Celecoxib inhibits cell proliferation through the activation of ERK and p38 MAPK in head and neck squamous cell carcinoma cell lines

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It has been observed that several cyclooxygenase-2 (COX-2) inhibitory chemicals might inhibit proliferation of various cancer cells through COX-2-independent action. We also identified that celecoxib more selectively kills cell lines derived from head and neck squamous cell carcinoma (HNSCC) than its non-cancerous counterparts, irrespective of COX-2 expression. Herein, we investigated whether the regulation of mitogen-activated protein kinases activity might be one of the main mechanisms related to a conspicuous COX-2-independent tumor-killing effect of celecoxib in HNSCC cell lines. We assessed the effect of celecoxib on extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase activity by a transcription factor activation assay then evaluated, if these factors might be involved in the COX-2-independent tumor-killing effect of celecoxib by blocking their activity. We found that the blocking activation of ERK and/or p38 could reverse the celecoxib-induced cell growth inhibition by 50–80% in HNSCC cell lines, but it was not tested in cancer cells of other types. In conclusion, our study suggests that most COX-2-independent tumor-killing action of celecoxib is mediated by the upregulation of ERK and/or p38 activity in HNSCC cells. These results encourage investigation on the underlying mechanisms and detailed outcomes of mitogen-activated protein kinases activation by celecoxib more concisely, for using its excellent tumor-killing effect more safely in the clinical field of cancer treatment. *Anti-Cancer Drugs* 21:823–830 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Earlier observations have pointed out the anti-cancer effects of cyclooxygenase-2 (COX-2) inhibitors resulting from various COX-2-independent mechanisms even in cancer cells without COX-2 expression and activity [1]. Furthermore, this effect is observed even in lower concentrations of COX-2 inhibitors than doses required to block COX-2 activity perfectly in some cancers [2,3]. In our own study with head and neck squamous cell carcinoma (HNSCC) cell lines, we observed that tumor-killing effects of several COX-2 inhibitory chemicals might be seen even in HNSCC cells without expression of COX-2, irrespective of inhibiting prostaglandins (PGs) produced by COX-2.

There are various COX-2 inhibitors; in particular, celecoxib has shown outstanding COX-2-independent tumor-killing action in several cancer models [4–6]. Some researchers regard it as a new anti-cancer drug or cell death-inducing agent, rather than a COX-2 inhibitor in the clinical field of cancer treatment [7,8]. Controversy remains regarding the mechanism of action of celecoxib, as there are different pathways of action even in cell lineages within the same type of cancer that are described [9,10]. The merits of celecoxib are many, and it may be beneficial to patients if it were to be used as an alternative anti-cancer agent. There are some reports on the characteristics of celecoxib in anti-cancer treatment [5,7,11]. To further clarify the utility of celecoxib, we attempt to identify the unique tumor-killing actions of celecoxib in HNSCC cells.

It has been suggested that the regulation of mitogen-activated protein kinases (MAPKs) activity might be one of the common events related to the anti-cancer effects of various cancer-killing agents [12,13]. It is known that activated MAPKs signaling might promote cell growth or induce cell death in different situations [14,15]. In cancer biology, MAPKs seem to be commonly regulated by various cancer cell-derived oncogenic factors and stress signals. Some groups have tried to develop MAPK regulators as anti-cancer agents [16]. However, according to many studies on the mechanisms of action of anti-cancer agents, activation of MAPKs seems to involve both

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tumor-promoting and tumor-killing action [17]. Interestingly, when PGs are produced by COX-2 from arachidonic acid and bound to each specific receptor by either the autocrine or paracrine route, they tend to change cyclic adenosine monophosphate and/or Ca^{2+} level in the intracellular environment. Cyclic adenosine monophosphate-dependent protein kinase and three MAPKs may be regulated through this event [18]. It has been reported earlier that activation and/or inhibition of MAPKs may be one of the COX-2-independent mechanisms of particular COX-2 inhibitors [19], and activation of MAPKs was a key response in the induction of cell death signaling by any stress stimulus in our own study with HNSCC cell lines.

Considering these points, it is possible that COX-2 inhibitory chemicals, including celecoxib, might affect MAPKs activity in treated cells. We focused on the role of MAPK activation in the unexpected tumor-killing actions of celecoxib in the HNSCC cell model. Finally, we examined the possibility of regulation of MAPKs as a novel strategy for cancer treatment by analyzing mechanisms of action of celecoxib in HNSCC.

Methods

Cell culture

SNU-1041 and SNU-1076 (HNSCC cell lines) were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea), whereas PCI-1, PCI-13, and PCI-50 (HNSCC cell lines) were obtained from the Pittsburgh Cancer Institute (University of Pittsburgh, Pennsylvania, USA) [20]. HOK-16B is a human immortalized cell line from normal pharyngeal mucosa. A549 is a cell line originating from a human lung carcinoma, and pancreatic cancer cell-1 (PANC-1), from a human carcinoma of the exocrine pancreas. The cells were maintained at 37°C in a humidified, 5% CO_2, 95% air atmosphere and were routinely subcultured using trypsin-EDTA (0.25% w/v). Unless otherwise stated, all cell culture reagents were obtained from Gibco BRL (Grand Island, New York, USA).

Chemicals

NS-398 and dup-697 (COX-2 specific inhibitor) were obtained from Cayman Chemical (Ann Arbor, Michigan, USA). U0126 [extracellular signal-regulated kinase (ERK) inhibitor] and SB202190 (p38 MAPK inhibitor) were obtained from Sigma Chemical Co. (St Louis, Missouri, USA). Celecoxib (COX-2 inhibitor) was a gift from Pharmacia Korea (Seoul, Korea). All chemicals were used according to the provided suggestion (IC_{50} and references).

Cell proliferation assay

The cells were seeded in 96-well plates and incubated for 24 h at 37°C and treated with specific drugs for an indicated time at 37°C. After drug treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (Sigma) and cell counting kit-8 (Dojindo Lab., Tokyo, Japan) were used for the investigation of cell proliferation according to the manufacturer's instructions.

Transfection of small interfering RNA

Individual small interfering RNA (siRNA) against COX-2 (D-004557-04) and non-targeting control (D-001210-01) were obtained from Dharmacon RNA Technologies (Lafayette, Colorado, USA). The best conditions of siRNA application (used doses and treatment time) were established beforehand by western blotting and enzyme immunoassay (Supplementary data 1). Cells were plated in 6-well, 12-well, or 24-well plates and grown to 50–70% confluence. After 24 h, the cells were transfected with siRNA (100–200 nmol/l) using lipofectamine-2000 reagent (Invitrogen, Carlsbad, California, USA) for 48–72 h, according to the manufacturer's instructions.

Western blot analysis

Denatured protein lysates were resolved by 4–12% NuPAGE gels (Invitrogen) and transferred to nitrocellulose membranes (Schleicher and Schuell, Dachen, Germany). The membranes were incubated with anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, California, USA) or monoclonal anti-α-tubulin (Sigma) for 2 h at room temperature or overnight at 4°C. Membranes were then washed (four times) with Tris-buffered saline–Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, Illinois, USA) for 1 h. Immunoreactive proteins were visualized by developing with Lumi-light western blotting substrate (Roche Diagnostics GmbH, Mannheim, Germany) followed by exposure in a LAS-3000 (Fuji Film Co., Tokyo, Japan) according to the manufacturer's instructions.

Quantification of prostaglandin E_2 production

The amount of prostaglandin E_2 (PGE_2) released by the cells was determined by using PGE_2 enzyme immunoassay kits (Cayman Chemical) according to the manufacturer's instructions.

Transcription factor activation assay

The fusion transactivator plasmids (pFA-CHOP, pFA2-Elk-1, and pFA2-cJun) consisting of the DNA binding domain of Gal4 (residues 1–147) and the transactivation domains of CHOP, Elk-1, or c-Jun were purchased from Stratagene (La Jolla, California, USA). pFA2-dbd, which contains only the DNA binding domain of Gal4 (residues 1–147), was used as the negative control. Experiments were performed according to the manufacturer's instructions. The cells were seeded at 10^5 cells per well in 12-well plates and grown to 70–80% confluence in complete growth media containing 10% fetal bovine serum (FBS). The cells were cotransfected with 0.05 μg of fusion transactivator plasmids, 0.5 μg pFR-Luc plasmid, and 0.3 μg pSV-β galactosidase control vector using Lipofectamine Plus according to the manufacturer's instructions

(Life Technologies, Gaithersburg, Maryland, USA). After 4 h, a medium containing 10% FBS was added and the cells were incubated for an additional 20 h. Subsequently, the cells were treated for 24 h with inhibitors as indicated. In the case of cotransfection with siRNA against COX-2, the cells were cotransfected with 0.05 μg fusion transactivator plasmid, 0.5 μg pFR-Luc plasmid, and 100–200 nmol/l siRNA. After a total of 48 h, the cells were analyzed using a transcription factor activation assay. Luciferase activity was measured using a TR717 microplate luminometer with a Bioluminescent Reporter Gene Assay kit, according to the manufacturer's instructions (Tropix, Bedford, Massachusetts, USA). Luciferase activity was normalized in relation to cotransfection with a pSV-β galactosidase control vector.

Transfection of MEK1 and MEK3 expressing plasmids

The MAPK/ERK kinase 1 (MEK1)-expressing and MEK3-expressing plasmids (pFC-MEK1 and pFC-MEK3) were purchased from Stratagene. The expression and activity of these plasmids was confirmed by transcription factor activation assays. The cells were transfected with 0.3 μg pFC-MEK1 (for activation of ERK) or pFC-MEK3 (for that of p38) using Lipofectamine Plus according to the manufacturer's instructions (Life Technologies). After 4 h, a medium containing 10% FBS was added and the cells were incubated for an additional 68 h. At 72 h, the cells were subjected to cell proliferation assays. To check ERK and p38 activation, the cells were cotransfected with 0.3 μg pFC-MEK1 or pFC-MEK3, 0.05 μg of the fusion transactivator plasmids, 0.5 μg pFR-Luc plasmid, and 0.3 μg pSV-β-galactosidase control vector using Lipofectamine Plus according to the manufacturer's instructions (Life Technologies). After 4 h, a medium containing 10% FBS was added and the cells were incubated for an additional 44 h. pFC2-dbd was used as the negative control.

Statistical analysis

The data are presented as the mean ± standard deviation (SD) of triplicates, or as a representative of three separate experiments. The levels of significance were determined between treated and untreated groups by the two-sided Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

Results

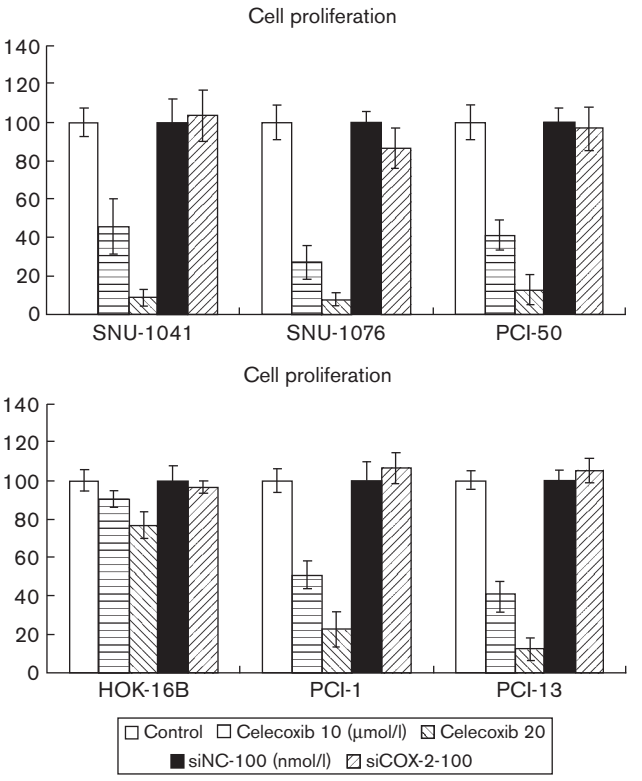
The COX-2-independent growth inhibitory effect of celecoxib in several HNSCC cell lines

We observed that the anti-cancer effect of celecoxib was not related to the degree of COX-2 expression in HNSCC cell lines. Among the cell lines tested, three had high COX-2 expression (SNU-1041, SNU-1076, and PCI-50) and the rest showed low COX-2 expression (PCI-1, PCI-13, and HOK16B). Although both siRNA of COX-2 and celecoxib completely inhibited PGE_2 synthesis (data not shown), only celecoxib showed growth-inhibitory effects irrespective of the degree of COX-2 expression in tested HNSCC cells (Fig. 1a and b). This direct growth-inhibitory effect of celecoxib was more outstanding in HNSCC cells than in HOK-16B, a non-cancerous cell line.

The effect of celecoxib on three MAPKs activity in HNSCC cells

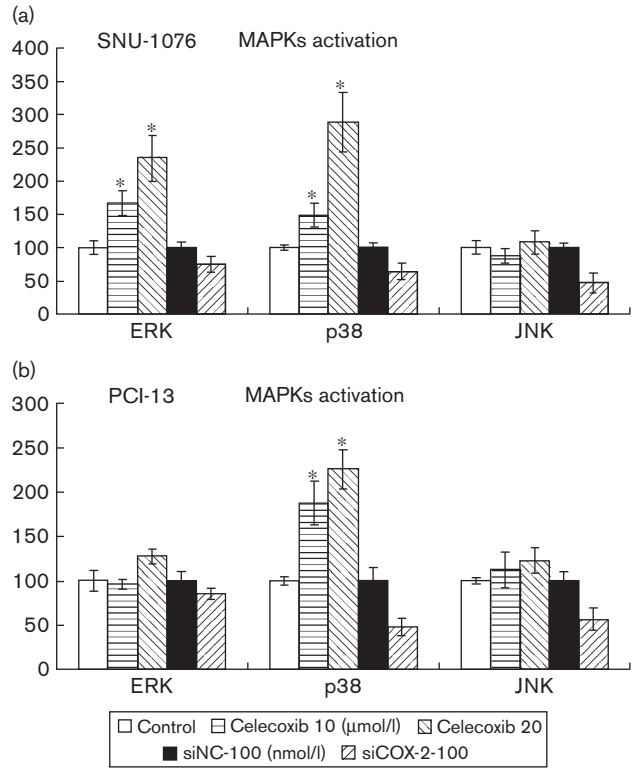
Considering our results shown in Fig. 1, to confirm that the regulation of MAPKs activity might be one of the common events related to anti-cancer effects of celecoxib in HNSCC cells, we investigated the effects of celecoxib on Elk-1 (for ERK), CHOP (for p38), and c-Jun (for c-Jun NH2-terminal kinase) through a transcription factor activation assay. Celecoxib increased the activity of ERK (by 70–170%) and p38 MAPK (by 50–250%) in a dose-dependent manner, but not c-Jun NH2-terminal kinase, whereas siRNA against COX-2 showed moderate inhibitory action (by 20–50%) on all three MAPKs in SNU-1076 (with high COX-2 expression) (Fig. 2a) and PCI-13 (with little COX-2 expression) (Fig. 2b).

Fig. 1



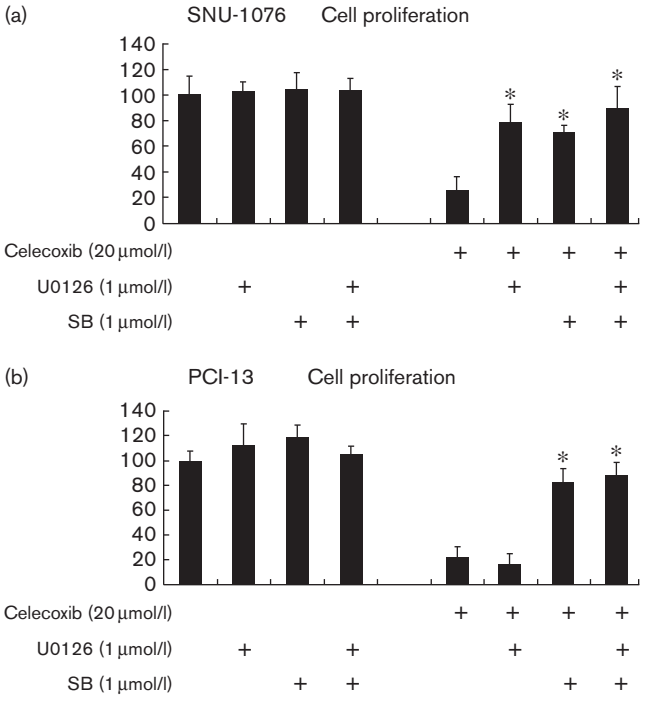
The cyclooxygenase-2 (COX-2)-independent growth inhibitory effect of celecoxib in several head and neck squamous cell carcinoma cell lines and HOK-16B. Cells were treated with celecoxib at indicated concentrations (micromole per liter) and transfected with the small interfering RNAs of COX-2 at 100 nmol/l doses. The small interfering RNA for negative control (siNC) was used for small interfering RNA control. At 72 h, cells were subjected to cell proliferation assays. Results are expressed as percentage relative to control (% of control) (HOK-16B is a non-cancerous cell line from pharyngeal mucosa).

Fig. 2



The effect of celecoxib on three mitogen-activated protein kinases activity in head and neck squamous cell carcinoma cells. The effect of cyclooxygenase-2 (COX-2) inhibition on mitogen-activated protein kinases was tested against SNU-1076 (a) and PCI-13 cells (b). Cells were transfected with reporter plasmids to detect signaling activity as described in the 'Methods' section. Then at 24 h, cells were treated with indicated doses of celecoxib for an additional 24 h. In the case of cotransfection with small interfering RNA (siRNA) against COX-2, cells were cotransfected by indicated siRNA and plasmids for detecting the activity of signaling as described in the 'Methods' section. The small interfering RNA for negative control (siNC) was used for siRNA control. At 24 h after transfection, culture media was replaced by fresh medium. Then, at 48 h, total cell lysates were prepared and used to determine luciferase activities. Results are expressed as percentage relative to control (% of control). *P* values were based on comparison with control (*$P<0.01$). ERK, extracellular signal-regulated kinase ; JNK, c-Jun NH2-terminal kinase.

Fig. 3



The role of mitogen-activated protein kinases (MAPKs) activation in the growth-inhibitory action of celecoxib in head and neck squamous cell carcinoma cell lines. The effect of MAPKs inhibition on the growth-inhibitory effects of celecoxib in SNU-1076 (a) and PCI-13 (b) was measured by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide assay. Cells were treated singly or in combination with celecoxib, U0126 (an extracellular signal-regulated kinase inhibitor), and/or SB202190 (a p38 MAPK inhibitor), with the indicated concentrations (micromole per liter). At 48 h, cells were subjected to cell proliferation assays. Results are expressed as percentage relative to control (% of control). *P* values were based on comparison with celecoxib-treated group (*$P<0.01$).

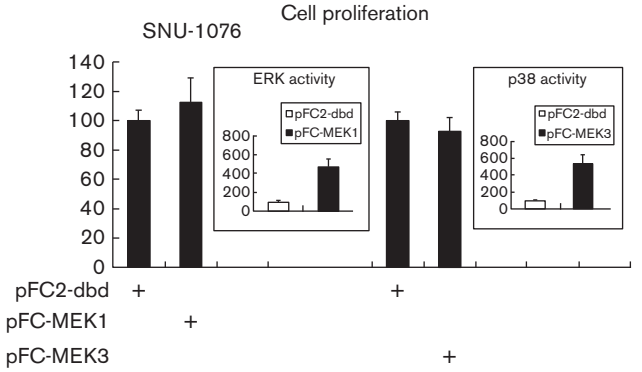
The role of MAPKs activation in the growth-inhibitory action of celecoxib in HNSCC cell lines

The possibility that MAPKs activation by celecoxib might be related to its tumor-killing effect in HNSCC cell lines was investigated. When U0126 (an ERK inhibitor) and SB202190 (a p38 inhibitor) were added to cells treated with celecoxib, both inhibitors decreased significantly (by 70–90%) the tumor-killing effect of celecoxib in SNU-1076 (Fig 3a). However, in PCI-13, p38 inhibition significantly decreased (by 70–90%) the tumor-killing effect of celecoxib, but not ERK inhibition (Fig. 3b). This is in agreement with findings that celecoxib increased ERK and p38 activity in SNU-1076 (Fig. 2a), whereas it activated only p38 MAPK in PCI-13 (Fig. 2b). From these observations, it seems that MAPKs activation has important roles in the growth-inhibitory action of celecoxib in HNSCC cells. ERK and p38 inhibition has little effect on cell proliferation of HNSCC cells under basic conditions. Effective inhibition of ERK and p38 activity by U0126 and SB202190 was assessed by an Elk-1 and CHOP transcription factor activation assay (Supplementary data 2).

Effect of ERK and p38 activation by their upstream inducers on HNSCC cell proliferation

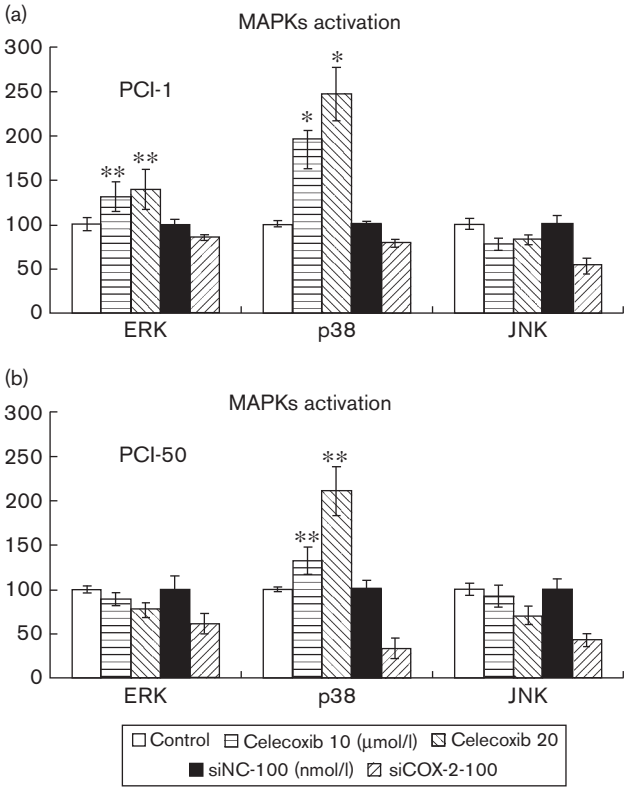
From the above findings, we wondered if excessive activation of ERK and/or p38 by any exogenous inducers might induce cell death of HNSCC cells. We attempted to activate them directly with the respective upstream inducers of the MAPKs signaling cascade (MEK1 for ERK and MEK3 for p38 activation). Then, ERK and p38 were activated by transfection with plasmids expressing MEK1 (pFC-MEK1) and MEK3 (pFC-MEK3), respectively. Even though activation of ERK and p38 (400–600%) was confirmed, we did not observe any cell growth inhibition in SNU-1076 (Fig. 4) and PCI-13 (Supplementary data 3).

Fig. 4



Effect of extracellular signal-regulated kinase (ERK) and p38 activation by their upstream inducers on head and neck squamous cell carcinoma cell proliferation. SNU-1076 cells were transfected with 0.3 μg pFC-MAPK/ERK kinase 1 (MEK1) (for activation of ERK) or pFC-MEK3 (for activation of p38). At 72 h, cells were subjected to cell proliferation assays. To check ERK and p38 activation (inner panels), SNU-1076 cells were cotransfected with 0.3 μg pFC-MEK1 (for activation of ERK) or pFC-MEK3 (for that of p38) and reporter plasmids to detect signaling activity as described in the 'Methods' section. Then, at 48 h, total cell lysates were prepared and used to determine luciferase activities. pFC2-dbd was used as the negative control. Results are expressed as percentage relative to control (% of control).

Fig. 5



The effect of celecoxib on three mitogen-activated protein kinases (MAPKs) activity in PCI-1 and PCI-50. The effect of cyclooxygenase-2 (COX-2) inhibition on MAPKs was tested against PCI-1 (a) and PCI-50 cells (b). Cells were transfected with reporter plasmids to detect signaling activity as described in the 'Methods' section. Then at 24 h, cells were treated with indicated doses of celecoxib for an additional 24 h. In the case of cotransfection with small interfering RNA (siRNA) against COX-2, cells were cotransfected by indicated siRNA and plasmids for detecting activity of signaling as described in the 'Methods' section. The small interfering RNA for negative control (siNC) was used for siRNA control. At 24 h after transfection, culture media was replaced by fresh medium. Then, at 48 h, total cell lysates were prepared and used to determine luciferase activities. Results are expressed as percentage relative to control (% of control). *P* values were based on comparison with control (*$P<0.01$; **$P<0.05$). ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase.

The role of MAPKs activation in the growth-inhibitory action of celecoxib in other HNSCC cell lines

In addition to comprehensively evaluating the key roles of ERK and p38 activation in COX-2-independent tumor-killing action of celecoxib in HNSCC cells, we checked ERK and p38 activation by celecoxib in PCI-1 (low COX-2 expression) and PCI-50 (high COX-2 expression). Similar to the results observed for SNU-1076 and PCI-13, celecoxib specifically increased the activity of p38 MAPK (by 50–170%) in both cell lines and slightly upregulated ERK activity (by 30–40%) in PCI-1 (Fig. 5a and b), whereas siRNA against COX-2 did not show any inducing action on MAPKs. Then, we observed that SB202190 (a p38 inhibitor) decreased the tumor-killing effect of celecoxib in both cell lines significantly (by 70–90%) (Fig. 6a and b). From these data, we identified that the outstanding tumor-killing effect of celecoxib occurred through at least p38 MAPK activation in HNSCC cells.

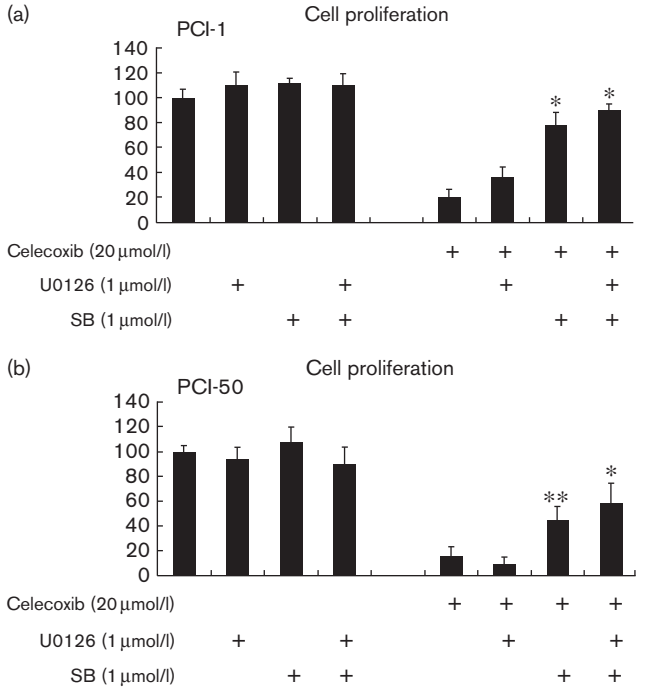
The effect of celecoxib on three MAPKs activity in pancreatic cancer cell-1 and A549 cells

As described in 'Methods' section, A549 is a cell line originating from a human lung carcinoma, and PANC-1 originating from a human carcinoma of the exocrine pancreas. We tested MAPKs activation (such as ERK and p38) as one pathway of COX-2-independent action of celecoxib in other types of cancer cells. Although celecoxib killed PANC-1 (low COX-2 expression) and A549 (moderate COX-2 expression) irrespective of the degree of COX-2 expression (Supplementary data 4), there was no significant activation of MAPKs in these cells (Fig. 7a and b).

The role of MAPKs activation in the growth-inhibitory action of dup-697 in HNSCC cell lines

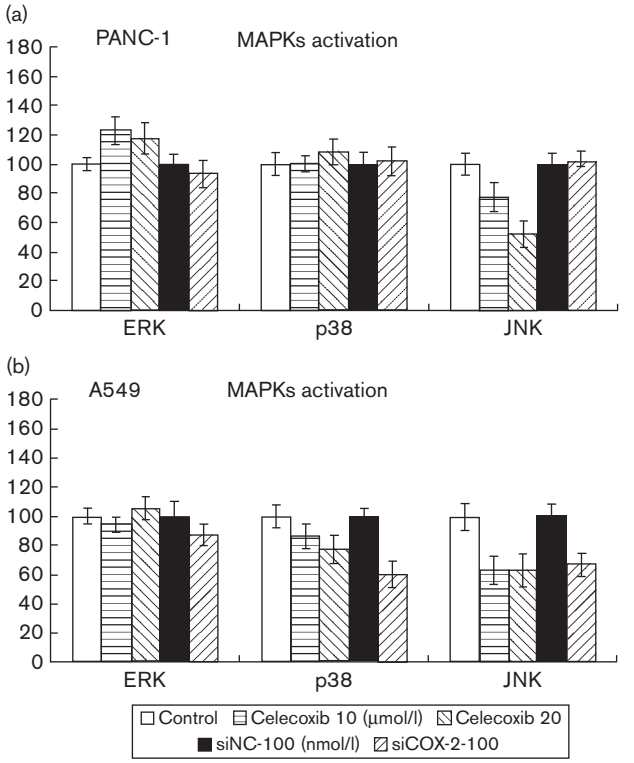
In contrast to the effects of celecoxib, dup-697, another COX-2 inhibitor, showed differential effects on MAPKs activity in HNSCC (Supplementary data 5). Although celecoxib-activated p38 MAPK, dup-697 increased p38 activity in SNU-1076, PCI-1, and PCI-50 but decreased slightly in PCI-13. In addition, dup-697 activated ERK in SNU-1041 and PCI-13. However, additional treatment of U0126 and SB201290 could not reverse the growth-inhibitory effects of dup-697, contrary to growth inhibition by ERK and p38 activation in the tumor-killing process of celecoxib (Supplementary data 6).

Fig. 6



The role of mitogen-activated protein kinases (MAPKs) activation in the growth-inhibitory action of celecoxib in other head and neck squamous cell carcinoma cell lines. The effect of MAPKs inhibition on the growth-inhibitory effects of celecoxib in PCI-1 (a) and PCI-50 (b) was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide assay. Cells were treated singly or in combination with celecoxib, U0126 (an extracellular signal-regulated kinase inhibitor), and SB202190 (a p38 MAPK inhibitor) with the indicated concentrations (micromole per liter). At 48 h, cells were subjected to cell proliferation assays. Results are expressed as percentage relative to control (% of control). *P* values were based on comparison with celecoxib-treated group (*$P<0.01$; **$P<0.05$).

Fig. 7



The effect of celecoxib on three mitogen-activated protein kinases (MAPKs) activity in pancreatic cancer cell-1 (PANC-1) and A549 cells. The effect of cyclooxygenase-2 (COX-2) inhibition on MAPKs was tested against PANC-1 (a) and A549 (b). Cells were transfected with reporter plasmids to detect signaling activity as described in the 'Methods' section. Then at 24 h, cells were treated with indicated doses of celecoxib for an additional 24 h. In the case of cotransfection with small interfering RNA (siRNA) against COX-2, cells were cotransfected by indicated siRNA and plasmids for detecting activity of signaling as described in the 'Methods' section. The small interfering RNA for negative control (siNC) was used for siRNA control. At 24 h after transfection, culture media was replaced by fresh medium. Then, at 48 h, total cell lysates were prepared and used to determine luciferase activities. Results are expressed as percentage relative to control (% of control). ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase.

Discussion

Earlier observations have shown that COX-2 inhibition showed several anti-cancer effects resulting from COX-2-independent action, followed by growth inhibition. These include the inhibition of β-catenin translocation to the nucleus, the activation and inhibition of MAPKs, blocking Akt activation, regulation of 15-lipoxygenase-1 and 13-S-HODE, induction of phosphodiesterases, activation of peroxisome proliferation activated receptor, regulation of nuclear factor-κB activity, and induction of nonsteroidal anti-inflammatory drug-associated genes [6,19,21,22]. Although some researchers have reported earlier that COX-independent action of COX-2 inhibitors may stimulate cell viability in pancreatic cancer without COX-2 expression [23], most of the anti-cancer effects by COX-independent action of COX-2 inhibitors were found to be direct cell growth inhibition. In contrast, promoting cell proliferation directly by COX-2 and PGs seemed to be restricted to very few types of cancer [24–26]. In our own experiment with HNSCC cell lines, we observed that tumor-killing effects of COX-2 inhibitors were mediated through COX-2-independent mechanisms, compared with the COX-2-dependent mechanisms of siRNA against COX-2.

Herein, focusing on MAPKs activation by COX-2 inhibition, we investigated the possible participation of MAPKs activation in COX-independent action of celecoxib in HNSCC cell lines. From the observations on important roles of MAPKs in the regulation of cell proliferation and apoptosis by a variety of endogenous and exogenous events [13–15], we assumed the possibility that various unexpected actions of celecoxib showing outstanding COX-independent actions, even in cells without COX-2 expression, might be mediated through the activation of some MAPKs. Earlier, some researchers reported that NS-398 induced apoptosis through ERK activation in cancer cells [19], whereas others observed an opposite finding that NS-398-activated ERK might have an inhibitory

effect on the anti-cancer effect of NS-398 [27]. In addition, these various actions were observed in cases of other COX-2 inhibitors [4,28].

In this study using HNSCC models, we showed upregulation of ERK and p38 related to the tumor-killing action of celecoxib, however, other groups have reported the possible involvement of the downregulated activity of MAPKs by celecoxib in its COX-2-independent action in other cancer models [29,30]. Considering earlier findings regarding the various effects of COX-2 inhibitors on MAPKs, it was thought that COX-2 inhibitors can affect MAPKs activity in different ways, depending on both the applied kinds of inhibitors and cell types. In addition, even if some MAPKs are activated by COX-2 inhibitors, altered MAPKs seem to function differently in the COX-independent action of COX-2 inhibitors. Although dup-697 and celecoxib activated ERK or p38 in some HNSCC cells (Supplementary data 5), blocking ERK or p38 activity reversed cell proliferation in celecoxib-treated groups but not in dup-697-treated groups, suggesting unknown differences in action between two inhibitors. The activation of p38 MAPK mediated celecoxib's action on cell growth-inhibition in all tested HNSCC cells; however, dup-697 showed promoting effects on p38 activity in some cell lines. In addition, in experiments using a PANC-1 and a lung cancer cell line (A549) available in our laboratory, it was observed that celecoxib generally activated p38 MAPK in HNSCC. Namely, it seems that MAPKs activation might have more important roles in HNSCC cells than in other cells for the growth-inhibitory action of celecoxib. Considering that excessive activation of ERK and/or p38 by any exogenous inducers could not induce cell death of HNSCC cells differently in the case of celecoxib (Fig. 4 and Supplementary data 3), concise investigation of the mechanisms of action of celecoxib-induced MAPKs activation will provide a good opportunity to find a new effective modality for cancer therapy.

A role of the upregulation of ERK and p38 by celecoxib has been described by few studies until now. In this study, we first suggest the participation of ERK and/or p38 activation in growth inhibition by a COX-independent action of celecoxib in HNSCC models. Our study encourages more concise investigation on underlying mechanisms and detailed outcomes of MAPKs activation by celecoxib for using its excellent tumor-killing effect more safely in the clinical field of cancer treatment such as HNSCC.

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