smith WL, Dewitt DL. Prostaglandin endoperoxide H synthases-1 and -2. *Adv Immunol* 1996; **62**: 167–215.
29. Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem* 1996; **271**: 33157–60.
30. Kinoshita T, Imamura J, Nagai H, Shimotohno K. Quantification of gene expression over a wide range by the polymerase chain reaction. *Anal Biochem*

1992; **206**: 231–5.
31. Miyagawa K, Chiba S, Shibuya K, Piao YF, Matsuki S, Yokota J, Terada M, Miyazono K, Takaku F. Frequent expression of receptors for granulocyte-macrophage colony-stimulating factor on human nonhematopoietic tumor cell lines. *J Cell Physiol* 1990; **143**: 483–7.
32. Tachibana M, Miyakawa A, Tazaki H, Nakamura K, Kubo A, Hata J, Nishi T, Amano Y. Autocrine growth of transitional cell carcinoma of the bladder induced by granulocyte-colony stimulating factor. *Cancer Res* 1995; **55**: 3438–43.
33. Hida T, Yatabe Y, Achiwa H, Muramatsu H, Kozaki K, Nakamura S, Ogawa M, Mitsudomi T, Sugiura T, Takahashi T. Increased expression of cyclooxigenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Res* 1998; **58**: 3761–4.
34. Achiwa H, Yatabe Y, Hida T, Kuroishi T, Kozaki K, Nakamura S, Ogawa M, Sugiura T, Mitsudomi T, Takahashi T. Prognostic significance of elevated cyclooxygenase 2 expression in primary, resected lung adenocarcinomas. *Clin Cancer Res* 1999; **5**: 1001–5.
35. Chang HC, Weng CF. Cyclooxygenase-2 level and culture conditions influence NS398-induced apoptosis and caspase activation in lung cancer cells. *Oncol Rep* 2001; **6**: 1321–5.
36. Cryer B, Feldman M. Cyclooxygenase-1 and cyclooxygenase-2 selectivity of widely used nonsteroidal anti-inflammatory drugs. *Am J Med* 1998; **104**: 413–21.
37. He TC, Chan TA, Vogelstein B, Kinzler KW. PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell* 1999; **99**: 335–45.
38. Ristimaki A, Garfinkel S, Wessendorf J, Maciag T, Hla T. Induction of cyclooxygenase-2 by interleukin-1 alpha. Evidence for post-transcriptional regulation. *J Biol Chem* 1994; **269**: 11769–75.
39. Chen CC, Sun YT, Chen JJ, Chiu KT. TNF-alpha-induced cyclooxygenase-2 expression in human lung epithelial cells: involvement of the phospholipase C-gamma 2, protein kinase C-alpha, tyrosine kinase, NF-kappa B-inducing kinase, and I-kappa B kinase 1/2 pathway. *J Immunol* 2000; **165**: 2719–28.
40. de Ledinghen V, Liu H, Zhang F, Lo CR, Subbaramaiah K, Dannenberg AJ, Czaja MJ. Induction of cyclooxygenase-2 by tumor promoters in transformed and cytochrome P450 2E1-expressing hepatocytes. *Carcinogenesis* 2002; **23**: 73–9.
41. Uemura Y, Nakata H, Kobayashi M, Harada R, Asahi Y, Taguchi H. Regulation of granulocyte colony-stimulating factor and parathyroid hormone-related protein production in lung carcinoma cell line OKa-C-1. *Jpn J Cancer Res* 2000; **91**: 911–7.
42. Dunn SM, Coles LS, Lang RK, Gerondakis S, Vadas MA, Shannon MF. Requirement for nuclear factor (NF)-kappaB p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. *Blood* 1994; **83**: 2469–79.
43. Gallois C, Habib A, Tao J, Moulin S, Maclouf J, Mallat A, Lotersztajn S. Role of NF-kappaB in the antiproliferative effect of endothelin-1 and tumor necrosis factor-alpha in human hepatic stellate cells. Involvement of cyclooxygenase-2. *J Biol Chem* 1998; **273**: 23183–90.
44. Crane IJ, Wallace CA, Forrester JV. Regulation of granulocyte-macrophage colony-stimulating factor in human retinal pigment epithelial cells by IL-1beta and IFN-gamma. *Cell Immunol* 2001; **209**: 132–9.
45. Milligan SA, Nopajaroonsri C. Inhibition of NF-kappa B with proteasome inhibitors enhances apoptosis in human lung adenocarcinoma cells *in vitro*. *Anticancer Res* 2001; **21**: 39–44.
46. Faour WH, He Y, He QW, de Ladurantaye M, Quintero M, Mancini A, Di Battista JA. Prostaglandin E(2) regulates the level and stability of cyclooxygenase-2 mRNA through activation of p38 mitogen-activated protein kinase in interleukin-1 beta-treated human synovial fibroblasts. *J Biol Chem* 2001; **276**: 31720–31.
47. Sheng H, Shao J, Dubois RN. K-Ras-mediated increase in cyclooxygenase 2 mRNA stability involves activation of the protein kinase B1. *Cancer Res* 2001; **61**: 2670–5.
48. Schmedtje JF Jr, Ji YS, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. *J Biol Chem* 1997; **272**: 601–8.
49. Miller C, Zhang M, He Y, Zhao J, Pelletier JP, Martel-Pelletier J, Di Battista JA. Transcriptional induction of cyclooxygenase-2 gene by okadaic acid inhibition of phosphatase activity in human chondrocytes: co-stimulation of AP-1 and CRE nuclear binding proteins. *J Cell Biochem* 1998; **69**: 392–413.
50. Kirtikara K, Raghow R, Laulederkind SJ, Goorha S, Kanekura T, Ballou LR. Mol transcriptional regulation of cyclooxygenase-2 in the human microvascular endothelial cell line, HMEC-1: control by the combinatorial actions of AP2, NF-IL-6 and CRE elements. *Mol Cell Biochem* 2000; **203**: 41–51.
51. Zhang Z, Sheng H, Shao J, Beauchamp RD, DuBois RN. Posttranscriptional regulation of cyclooxygenase-2 in rat intestinal epithelial cells. *Neoplasia* 2000; **2**: 523–30.