Abstract. We examined the effects of granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on the lung cancer cell lines PC-9, LA-1 and A549. In addition, we examined if the effects of the cytokines on the cell lines are mediated by activation of cyclooxygenase (COX)-2. The three cell lines did not constitutively produce either G-CSF or GM-CSF. G-CSF did not influence cell growth in the three cell lines, while GM-CSF increased cell growth in the A549 and LA-1 lines. G-CSF and GM-CSF dose-dependently decreased cell death in the three cell lines. RT-PCR demonstrated GM-CSF receptor expression in the three lung cancer cell lines, whereas the G-CSF receptor exists only in the PC-9 line. We suggest that G-CSF might rescue the tumor cells from cytotoxicity due to serum deprivation through cellular pathways independent of the G-CSF receptor. G-CSF and GM-CSF increased cyclooxygenase-2 (COX-2) expression in PC-9 and LA-1 cells whereas they decreased COX-2 expression in A549 cells. The COX-2 inhibitor NS-398 increased cell death in PC-9 and LA-1 cells, whereas it decreased cell death in A549 cells. PC-9 and LA-1 clones transfected with sense G-CSF- or GM-CSF showed an increase in COX-2 expression, while COX-2 expression was decreased in transfected A549 clones. COX-2 expression was increased in anti-sense G-CSF- and GM-CSF-transfected A549 clones. Thus, although COX-2 activation seems to induce different biological behavior depending on the cell type, we propose that G-CSF and GM-CSF might accelerate tumor progression by directly regulating COX-2 expression, independently of an autocrine mechanism.

Introduction

A significantly increased expression of cyclooxygenase-2 (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. Such an increase in COX-2 expression may be a clinically significant prognostic factor for patients undergoing surgical resection of early-stage adenocarcinomas (1,2). Some COX-2 inhibitors induce growth inhibition and apoptosis, indicating that increased COX-2 activity is closely associated with tumor growth in lung cancer (3). In advanced clinical stages, many cases of lung cancer constitutively produce colony-stimulating factors (CSFs), including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), in amounts large enough to cause a significant hematopoietic effect (4-6). The prognosis is significantly worse in patients with non-small cell lung cancer (NSCLC) showing G-CSF gene expression (7). Some studies suggest that CSFs produced by lung cancer cells might promote tumor progression via an autocrine mechanism (8,9). However, G-CSF-producing lung tumor cells do not necessarily have G-CSF receptors (10). We hypothesize that G-CSF might promote tumor progression through a mechanism separate from an autocrine growth loop. We previously reported that a COX-2 inhibitor decreases constitutive production of G-CSF and GM-CSF from lung cancer cells, resulting in inhibition of cell growth (11). These data suggested that the effects of G-CSF and GM-CSF on the tumor progression might be closely associated with COX-2 activity in the lung cancer with an autocrine growth loop. In this study, we also examined the effects of G-CSF and GM-CSF on the biological behavior in lung cancer cells not constitutively producing G-CSF and GM-CSF.

Materials and methods

Reagents and supplies. Enzyme-linked immunosorbent assay (ELISA) kits for measuring the contents of G-CSF and GM-CSF in culture medium were from TECHNE (Minneapolis,
were isolated. Successful transfection was confirmed by G418 as a selection marker. Clonal transfected populations DNA in the presence of 15 μl of TransIT-LT1 transfection reagent. Subconfluent cells clone and named Mock. DNA transfection was carried out clone AGM. pUSEamp without insert was used as a control sense GM-CSF clone SGM, and the anti-sense GM-CSF by restriction digests and sequence analysis. We named the cloning site of the expression vector pUSEamp and verified in our laboratory. Subcultures were carried out twice weekly line was established from a patient with large cell carcinoma. The PC-9 cell line was kindly provided by Professor Jiro. A549, PC-9 and LA-1 lung cancer cell lines were handled in subdued light conditions. dimethyl sulphoxide (DMSO) as a 1000X stock and was obtained from Mirus (Madison, WI). Human G-CSF cDNA in pBRV2 was kindly provided by Chugai Pharmaceutical Co. (Tokyo, Japan) and human GM-CSF cDNA in pCSF-1 was obtained from American Type Culture Collection (Manassas, VA). Empty expression vector pUSEamp was purchased from Upstate Biotechnology. N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398) was purchased from Alexis Biochemicals (San Diego, CA). NS-398 was prepared in dimethyl sulphoxide (DMSO) as a 1000X stock and was handled in subdued light conditions.

Cell lines. A549, PC-9 and LA-1 lung cancer cell lines were used and maintained in Dulbeco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) at 37˚C in an incubator under a 100% humidified 5% CO2 atmosphere. The PC-9 cell line was kindly provided by Professor Jiro Hujita (Rykyu University, Okinawa, Japan). The LA-1 cell line was established by a patient with large cell carcinoma in our laboratory. Subcultures were carried out twice weekly by removing the cells from 75 cm2 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. The cells were harvested with a cell scraper (Beckton Dickinson, Lincoln Park, NJ) for RNA and protein analysis. The medium was changed to serum-free medium. After 48 h, the culture medium was collected 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.

Plasmids and DNA transfection. EcoRI-digested cDNA fragments of G-CSF or GM-CSF were ligated into the multiple cloning site of the expression vector pUSEamp and verified by restriction digests and sequence analysis. We named the sense G-CSF clone SG, the antisense G-CSF clone AG, the sense GM-CSF clone SGM, and the anti-sense GM-CSF clone AGM. pUSEamp without insert was used as a control clone and named Mock. DNA transfection was carried out using TransIT-LT1 transfection reagent. Subconfluent cells in a 60-mm tissue culture dish were transfected with 5 μg DNA in the presence of 15 μl of TransIT-LT1 transfection reagent in 2 ml of media containing 5% FCS. At 48 h after transfection, the medium was changed to new one containing G418 as a selection marker. Clonal transfected populations were isolated. Successful transfection was confirmed by immunoblot analysis using anti-neomycin phosphotransferase II Ab.

Detection of G-CSF and GM-CSF. The concentrations of G-CSF or GM-CSF in the culture medium were determined using an ELISA kit according to the supplier’s instructions. The sensitivities for these kits are 0.4 and 3.0 pg/ml for G-CSF and GM-CSF, respectively. The values of G-CSF and GM-CSF in the conditioned medium were expressed as pg/106 cells. G-CSF or GM-CSF levels in the fresh medium were less than the sensitivity for these kits.

RT-PCR. Total RNA was extracted from the cultured cells by the guanidine-thiocyanate method using Isogen solution (Nippon Gene Co., Tokyo). RNA (1 μm) was converted to cDNA with Moloney 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 μM of each specific primer, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTP, and 1 unit Taq polymerase. The G-CSF 328-bp fragment was detected by PCR (30 cycles at 94˚C for 1 min, 58˚C for 1 min and 72˚C for 1 min) with 5'TAGAGGAAAGTGAAGAAGAAGTCGGAGGACCTCATCATCT GTCAGGACAGT-3' (forward primer) and 5'AGTTTCTCCATCTG CTGCAAGAGATAG-3' (reverse primer). The GM-CSF 424-bp fragment was detected by PCR (30 cycles at 95˚C for 1 min, 60˚C for 1 min and 73˚C for 1 min) with 5'ATGGGTCGCTCAGAGGCGCTGCCTGCAGACC-3' (forward primer) and 5'CTGGCTC CAAGGAA-3' (reverse primer). The G-CSF receptor 668-bp fragment was detected by PCR (30 cycles at 95˚C for 55 sec, 72˚C for 70 sec, 65˚C for 95 sec, and one cycle of 435 sec at 72˚C) with 5'TGGACTGAGCAGCCTGTT CTCCAGGAAC-3' (forward primer) and 5'GGTCTGACAGT TGCCCCGCTC-3' (reverse primer). The GM-CSF receptor α 546-bp fragment was detected by PCR (30 cycles at 94˚C for 1 min, 60˚C for 1 min and 72˚C for 1 min) with 5'CTTCTC TCTGACCCAGCA-3' (forward primer) and 5'ACATGGG TTCTGGATCCTGC-3' (reverse primer). The β-actin 309-bp fragment was detected by PCR (20 cycles at 94˚C for 1 min, 58˚C for 1 min and 72˚C for 1 min) with 5'ACCTCTCAACA CCCAGCCATG-3' (forward primer) and 5'GGCCATCTT GTGTCGAAGTGC-3' (reverse primer). PCR product (10 μl) was electrophoresed on a 1% NuSieve GTG agarose gel and stained with ethidium bromide. Human G-CSF cDNA in pBRV2 and human GM-CSF cDNA in pCSF-1 were used as positive controls for the mRNA expression of G-CSF and GM-CSF. U937 and BALL-2 cells were used as positive and negative controls for the mRNA expression of G-CSF receptor or GM-CSF receptor α respectively. The identities of the PCR amplification products were confirmed by size and restriction digests. The intensity of the bands was evaluated using a UV-light box imaging system (Atto, Tokyo).

Western blot analysis. The medium was removed and cell monolayers were washed 3 times with ice-cold PBS and lysed in ice-cold buffer (50 mM Tris-buffered saline (pH 8.0), 150 mM NaCl, 0.002% sodium azide, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, 200 mM sodium orthovandate, 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 by a Bradford assay (Bio-Rad, Hercules, CA). Protein (10 μg) was separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad). The membrane was blocked overnight at 4°C using I-block (Tropix, Bedford, MA). Membranes were incubated with 1:2000 dilutions of rabbit anti-neomycin phosphotransferase II Ab and mouse anti-COX-2 Ab and a 1:10,000 dilution of mouse anti-ß-actin Ab. Chemiluminescence was determined using the Western-Star kit (Tropix) according to the manufacturer’s instructions.

Cell proliferation and cytotoxicity assay. Cells (1x10^5) were plated in 96-well plates and then cultured in serum-free medium for 24 h to deprive them of FCS. The medium was changed to a new one containing 1% FCS. After 48 h, cell growth and death were analyzed using cell proliferation and cytotoxicity assay kits. NFS-60 (ATCC CRL-1838) and TF-1 (ATCC CRL-2003) cells, which proliferate in response to G-CSF and GM-CSF, respectively, were used as positive controls. G-CSF did not influence cell growth in PC-9, LA-1 or A549 cells at any concentration. GM-CSF dose-dependently stimulated cell growth in LA-1 and A549 cells, while it did not influence PC-9 cells at any concentration. The maximal stimulatory effect of GM-CSF in A549 cells was observed at 1 ng/ml (Fig. 1A). Both G-CSF and GM-CSF decreased cell death in a dose-dependent manner in the three cell lines. The maximal effects of G-CSF and GM-CSF were observed at 10 IU/ml and 0.1 ng/ml, respectively, in LA-1 cells (Fig. 1B). The data represent the mean ± SE of four wells from three independent experiments.

Results

Effects of exogenous G-CSF and GM-CSF on cell growth and death. After 48 h of serum deprivation, 70% confluent cells were incubated with various concentrations of G-CSF (0-10^4 IU/ml) and GM-CSF (0-10^2 ng/ml) in media containing 1% FCS. After 48 h, cell growth and death were analyzed using cell proliferation and cytotoxicity assay kits. NFS-60 (ATCC CRL-1838) and TF-1 (ATCC CRL-2003) cells, which proliferate in response to G-CSF and GM-CSF, respectively, were used as positive controls. G-CSF did not influence cell growth in PC-9, LA-1 or A549 cells at any concentration. GM-CSF dose-dependently stimulated cell growth in LA-1 and A549 cells, while it did not influence PC-9 cells at any concentration. The maximal stimulatory effect of GM-CSF in A549 cells was observed at 1 ng/ml (Fig. 1A). Both G-CSF and GM-CSF decreased cell death in a dose-dependent manner in the three cell lines. The maximal effects of G-CSF and GM-CSF were observed at 10 IU/ml and 0.1 ng/ml, respectively, in LA-1 cells (Fig. 1B). The data represent the mean ± SE of four wells from three independent experiments.

Effect of a neutralizing human G-CSF Ab and a human GM-CSF Ab on cell growth and death. To confirm the growth advantages of G-CSF and GM-CSF, cells were incubated with various concentrations of neutralizing human G-CSF mouse monoclonal Ab or human GM-CSF goat Ab in the medium containing 1% FCS. Mouse IgG1 isotype control and normal goat IgG were used as controls for anti-G-CSF Ab and anti-GM-CSF Ab, respectively. NFS-60 and TF-1 cells, in which cell growth is decreased in response to anti-G-CSF Ab and anti-GM-CSF Ab, respectively, were used as positive controls. After serum deprivation, 70% confluent cells were incubated with various concentrations of anti-G-CSF Ab (0-50 μg/ml) and anti-GM-CSF Ab (0-10 μg/ml) in the media containing 1% FCS. After 48 h, cell growth and death were analyzed as described above. Anti-G-CSF Ab did not alter cell growth in the three cell lines, whereas anti-GM-CSF Ab dose-dependently inhibited cell growth (Fig. 2A).

Anti-G-CSF Ab also did not alter cell death in the three cell lines, whereas anti-GM-CSF Ab dose-dependently increased cell death (Fig. 2B). The data represent the mean ± SE of four wells from three independent experiments.

Expression of G-CSF and GM-CSF receptors. Expression of the G-CSF receptor or GM-CSF receptor was examined by RT-PCR in the cell lines. U937 cells were used as positive controls for both receptors and BALL-2 cells were used as negative controls. G-CSF receptor expression was detected in only PC-9 cells. GM-CSF receptor was detected in PC-9, A549 and LA-1 cells (Fig. 3). Results are representative of three independent experiments.
Effects of G-CSF and GM-CSF on COX-2 expression. We examined the effect of exogenous G-CSF and GM-CSF on COX-2 expression in the cell lines. After 48 h of serum deprivation, 70% confluent cells of A549 and PC-9 cells were incubated with $10^3$ IU/ml G-CSF or 10 ng/ml GM-CSF in serum-free media and LA-1 were incubated with 10 IU/ml G-CSF or 0.1 ng/ml GM-CSF. After a 24 h incubation, cells were harvested and then assayed for COX-2 expression by Western blot analysis. Exogenous G-CSF and GM-CSF increased COX-2 expression in LA-1 and PC-9 cells, whereas they oppositely inhibited expression in A549 cells (Fig. 4).

The data represent the mean ± SE of four wells from three independent experiments.

Effect of the COX-2 inhibitor NS-398 on cell growth and cell death. We examined the effect of NS-398 on cell growth and cell death. After serum deprivation, the 70% confluent cells were incubated with 10·10^3 IU/ml G-CSF or 10 ng/ml GM-CSF in serum-free media, and LA-1 cells were incubated with 10 IU/ml G-CSF or 0.1 ng/ml GM-CSF. After a 24 h incubation, harvested cells were analyzed for COX-2 expression by Western blot analysis. Both G-CSF and GM-CSF increased COX-2 expression in PC-9 and LA-1 cells, whereas they decreased expression in A549 cells. Results are representative of three independent experiments.

Transfection with G-CSF or GM-CSF cDNA and expression of these proteins and their receptors. Clonal transfected
populations were isolated, and positive cell clones were identified by Western blot analysis using anti-neomycin phosphotransferase II Ab (Fig. 6). RT-PCR showed increased expression of G-CSF or GM-CSF in positive cell clones of each cell line (Fig. 7A and B). Messenger RNA expression data were confirmed by the protein levels in conditioned media using ELISAs for G-CSF and GM-CSF (Table I). Levels of G-CSF or GM-CSF that were constitutively produced by A549, LA-1 and PC-9 parent cells were <10 pg/10⁶ cells. Sense clones of G-CSF and GM-CSF constitutively produced G-CSF and GM-CSF, respectively, in the three cell lines, whereas Mock transfecant and anti-sense
clones of G-CSF and GM-CSF did not. To confirm the maintenance of the G-CSF or GM-CSF receptor expression in the transfected clones, we analyzed the expression of these receptors by RT-PCR. G-CSF receptor expression in PC-9 cells and GM-CSF receptor expression in each of the three cell lines was maintained even after transfection of each clone (Fig. 7C). The data represent the mean ± SE of four wells from three independent experiments. Results are representative of three independent experiments.

**Table I. Protein expression of G-CSF and GM-CSF.**

<table>
<thead>
<tr>
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<th>G-CSF (pg/10^6 cells)</th>
<th>GM-CSF (pg/10^6 cells)</th>
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<tr>
<td>A549-Mock PB</td>
<td>A549-Mock PB</td>
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<tr>
<td>A549-AG PB</td>
<td>A549-AGM PB</td>
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<td>A549-SG 120±11</td>
<td>A549-SGM 310±21</td>
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<tr>
<td>LA-1SG 210±18</td>
<td>LA-1SGM 410±51</td>
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<tr>
<td>PC-9-Mock PB</td>
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<td>PC-9-AG PB</td>
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<tr>
<td>PC-9-SG 187±14</td>
<td>PC-9-SGM 280±28</td>
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*PB, protein below detection level.*

**Figure 8.** COX-2 expression in the transfected clones. COX-2 expression was increased in SG and SGM transfected clones of PC-9 and LA-1 cells, whereas it was decreased in AG and AGM. It was increased in AG and AGM transfected clones of A549 cells, whereas it was decreased in SG and SGM. Results are representative of three independent experiments.

**Figure 9.** Cell growth and cell death in the transfected clones. Cell growth was not altered in SG and AG clones of PC-9, A549 and LA-1 cells. It was increased in SGM of A549 and LA-1 cells, whereas it was decreased in AGM. Cell death was decreased in SG and SGM in the three cell lines, whereas it was increased in AG and AGM. Results are representative of three independent experiments.

**Discussion**

It is known that the prognosis is significantly worse in patients with non-small-cell lung cancer (NSCLC) showing G-CSF gene expression (7). Several studies have demonstrated the presence of an autocrine growth loop for G-CSF or GM-CSF in nonhematopoietic tumor cells involving lung cancer (12-15). Exogenous G-CSF and GM-CSF also stimulate invasion by lung cancer cells as well as their endogenous counterparts (16,17). These studies indicate that tumor progression might be critically controlled by alterations in the microenvironment often caused by an aberrant expression of growth factors and receptors. However, a G-CSF autocrine loop is not necessarily involved in the growth of these G-CSF-producing tumors (10), suggesting that exogenous G-CSF or endogenous G-CSF gene expression might progress tumor growth by mechanisms besides an autocrine growth loop.

In our study, exogenous and endogenous GM-CSF increased cell growth and decreased cell death in PC-9, A549 and LA-1 cells. The three cell lines expressed the GM-CSF receptor, suggesting that they might have a growth advantage via an autocrine growth loop for GM-CSF. Exogenous and endogenous G-CSF decreased cell death in the three cell lines, but could not, however, influence cell growth. We were not able to demonstrate the presence of the G-CSF receptor in A549 and LA-1 cells by RT-PCR. These results suggested that inhibition of cell death by G-CSF might be induced through mechanisms independent of the G-CSF receptor in the cell lines.

Many studies report that COX-2 inhibitors can inhibit tumor progression through several pathways, for example matrix metalloproteinase or mitogen-activated protein kinase/
ERK (18-20). We previously showed that COX-2 activity might be closely associated with tumor growth via an autocrine growth loop for G-CSF and GM-CSF in lung cancer (11). COX-2 expression was activated by exogenous stimulation and endogenous expression of G-CSF and GM-CSF in PC-9 and LA-1 cells. Although NS398 did not influence cell growth in the three cell lines, it increased cell death in PC-9 and LA-1 cells. G-CSF and GM-CSF transfected clones of PC-9 or LA-1 cells also showed increased COX-2 expression, resulting in inhibition of cell death. G-CSF and GM-CSF decreased COX-2 expression in A549 cells and, in addition, NS398 decreased cell death. COX-2 expression was increased in anti-sense clones of G-CSF and GM-CSF in A549 cells, whereas it was decreased in the sense-clones of A549 cells. Duan et al reported that NS398 (0.2-20 μM) was not able to inhibit A549 cell proliferation, on the contrary, NS398 promoted cell growth (21). Although we could not show a growth effect for NS398 in A549 cells, NS398 appeared to inhibit cell death, resulting in increased tumor progression. Thus, we proved that exogenous and endogenous G-CSF and GM-CSF could regulate cell growth and death via COX-2 expression. In particular, the inhibitory effect of G-CSF on cell death appeared to be induced regardless of G-CSF receptor expression in some types of lung cancer. However, it is not clear whether the inhibitory effect of NS398 on cell death is restricted to A549 cells or a common phenomenon in lung cancer. It is known that GM-CSF promotes tumor growth via an autocrine mechanism in lung cancer (14). In our study, GM-CSF also had a growth advantage through COX-2 expression. However, it is not clear whether GM-CSF could also induce COX-2 expression without the aid of the GM-CSF receptor because we did not examine this using cell lines lacking the GM-CSF receptor in this study. To clarify whether the biological effect of NS398 is dependent on cell type and whether the effects of G-CSF and GM-CSF on COX-2 expression are independent of the receptors for each, many more types of lung cancer cells need to be examined.

References