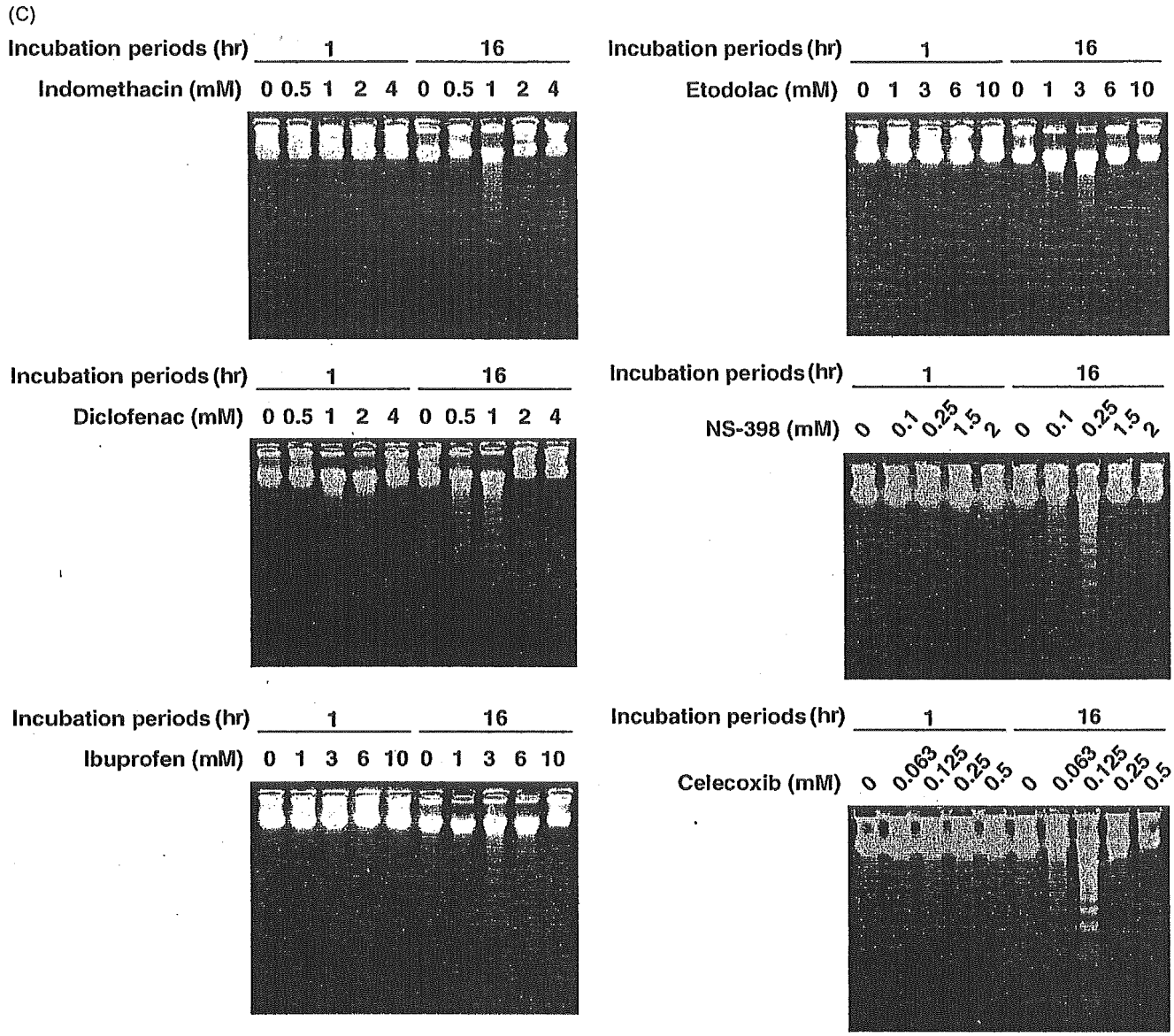

Fig. 1. (*Continued*).

2.5. Statistical analysis

All values are expressed as the mean ± standard error (SEM). One-way ANOVA followed by Scheffe's multiple comparison was used for evaluation of differences between the groups. A Student's t test for unpaired results was performed for the evaluation of differences between two groups. Differences were considered to be significant for values of $P < 0.05$.

3. Results

3.1. In vitro necrosis and apoptosis induced by various NSAIDs, and their relationship with COX inhibition

We previously reported that short-term (1 hr) treatment of primary cultures of guinea pig gastric mucosal cells with high concentrations of NSAIDs (indomethacin, 2.5 mM) and long-term (16 hr) treatment of these cells with low concentrations of NSAIDs (indomethacin, 1 mM) induced necrosis and apoptosis, respectively [30]. In the present study, selective COX-2 inhibitors (etodolac, NS-398, celecoxib, and rofecoxib) and non-selective NSAIDs (or slightly selective NSAIDs for COX-1 or COX-2) (indomethacin, diclofenac, and ibuprofen) were tested for their ability to induce necrosis and apoptosis *in vitro*. Necrosis and apoptosis were assessed on the basis of the presence and absence, respectively, of apoptotic DNA fragmentation and caspase 3 activation. The decrease in cell viability with short-term (1 hr) NSAIDs treatment (Fig. 1A) is not associated with apoptotic DNA fragmentation (Fig. 1C) and caspase 3 activation (Table 1), suggesting that it is mediated by necrosis. To confirm this finding, we carried out double-staining experiments with propidium iodide and Hoechst 33342. Since necrotic cells lose

Table 1
Activation of caspase 3 by NSAIDs

Incubation (hr)	NSAIDs	Caspase 3-like activity (U/mg protein)
1	Control	10 ± 3
	4 mM indomethacin	11 ± 1
	4 mM diclofenac	13 ± 6
	10 mM ibuprofen	13 ± 2
	10 mM etodolac	10 ± 3
	2 mM NS-398	11 ± 5
	0.5 mM celecoxib	10 ± 5
	6 mM rofecoxib	10 ± 4
16	Control	14 ± 4
	1 mM indomethacin	525 ± 23***
	1 mM diclofenac	564 ± 50***
	3 mM ibuprofen	591 ± 32***
	3 mM etodolac	455 ± 29***
	0.25 mM NS-398	473 ± 33***
	0.125 mM celecoxib	637 ± 61***
	3 mM rofecoxib	29 ± 8

Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of various NSAIDs for 1 or 16 hr. Activities of caspase 3 were examined by the use of specific fluorogenic peptide substrates (Ac-DEVD-MCA). Values are mean ± SEM (N = 3).
***$P < 0.001$.

their membrane integrity, propidium iodide staining causes pink nuclear staining in necrotic cells, whereas living cells and apoptotic cells are not stained with propidium iodide. We previously reported that 1 hr treatment with 2.5 mM indomethacin caused pink nuclear staining [30]. We here performed the same type of experiment for other NSAIDs used in Fig. 1 and found that 1 hr treatment with 4 mM indomethacin, 4 mM diclofenac, 10 mM ibuprofen, 10 mM etodolac, 2 mM NS-398, or 0.5 mM celecoxib caused pink nuclear staining (data not shown), strongly suggesting that cell death shown in Fig. 1A is mediated through necrosis. In contrast, the decrease in cell viability with long-term (16 hr) NSAIDs treatment (Fig. 1B) is associated with apoptotic DNA fragmentation (Fig. 1C) and caspase 3 activation (Table 1), suggesting that it is mediated by apoptosis. Among all of NSAIDs tested here, only rofecoxib induced neither necrosis nor apoptosis (Fig. 1). Interestingly, apoptosis induced by NS-398 was greatest at a concentration of 0.25 mM and the higher concentrations of NS-398 caused the less induction. We have no explanation for this phenomenon at present. We consider that the concentrations of NSAIDs required for necrosis and apoptosis *in vitro* are possible *in vivo* associating with gastric ulceration in animal models, as discussed in our previous paper [30]. However, it is unclear whether gastric mucosal cells can be exposed to NSAIDs as long as 16 hr in animal models. Furthermore, it is also unclear whether these concentrations are relevant for clinical use of NSAIDs. There seemed to be no direct relationship between the cytotoxicity (concentrations of NSAIDs required for necrosis and apoptosis) and the selectivity for COX-2 of NSAIDs.

The level of PGE_2 in the medium upon treatment of cells with various NSAIDs was measured. Compared to non-selective NSAIDs, higher concentrations of selective COX-2 inhibitors were required for inhibiting PGE_2 synthesis, being consistent with the idea that the majority of COX activity is derived from COX-1 activity in gastric mucosal cells [10,11]. Comparing results (Figs. 1 and 2), it is clear that there is no relationship between them in terms of their cytotoxicity and their ability to inhibit PGE_2 synthesis. For example, celecoxib was the strongest compound in terms of cytotoxicity, but the weakest for inhibiting PGE_2 synthesis. Therefore, it seems that the cytotoxic

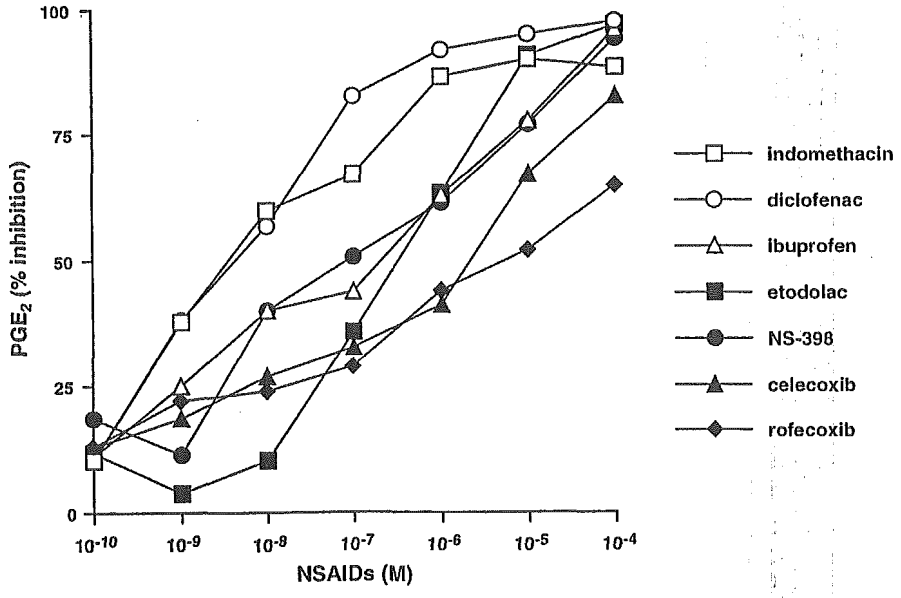

Fig. 2. Inhibition of PGE_2 synthesis by exposure to various NSAIDs in cultured cells. Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of various NSAIDs for 30 min, following which the levels of PGE_2 in the media were determined by ELISA. Values are expressed as relative to control (without NSAIDs) (2×10^{-9} M).

effects of NSAIDs (necrosis and apoptosis) are independent of their ability to inhibit COX. For further confirmation of this point, we examined the effect of exogenously added PGE_2 on necrosis and apoptosis induced by indomethacin. Exogenously added PGE_2 did not affect the extent of cell death by short-term or long-term treatment with indomethacin (necrosis or apoptosis, respectively) even at higher concentrations of PGE_2 than is present endogenously in medium (10^{-9} M) (Fig. 3).

It was recently reported that prostacyclin (PGI_2) protects cells from apoptosis [36,37]. Therefore, we also measured the level of PGI_2 in the medium. Since PGI_2 is very unstable in medium, we determined the level of 6-keto-$PGF_{1\alpha}$ (metabolite of PGI_2) instead of PGI_2. In the absence of NSAIDs, the concentration of 6-keto-$PGF_{1\alpha}$ in the medium was 0.7 nM. The IC_{50} value of indomethacin and celecoxib for inhibiting 6-keto-$PGF_{1\alpha}$ synthesis was about 5×10^{-9} M. We also examined the effect of exogenously added PGI_2 on necrosis and apoptosis induced by indomethacin. Due to the instability of PGI_2 in medium, we used carbaprostacyclin (stable analogue of PGI_2) instead of PGI_2. Exogenously added carbaprostacyclin did not affect the extent of cell death by short-term or long-term treatment with indomethacin (necrosis or apoptosis, respectively) (Fig. 3C and D). These results suggest that inhibition of PGI_2 synthesis by NSAIDs is not involved in NSAID-induced necrosis and apoptosis.

3.2. Development of gastric lesions by a combination of the oral administration of selective COX-2 inhibitors with the intravenous administration of non-selective NSAIDs

We considered that not only COX inhibition (inhibition of PG synthesis) but also the COX-independent direct cytotoxic effect of NSAIDs is involved in the development of gastrointestinal lesions *in vivo*. For testing this idea by pharmacological experiments, it is necessary to separate

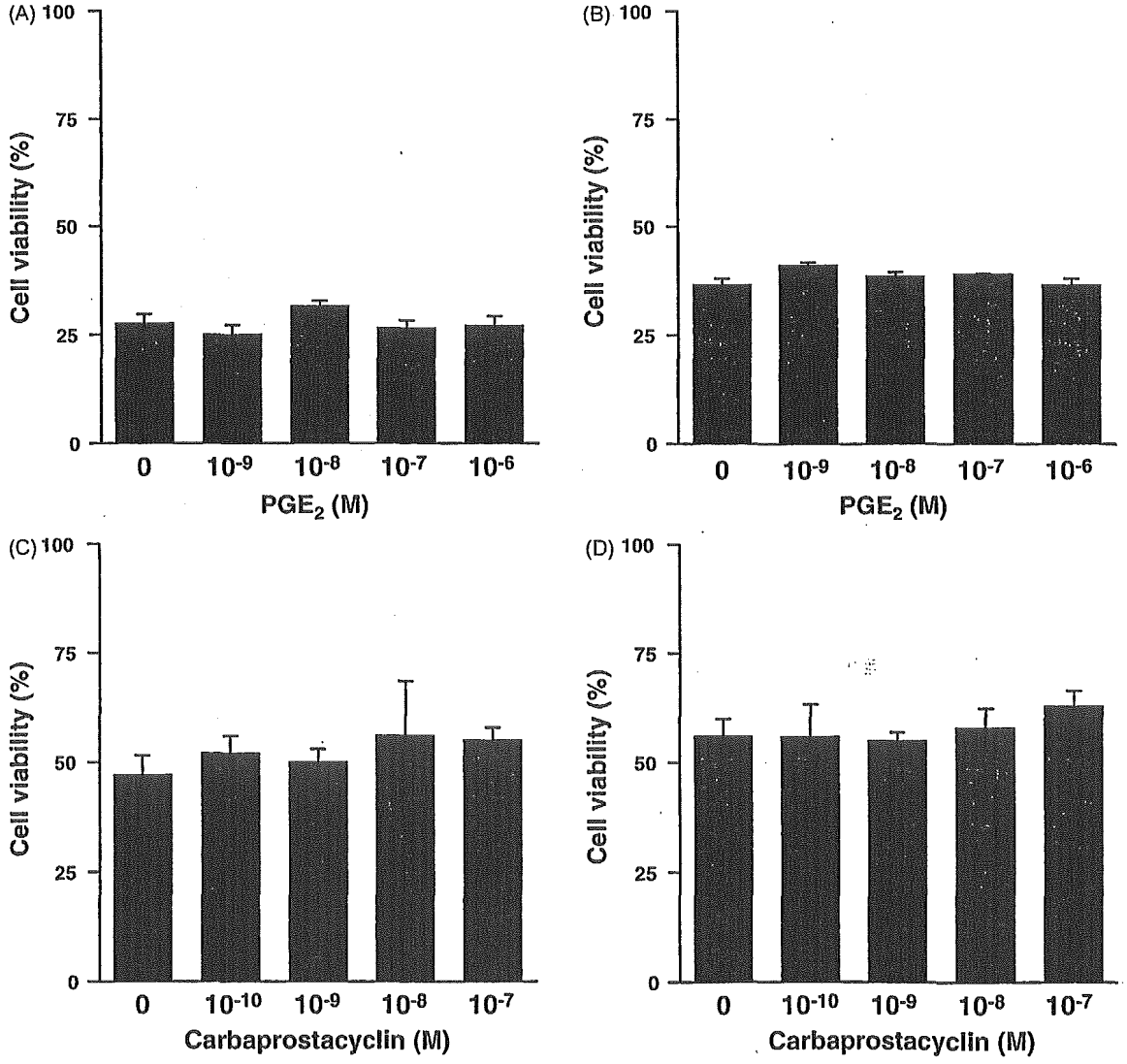

Fig. 3. Effect of PGE_2 on necrosis and apoptosis induced by NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with 2.5 mM (A, C) or 0.9 mM (B, D) indomethacin for 1 hr (A, C) or 16 hr (B, D) in the presence of indicated concentrations of PGE_2 (A, B) or carbaprostacyclin (C, D). Cell viability was determined by the trypan blue exclusion test. Values are mean ± SEM (N = 3). Similar results were obtained by MTT assay.

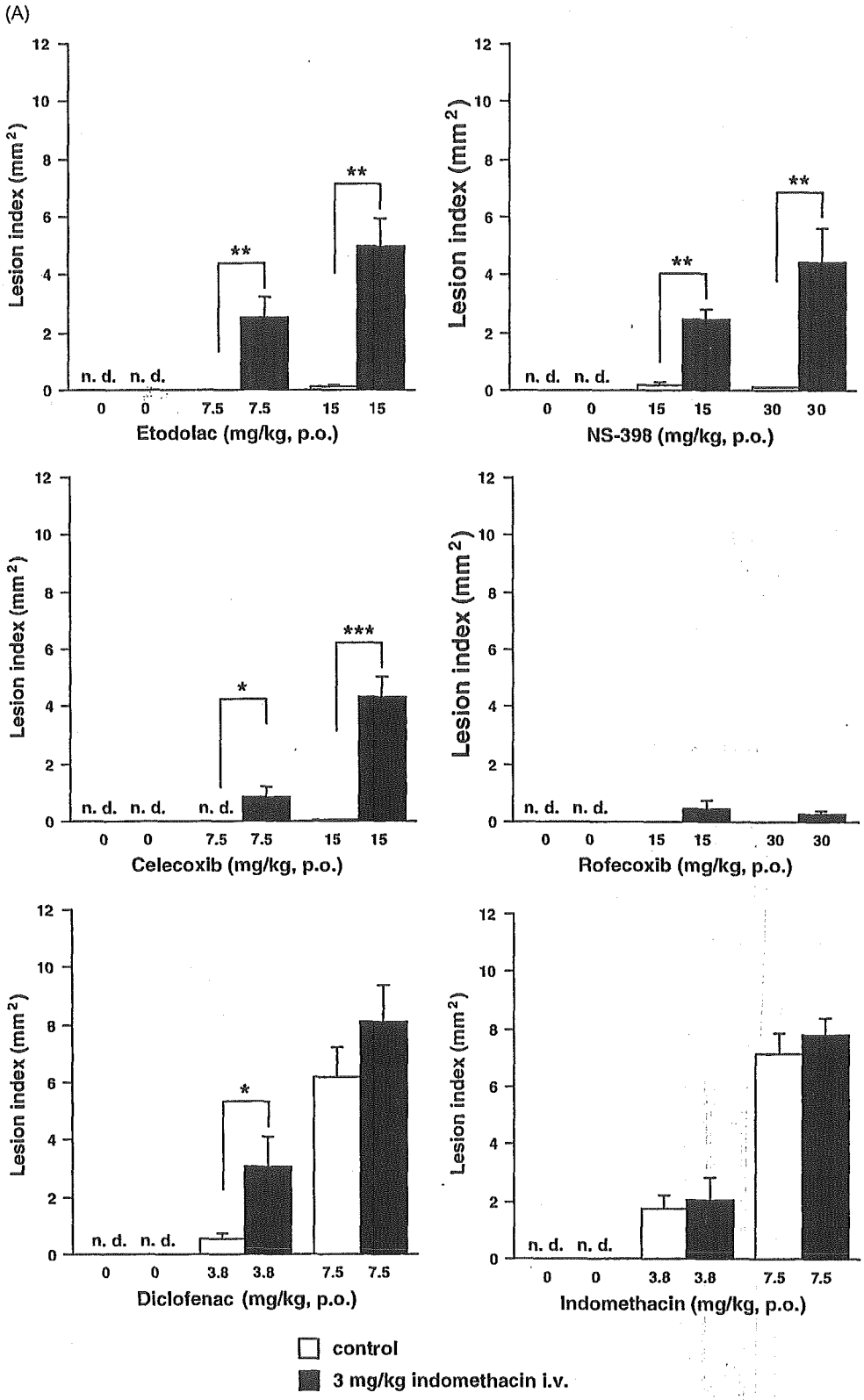

Fig. 4. Production of gastric lesions in rats. Rats were intravenously administered with 3 mg/kg indomethacin (A), both 3 mg/kg indomethacin and indicated dose of etodolac (B), 100 mg/kg aspirin (C), or vehicle. After 1 hr, animals were orally administered with NSAIDs as indicated or vehicle (A, C) (no oral administration (B)). After 6 hr, the stomach was removed and scored for hemorrhagic damage. Values are mean ± SEM (N = 6). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ (both intravenously and orally administered groups vs. only orally administered groups). n.d.; not detected.

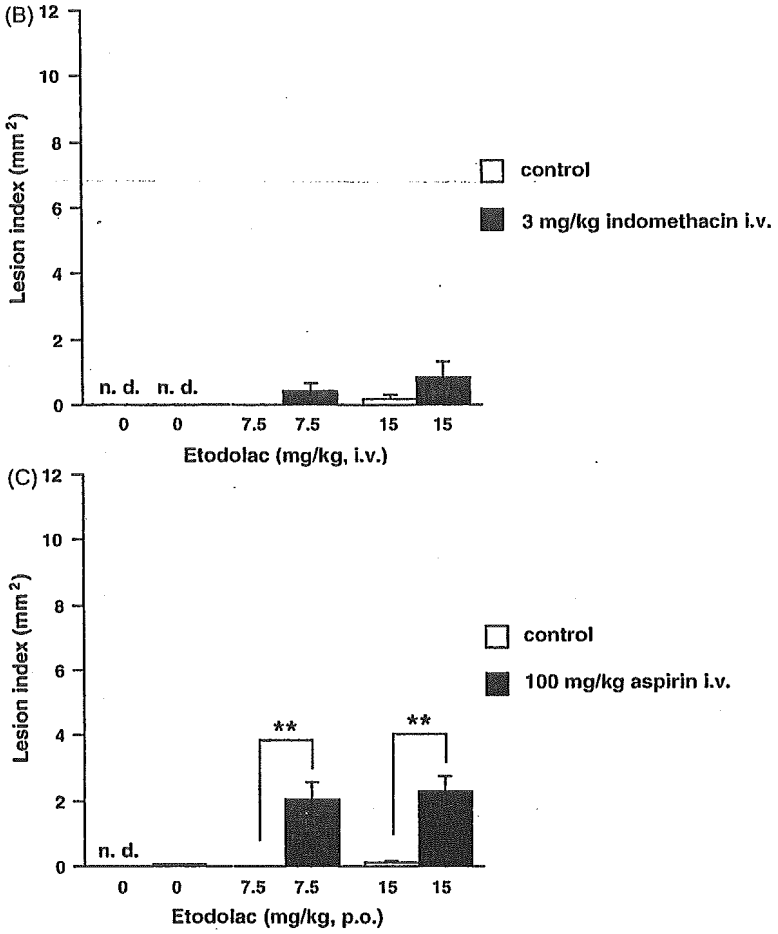

Fig. 4. (*Continued*).

these two properties of NSAIDs (i.e. COX inhibition and direct cytotoxicity) in the model of NSAID-induced gastric lesions *in vivo*. We tried to achieve this by employing intravenous administration of a non-selective NSAID (indomethacin) and oral administration of selective COX-2 inhibitors in rats. Intravenous administration of non-selective NSAIDs may cause inhibition of both COX-1 and COX-2 (thus inhibition of PG synthesis) at the gastric mucosa without any direct cytotoxicity to the gastric mucosa, because the concentration of NSAIDs at the gastric mucosa following intravenous administration is much lower compared to when NSAIDs are orally administered. On the other hand, oral administration of selective COX-2 inhibitors (except rofecoxib) may cause direct cytotoxicity to the gastric mucosa without inhibition of COX-1, and thus PG synthesis may be maintained.

Intravenous administration of indomethacin (3 mg/kg) in rats did not produce gastric lesions (Fig. 4A) even though the level of PGE_2 at the gastric mucosa was reduced by more than 90% (Table 2). These data suggest that inhibition of COX is not sufficient to produce gastric lesions. On the other hand, oral administration of selective COX-2 inhibitors (etodolac, NS-398, celecoxib, and rofecoxib) did not by themselves (i.e. without intravenous administration of indomethacin) produce gastric lesions (Fig. 4A). PGE_2 synthesis at the gastric mucosa was not inhibited by the oral administration of these selective COX-2 inhibitors (except for weak inhibition by 15 mg/kg etodolac) (Table 1). Therefore, the absence of gastric lesions only by oral administration of these selective COX-2 inhibitors can be explained by the fact that inhibition of PG synthesis is required for the development of gastric lesions by NSAIDs.

Table 2
Inhibition of gastric PGE_2 synthesis by NSAIDs *in vivo*

NSAIDs	Gastric PGE_2 (ng/g tissue)
Control	26.8 ± 1.0
3 mg/kg indomethacin i.v.	1.4 ± 0.4***
100 mg/kg aspirin i.v.	1.4 ± 0.2***
3.8 mg/kg indomethacin p.o.	2.3 ± 0.4***
3.8 mg/kg diclofenac p.o.	12.2 ± 1.1***
7.5 mg/kg diclofenac p.o.	3.5 ± 1.5***
7.5 mg/kg etodolac p.o.	18.2 ± 3.8
15 mg/kg etodolac p.o.	5.1 ± 1.4***
30 mg/kg NS-398 p.o.	20.2 ± 3.2
15 mg/kg celecoxib p.o.	25.3 ± 1.3
30 mg/kg rofecoxib p.o.	19.9 ± 4.1

Rats were intravenously (i.v.) or orally (p.o.) administered with indicated doses of NSAIDs. After 6 hr (p.o.) or 7 hr (i.v.), the level of PGE_2 in gastric mucosa was determined by ELISA. Values are mean ± SEM (N = 4–6).
***$P < 0.001$.

Interestingly, a combination of intravenous administration of indomethacin and oral administration of COX-2-selective inhibitors (except rofecoxib) clearly produced gastric lesions (Fig. 4A). On the other hand, a combination of intravenous administration of indomethacin and oral administration of rofecoxib did not significantly produce gastric lesions (Fig. 4A). We repeated experiments in Fig. 4A using piroxicam instead of indomethacin and obtained similar results (data not shown). Since among all of COX-2-selective inhibitors, only rofecoxib did not show direct cytotoxicity *in vitro* (Fig. 1), results in Fig. 4A suggest that direct cytotoxicity of NSAIDs is involved in production of gastric lesions. However, since it was recently proposed that inhibition of both COX-1 and COX-2 is required for the development of gastric lesions by NSAIDs [17,19,20], and indomethacin has a weak selectivity for COX-1 (1:0.3) [38], one can argue that the inhibition of COX-2 by intravenously administered indomethacin was not enough and that orally administered selective COX-2 inhibitors inhibited the remaining COX-2 activity, thereby resulting in the development of gastric lesions. However, this possibility was ruled out by an experiment which showed that intravenous administration of both indomethacin (3 mg/kg) and etodolac (15 mg/kg), which must inhibit both COX-1 and COX-2 [39], did not produce gastric lesions (Fig. 4B). We also showed that a combination of intravenous administration of indomethacin and oral administration of SC-560 (selective COX-1 inhibitor) produced gastric lesions (data not shown). Furthermore, we confirmed that the level of PGE_2 at the gastric mucosa with intravenous administration of indomethacin was not further decreased by oral administration of COX-2-selective inhibitors (data not shown). These combined results support our idea that not only COX inhibition (inhibition of PG synthesis) but also the COX-independent direct cytotoxic effect of NSAIDs is involved in the development of gastric lesions *in vivo*.

On the other hand, oral administration of indomethacin did produce gastric lesions without intravenous administration of indomethacin (Fig. 4A). Based on the hypothesis described above, these data can be explained by the fact that orally administered indomethacin had not only a direct cytotoxic effect but also resulted in COX inhibition (inhibition of PG synthesis) (Table 2), and thus produced gastric lesions without intravenous administration of indomethacin. Production of gastric lesions by oral administration of 3.8 but not 7.5 mg/kg diclofenac was increased by the prior intravenous administration of indomethacin (Fig. 4A), which may be related to the fact that orally administration of 7.5 mg/kg but not 3.8 mg/kg diclofenac inhibited PG synthesis completely (about 90%) (Table 2).

We also intravenously administered aspirin, a non-selective NSAID, instead of indomethacin (Fig. 4C). Administration of aspirin alone in this way did not produce gastric lesions, but lesions were produced when etodolac was administered orally in conjunction with intravenously administered aspirin (Fig. 4C). Similar results were obtained for NS-398 and celecoxib (data not shown). This result not only supports our hypothesis but also provides us with an important suggestion for the clinical use of selective COX-2 inhibitors (see Section 4).

4. Discussion

In this study, we have shown that the cytotoxic effects (necrosis and apoptosis) of NSAIDs on gastric mucosal cells *in vitro* are independent of COX inhibition by those NSAIDs. Furthermore, *in vivo* analysis using both oral and intravenous administration of NSAIDs suggested that not only COX inhibition but also the COX-independent direct cytotoxic effect of NSAIDs is involved in the development of gastric lesions. Both increase in aggressive factors and decrease in defensive factors cause gastropathy. As for NSAID-induced gastropathy, the decrease in defensive factors by NSAIDs (inhibition of PG synthesis) had been paid much attention. Results in this paper suggested that increase in aggressive factors by NSAIDs (direct cytotoxic effect of NSAIDs) is also involved in NSAID-induced gastropathy. This finding can be used to explain the previously unsolved issue that the decrease in PG levels and gastrointestinal lesions by NSAIDs are not always linked (see Section 1). Our findings can explain the fact that higher doses of NSAIDs were required for producing gastric lesions than those for inhibiting COX at the gastric mucosa [21,22], because higher concentrations were required for inducing the direct cytotoxic effect of NSAIDs than were required for inhibiting PG synthesis (Figs. 1 and 2). On the other hand, the fact that parenterally administered NSAIDs causes gastric and duodenal lesions [40–42] and that immunoneutralization of PGs causes gastric lesions [43] show that the direct cytotoxicity of NSAIDs is not essential for the development of gastric lesions by NSAIDs. Furthermore, at present, it is possible that mechanisms other than direct cytotoxicity and COX inhibition (such as increase in gastric motility by NSAIDs) are involved in results in Fig. 4. For example, the beneficial roles of COX-2 at gastric mucosa, such as stimulation of wound healing and resolution of inflammation, were reported [44]. Therefore, inhibition of these beneficial roles of COX-2 by oral administration of selective COX-2 inhibitors may be partly involved in results in Fig. 4. The mechanism of the direct cytotoxicity of NSAIDs and lack of the direct cytotoxicity in rofecoxib are also unclear at present. Furthermore, we have no direct evidence that necrosis and apoptosis are induced, accompanying with production of gastric lesions by NSAIDs *in vivo*.

A recently raised issue concerning the use of selective COX-2 inhibitors is their potential risk for cardiovascular thrombotic events [24,25], although there are still discussions on this point. PGI_2, a potent anti-aggregator of

platelets and a vasodilator, is mainly produced by COX-2 in vascular endothelial cells, while thromboxane A_2, a potent aggregator of platelets and a vasoconstrictor, is mainly produced by COX-1 in platelets [45–47]. Therefore, selective COX-2 inhibitors, but not non-selective NSAIDs, may lead to increased prothrombotic activity. Recent genetic studies using knockout mice for the receptor of PGI_2 or thromboxane A_2 supported this notion [48,49]. Furthermore, both animal and clinical data suggest that, compared to non-selective NSAIDs, selective COX-2 inhibitors increase cardiovascular thrombotic events [24,50,51]. Therefore, the method for decreasing the gastric side effects of NSAIDs other than increasing their selectivity for COX-2 may be useful in order to develop safer NSAIDs for both gastrointestine and cardiovascular. Considering our hypothesis described above, NSAIDs that do not exhibit direct cytotoxicity on gastric mucosal cells (i.e. NSAIDs that do not induce necrosis and apoptosis in gastric mucosal cells) may be safe for the gastrointestinal tract even if they do not have high selectivity for COX-2.

Low doses of aspirin are widely used for preventing thrombosis. Therefore, it is not unusual that patients who use aspirin chronically for preventing thrombosis are further administered with selective COX-2 inhibitors as anti-inflammatory drugs. Results (Fig. 4C) suggest that the oral administration of selective COX-2 inhibitors into chronic aspirin users causes gastric lesions, even though such administration into non-aspirin users is safe. Similar results were reported recently using simultaneous oral administration of aspirin and selective COX-2 inhibitors [52]. In fact, the Celecoxib Long-term Arthritis Safety Study (CLASS) showed that, compared to non-selective NSAIDs, celecoxib (at dosages greater than those indicated clinically) was clearly associated with a lower incidence of symptomatic lesions and lesion complication for patients not taking aspirin concomitantly. The difference in gastric side effects between them (non-selective NSAIDs and celecoxib) was not so clear for patients taking aspirin concomitantly [13]. Therefore, much attention should be paid to the concomitant use of both aspirin and selective COX-2 inhibitors.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare of Japan.

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Research Article

Induction of Claudin-4 by Nonsteroidal Anti-inflammatory Drugs and Its Contribution to Their Chemopreventive Effect

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAID) have shown chemopreventive effects in both preclinical and clinical studies; however, the precise molecular mechanism governing this response remains unclear. We used DNA microarray techniques to search for genes whose expression is induced by the NSAID indomethacin in human gastric carcinoma (AGS) cells. Among identified genes, we focused on those related to tight junction function (*claudin-4*, *claudin-1*, and *occludin*), particularly *claudin-4*. Induction of claudin-4 by indomethacin was confirmed at both mRNA and protein levels. NSAIDs, other than indomethacin (diclofenac and celecoxib), also induced claudin-4. All of the tested NSAIDs increased the intracellular Ca^{2+} concentration. Other drugs that increased the intracellular Ca^{2+} concentration (thapsigargin and ionomycin) also induced claudin-4. Furthermore, an intracellular Ca^{2+} chelator [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] inhibited the indomethacin-dependent induction of claudin-4. These results strongly suggest that induction of claudin-4 by indomethacin is mediated through an increase in the intracellular Ca^{2+} concentration. Overexpression of claudin-4 in AGS cells did not affect cell growth or the induction of apoptosis by indomethacin. On the other hand, addition of indomethacin or overexpression of claudin-4 inhibited cell migration. Colony formation in soft agar was also inhibited. Suppression of claudin-4 expression by small interfering RNA restored the migration activity of AGS cells in the presence of indomethacin. Based on these results, we consider that the induction of *claudin-4* and other tight junction–related genes by NSAIDs may be involved in the chemopreventive effect of NSAIDs through the suppression of anchorage-independent growth and cell migration. (Cancer Res 2005; 65(5): 1-9)

Introduction

Nonsteroidal anti-inflammatory drugs (NSAID) are the most widely used therapeutic agents in the treatment of pain, inflammation, and fever (1). Recent epidemiologic studies clearly show that NSAID use is associated with a reduced risk of cancer, and preclinical and clinical studies have shown that some NSAIDs are effective for the treatment and prevention of cancer. This effect is particularly well documented in relation to colon and rectal cancer. Recent studies have also shown that NSAID use reduces the risk of stomach cancer (2, 3). Several different effects of NSAIDs on cancer cells, such as stimulation of apoptosis, cell growth suppression, inhibition of angiogenesis, and inhibition of metastasis, have been proposed to play important roles in NSAID-mediated chemoprevention (4, 5). However, the precise molecular mechanisms governing these effects of NSAIDs have not been elucidated.

The anti-inflammatory action of NSAIDs is mediated through its inhibition of cyclooxygenase (COX). COX is an enzyme essential for the synthesis of prostaglandins, which have a strong propensity for inducing inflammation. Prostaglandins, such as prostaglandin E_2 (PGE_2), inhibit apoptosis and stimulate cell growth, angiogenesis, and metastasis (6–8). Furthermore, overexpression of COX-2 (a subtype of COX) has been reported in various tumor cells and tissues (9, 10). Therefore, the inhibition of COX by NSAIDs was thought previously to be the sole explanation for their chemopreventive effect. However, several lines of evidence suggest that chemoprevention by NSAIDs also involves COX-independent mechanisms. Sulindac sulfone, a derivative of the NSAID sulindac, does not inhibit COX activity and has been shown to display antitumor activity *in vivo* as well as induce apoptosis and inhibit cell growth in tumor cells *in vitro* (11, 12). Moreover, the induction by NSAIDs of apoptosis and the inhibition of cell growth in COX-null fibroblasts and tumor cells in which COX expression was absent have been reported (13, 14). Therefore, it is important that the COX-independent mechanisms for chemoprevention by NSAIDs are elucidated to develop more effective NSAIDs.

Tight junctions are the most apical intercellular structure in epithelial and endothelial cells and create a physiologic barrier separating the apical and basolateral spaces; in other words, they create a paracellular permeability barrier. Tight junctions contain the transmembrane proteins occludin and claudin, which are connected to the cytoskeleton via zonula occludens (ZO-1; ref. 15). Several studies have shown a correlation between a reduction in tight junction function and tumor progression. A loss of tight junction structure is frequently observed in epithelium-derived cancers, whereas some tumor-promoting agents are known to disrupt tight junctions (16, 17). Furthermore, overexpression of tight junction–related proteins (such as claudin-1, claudin-4, and occludin) in cancer cells has been reported to induce apoptosis and suppress the invasive potential of these cells (18, 19).

NSAIDs affect the expression of several genes in a COX-independent manner. For example, NSAIDs induce NAG-1, a transforming growth factor-β superfamily member protein, which is involved in the induction of apoptosis by NSAIDs (20). We reported recently that NSAIDs induce CCAAT/enhancer binding protein homologous transcription factor, which is involved

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evaluation of differences between the groups. The Student's t test for unpaired results was done for the evaluation of differences between two groups, which were considered to be significant for values of $P < 0.05$.

Results

DNA Microarray Analysis for Gene Expression in the Presence of Indomethacin. We used the DNA microarray technique and AGS cells to identify genes whose expression is altered by indomethacin. AGS cells were treated with 0.3 mmol/L indomethacin for 4 hours before microarray analysis. As shown in F1 Fig. 1*A*, this treatment did not affect cell viability. We did microarray analysis four times (four hybridizations) and selected genes that were induced by indomethacin based on the criteria that the induction was observed in all four hybridizations and that the mean value (fold change) of four hybridizations was >2.0. As shown in Table 1, 34 genes were identified. Induction of some of these genes, such as *CCAAT/enhancer binding protein* β and *prostate differentiation factor* (*NAG-1*), by NSAIDs in other cancer cell types has been reported previously (20, 27). Among these genes, we focused our attention on genes related to tight junction function (*claudin-1*, *claudin-4*, and *occludin*), particularly on *claudin-4*, because the induction was relatively clear, its expression in gastric mucosal cells has been confirmed previously (28), and a recent report showed that overexpression of claudin-4 suppressed anchorage-independent growth and the invasive potential of pancreatic cancer cells (19). Nineteen genes were identified whose expression was repressed by the indomethacin treatment (data not shown). T1

Changes in the indomethacin-induced expression of these genes were then verified by RT-PCR. As shown in Fig. 1*B*, the induction of claudin-1, claudin-4, and occludin was confirmed. Results of the

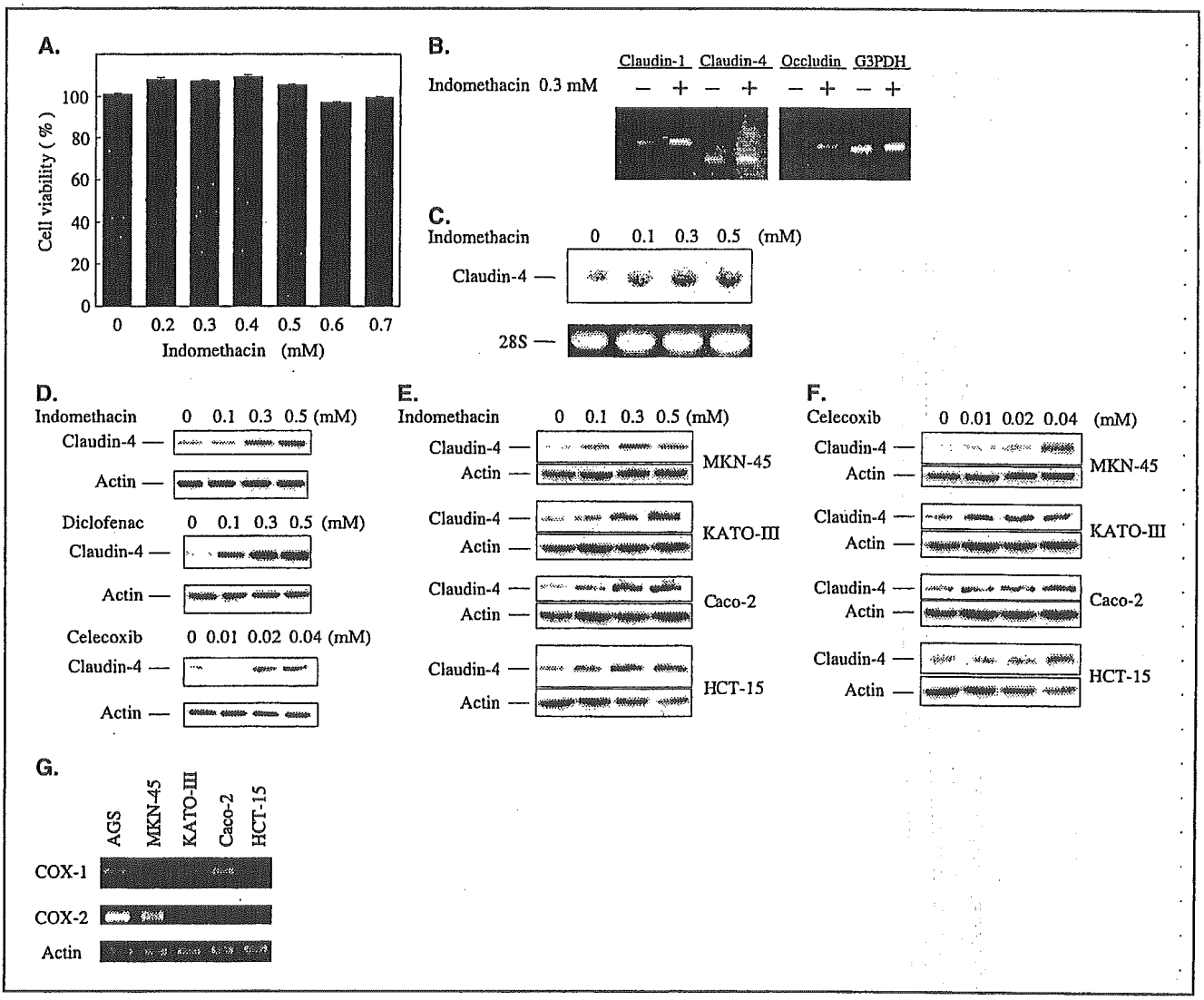

Figure 1. Induction of tight junction–related genes by NSAIDs. AGS (*A-D* and *G*) or MKN-45, KATO-III, Caco-2, and HCT-15 (*E-G*) cells were incubated with indicated concentrations of NSAIDs for 4 hours (*A-C*) or 24 hours (*D-F*). Results for cells cultured without NSAIDs (*G*). Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. *Columns*, mean (n = 3); *bars*, SE (*A*). Total RNA was extracted and subjected to RT-PCR by use of a specific primer for each gene. GAPDH (*G3PDH*; *B*) or actin (*G*) was used as a control. Reaction products were analyzed by agarose (1%) gel electrophoresis (*B* and *G*). Total RNA samples were analyzed by Northern blotting experiments using a specific DNA probe for claudin-4. Bands of rRNA (28S) stained with ethidium bromide (*C*). Whole cell extracts (2.5 μg protein) were analyzed by immunoblotting with an antibody against claudin-4 or actin (*D-F*).

Table 1. List of genes overexpressed in AGS cells following treatment with indomethacin

Gene name	Accession no.	Function	Fold change
Claudin-1	AF115546	Tight junction	2.00
Claudin-4	AK026651	Tight junction	2.54
Occludin	U49184	Tight junction	2.24
Tissue factor pathway inhibitor 2	NM_006528	Blood coagulation	2.46
Zinedin	AF212940	Calmodulin binding protein	2.01
Arginine-rich protein	AA582041	Carcinogenicity	2.00
Human urokinase-type plasminogen receptor, exon 7	U09937	Cell surface plasminogen activation	2.81
Chromobox homologue 4 (Drosophila Pc class)	AF013956	Cellular memory system	2.30
Human low-density lipoprotein receptor gene, exon 18	L00352	Cholesterol homeostasis	3.43
Low-density lipoprotein receptor (familial hypercholesterolemia)	NM_000527	Cholesterol homeostasis	2.75
Epithelial protein lost in neoplasm β	AA594624	Cytoskeleton	2.58
Keratin 8	AI978932	Cytoskeleton	2.37
Immediate early response 3	AI022951	Differentiation	2.36
Prostate differentiation factor	AB000584	Differentiation	2.00
Procollagen-proline	J02783	Disulfide isomerase/oxidoreductase	2.50
Glucosidase β, acid (includes glucosylceramidase)	AF023268	Glucocerebrosidase	2.01
Tumor necrosis factor--induced protein 6	M31165	Hyaluronan binding protein family	2.14
Basigin	X64364	Immunoglobulin superfamily	2.15
Solute carrier family 7	M80244	L-Amino acid transporter	3.00
Cathepsin D	M11233	Lysosomal proteinase	2.40
Pim-1 oncogene	M24779	Protein kinase	2.52
Cytochrome c oxidase subunit VIII	J04823	Respiratory	2.90
3,4-Dihydroxy-L-phenylalanine decarboxylase (aromatic l-amino acid decarboxylase)	M76180	Synthesis of dopamine and serotonin	2.27
CCAAT/enhancer binding protein β	W93514	Transcription factor	4.76
Predicted using Genefinder, preliminary prediction	CAB60892	Tumor protein p53	2.18
ATPase, H^+ transporting, lysosomal (vacuolar proton pump) 21 kDa	AI567477	Vacuolar proton pump	2.39
Ribosomal protein S21	BE221408	Unknown	2.34
Human genomic DNA, chromosome 22q11.2, BCRL2 region	AP000553	Unknown	2.23
Ubiquinol-*cytochrome c* reductase (6.4 kDa) subunit	AW163002	Unknown	2.13
IFN-induced transmembrane protein 3 (1-8U)	X57352	Unknown	2.10
Conserved hypothetical protein	AAF96700	Unknown	2.07
KIAA0316 gene product	AB002314	Unknown	2.06
Sequence 100 from patent WO9951727	AX015425	Unknown	2.03
Ribosomal protein S28	AW161288	Unknown	2.03

NOTE: Fold changes in gene expression by indomethacin compared with untreated cells. Mean values from four independent hybridizations. AGS cells were treated with or without 0.3 mmol/L indomethacin for 4 hours and subjected to DNA microarray analysis.

real-time RT-PCR experiments used to determine the extent of the induction yielded fold changes in copy number of 2.3, 3.0, and 1.5 for claudin-1, claudin-4, and occludin mRNA, respectively, in response to treatment of cells for 4 hours with 0.3 mmol/L indomethacin. In addition, the induction by indomethacin of claudin-4 mRNA or claudin-4 protein was confirmed using Northern blot analysis (Fig. 1*C*) and immunoblot analysis (Fig. 1*D*), respectively.

We then examined whether the induction of claudin-4 by indomethacin is specific to AGS cells or is a general property also observed in other cell types. We used MKN-45 and KATO-III cells (derived from gastric cancer tissue) and Caco-2 and HCT-15 cells (derived from colon cancer tissue) to test this effect. As shown in Fig. 1*E*, indomethacin induced claudin-4 in each of the cell lines tested, with the concentration of indomethacin required for the induction being similar for each cell line.

Diclofenac, another NSAID, also induced claudin-4 in a dose-dependent manner (Fig. 1*D*). Some NSAIDs are specific in their effect on COX, which exists in two forms, COX-1 and COX-2. Celecoxib, a COX-2-specific NSAID, induced claudin-4 not only in AGS cells (Fig. 1*D*) but also in the other cell lines tested (Fig. 1*F*). These results suggest that NSAIDs induce claudin-4 irrespective of whether they are specific for COX-2. It has been reported that both COX-1 and COX-2 mRNA are expressed in AGS, MKN-45, and Caco-2 cells, whereas COX-2 mRNA expression is very low in KATO-III and HCT-15 cells (29–33). COX-1 mRNA expression was confirmed by RT-PCR in each of the cell lines tested, whereas COX-2 mRNA expression was detected only in AGS, MKN-45, and Caco-2 cells (Fig. 1*G*). Therefore, COX-2-specific NSAIDs (in this case, celecoxib) induce claudin-4 not only in COX-2-expressing cells but also in cells lacking COX-2 expression. Furthermore, whereas indomethacin inhibited both COX-1 and COX-2 at a concentration of <1 mol/L (34), the induction of claudin-4 required higher concentrations (Fig. 1). These findings strongly suggest that NSAIDs induce claudin-4 independently of COX-inhibition.

Mechanism for Induction of Claudin-4 by Indomethacin. For further confirmation that NSAIDs induce claudin-4 independently of COX-inhibition, we examined the effect of PGE_2, a major prostaglandin in the gastric mucosa, on the induction of claudin-4 by indomethacin. PGE_2 (0.1-10 μmol/L) did not affect the level of F2 claudin-4 in the presence and absence of indomethacin (Fig. 2*A*). We determined previously the level of PGE_2 in the culture medium of AGS cells to be ~10 nmol/L (23). Therefore, inhibition of PGE_2 synthesis by indomethacin does not seem to be involved in the induction of claudin-4 by indomethacin.

Recent studies suggest that indomethacin and other NSAIDs act as agonists of the peroxisome proliferator-activated receptor-γ (35). To test the contribution of this activity to the induction of claudin-4 by indomethacin, we examined the effect of a peroxisome proliferator-activated receptor-γ antagonist (GW9662) on the induction of claudin-4 by indomethacin. As shown in Fig. 2*B*, GW9662 did not inhibit but rather slightly heightened the induction of claudin-4 by indomethacin. The different concentrations of GW9662 tested did not affect cell viability (data not shown), but based on data from a previous report, these concentrations are considered sufficient to inhibit agonist binding to peroxisome proliferator-activated receptor-γ (36). Therefore, peroxisome proliferator-activated receptor-γ does not seem to be associated with the induction of claudin-4 by indomethacin.

It has been reported that some NSAIDs increase reactive oxygen species production (37). To test the contribution of reactive oxygen species to the induction of claudin-4 by indomethacin, we examined the effects of the antioxidants *N*-acetylcysteine and SOD. As shown in Fig. 2*C* and *D*, neither *N*-acetylcysteine nor SOD affected claudin-4 expression in either the presence or the absence of indomethacin. Activation of the extracellular signal-regulated kinase pathway—one of the mitogen-activated protein kinase pathways—has been reported to stimulate the expression of claudin-4. Although some NSAIDs have been reported to activate the extracellular signal-regulated kinase pathway (19, 38), an inhibitor of extracellular signal-regulated kinase (PD98059) did not affect the expression of claudin-4 in either the presence or the absence of indomethacin (Fig. 2*E*). *N*-acetylcysteine, SOD, and PD98059 did not affect cell viability at the concentrations used (data not shown). These results suggest that neither reactive oxygen species nor extracellular signal-regulated kinase is responsible for the induction of claudin-4 by indomethacin.

Some NSAIDs have been reported to increase the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ (39, 40). In this study, we tested whether an increase in $[Ca^{2+}]_i$ by NSAIDs is responsible for the induction of claudin-4. Firstly, we confirmed that a NSAID-induced increase in $[Ca^{2+}]_i$ occurred under the same conditions as those in which the induction of claudin-4 in AGS cells was observed. As shown in Fig. 3*A*, all NSAIDs tested (indomethacin, diclofenac, and celecoxib) F3 increased $[Ca^{2+}]_i$ at the same NSAID concentrations that caused the induction of claudin-4.

Some drugs that are known to increase $[Ca^{2+}]_i$ were examined for their capacity to induce claudin-4 expression. The actions of thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase, and the Ca^{2+} ionophore ionomycin were thus tested on AGS cells. As shown in Fig. 3*A-C*, in addition to increasing $[Ca^{2+}]_i$, both thapsigargin and ionomycin induced claudin-4 in a dose-dependent manner. Furthermore, an intracellular Ca^{2+} chelator, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, was found to inhibit the induction of claudin-4 not only by ionomycin but also by indomethacin (Fig. 3*D*). 1,2-Bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid did not affect cell viability at the concentration used in these experiments (data not

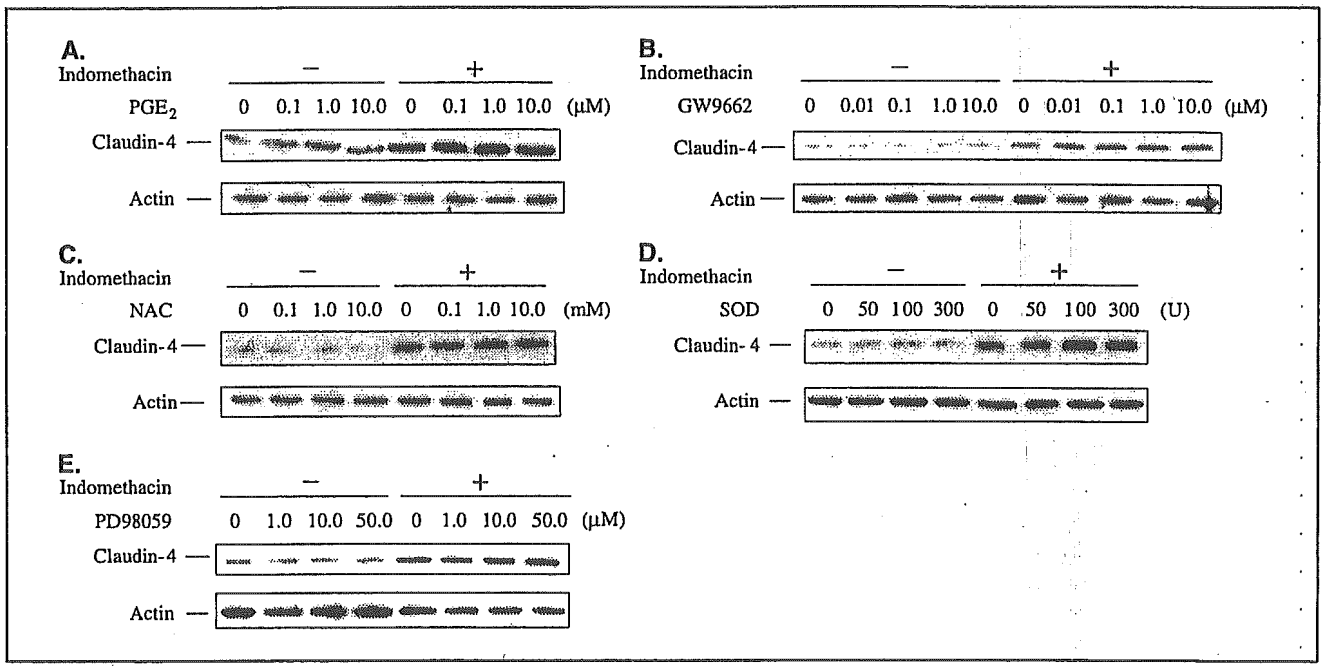

Figure 2. Mechanism for the induction of claudin-4 by indomethacin. AGS cells were incubated with or without 0.3 mmol/L indomethacin for 24 hours in the presence of indicated concentrations of PGE_2 (*A*), GW9662 (*B*), *N*-acetylcysteine (*NAC*; *C*), SOD (*D*), or PD98059 (*E*). Levels of claudin-4 and actin were estimated by immunoblotting experiments as described in Fig. 1. One unit of SOD was evaluated based on its inhibitory effect on the reduction of cytochrome *c* as described in the manufacturer's instructions.

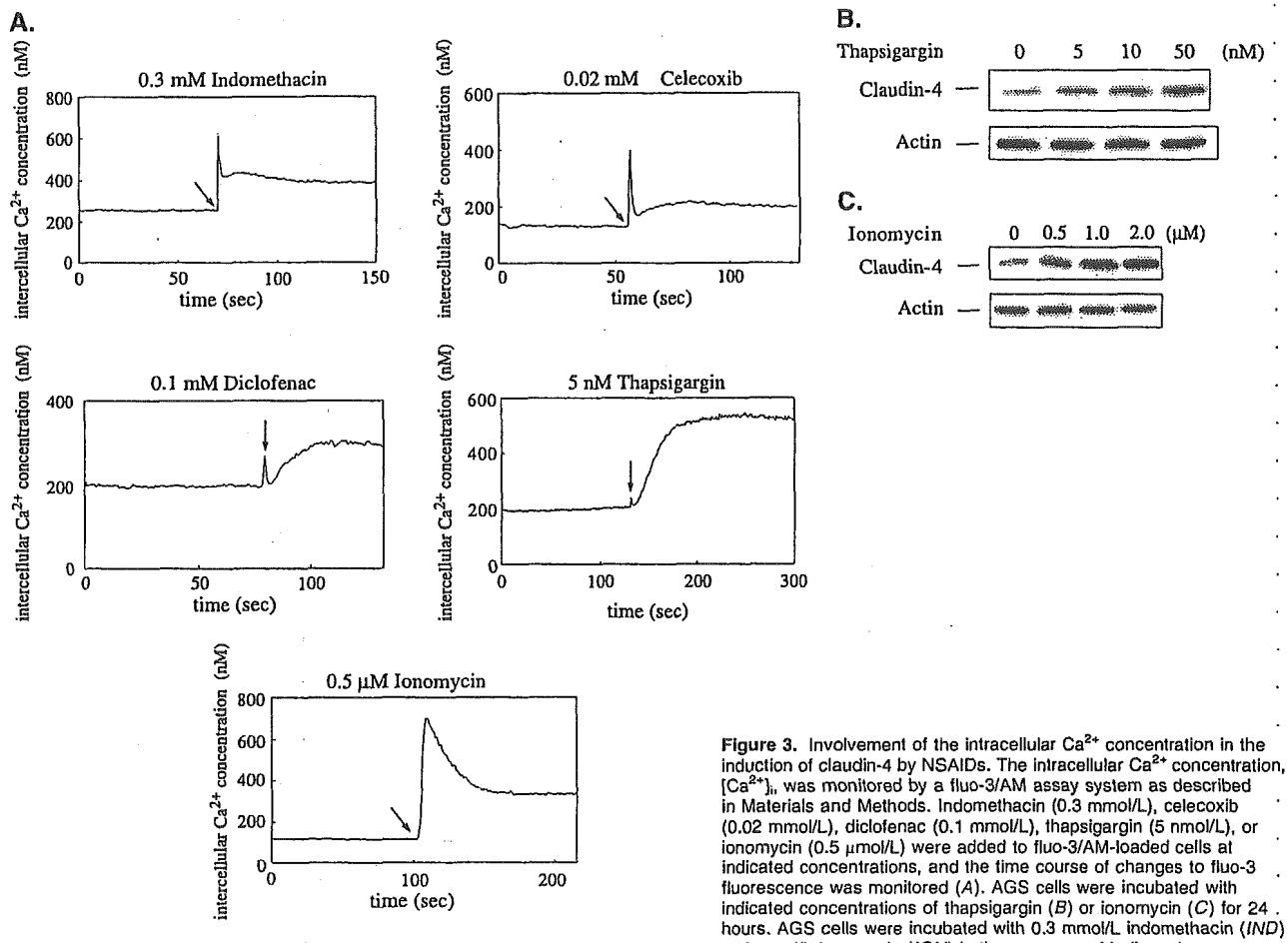

Figure 3. Involvement of the intracellular Ca^{2+} concentration in the induction of claudin-4 by NSAIDs. The intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, was monitored by a fluo-3/AM assay system as described in Materials and Methods. Indomethacin (0.3 mmol/L), celecoxib (0.02 mmol/L), diclofenac (0.1 mmol/L), thapsigargin (5 nmol/L), or ionomycin (0.5 μmol/L) were added to fluo-3/AM-loaded cells at indicated concentrations, and the time course of changes to fluo-3 fluorescence was monitored (*A*). AGS cells were incubated with indicated concentrations of thapsigargin (*B*) or ionomycin (*C*) for 24 hours. AGS cells were incubated with 0.3 mmol/L indomethacin (*IND*) or 2 μmol/L ionomycin (*ION*) in the presence of indicated concentrations of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (*BAPTA-AM*) for 24 hours (*D*). Levels of claudin-4 and actin were estimated by immunoblotting experiments as described in Fig. 1 (*B-D*).

shown). These results strongly suggest that induction of claudin-4 by indomethacin is mediated via an increase in $[Ca^{2+}]_i$.

Role of Claudin-4 Induction in the *In vitro* Antitumor Action of NSAIDs. As described in Introduction, various mechanisms have been proposed for the chemopreventive action of NSAIDs; these include the inhibition of cell growth, stimulation of apoptosis, and inhibition of metastasis. Here, we examined the contribution that NSAID induction of claudin-4 makes to the antitumor effect of NSAIDs *in vitro*. We constructed stable transfectants of AGS cells that continuously overexpress claudin-4 and selected four clones (clones 1, 6, 7, and 11) in which the level of expression of claudin-4 varied (clone 7 > clone 11 > clone 1 > clone 6; Fig. 4*A*).

Figure 4*B* shows the cell growth curve for each clone. The growth of cells from each clone was indistinguishable from that of the mock transfectant control, demonstrating that overexpression of claudin-4 did not affect the growth of AGS cells. Therefore, induction of claudin-4 by NSAIDs does not seem to be involved in the inhibition of cell growth by NSAIDs.

We also examined the effect of overexpression of claudin-4 on the induction of apoptosis. In the absence of indomethacin, the cell viability of each clone, as determined by the trypan blue exclusion test, was close to 100%, showing that expression of claudin-4 does not affect cell viability. As shown in Fig. 4*C*, the dose-response curve for the decrease in cell viability by indomethacin was indistinguishable between each of the claudin-4-overexpressing clones and the mock transfectant control. Further, we confirmed that the cell death (Fig. 4*C*) was mediated by apoptosis as evidenced by apoptotic DNA fragmentation, activation of caspase-3, and chromatin condensation (data not

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shown). The results presented in Fig. 4C show that claudin-4 overexpression does not affect the indomethacin-induced cell apoptosis. Therefore, the induction of claudin-4 by NSAIDs does not seem to be involved in NSAID-mediated apoptosis.

The anchorage-independent growth of tumor cells, which can be measured by colony formation in soft agar, is important for tumor progression. NSAIDs are known to inhibit colony formation of some cancer cells in soft agar (13); recently, it was reported that overexpression of claudin-4 in pancreatic cancer cells inhibited colony formation in soft agar (19). In this study, we examined the effect of claudin-4 overexpression and the presence of indomethacin on the anchorage-independent growth of AGS cells. We first examined the colony-forming ability of each of the claudin-4-overexpressing clones in soft agar. All clones showed less activity for colony formation in soft agar than the mock transfectant control (Fig. 4D), which is consistent with previous results obtained using pancreatic cancer cells (19). We compared the extent of inhibition of colony formation in soft agar with the degree of claudin-4 overproduction in these clones and found a close correlation between the two (Fig. 4A and D).

We also examined the effect of indomethacin on colony formation of AGS cells in soft agar. Because a long incubation period (10 days) was required for this assay, relatively low concentrations of indomethacin were used. As shown in Fig. 4E, indomethacin (100 mol/L) significantly decreased the colony-forming ability of AGS cells in soft agar. Real-time RT-PCR experiments confirmed that claudin-4 mRNA expression in AGS cells was induced at the concentration of indomethacin used (Fig. 4F). These results suggest that the induction of claudin-4 is involved in the indomethacin-dependent inhibition of AGS cell colony formation in soft agar.

The migration activity of tumor cells is also very important for tumor progression. We examined the relationship between expression of claudin-4 and migration activity in AGS cells. Wound healing assays were carried out in which the cell-free area was measured at the time a wound was made and then 24 hours later. Because neither claudin-4 overexpression nor addition of NSAIDs affected the growth of AGS cells (Fig. 4B; data not shown), a smaller cell-free area is indicative of a higher activity for cell migration. As shown in Fig. 5A, claudin-4- F5 overexpresing cells (clone 7) showed less cell migration activity

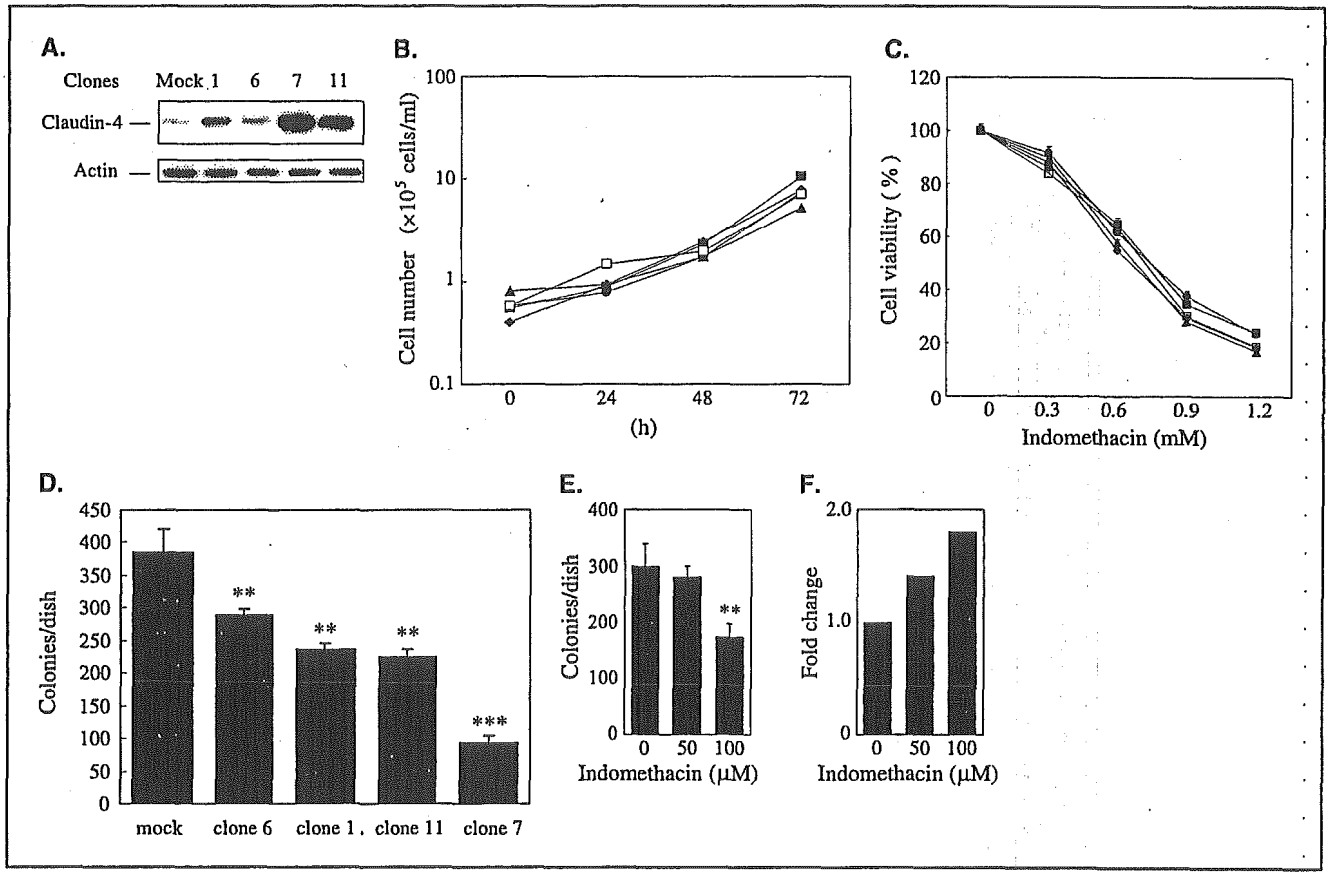

Figure 4. Effect of overexpression of claudin-4 on cell growth, apoptosis, and colony formation of AGS cells in soft agar. The extent of expression of claudin-4 in each clone (stable transfectant of claudin-4 expression plasmid) was estimated by immunoblotting experiments as described in Fig. 1 (*A*). Cells of each clone were cultured for indicated periods, and cell numbers were determined by direct cell counting (*B*). Cells of each clone were cultured in the presence of indicated concentrations of indomethacin for 24 hours and cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (*C*). Cells of each clone (*D*) or nontransfected AGS cells (*E*) were layered over soft agar in the presence (*E*) or absence (*D*) of indicated concentrations of indomethacin. After 10 days, cells were stained with crystal violet and colonies were counted (*D* and *E*). Expression of claudin-4 mRNA after treatment of cells with indicated concentrations of indomethacin for 24 hours was monitored by real-time RT-PCR (*F*). Points, mean ($n = 3$); bars, SE (C). *Columns*, mean (n = 3); *bars*, SE (*D and E*). ***, $P < 0.001$; **, $P < 0.01$ (*D and E*).

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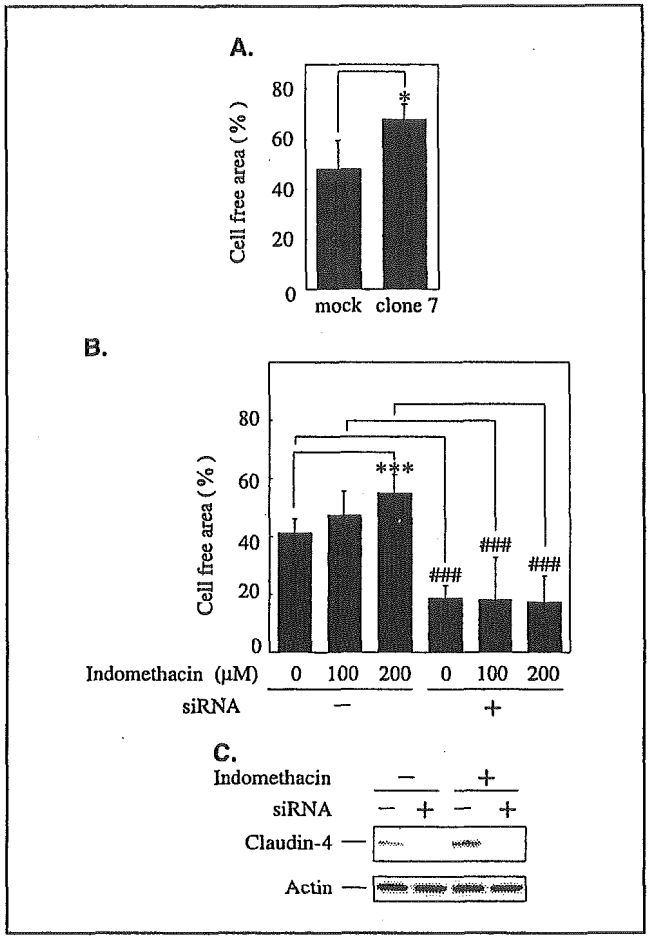

Figure 5. Effect of claudin-4 overexpression or of indomethacin on AGS cell migration. AGS cells of stable transfectant of claudin-4 expression plasmid (clone 7 in Fig. 4) and mock transfectant control AGS cells (*A*) or AGS cells transfected or nontransfected with siRNA for claudin-4 (*B*) were wounded and cultured for 24 hours in the presence (*B*) or absence (*A*) of indicated concentrations of indomethacin. The cell-free area was measured after 24 hours of incubation and expressed as relative to that before incubation. *Columns*, mean ($n = 3$); *bars*, SE. ***, $P < 0.001$ or ###, $P < 0.001$; *$P < 0.05$ (*A* and *B*). AGS cells transfected or nontransfected with siRNA for claudin-4 were cultured for 24 hours in the presence or absence of 0.3 mmol/L indomethacin for 24 hours. Levels of claudin-4 and actin were estimated by immunoblotting experiments as described in Fig. 1.

than the mock transfectant control. Furthermore, transfection of siRNA for claudin-4 stimulated the migration activity of AGS cells even in the absence of indomethacin (Fig. 5*B*). We confirmed that the transfection almost completely inhibited the expression of claudin-4 in AGS cells (Fig. 5*C*). These results suggest that the migration activity of AGS cells decreases as claudin-4 expression increases.

As shown in Fig. 5*B*, indomethacin inhibited the activity of AGS cells for cell migration and this inhibitory effect was almost completely suppressed by the transfection of siRNA for claudin-4. We confirmed that transfection of siRNA almost completely inhibited the induction of claudin-4 by indomethacin (Fig. 5*C*). Taken together, these results support the hypothesis that inhibition of cell migration by indomethacin is mediated through the induction of claudin-4.

Discussion

We have shown here that some tight junction–related genes, especially *claudin-4*, are induced by NSAIDs. Although NSAIDs and tight junctions are closely associated in relation to cancer progression, this is the first time that a connection between NSAIDs and tight junctions has been shown at the molecular level.

It is known that various factors disrupt or stimulate the function of tight junctions. For example, tumor necrosis factor-α, transforming growth factor-α, and interleukin-1 disrupt tight junctions, whereas transforming growth factor-β, interleukin-10, and PGE_2 are known to stimulate the function of tight junctions (41). However, the effect of these factors on the expression of components of tight junctions (such as claudin-4) has not been examined to the same extent. It seems that the alteration of tight junction function is not always correlated with an alteration in the expression of tight junction components. For example, we have found that PGE_2, which is known to stimulate the function of tight junctions, does not induce claudin-4. Because the expression of claudin-4 affects various aspects of cancer progression (see below), we consider that the effect of cancer-promoting agents or anticancer drugs on claudin-4 expression should be examined more extensively.

As for a mechanism of claudin-4 induction by NSAIDs, we postulate that it is mediated by an increase in $[Ca^{2+}]_i$ based on the following observations: (*a*) NSAIDs increased $[Ca^{2+}]_i$ and induced claudin-4 simultaneously, (*b*) thapsigargin and ionomycin increased $[Ca^{2+}]_i$ and induced claudin-4, and (*c*) the intracellular Ca^{2+} chelator [1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid] attenuated the indomethacin-dependent induction of claudin-4. As for the mechanism for the increase in $[Ca^{2+}]_i$ by NSAIDs, both inhibition of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (endoplasmic reticulum–located Ca^{2+} pump that is responsible for accumulation of Ca^{2+} in the endoplasmic reticulum) and stimulation of the influx of extracellular Ca^{2+} have been proposed (40). We found recently that all of the NSAIDs tested permeabilize the membranes of both erythrocytes and liposomes (42). This activity of NSAIDs was found to be closely related to their ability to increase $[Ca^{2+}]_i$, suggesting that NSAIDs permeabilize membranes and stimulate the influx of extracellular Ca^{2+} (42).

NSAIDs seem to achieve their chemopreventive effect via several mechanisms, such as stimulation of apoptosis, cell growth suppression, inhibition of angiogenesis, and inhibition of metastasis (4, 5). In this study, we examined the contribution of claudin-4 induction to the antitumor activity of NSAIDs *in vitro*. Experiments using claudin-4–overproducing AGS cells and siRNA for claudin-4 suggested that NSAID-induced claudin-4 is involved in the NSAID-dependent suppression of anchorage-independent tumor growth and tumor cell migration but not in stimulation of apoptosis and cell growth suppression. As for cell migration, this is the first evidence showing not only that NSAIDs inhibit of cancer cell migration but also that claudin-4 is involved in cell migration. It was reported recently that overexpression of claudin-4 suppressed the invasive potential of pancreatic cancer cells (19); therefore, if NSAIDs also induce claudin-4 *in vivo*, then suppression of the invasive potential of tumor cells by NSAID-induced claudin-4 may be one of the mechanisms involved in the inhibition of metastasis by NSAIDs. It is also possible that the induction of claudin-4 by NSAIDs contributes to their antitumor activity through other

mechanisms. Tight junctions act as a barrier for diffusion of molecules that include nutrients and growth factors. It is well known that the constitutive accessibility of nutrients and growth factors is very important for tumor progression. Therefore, if NSAIDs also induce claudin-4 *in vivo*, then the supply of nutrients and growth factors to a tumor may be retarded or inhibited, thereby suppressing tumor progression.

Acknowledgments

Received 8/3/2004; revised 11/22/2004; accepted 12/20/2004.

Grant support: Ministry of Health, Labor, and Welfare of Japan Grants-in-Aid for Scientific Research, Suzuken Memorial Foundation, and Japan Research Foundation for Clinical Pharmacology.

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Low Direct Cytotoxicity of Nabumetone on Gastric Mucosal Cells

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This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare of Japan, as well as by the Suzuken Memorial Foundation, and the Japan Research Foundation for Clinical Pharmacology.

Running head: Cytotoxicity of nabumetone

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Prodrugs of non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for clinical purposes because they are not harmful to the gastrointestinal mucosa. We recently showed that NSAIDs have direct cytotoxicity in NSAID-induced gastric lesions. We show here that under conditions where the NSAIDs indomethacin and celecoxib clearly induce cell death, an NSAID prodrug, nabumetone, and its active metabolite 6-methoxy-2-naphthylacetic acid (6MNA), did not have such effects. Moreover, nabumetone and 6MNA exhibited much lower membrane permeabilizing activities than did indomethacin and celecoxib. We recently reported that when an orally administered NSAID was used in combination with a low dose of intravenously administered indomethacin, the severity of gastric lesions produced in rats depended on the cytotoxicity of the orally administered NSAID. Using a similar protocol, we show here that gastric lesions were produced when the orally administered NSAID was celecoxib, but not when nabumetone was used. We thus propose that the low direct cytotoxicity of nabumetone observed *in vitro* is maintained *in vivo*, and that the use of nabumetone does not harm the gastric mucosa.

KEY WORDS: nabumetone; gastric mucosal cells; membrane permeabilization; gastric lesions

Non-steroidal anti-inflammatory drugs (NSAIDs) are very popular and effective medicines used in the treatment of pain, inflammation and fever. The anti-inflammatory action of NSAIDs is mediated by their inhibition of cyclooxygenase (COX) activity. COX is an enzyme that is essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. On the downside, the use of NSAIDs is associated with gastrointestinal side-effects (1), with about 15-30% of chronic users of NSAIDs suffering from gastrointestinal ulcers and bleeding (2, 3). This negative aspect of NSAID use was previously thought to be due only to the inhibition of COX, because PGs have a strong protective effect on the gastrointestinal mucosa (4). In order to overcome the gastrointestinal side-effects of NSAID use, NSAIDs that inhibit COX activity in inflammatory tissues but not in the gastric mucosa are therefore required. Selective COX-2 inhibitors belong to such a category of NSAIDs. COX has two subtypes, COX-1 and COX-2, which are responsible for the majority of COX activity in the gastric mucosa and in inflamed tissues, respectively (5, 6). While a greatly reduced incidence of gastroduodenal lesions was reported for selective COX-2 inhibitors (such as rofecoxib and celecoxib) both in animal and clinical data (7, 8), their use however has been recently questioned because of their potential for causing cardiovascular thrombotic events owing to their specificity for COX-2 (9-12).

NSAID prodrugs (such as loxoprofen sodium and nabumetone) are generally safe for use on the gastrointestinal mucosa and are widely used for clinical purposes, especially in Japan where highly specific COX-2 inhibitors (such as celecoxib) are not presently available