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): 1868-76)

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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in the induction of apoptosis by endoplasmic reticulum stressors. By using a CCAAT/enhancer binding protein homologous transcription factor–deficient mouse, we showed that this induction is essential for NSAID-induced apoptosis (21). Therefore, systematic screening of genes whose expression is induced by NSAIDs is important for understanding the COX-independent mechanism of chemoprevention by NSAIDs. In this study, we searched for genes in human gastric carcinoma (AGS) cells whose expression is induced by indomethacin. We found that claudin-4, claudin-1, and occludin were induced in these cells in the presence of indomethacin. We propose that the induction of claudin-4 is mediated by an increase in the intracellular Ca^{2+} concentration. Moreover, by using claudin-4-overexpressing cells and small interfering RNA (siRNA), we show that claudin-4 is involved in the NSAID-mediated suppression of anchorage-independent growth and cell migration.

Materials and Methods

Chemicals and Media. Ham's F-12 and RPMI 1640 were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum was purchased from Life Technologies (Tokyo, Japan). 1,2-Bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid was purchased from Dojindo Co. (Tokyo, Japan). Thapsigargin, ionomycin, G418, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, diclofenac, and cycloheximide were purchased from Sigma Co. (Tokyo, Japan). Indomethacin, *N*-acetylcysteine, and superoxide dismutase (SOD) were from Wako Co. (Tokyo, Japan). Celecoxib was purchased from LKT Laboratories, Inc. (St. Paul, MN). Antibodies against claudin-4 and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture and Overexpression of Claudin-4. AGS cells were cultured in Ham's F-12 medium containing 10% fetal bovine serum. Other cell types (MKN-45, KATO-III, Caco-2, and HCT-15) were cultured in RPMI 1640 containing 10% fetal bovine serum. Cells (2×10^5 per well in a 24-well plate) were cultured for 24 hours and used in the experiments. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method as described previously (22).

A full-length human claudin-4 cDNA was PCR amplified from the cDNA of AGS cells and cloned into pcDNA3.1(-) (Invitrogen, Carlsbad, CA). Transfection of AGS cells with plasmids was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. The stable transfectants expressing claudin-4 were selected by immunoblotting analysis. Positive clones were maintained in the presence of 300 μg/mL G418.

DNA Microarray Analysis. Total RNA was extracted from cells treated with 0.3 mmol/L indomethacin for 4 hours or nontreated cells using a RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Samples (10 μg RNA) were labeled with cyanine 3- or cyanine 5-conjugated dUTP with the use of an Agilent cDNA labeling kit. The fluorescent-labeled cDNAs were mixed and hybridized simultaneously to Agilent cDNA microarray human I. The microarray was scanned with a DNA Microarray Scanner (Agilent, Palo Alto, CA) using laser excitation at 532 and 635 nm wavelengths for the cyanine 3 and cyanine 5 labels, respectively. The raw pixel intensity images were analyzed using the Feature Extraction and Analysis Software version 7.5 (Agilent). After pixel intensity determination and background subtraction, the ratio of the intensity of the treated cells to the intensity of the control was calculated following normalization.

Reverse Transcription. Total RNA was extracted from cells using a RNeasy kit according to the manufacturer's protocols. Samples (10 μg RNA) were reverse transcribed using a first-strand cDNA synthesis kit (Amersham, Tokyo, Japan) according to the manufacturer's instructions. For traditional reverse transcription–coupled PCR (RT-PCR), synthesized cDNA was amplified by PCR [Takara (Shiga, Japan) PCR Thermal Cycler] using KOD Plus Polymerase (Toyobo, Osaka, Japan), and reaction products were analyzed by agarose gel electrophoresis. For real-time RT-PCR, synthesized cDNA was applied to real-time RT-PCR (ABI PRISM 7700) using SYBR Green PCR Master Mix (ABI) and analyzed with ABI PRISM 7700 Sequence Detection Software according to the manufacturer's instructions. Real-time cycle conditions were 2 minutes at 50°C followed by 10 minutes at 90°C and then for 45 cycles at 95°C for 30 seconds and 63°C for 60 seconds. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template-free or reverse transcriptase–free controls. To normalize the amount of total RNA present in each reaction, *GAPDH* or *actin* genes were used as an internal standard.

Primer Design. Primers were designed using the Primer3 Web site (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers are listed as gene name, forward primer, reverse primer. For RT-PCR: *claudin-1*, CCGTTGGCATGAAGTGTATG, CCAGTGAAGAGAGCCTGACC; *claudin-4*, CTCTGTGGCCTCAGGACTCT, CAGGACTTCCAAGGGTGAAG; *occludin*, TCCAATGGCAAAGTGAATGA, GCAGGTGCTCTTTTTGAAGG; *COX-1*, CTGGCTCCGGAATTCACT, CATCTGGCAACTGCTTCTTC; *COX-2*, CCACCAACTTACAATGCTGC, CACCAGACCAAAGACCTCC; and *actin*, GGACTTCGAGCAAGAGATGG, AGCACTGTGTTGGCGTACAG. For PCR cloning: *claudin-4*, CGGGATCCCTGACAATGGCCTCCATGGGGCT, GCTCTAGATTACACGTAGTTGCTGGCAGC.

Immunoblotting and Northern Blotting Analyses. Whole cell extracts were prepared as described previously (23). The protein concentration of samples was determined by the Bradford method. Samples were applied to 12% SDS-PAGE gels and subjected to electrophoresis, and proteins were then immunoblotted with respective antibodies.

Total RNA was extracted from the cells using a RNeasy kit according to the manufacturer's protocols. Samples (5 μg RNA) were separated by agarose (1%) gel electrophoresis in the presence of 6.3% formaldehyde and blotted onto nylon membranes. DNA probes for claudin-4 were amplified by PCR and labeled with [α-^{32}P]dCTP (6,000 Ci/mmol, Amersham) using the Rediprime II DNA Labeling System (Amersham) according to the manufacturer's instructions. After hybridization and washing, membranes were analyzed with BAS2000A (Fujix, Kanagawa, Japan).

Measurement of the Intracellular Ca^{2+} Concentration, $[Ca^{2+}]_i$. The intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, was monitored according to manufacturer's protocols (24). Cells were washed with assay buffer containing 115 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.8 mmol/L $MgCl_2$, 20 mmol/L HEPES, and 13.8 mmol/L glucose. Cells were then incubated with 4 μmol/L fluo-3/AM in the assay buffer containing 0.1% bovine serum albumin, 0.04% Pluronic F127, and 2 mmol/L probenecid for 40 minutes at 37°C. After washing twice with the assay buffer, cells were suspended in assay buffer containing 2 mmol/L probenecid. Fluo-3 fluorescence of cells in a water-jacketed cuvette (1.6×10^6 cells per cuvette) was measured with a Hitachi (Tokyo, Japan) F-4500 spectrofluorophotometer by recording excitation signals at 490 nm and the emission signal at 530 nm at 1-second intervals. Maximum and minimum fluorescence values (F_{max} and F_{min}) were obtained by adding 10 mol/L ionomycin and 10 mol/L ionomycin plus 5 mmol/L EGTA (in Ca^{2+}-free medium), respectively. $[Ca^{2+}]_i$ was calculated according to the following equation: $[Ca^{2+}]_i = K_d(F - F_{min}) / (F_{max} - F)$, where K_d is the apparent dissociation constant (400 nmol/L) of the fluorescence dye-Ca^{2+} complex (24).

Cell Migration Assays. *In vitro* wound healing assays were used to assess cell migration as described previously (25). Confluent AGS cells on a 24-well plate were used. Two linear wounds were scratched with a p200 pipette tip. The cell-free area was measured before and after 24 hours of incubation (healing step) using Scion Image software (Scion Corp., Frederick, MD).

Soft Agar Assay. Soft agar assay was done as described previously (26). Cells (2×10^4 per dish) were suspended in 0.5 mL of 0.3% Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 mL of a 0.8% agar medium base layer in 35 mm culture dishes (Iwaki, Chiba, Japan). After 10 days, cells were stained with crystal violet and colonies were counted.

siRNA Targeting of Claudin-4. Synthetic siRNAs were purchased from Qiagen. The target DNA sequence of claudin-4 is CCCGCACAGACAAGCCTTACT and siRNA 5'-CGCACAGACAAGCCUUACUUU-3' and 5'-AGUAAGGCUUGUCUGUGCGGG-3' were used as annealed oligonucleotides. AGS cells were transfected with siRNA using RNAiFect Transfection Reagent (Qiagen) according to the manufacturer's instructions.

Statistical Analysis. All values are expressed as mean ± SE. One-way ANOVA followed by Scheffe's multiple comparison test was used for 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 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 Table 1, 34 genes were identified. Induction of some of these ge such as *CCAAT/enhancer binding protein* β and *prostate differe ation factor* (*NAG-1*), by NSAIDs in other cancer cell types has b reported previously (20, 27). Among these genes, we focused attention on genes related to tight junction function (*claudin claudin-4*, and *occludin*), particularly on *claudin-4*, because induction was relatively clear, its expression in gastric mucosal ce has been confirmed previously (28), and a recent report showed th overexpression of claudin-4 suppressed anchorage-independe growth and the invasive potential of pancreatic cancer cells (1 Nineteen genes were identified whose expression was repressed the indomethacin treatment (data not shown).

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

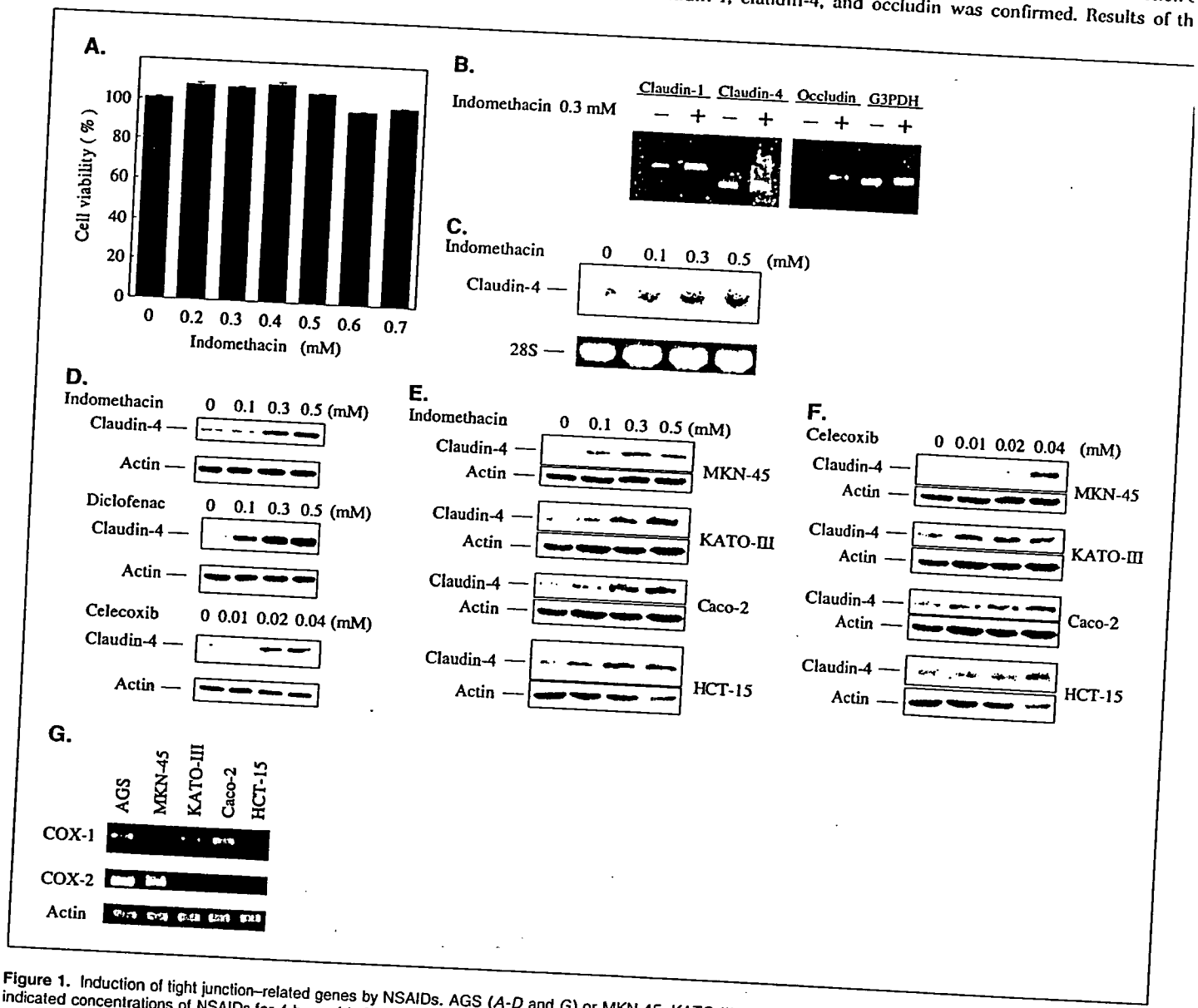

Figure 1. Induction of tight junction–related genes by NSAIDs. AGS (*A-D* and *G*) or MKN-45, KATO-III, Caco-2, and HCT-15 (*E-G*) cells were incubated with indicated concentrations of NSAIDs for 4 hours (*A-C*) or 24 hours (*D-F*). Results for cells cultured without NSAIDs (*G*). Cell viability was 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 nmol/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 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*-acetylcystein SOD, and PD98059 did not affect cell viability at the concentratio used (data not shown). These results suggest that neither reacti oxygen species nor extracellular signal-regulated kinase is responsib for the induction of claudin-4 by indomethacin.

Some NSAIDs have been reported to increase the intracellula Ca^{2+} concentration, $[Ca^{2+}]_i$ (39, 40). In this study, we tested whethe an increase in $[Ca^{2+}]_i$ by NSAIDs is responsible for the induction o claudin-4. Firstly, we confirmed that a NSAID-induced increase i $[Ca^{2+}]_i$ 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) 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 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

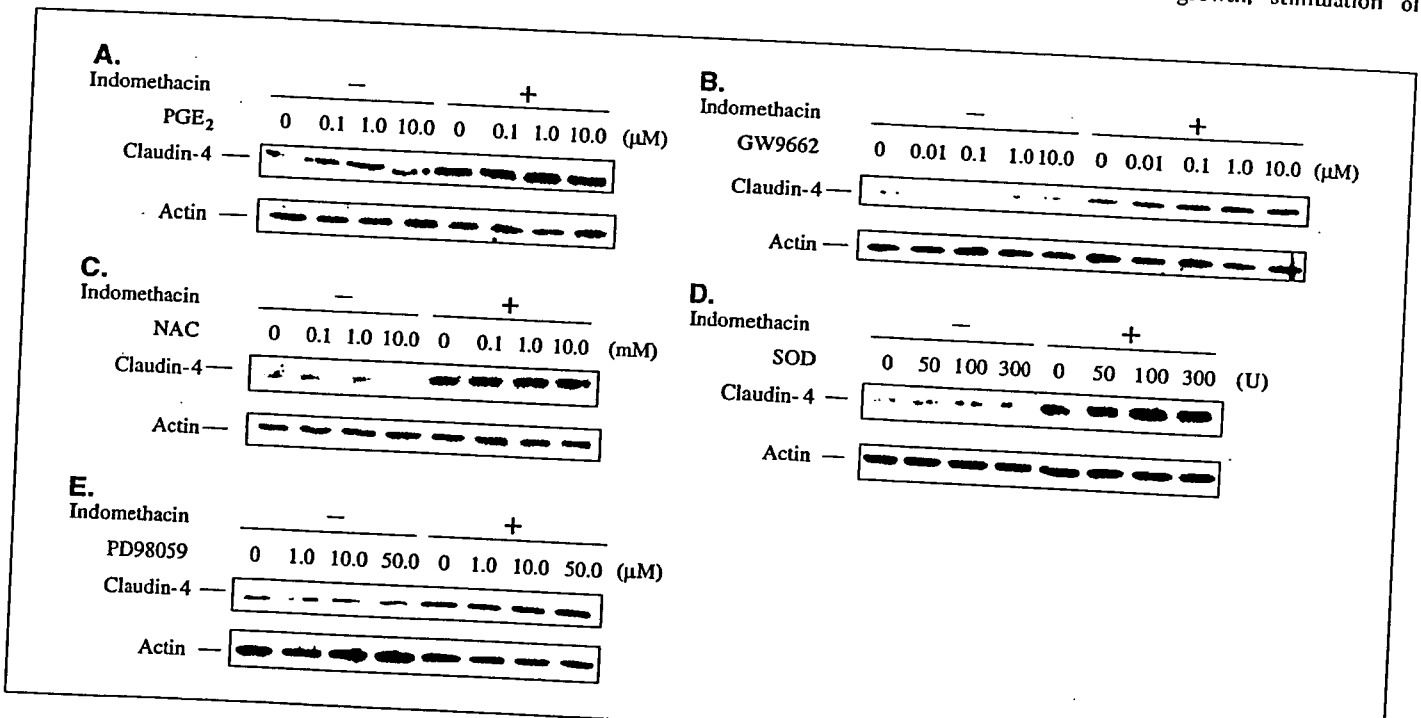

Figure 2. Mechanism for the induction of claudin-4 by indomethacin. AGS cells were incubated with or without 0.3 mmol/L indomethacin for 24 hours in the presence of indicated concentrations of PGE_2 (*A*), GW9662 (*B*), *N*-acetylcysteine (*NAC*; *C*), SOD (*D*), or PD98059 (*E*). Levels of claudin-4 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.

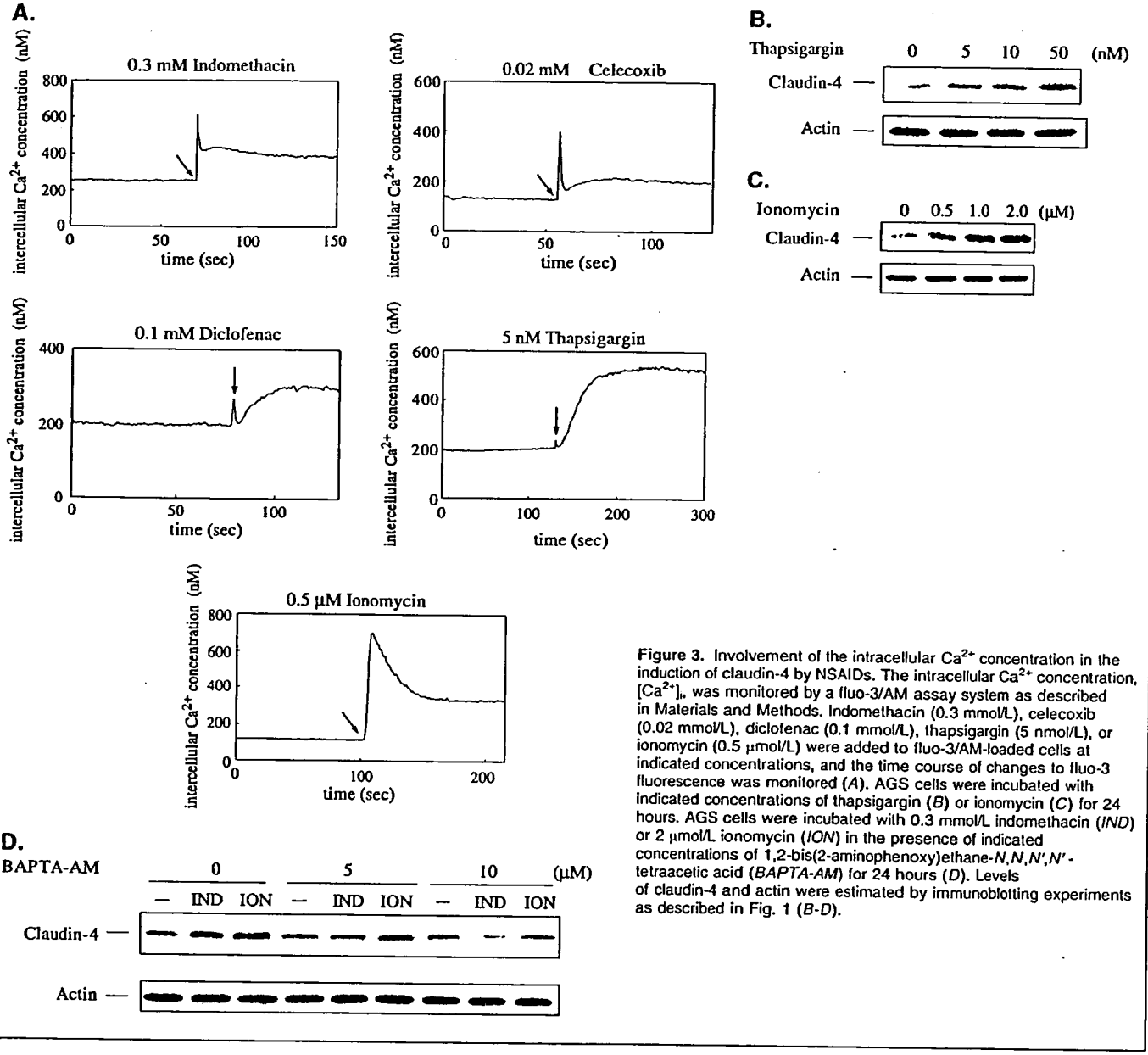

Figure 3. Involvement of the intracellular Ca^{2+} concentration in the induction of claudin-4 by NSAIDs. The intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, was monitored by a fluo-3/AM assay system as described in Materials and Methods. Indomethacin (0.3 mmol/L), celecoxib (0.02 mmol/L), diclofenac (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*).

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 shown). The results presented in Fig. 4*C* 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. 4*D*), 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. 4*A* 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. 4*E*, indomethacin (100 μmol/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. 4*F*). These results suggest that the induction of claudin-4 is involved in the indomethacin-dependent inhibition of AGS colony formation in soft agar.

The migration activity of tumor cells is also very impor for tumor progression. We examined the relationship betv expression of claudin-4 and migration activity in AGS c Wound healing assays were carried out in which the cell-area was measured at the time a wound was made then 24 hours later. Because neither claudin-4 overexpress nor addition of NSAIDs affected the growth of AGS cells (Fig. data not shown), a smaller cell-free area is indicative a higher activity for cell migration. As shown in Fig. claudin-4-overexpresing cells (clone 7) showed less cell migrati activity than the mock transfectant control. Furthermo transfection of siRNA for claudin-4 stimulated the migrati activity of AGS cells even in the absence of indomethacin (F 5*B*). We confirmed that the transfection almost complete inhibited the expression of claudin-4 in AGS cells (Fig. 5C These results suggest that the migration activity of AGS cel decreases as claudin-4 expression increases.

As shown in Fig. 5*B*, indomethacin inhibited the activity of AG cells for cell migration and this inhibitory effect was almos completely suppressed by the transfection of siRNA for claudin-4 We confirmed that transfection of siRNA almost completel

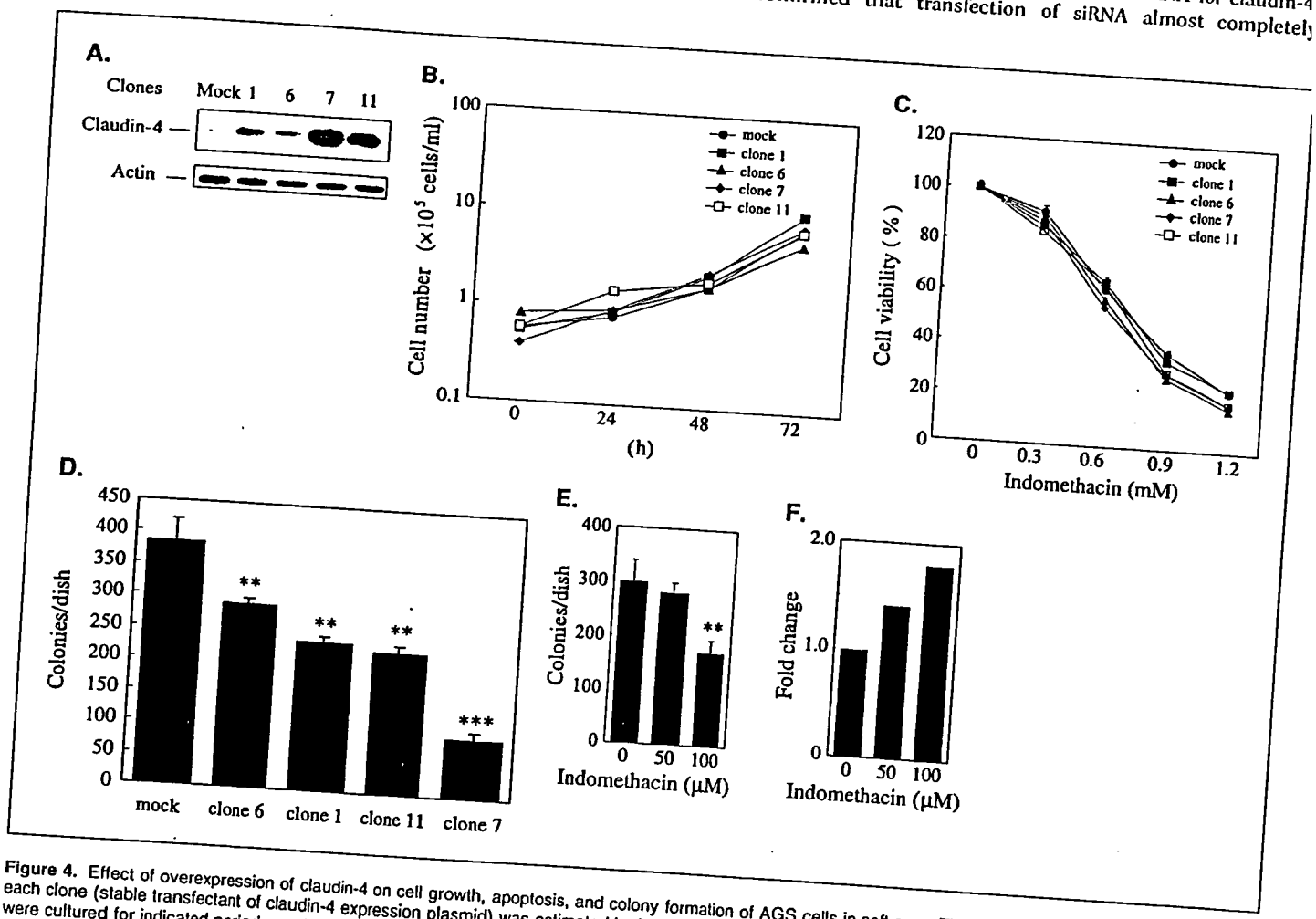

Figure 4. Effect of overexpression of claudin-4 on cell growth, apoptosis, and colony formation of AGS cells in soft agar. The extent of expression of claudin-4 in each clone (stable transfectant of claudin-4 expression plasmid) was estimated by immunoblotting experiments as described in Fig. 1 (*A*). 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*).

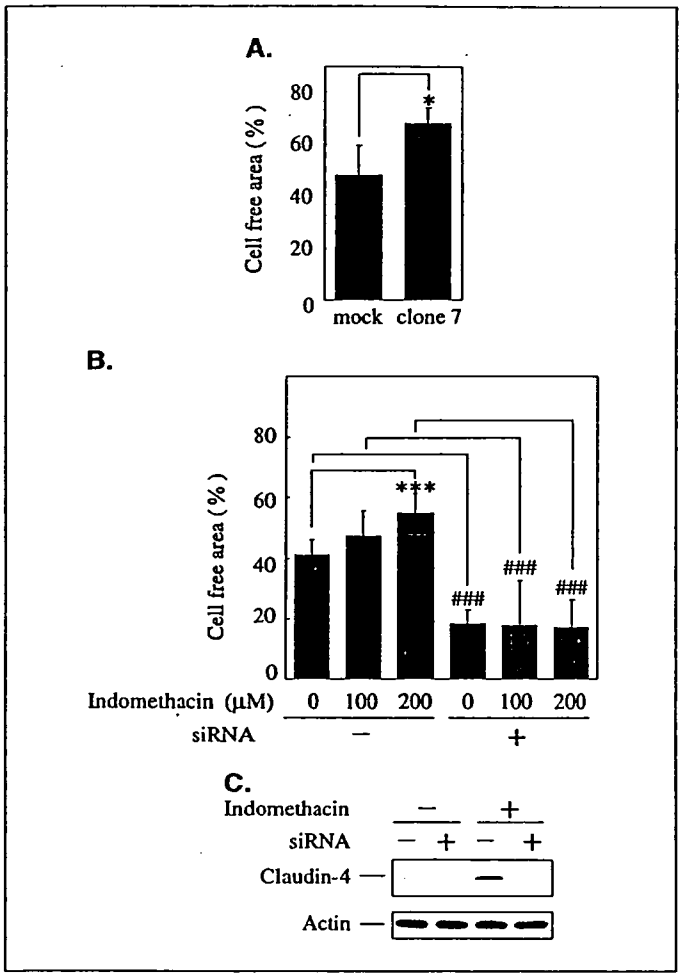

Figure 5. Effect of claudin-4 overexpression or of indomethacin on AGS cell migration. AGS cells of stable transfectant of claudin-4 expression plasmid (clone 7 in Fig. 4) and mock transfectant control AGS cells (*A*) or AGS cells transfected or nontransfected with siRNA for claudin-4 (*B*) were wounded 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.

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.

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Low Direct Cytotoxicity of Nabumetone on Gastric Mucosal Cells

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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).

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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 in the market. Because most NSAID prodrugs do not possess any significant specificity for COX-2, these prodrugs may become very important as NSAIDs, considering the potential risk for cardiovascular thrombotic events of selective COX-2 inhibitors.

NSAIDs have a direct cytotoxic effect on gastrointestinal mucosal cells (13, 14) and we recently demonstrated that NSAIDs induce both necrosis and apoptosis in cultured gastric mucosal cells in a manner independent of COX inhibition (15–17). We also found that NSAIDs cause membrane permeabilization, which in turn is implicated in their direct cytotoxicity; that is, liposomal membranes are directly permeabilized by NSAIDs at concentrations closely related to those which result in cytotoxicity (18). Furthermore, we recently suggested that the combined effect of COX inhibition and the direct cytotoxic effect of NSAIDs (direct cell damage) on the gastric mucosa induces the production of gastric lesions (19). Therefore, the direct cytotoxicity of individual NSAIDs is a key factor to be determined in assessing their harmfulness on the gastric mucosa.

Since the direct cytotoxicity of NSAID prodrugs has not been studied at all, we examined here the direct cytotoxicity of nabumetone which, along with its active metabolite 6-methoxy-2-naphthylacetic acid (6MNA), was found to not harm the gastrointestinal mucosa in clinical studies on humans and in animal models (20, 21). Compared to indomethacin and celecoxib, both nabumetone and 6MNA showed very low activities for inducing necrosis, apoptosis and membrane permeabilization. Furthermore, in combination with the intravenous administration of a low dose of indomethacin (conditions under which gastric mucosal COX activity is completely inhibited), the oral administration of nabumetone did not result in the production of gastric lesions, which is in contrast to results obtained following the oral administration of celecoxib. Based on these observations, we consider that the low direct cytotoxicity of nabumetone will render its use safe on the gastrointestinal mucosa in vivo.

MATERIALS AND METHODS

Chemicals and Media. Fetal bovine serum (FBS) was from Gibco Co. 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was from Sigma Co. Nabumetone and 6MNA were kindly gifted from Sanwa Kagaku Kenkyusho Co. Indomethacin was from Wako Co. Celecoxib was from LKT Laboratories Inc. Egg phosphatidylcholine (PC) was from Kanto Chemicals Co. The ELISA kit for PGE_2 quantitation was from Cayman Chemical Co. Male Wistar rats weighing 160–200 g and male guinea pigs weighing 200–300 g were purchased from Shimizu Co. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health and were approved by the Animal Care Committee of Kumamoto University.

In Vitro Assay of Cytotoxicity of NSAIDs

Gastric mucosal cells were isolated from guinea pig fundic glands as described previously (22, 23). Isolated gastric mucosal cells were cultured for 12 hr in RPMI 1640 containing 0.3% v/v FBS, 100 U/ml ampicillin and 100 μg/ml streptomycin in type-I collagen-coated plastic culture plates under the conditions of 5% CO_2/95% air and 37 C. After removing non-adherent cells, cells attached to the plate were used. Guinea pig gastric mucosal cells prepared under these conditions were previously characterized, with the majority (about 90%) of cells being identified as pit cells (22, 24).

NSAIDs were dissolved in DMSO. Cells were exposed to NSAIDs by changing the entire bathing medium.

We used MTT assay for monitoring cell viability. Cells were incubated for 2 hr with MTT solution at a final concentration of 0.5 mg/ml. Isopropanol and hydrochloric acid were added to the culture medium at final concentrations of 50% and 20 mM, respectively. The optical density of each sample at 570 nm was determined spectrophotometrically using a reference wavelength of 630 nm (25).

Gastric Damage Assay. Gastric damage assays were performed as described previously (19). Rats (24 hr fasted) were administered orally with NSAIDs in 1% methylcellulose in a volume of 5 ml/kg. In some experiments, indomethacin in PBS was administered intravenously 1 hr before the oral administration. Six hours after the oral administration, the rats were anesthetized and the stomach was removed and scored for hemorrhagic damage by an observer unaware of the treatment the rats had received. The score involved measuring the area of all lesions in millimeters squared and summing the values to give an overall gastric lesion index. Determination of PGE_2 levels at the gastric mucosa was done by ELISA as previously described (26).

Assay for Erythrocyte Hemolysis. Hemolysis in erythrocytes were monitored as described (18). Human erythrocytes were washed twice with buffer A (5 mM HEPES/NaOH (pH 7.4) and 150 mM NaCl) and then suspended in fresh buffer A at a final concentration of 0.5% hematocrit (5×10^7 cells/ml). After incubation with NSAIDs for 10 min at 30 C, hemolysis was estimated by measuring the absorbance at 540 nm.

Membrane Permeability Assay. Membrane permeability assays were performed as described previously (18). Liposomes were prepared using the reversed-phase evaporation method. Egg PC (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2, v/v), dried, and dissolved in 1.5 ml of diethyl ether. This was followed by the addition of 1 ml of 100 mM calcein-NaOH (pH 7.4). The mixture was sonicated to obtain a homogenous emulsion. The diethyl ether solvent was removed using a conventional rotary evaporator under reduced pressure at 25 C. The resulting suspension of liposome was centrifuged and washed twice with fresh buffer A (10 mM phosphate buffer, containing 150 mM NaCl) to remove untrapped calcein. The final liposome

precipitate was re-suspended in 5 ml buffer A. A 0.3 ml aliquot of this suspension was diluted with 19.7 ml of buffer A, following which 400 l of this suspension was incubated at 30 C for 10 min in the presence of the NSAID under investigation. The release of calcein from liposomes (the amount of calcein outside the liposomes) was determined by measuring fluorescence intensity at 520 nm (excitation at 490 nm), because the calcein fluoresces very weakly when at high concentrations (when calcein is trapped in liposomes) due to self-quenching.

Statistical Analyses. All values are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test was used for the 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 $P < 0.05$.

RESULTS AND DISCUSSION

Necrosis- and Apoptosis-Inducing Activities of Nabumetone and 6MNA. We previously reported that NSAIDs induced either necrosis or apoptosis depending on treatment conditions; short-term (1 hr) treatment of primary cultures of guinea pig gastric mucosal cells with relatively high concentrations of NSAIDs (2.5 mM for indomethacin and 0.2 mM for celecoxib) and long-term (16 hr) treatment of these cells with relatively low concentrations of NSAIDs (1 mM for indomethacin and 0.05 mM for celecoxib) induces necrosis and apoptosis, respectively (15, 18, 19). Nabumetone and 6MNA were tested here for their ability to induce necrosis and apoptosis. Consistent with previous reports (15, 18, 19), cell viability decreased in a dose-dependent manner when guinea pig gastric mucosal cells in primary culture were treated with indomethacin or celecoxib for 1 hr. In contrast, nabumetone and 6MNA decreased cell viability to a much lesser extent under the same experimental conditions (Figure 1A), with the necrosis- and apoptosis-inducing effects of nabumetone being slightly but significantly lower than those of 6MNA (Figure 1B). We confirmed that cell death highlighted in Figure 1 was mediated by necrosis given that no accompanying apoptotic DNA fragmentation or chromatin condensation were evident (data not shown).

Similar results to the above were obtained when apoptosis was induced. Treatment of cells for 16 hr with indomethacin or celecoxib decreased cell viability in a dose-dependent manner (Figure 2A), which is also consistent with previous reports (15, 18, 19). Nabumetone and 6MNA showed very low activities for decreasing cell viability under these conditions (Figure 2A), and nabumetone was again slightly but significantly less damaging than 6MNA (Figure 2B). Because cell death as highlighted in Figure 2 was accompanied by apoptotic DNA fragmentation and chromatin condensation (data not shown), it is

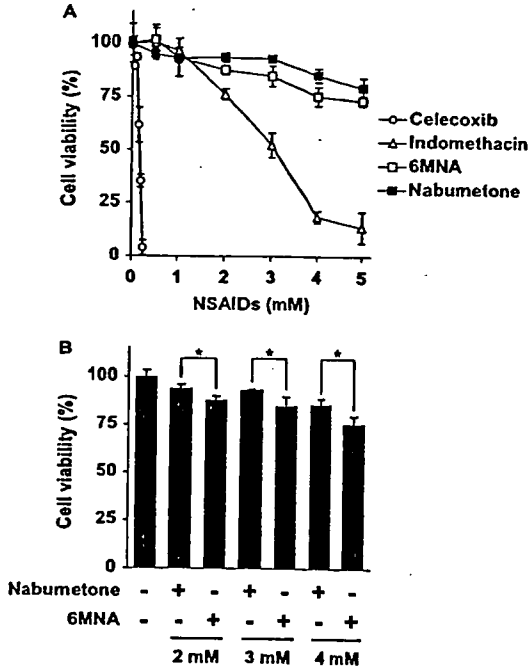

Fig 1. Necrosis induced by NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NSAIDs for 1 hr. Cell viability was determined by the MTT method. Values are mean ± SD ($n = 3$). $^*P < 0.05$.

most likely to have been mediated by apoptosis. Overall, the results in Figures 1 and 2 show that nabumetone and 6MNA induce necrosis and apoptosis to a lesser extent than do indomethacin and celecoxib. Furthermore, although the metabolic conversion of nabumetone to 6MNA drastically increases the inhibition of COX activity, this conversion does not seem to be associated with a similar increase in direct cytotoxicity.

Membrane Permeabilization Activities of Nabumetone and 6MNA. The ability of nabumetone and 6MNA to permeabilize the membranes of calcein-loaded liposomes was examined. Calcein fluoresces very weakly when at high concentrations due to self-quenching. Thus, the addition of membrane permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by releasing calcein trapped within the liposomes and thus lower the calcein concentration (18). As shown in Figure 3, indomethacin and celecoxib increased the calcein fluorescence in a dose-dependent manner, which is consistent with previous findings (18). Nabumetone and 6MNA also increased the calcein fluorescence, suggesting that they

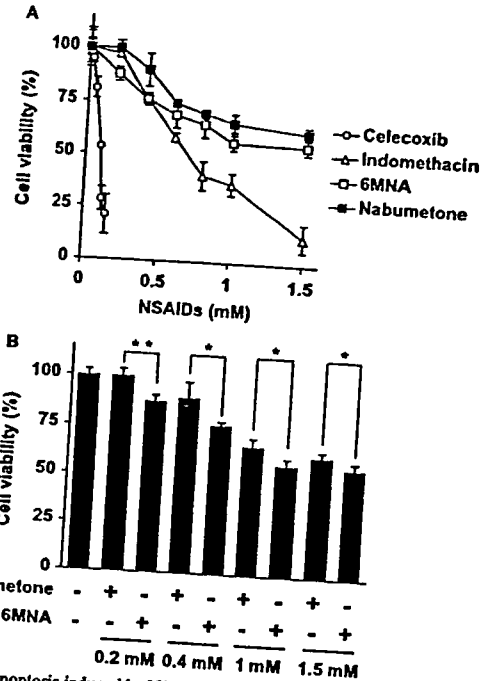

Fig 2. Apoptosis induced by NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NSAIDs for 16 hr. Cell viability was determined by the MTT method. Values are mean ± SD ($n = 3$). $^{**}P < 0.01$; $^{*}P < 0.05$.

caused membrane permeabilization; however, as the concentrations of nabumetone and 6MNA required for membrane permeabilization were much higher than those of indomethacin and celecoxib, their abilities to permeabilize membranes were thus very weak. The results shown in Figure 3 suggest that the low direct cytotoxicity of

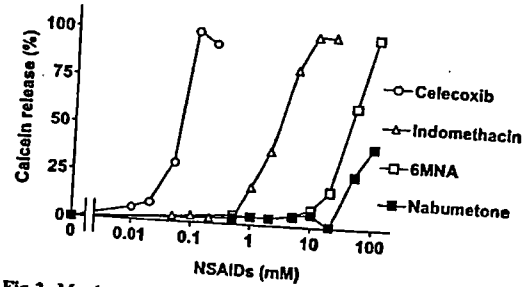

Fig 3. Membrane permeabilization by NSAIDs. Calcein-loaded liposomes were incubated for 10 min at 30 C with indicated concentrations of each NSAID. The release of calcein from liposomes was determined by measuring fluorescence intensity. Melittin (10 μM) was used to determine the 100% level of membrane permeabilization.

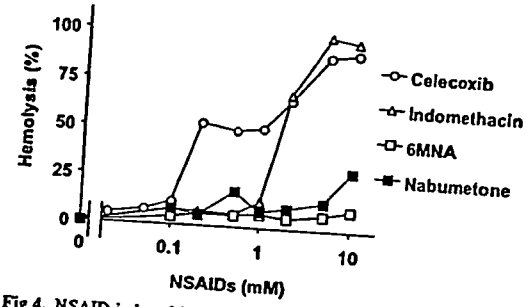

Fig 4. NSAID-induced hemolysis from erythrocytes. Human erythrocytes were incubated in the presence of each of NSAIDs for 10 min at 30 C. Hemolysis was estimated by measuring the absorbance at 540 nm. Melittin (10 μM), a membrane permeabilizing reagent, was used to determine the 100% level of hemolysis (33).

nabumetone and 6MNA is due to their low membrane permeabilizing effects. When nabumetone and 6MNA were compared, 6MNA had a higher membrane permeabilizing effect than nabumetone (Figure 3), which is consistent with the results describing their direct cytotoxicity (Figures 1 and 2).

We also examined the membrane permeabilization activities of NSAIDs by measuring hemolysis. As shown in Figure 4, results similar to calcein release (Figure 3) were obtained, suggesting that NSAIDs cause membrane permeabilization not only in liposomes but also in cells. Membrane permeabilization (Figures 3 and 4) was observed at relatively higher concentrations of NSAIDs than those required for decrease in cell viability (Figures 1 and 2), being consistent with our previous report (18). This may be due to the difference in assay conditions.

Activities of Nabumetone and 6MNA for Production of Gastric Lesions. As described in the Introduction, we recently found that gastric lesions develop in a manner that depends both on intravenously administered low doses of indomethacin and on orally administered cytotoxic NSAIDs, such as celecoxib (19). Using this model, the ability of nabumetone to produce gastric lesions was compared to that of celecoxib. As shown in Figure 5, in the absence of prior intravenous administration of indomethacin, the oral administration of either nabumetone or celecoxib did not clearly produce gastric lesions, which is consistent with previous results (27, 28). Oral administration of either nabumetone or celecoxib did not significantly reduce the level of PGE_2 (Table 1). The intravenous administration of a low dose (5 mg/kg) of indomethacin alone also did not produce gastric lesions (Figure 5), but did bring about a reduction of more than 90% in the level of PGE_2 (Table 1). A combination of the oral administration of celecoxib and the intravenous administration of

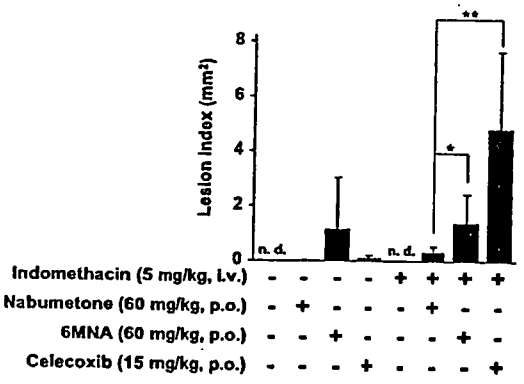

Fig 5. Production of gastric lesions by NSAIDs. Rats were intravenously administered with indomethacin or vehicle. After 1 hr, animals were administered orally with nabumetone, celecoxib, 6MNA or vehicle. After 6 hr, the stomach was removed and scored for hemorrhagic damage as described in Materials and Methods section. Values are mean ± SD ($n = 5$–6). $^{**}P < 0.01$; $^{*}P < 0.05$. n.d.; not detected.

indomethacin clearly gave rise to the production of gastric lesions (Figure 5) as previously reported (19). In contrast, gastric lesions were not so evident when the oral administration of nabumetone and the intravenous administration of indomethacin were used in combination (Figure 5). Oral administration of 6MNA produced a little but significant gastric lesions both in the presence or absence of intravenous administration of indomethacin (Figure 5). This may be due to that oral administration of 6MNA, itself, significantly reduced the level of PGE_2 (Table 1). We consider from the results presented in Figure 5 that nabumetone also has a low level of direct cytotoxicity in vivo.

In summary, we show here that nabumetone has a very low level of direct cytotoxicity on gastric mucosal cells in vitro and suggest that this is also the case in vivo. As described earlier, it is well known that nabumetone is experimentally and clinically safe and that its use is not as harmful to the gastric mucosa compared to other NSAIDs such as indomethacin and aspirin (20, 29). In addition to its inability to inhibit gastric mucosal COX activity soon after oral administration, its inhibitory effect on neutrophil functions was also recently suggested (30). We propose here that in addition to these mechanisms, the low direct cytotoxicity of nabumetone make it far less harmful on the gastric mucosa and therefore much safer for clinical use.

It is known that non-selective NSAIDs modulate the gastric acid secretion and inhibit bicarbonate secretion (31, 32). Although we did not examine the effect of nabumetone on these processes, it is possible that these process also involve the safety of this drug on gastric mucosa in vivo.

TABLE 1. INHIBITION OF GASTRIC PGE_2 SYNTHESIS BY NSAIDS

NSAIDs	*Gastric PGE_2 (ng/g tissue)*
Control	29.3 ± 2.6
5 mg/kg indomethacin i.v.	2.4 ± 0.4***
15 mg/kg celecoxib p.o.	32.4 ± 11.8
60 mg/kg nabumetone p.o.	19.1 ± 9.6
60 mg/kg 6MNA p.o.	2.8 ± 0.6***

Note. Rats were intravenously (i.v.) or orally (p.o.) administered with indicted 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 ($n = 3$).
$^{***}P < 0.001$.

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Low Direct Cytotoxicity and Cytoprotective Effects of Nitric Oxide Releasing Indomethacin

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Nitric oxide (NO) releasing non-steroidal anti-inflammatory drugs (NSAIDs) have shown a marked reduction of gastrointestinal side effects and we here examined the cytotoxicity of NCX 530 (NO-indomethacin). Under conditions where indomethacin clearly induced both necrosis and apoptosis, NCX 530 induced neither. NCX 530 protected cells from celecoxib-induced necrosis and apoptosis. NCX 530 partially suppressed celecoxib-dependent membrane permeabilization and an inhibitor for guanylate cyclase suppressed the cytoprotective effect of NCX 530 against celecoxib. In vivo, NCX 530 alone produced fewer gastric lesions in rats than did indomethacin. A combination of the oral administration of celecoxib together with the intraperitoneal administration of indomethacin, but not of NCX 530, clearly resulted in the production of gastric lesions. The low direct cytotoxicity and the cytoprotective effect of NCX 530 observed in vitro may also act in vivo, thus ensuring that NCX 530 is safe for use on the gastric mucosa.

KEY WORDS: direct cytotoxicity; cyclooxygenase; NO-NSAID; indomethacin; gastric lesion; selective cyclooxygenase-2 inhibitor.

Non-steroidal anti-inflammatory drugs (NSAIDs) are a very popular class of medicines that account for nearly 5% of all prescribed medications (1). The anti-inflammatory action of NSAIDs is mediated through their inhibition of cyclooxygenase (COX) activity. COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. On the reverse side, NSAID use is associated with gastrointestinal complications (2), with about 15–30% of chronic users of NSAIDs suffering from gastrointestinal ulcers and bleeding (3–6). In the United States, about 16,500 people die per year as a result of NSAID-associated gastrointestinal complications (7).

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The inhibition of COX by NSAIDs was previously thought to be the sole explanation for their associated gastrointestinal side effects (8), given that PGs have a strong protective effect on the gastrointestinal mucosa (9). However, since the increased incidence of gastrointestinal lesions and the decrease in PG levels induced by NSAIDs do not always occur in parallel (10, 11), it was proposed that the induction of gastrointestinal lesions by NSAIDs involved additional mechanisms (12). It is well known that NSAIDs have a direct cytotoxic effect on gastrointestinal mucosal cells (12–14). We recently demonstrated that NSAIDs induce both necrosis and apoptosis in cultured gastric mucosal cells in a manner independent of COX inhibition (15, 16). Furthermore, we recently proposed that both COX inhibition and the direct cytotoxic effect of NSAIDs (direct cell damage) on the gastric mucosa are involved in the production of gastric lesions in vivo; gastric lesions develop in a manner that depends on both an intravenously administered low dose of indomethacin (inhibition of COX activity without direct gastric mucosal cell damage) and an orally administered cytotoxic

COX-2-selective inhibitor, such as celecoxib (direct cell damage without inhibition of COX at the gastric mucosa) (16). Therefore, the direct cytotoxicity of individual NSAIDs is one of key factors to determine the safety of their use on the gastric mucosa.

Nitric oxide (NO) releasing NSAIDs (NO-NSAIDs) are a newly developed group of NSAIDs, consisting of a traditional NSAID to which a group donating NO has been covalently attached via spacers. NO-NSAIDs show a marked reduction of gastrointestinal side effects but maintain, and in some cases extend, anti-inflammatory properties both in clinical studies on humans and in animal models (17–21). Further to this, given that the anti-thrombotic effect of NO-aspirin was reported to be superior to that of aspirin (22), much attention is now being paid to NO-NSAIDs as alternatives in treatment protocols.

Various mechanisms have been proposed for the gastrointestinal safety of NO-NSAIDs, such as inhibition of neutrophil adherence, promotion of mucosal blood flow and stimulation of mucin and bicarbonate secretion by NO (21, 23). In addition to these mechanisms, a lower cytotoxicity on gastric mucosal cells was reported for one NO-NSAID, NO-flurbiprofen, whose use resulted in reduced apoptosis compared to that seen with standard flurbiprofen (24). Furthermore, NO-flurbiprofen suppressed the extent of TNFα- or ceramide-induced apoptosis by inhibiting caspases in vitro (24, 25). However, necrosis induced by NO-NSAIDs and apoptosis induced by NO-NSAIDs other than NO-flurbiprofen are yet to have been studied. Furthermore, although more than two species of NSAIDs are usually used simultaneously in a clinical setting, the effect of NO-NSAIDs on cell death induced by other NSAIDs is also yet to be examined.

In this study, we used primary cultures of guinea pig gastric mucosal cells to examine the direct cytotoxicity of NCX 530 (NO-indomethacin), which according to one animal study (17) is safe from the perspective of the gastric mucosa. We also examined the effect of NCX 530 on cell death induced by other gastric irritants, including NSAIDs. NCX 530 not only showed a lower propensity than indomethacin for inducing both necrosis and apoptosis, but also protected cells from necrosis and apoptosis induced by celecoxib or ethanol. In vivo, NCX 530 alone or in combination with orally administered celecoxib or ethanol resulted in the production of fewer gastric lesions than did indomethacin. Furthermore, NCX 530 suppressed the production of gastric lesions caused by other gastric irritants. These results concerning the safety of NCX 530 use *in vivo* are probably related to the low direct cytotoxicity and cytoprotective effects of NCX 530 observed in vitro.

MATERIALS AND METHODS

Chemicals and Media. Fetal bovine serum (FBS) was from Gibco Co. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 1H-[1,2,4-]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and cycloheximide were from Sigma Co. Celecoxib was from LKT Laboratories Inc. Indomethacin and NCX 530 were kindly provided by NicOx S. A. Egg phosphatidylcholine (PC) was from Kanto Chemicals Co. Male Wistar rats weighing 160–200 g and male guinea pigs weighing 200–300 g were purchased from Shimizu Co. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health and were approved by the Animal Care Committee of Kumamoto University.

In vitro Assay of Cytotoxicity and DNA Fragmentation. Gastric mucosal cells were isolated from guinea pig fundic glands as described previously (26, 27). Isolated gastric mucosal cells were cultured for 12 hr in RPMI 1640 containing 0.3% v/v FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in type-I collagen-coated plastic culture plates under the conditions of 5% CO_2/95% air and 37°C. After removing non-adherent cells, cells attached to the plate were used. Guinea pig gastric mucosal cells prepared under these conditions were previously characterized, with the majority (about 90%) of cells being identified as pit cells (26, 28). NSAIDs were dissolved in DMSO and control experiments (without NSAIDs) were performed in the presence of same concentrations of DMSO. Cells were exposed to NSAIDs by changing the entire bathing medium. Cell viability was determined by the MTT method.

Apoptotic DNA fragmentation was monitored as previously described (29). Cells were collected using a rubber policeman and suspended in 50 μL of lysis buffer, consisting of 50 mM Tris–HCl (pH 7.8), 10 mM EDTA, and 0.5% sodium-*N*-lauroylsarcosinate. Proteinase K was added to a final concentration of 1 mg/mL, and the lysate incubated at 50°C for 2 hr. RNaseA was then added to a final concentration of 0.5 mg/mL and incubated at 50°C for 30 min. These samples were analyzed by 2% agarose gel electrophoresis in the presence of 0.5 μg/mL ethidium bromide.

Gastric Damage Assay. Gastric damage assays were performed as described previously (16). Rats (24 hr fasted) were administered orally with ethanol or NSAIDs in 1% methylcellulose in a volume of 5 mL/kg. In some experiments, indomethacin or NCX 530 in 1% methylcellulose was administered intraperitoneally 1 hr before the oral administration. Six hours after the oral administration, the rats were anesthetized and the stomach was removed and scored for hemorrhagic damage by an observer unaware of the treatment the rats had received. The score involved measuring the area of all lesions in millimeters squared and summing the values to give an overall gastric lesion index.

Membrane Permeability Assay. Liposomes were prepared using the reversed-phase evaporation method (30, 31). Egg PC (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1:2, v/v), dried, and dissolved in 1.5 mL of diethyl ether. This was followed by the addition of 1 mL of 100 mM calcein-NaOH (pH 7.4). The mixture was sonicated to obtain a homogenous emulsion. The diethyl ether solvent was removed using a conventional rotary evaporator under reduced pressure at 25°C. The resulting suspension of liposome was centrifuged and washed twice with fresh buffer A (10 mM potassium buffer, containing 150 mM

NaCl) to remove untrapped calcein. The final liposome precipitate was re-suspended in 5 mL buffer A. A 0.3 mL aliquot of this suspension was diluted with 19.7 mL of buffer A, following which 400 L of this suspension was incubated at 30°C for 10 min in the presence of the NSAID under investigation. The final PC concentration was 30 μM. The release of calcein from liposomes was determined by measuring fluorescence intensity at 520 nm (excitation at 490 nm).

Statistical Analyses. All values are expressed as the mean ± standard error (S.E.M.). One-way analysis of variance (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 $P < 0.05$.

RESULTS

Comparison of Necrosis- and Apoptosis-Inducing Activities of NCX 530 and Indomethacin. We previously reported that short-term (1 hr) treatment of primary cultures of guinea pig gastric mucosal cells with relatively high concentrations of NSAIDs and long-term (16 hr) treatment of these cells with relatively low concentrations of NSAIDs induces necrosis and apoptosis, respectively (15). The results of experiments in which guinea pig gastric mucosal cells in primary culture were treated with NCX 530 or indomethacin for 1 hr are shown in Figure 1A. Indomethacin decreased cell viability in a dose-dependent manner, while NCX 530 did not affect cell viability at concentrations lower than 5 mM. We confirmed that cell death induced by indomethacin (Figure 1A) was mediated by necrosis as no accompanying apoptotic DNA fragmentation or chromatin condensation was evident (data not shown). The findings of experiments in which cells were treated with NCX 530 or indomethacin for 16 hr are summarized in Figure 1B. Again, indomethacin but not NCX 530 induced cell death. Because cell death under these conditions was accompanied by apoptotic DNA fragmentation (Figure 1C), it is most likely to have been mediated by apoptosis. Overall, the results in Figure 1 show that NCX 530 had a lower capacity than indomethacin for inducing necrosis and apoptosis.

Effect of NCX 530 on Cell Death Induced by Other Gastric Irritants. We next examined the cytoprotective effect of NCX 530 by pre-treating gastric mucosal cells with NCX 530 for 1 hr (pre-incubation step) and then with one of several of gastric irritants (celecoxib, ethanol, indomethacin or hydrogen peroxide) for 16 hr (apoptotic conditions) (Figure 2) or 1 hr (Figure 3) (necrotic conditions) (incubation step). As shown in Figure 2A, treatment of cells with 0.1 mM celecoxib for 16 hr induced cell death which could be partially suppressed by the pre-treatment of cells with NCX 530. A similar cytoprotective effect of NCX 530 was observed for cell death induced by exposure of cells to 3% ethanol for 16 hr (Figure 2B). Under these conditions (Figure 2), the cell death was mediated by apoptosis given that apoptotic DNA fragmentation and chromatin condensation were also present (data not shown).

As shown in Figure 3A and B, NCX 530 partially suppressed the cell death induced by treatment of cells with 0.18 mM celecoxib or 8% ethanol for 1 hr. Cell death under these conditions was mediated by necrosis as apoptotic DNA fragmentation and chromatin condensation were not in evidence (data not shown). NCX 530 was included in both the pre-incubation and incubation steps of experiments whose results are detailed in Figures 2 and 3. When NCX 530 was omitted in the pre-incubation step, its cytoprotective effect was similar to that when it was used in both the pre-incubation and incubation steps (data not shown). On the other hand, when NCX 530 was omitted from the incubation step (but included in the pre-incubation step), its cytoprotective effect was not as clear-cut. Thus, it seems that NCX 530 must be present simultaneously with celecoxib or ethanol in order to exert fully its cytoprotective effect.

The cytoprotective effect of NCX 530 was not observed for all gastric irritants tested. For example, NCX 530 affected neither apoptosis (Figure 2C and D) nor necrosis (Figure 3C and D) induced by indomethacin or hydrogen peroxide. Similar results were obtained when NCX 530 was omitted in the pre-incubation or incubation steps (data not shown). Based on these findings, it appears that NCX 530 protects gastric mucosal cells from necrosis and apoptosis induced by celecoxib or ethanol but not by indomethacin or hydrogen peroxide.

Mechanism for the Low Cytotoxicity and Cytoprotective Effect of NCX 530. We recently found that NSAIDs cause membrane permeabilization, which, in turn, is implicated in their cytotoxicity (induction of necrosis and apoptosis); that is, NSAIDs directly permeabilize the membranes of liposomes, with concentrations of NSAIDs required for this effect being closely related to those which result in cytotoxicity (32). In further experiments, the ability of NCX 530 and indomethacin to permeabilize the membranes of calcein-loaded liposomes was compared. Calcein fluoresces very weakly at high concentrations due to self-quenching. Thus, the addition of membrane permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by releasing calcein trapped within the liposomes (30, 31). As shown in Figure 4A, NCX 530 and indomethacin showed similar dose–response curves for the increase in calcein fluorescence, suggesting that their ability to cause membrane permeabilization is virtually indistinguishable. Therefore, it seems that the low cytotoxicity

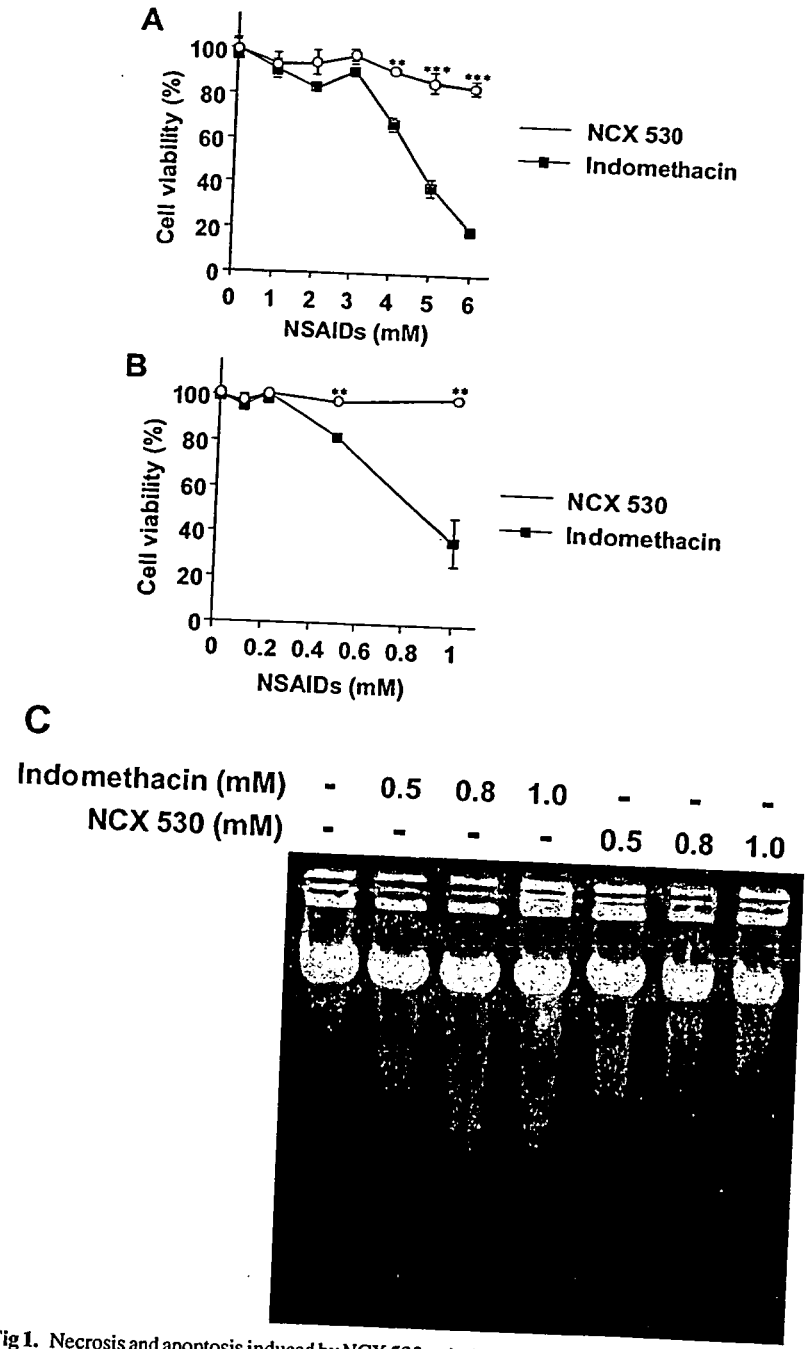
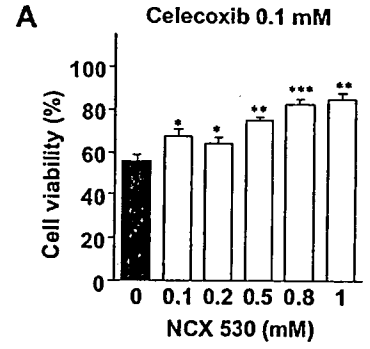
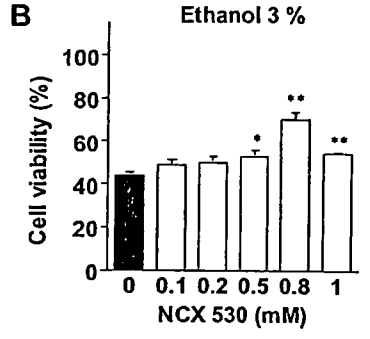
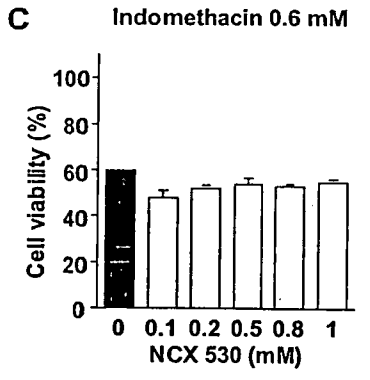


Fig 1. Necrosis and apoptosis induced by NCX 530 or indomethacin. Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NCX 530 or indomethacin for 1 hr (A) (necrotic conditions) or 16 hr (B, C) (apoptotic conditions). Cell viability was determined by the MTT method (A, B). Values are mean ± S.E.M. ($n = 3$). ***$P < 0.001$; **$P < 0.01$. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (C).

of NCX 530 cannot be explained on the basis of its membrane permeabilization activity. Celecoxib caused membrane permeabilization at very low concentrations, as found previously (32), which could be partially suppressed by NCX 530 (Figure 4B) but not by indomethacin (data not shown). On the other hand, NCX 530 did not affect the indomethacin-dependent membrane permeabilization (data not shown). Therefore, the cytoprotective effect of







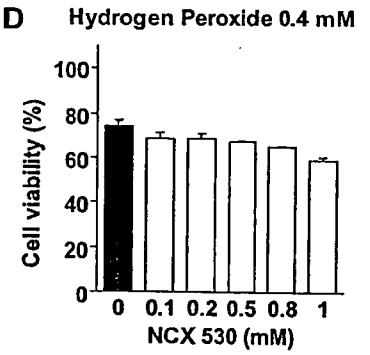
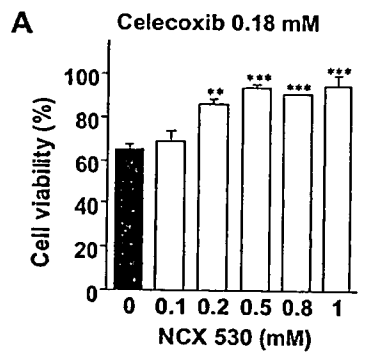
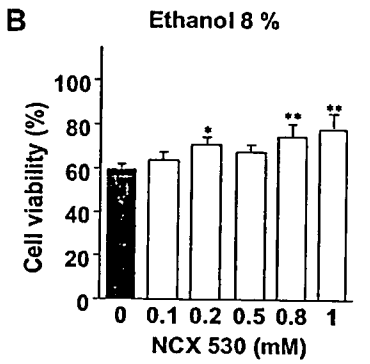
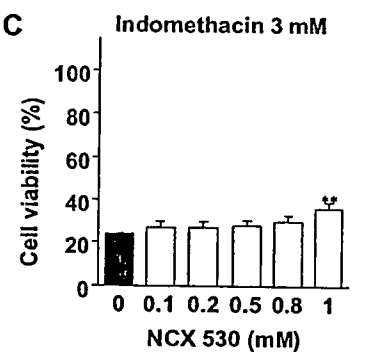


Fig 2. Effect of NCX 530 on apoptosis induced by various gastric irritants. Cultured guinea pig gastric mucosal cells were pre-incubated with indicated concentrations of NCX 530 for 1 hr and further incubated with 0.1 mM celecoxib (A), 3% ethanol (B), 0.6 mM indomethacin (C) or 0.4 mM hydrogen peroxide (D) in the presence of indicated concentrations of NCX 530 for 16 hr (apoptotic conditions). Cell viability was determined by the MTT method. Values are mean ± S.E.M. ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.







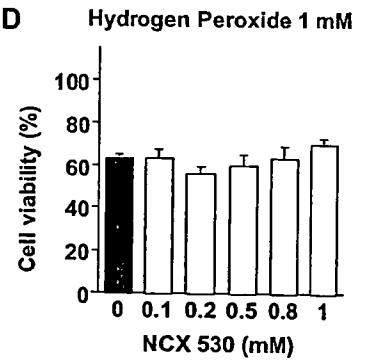


Fig 3. Effect of NCX 530 on necrosis induced by various gastric irritants. Cultured guinea pig gastric mucosal cells were pre-incubated with indicated concentrations of NCX 530 for 1 hr and further incubated with 0.18 mM celecoxib (A), 8% ethanol (B), 3 mM indomethacin (C) or 1 mM hydrogen peroxide (D) in the presence of indicated concentrations of NCX 530 for 1 hr (necrotic conditions). Cell viability was determined by the MTT method. Values are mean ± S.E.M. ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.