

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 Ca²⁺ concentration. Other drugs that increased the intracellular Ca²⁺ concentration (thapsigargin and ionomycin) also induced *claudin-4*. Furthermore, an intracellular Ca²⁺ 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 Ca²⁺ 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 E₂ (PGE₂), 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 homologue 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 $\mu\text{g}/\text{mL}$ 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, CCACTGAAGAGAGCCTGACC; *claudin-4*, CTCTGTGGCCACAGGACTCT, CAGGACTTCCAAGGGTGAAG; *occludin*, TCCAATGGCAAAGTGAATGA, GCAGTGCTCTTTTGAAGG; *COX-1*, CTGGCTCCGGAATTCACCT, 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, $[\text{Ca}^{2+}]_i$. The intracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, 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 $\mu\text{mol}/\text{L}$ 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. $[\text{Ca}^{2+}]_i$ was calculated according to the following equation: $[\text{Ca}^{2+}]_i = K_d(F - F_{\text{min}}) / (F_{\text{max}} - F)$, 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 CCCGCACAGACAAGCCT-TACT and siRNA 5'-CGCACAGACAAGCCUACUUU-3' and 5'-AGUAAAGG-CUUGUCUGUGCGGG-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 \pm 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. 1A, 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).

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

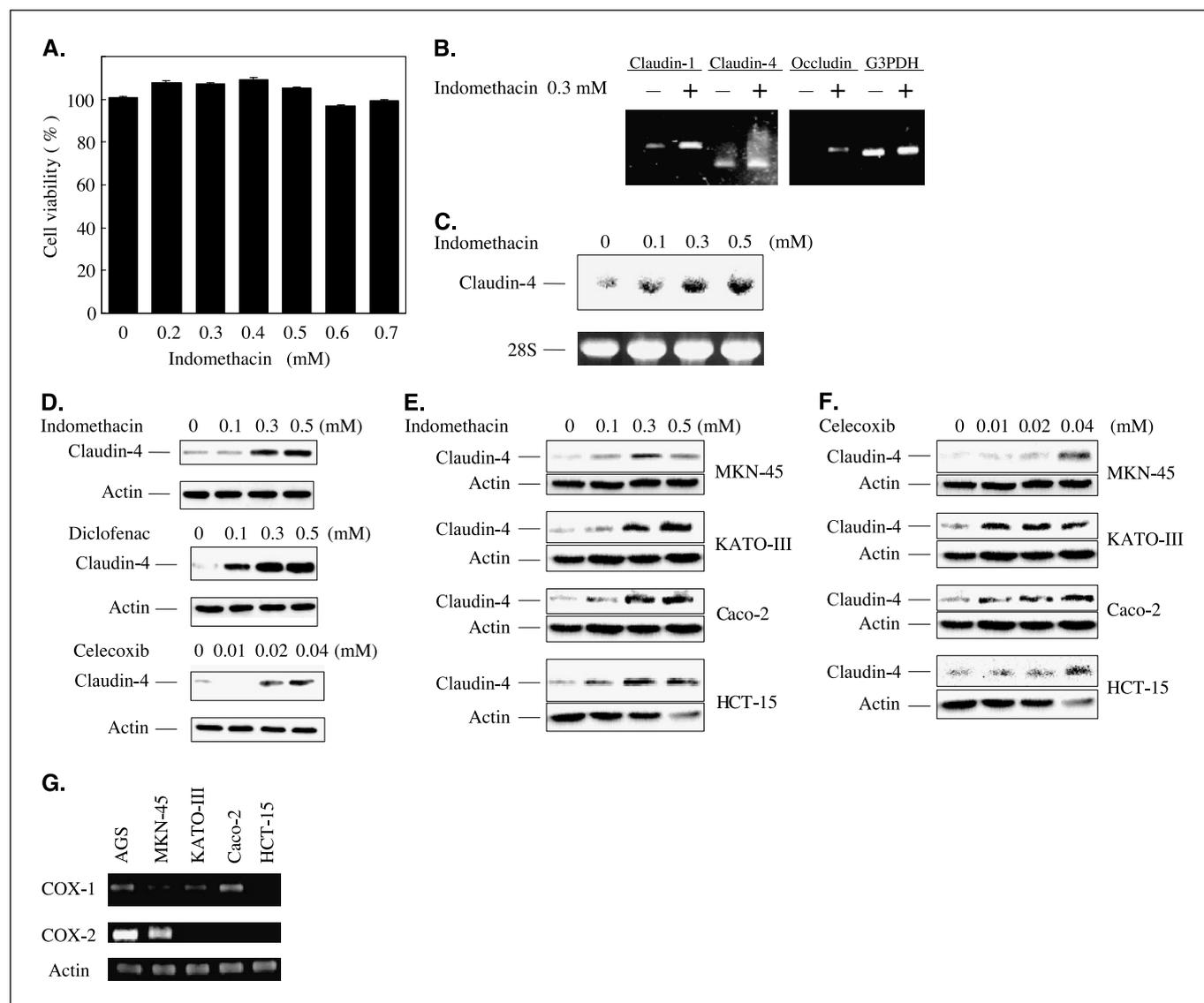


Figure 1. Induction of tight junction-related genes by NSAIDs. AGS (A-D and G) or MKN-45, KATO-III, Caco-2, and HCT-15 (E-G) cells were incubated with indicated concentrations of NSAIDs for 4 hours (A-C) or 24 hours (D-F). Results for cells cultured without NSAIDs (G). Cell viability was determined by 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 μ g 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. 1C) and immunoblot analysis (Fig. 1D), 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. 1E, 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. 1D). 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. 1D) but also in the other cell lines tested (Fig. 1F). 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. 1G). 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 PGE₂, a major prostaglandin in the gastric mucosa, on the induction of claudin-4

by indomethacin. PGE₂ (0.1-10 μmol/L) did not affect the level of claudin-4 in the presence and absence of indomethacin (Fig. 2A). We determined previously the level of PGE₂ in the culture medium of AGS cells to be ~10 nmol/L (23). Therefore, inhibition of PGE₂ 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. 2B, 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. 2C 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. 2E). *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 Ca²⁺ concentration, [Ca²⁺]_i (39, 40). In this study, we tested whether an increase in [Ca²⁺]_i by NSAIDs is responsible for the induction of claudin-4. Firstly, we confirmed that a NSAID-induced increase in [Ca²⁺]_i occurred under the same conditions as those in which the induction of claudin-4 in AGS cells was observed. As shown in Fig. 3A, all NSAIDs tested (indomethacin, diclofenac, and celecoxib) increased [Ca²⁺]_i at the same NSAID concentrations that caused the induction of claudin-4.

Some drugs that are known to increase [Ca²⁺]_i were examined for their capacity to induce claudin-4 expression. The actions of thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase, and the Ca²⁺ ionophore ionomycin were thus tested on AGS cells. As shown in Fig. 3A-C, in addition to increasing [Ca²⁺]_i, both thapsigargin and ionomycin induced claudin-4 in a dose-dependent manner. Furthermore, an intracellular Ca²⁺ 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. 3D). 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 [Ca²⁺]_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

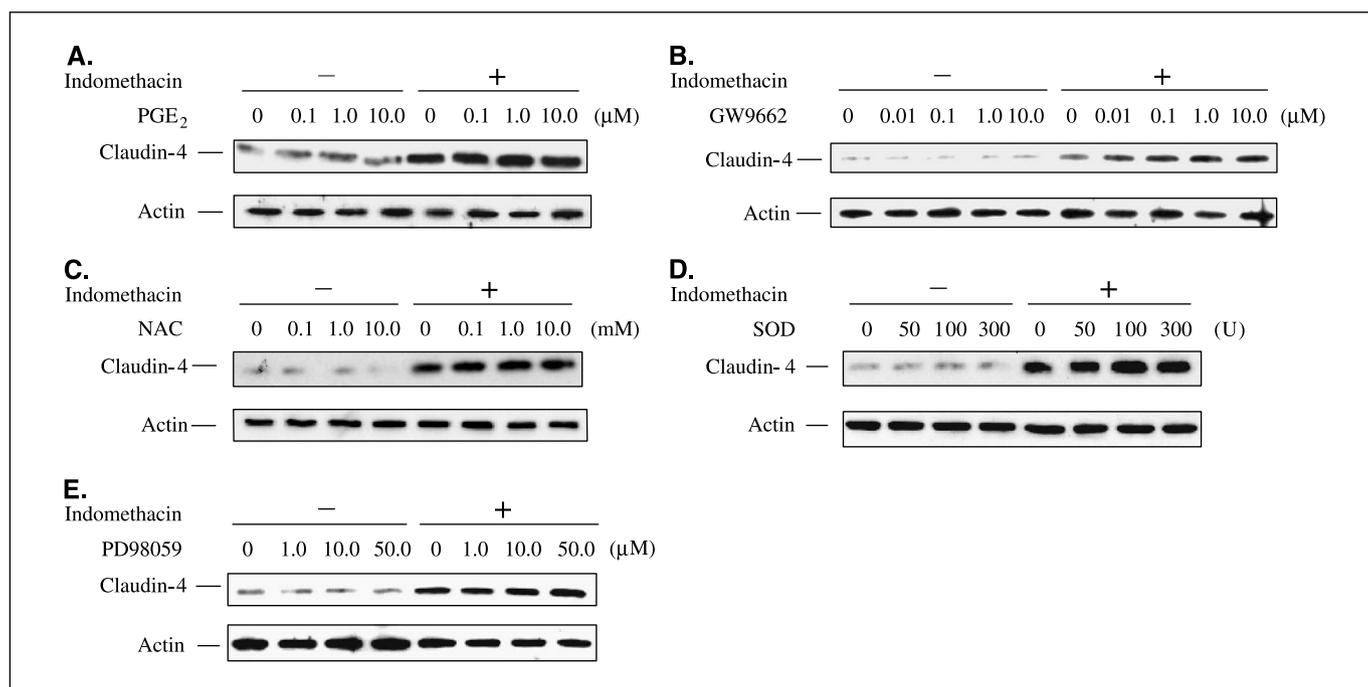


Figure 2. Mechanism for the induction of claudin-4 by indomethacin. AGS cells were incubated with or without 0.3 mmol/L indomethacin for 24 hours in the presence of indicated concentrations of PGE₂ (A), GW9662 (B), *N*-acetylcysteine (NAC; C), SOD (D), or PD98059 (E). Levels of claudin-4 and actin were 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.

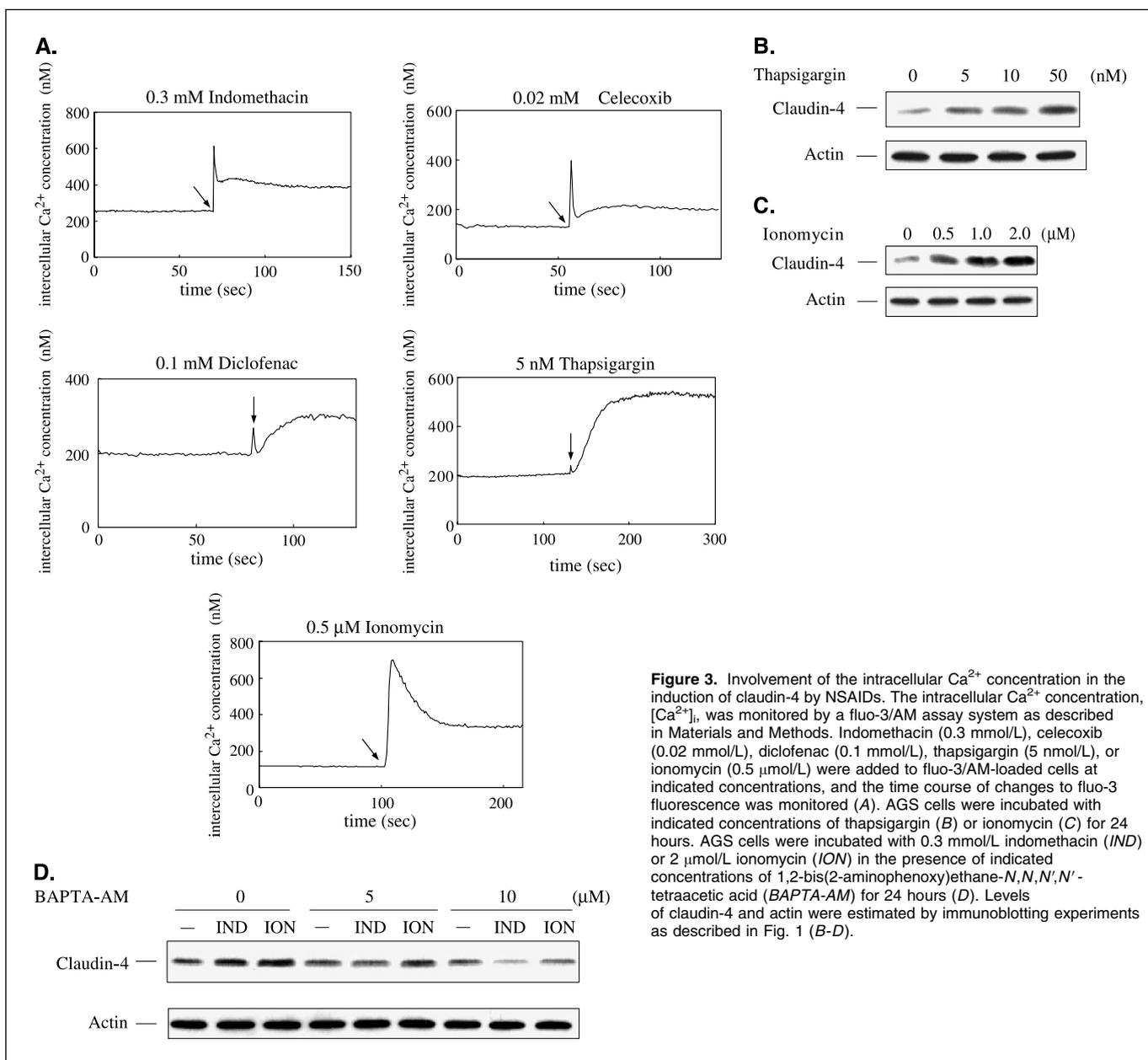


Figure 3. Involvement of the intracellular Ca^{2+} concentration in the induction of claudin-4 by NSAIDs. The intracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, was monitored by a fluo-3/AM assay system as described in Materials and Methods. Indomethacin (0.3 mmol/L), celecoxib (0.02 mmol/L), diclofenac (0.1 mmol/L), thapsigargin (5 nmol/L), or ionomycin (0.5 $\mu\text{mol/L}$) 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 $\mu\text{mol/L}$ 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. 4A).

Figure 4B 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. 4C, 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. 4C) 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. 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 transfected 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 μM /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-overexpressing cells (clone 7) showed less cell migration activity than the mock transfected control. Furthermore, transfection of siRNA for claudin-4 stimulated the migration activity of AGS cells even in the absence of indomethacin (Fig. 5B). We confirmed that the transfection almost completely inhibited the expression of claudin-4 in AGS cells (Fig. 5C). These results suggest that the migration activity of AGS cells decreases as claudin-4 expression increases.

As shown in Fig. 5B, 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

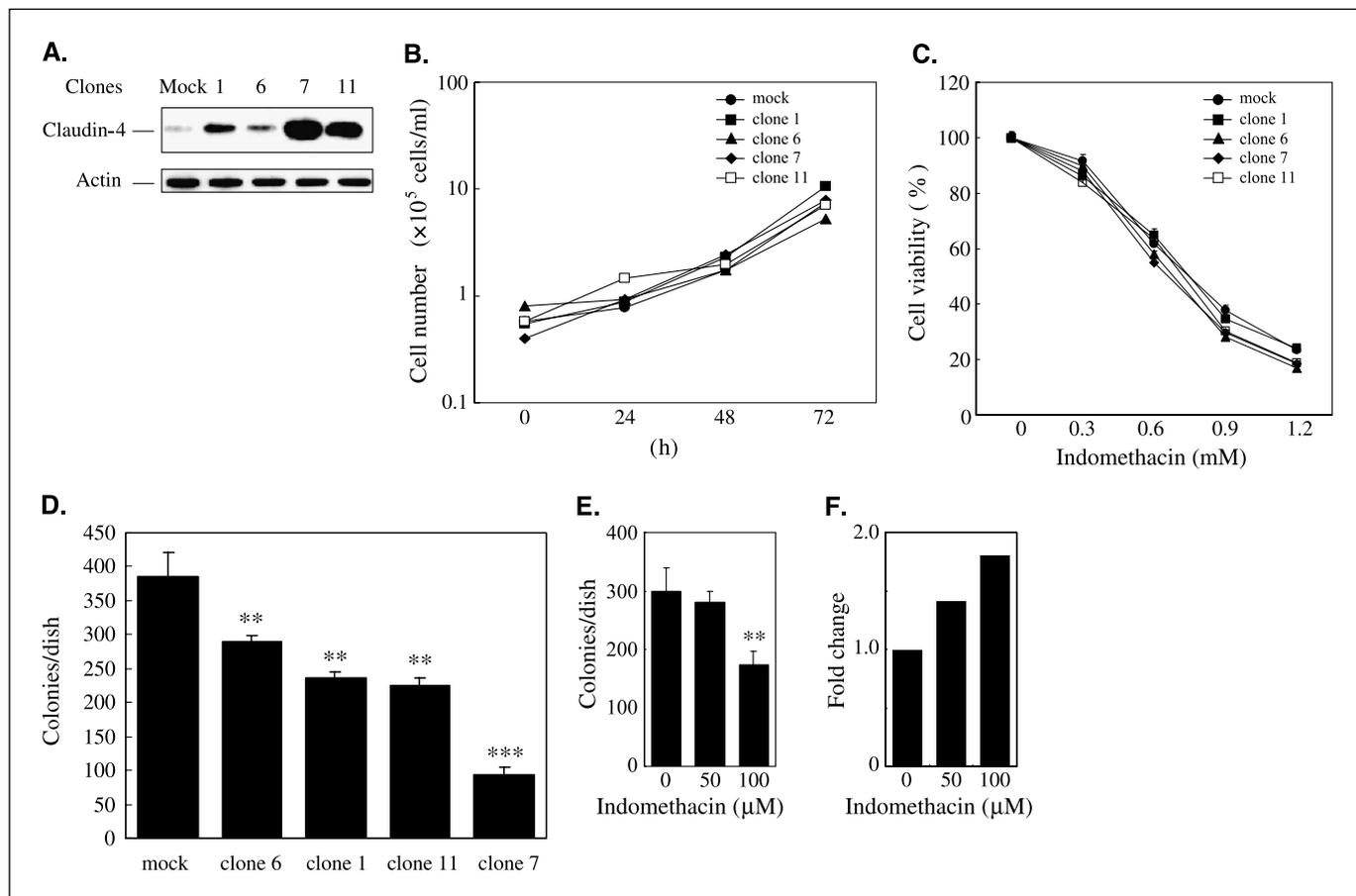


Figure 4. Effect of overexpression of claudin-4 on cell growth, apoptosis, and colony formation of AGS cells in soft agar. The extent of expression of claudin-4 in each clone (stable transfectant of claudin-4 expression plasmid) was estimated by immunoblotting experiments as described in Fig. 1 (A).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

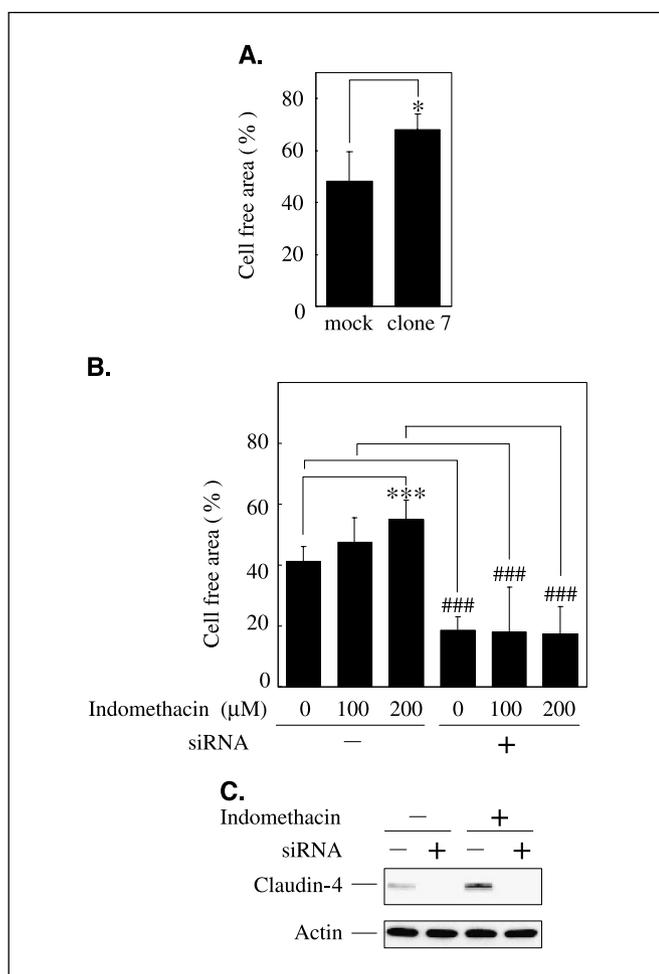


Figure 5. Effect of claudin-4 overexpression or of indomethacin on AGS cell migration. AGS cells of stable transfectant of claudin-4 expression plasmid (clone 7 in Fig. 4) and mock transfectant control AGS cells (A) or AGS cells transfected or nontransfected with siRNA for claudin-4 (B) were wounded 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. 5C). 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 PGE₂

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 PGE₂, 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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References

1. Smalley WE, Ray WA, Daugherty JR, Griffin MR. Nonsteroidal anti-inflammatory drugs and the incidence of hospitalizations for peptic ulcer disease in elderly persons. *Am J Epidemiol* 1995;141:539-45.
2. Farrow DC, Vaughan TL, Hansten PD, et al. Use of aspirin and other nonsteroidal anti-inflammatory drugs and risk of esophageal and gastric cancer. *Cancer Epidemiol Biomarkers Prev* 1998;7:97-102.
3. Sorensen HT, Friis S, Norgard B, et al. Risk of cancer in a large cohort of nonaspirin NSAID users: a population-based study. *Br J Cancer* 2003;88:1687-92.
4. Gupta RA, Dubois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat Rev Cancer* 2001;1:11-21.
5. Kismet K, Akay MT, Abbasoglu O, Ercan A. Celecoxib: a potent cyclooxygenase-2 inhibitor in cancer prevention. *Cancer Detect Prev* 2004;28:127-42.
6. Hoshino T, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T, Mizushima T. Prostaglandin E₂ protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation. *J Biol Chem* 2003;278:12752-8.
7. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 1998;93:705-16.
8. Rolland PH, Martin PM, Jacquemier J, Rolland AM, Toga M. Prostaglandin in human breast cancer: evidence suggesting that an elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells. *J Natl Cancer Inst* 1980;64:1061-70.
9. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994;107:1183-8.
10. Ristimaki A, Honkanen N, Jankala H, Sipponen P, Harkonen M. Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Res* 1997;57:1276-80.
11. Piazza GA, Alberts DS, Hixson LJ, et al. Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Res* 1997;57:2909-15.
12. Reddy BS, Kawamori T, Lubet RA, Steele VE, Kelloff GJ, Rao CV. Chemopreventive efficacy of sulindac sulfone against colon cancer depends on time of administration during carcinogenic process. *Cancer Res* 1999;59:3387-91.
13. Zhang X, Morham SG, Langenbach R, Young DA. Malignant transformation and antineoplastic actions of nonsteroidal antiinflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblasts. *J Exp Med* 1999;190:451-9.
14. Hanif R, Pittas A, Feng Y, et al. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol* 1996;52:237-45.
15. Anderson JM, Van Itallie CM. Tight junctions and the molecular basis for regulation of paracellular permeability. *Am J Physiol* 1995;269:G467-5.
16. Soler AP, Miller RD, Laughlin KV, Carp NZ, Klurfeld DM, Mullin JM. Increased tight junctional permeability is associated with the development of colon cancer. *Carcinogenesis* 1999;20:1425-31.
17. Li D, Mrsny RJ. Oncogenic Raf-1 disrupts epithelial tight junctions via downregulation of occludin. *J Cell Biol* 2000;148:791-800.
18. Hoevel T, Macek R, Swisshelm K, Kubbies M. Reexpression of the TJ protein CLDN1 induces apoptosis in breast tumor spheroids. *Int J Cancer* 2004;108:374-83.
19. Michl P, Barth C, Buchholz M, et al. Claudin-4 expression decreases invasiveness and metastatic potential of pancreatic cancer. *Cancer Res* 2003;63:6265-71.
20. Baek SJ, Kim KS, Nixon JB, Wilson LC, Eling TE. Cyclooxygenase inhibitors regulate the expression of a TGF- β superfamily member that has proapoptotic and antitumorogenic activities. *Mol Pharmacol* 2001;59:901-8.
21. Tsutsumi S, Gotoh T, Tomisato W, et al. Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. *Cell Death Differ* 2004;11:1009-16.
22. Tsutsumi S, Tomisato W, Hoshino T, Tsuchiya T, Mizushima T. Transforming growth factor- β 1 is responsible for maturation-dependent spontaneous apoptosis of cultured gastric pit cells. *Exp Biol Med* (Maywood) 2002;227:402-11.
23. Tsutsumi S, Tomisato W, Takano T, Rokutan K, Tsuchiya T, Mizushima T. Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture. *Biochim Biophys Acta* 2002;1589:168-80.
24. Kao JP, Harootyan AT, Tsien RY. Photochemically generated cytosolic calcium pulses and their detection by fluo-3. *J Biol Chem* 1989;264:8179-84.
25. Wittchen ES, Haskins J, Stevenson BR. NZO-3 expression causes global changes to actin cytoskeleton in Madin-Darby canine kidney cells: linking a tight junction protein to Rho GTPases. *Mol Biol Cell* 2003;14:1757-68.
26. Ciardiello F, Caputo R, Damiano V, et al. Antitumor effects of ZD6474, a small molecule vascular endothelial growth factor receptor tyrosine kinase inhibitor, with additional activity against epidermal growth factor receptor tyrosine kinase. *Clin Cancer Res* 2003;9:1546-56.
27. Bottone FG Jr, Martinez JM, Collins JB, Afshari CA, Eling TE. Gene modulation by the cyclooxygenase inhibitor, sulindac sulfide, in human colorectal carcinoma cells: possible link to apoptosis. *J Biol Chem* 2003;278:25790-801.
28. Rahner C, Mitic LL, Anderson JM. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. *Gastroenterology* 2001;120:411-22.
29. Lim JW, Kim H, Kim KH. Nuclear factor- κ B regulates cyclooxygenase-2 expression and cell proliferation in human gastric cancer cells. *Lab Invest* 2001;81:349-60.
30. Fan XM, Wong BC, Lin MC, et al. Interleukin-1 β induces cyclo-oxygenase-2 expression in gastric cancer cells by the p38 and p44/42 mitogen-activated protein kinase signaling pathways. *J Gastroenterol Hepatol* 2001;16:1098-104.
31. Tsuji S, Kawano S, Sawaoka H, et al. Evidences for involvement of cyclooxygenase-2 in proliferation of two gastrointestinal cancer cell lines. *Prostaglandins Leukot Essent Fatty Acids* 1996;55:179-83.
32. Tsujii M, Kawano S, DuBois RN. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci U S A* 1997;94:3336-40.
33. Merritt G, Aliprandi ET, Prada F, Rigas B, Kashfi K. The retinoid fenretinide inhibits proliferation and downregulates cyclooxygenase-2 gene expression in human colon adenocarcinoma cell lines. *Cancer Lett* 2001;164:15-23.
34. Kawai S, Nishida S, Kato M, et al. Comparison of cyclooxygenase-1 and -2 inhibitory activities of various nonsteroidal anti-inflammatory drugs using human platelets and synovial cells. *Eur J Pharmacol* 1998;347:87-94.
35. Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA. Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other nonsteroidal anti-inflammatory drugs. *J Biol Chem* 1997;272:3406-10.
36. Huang JT, Welch JS, Ricote M, et al. Interleukin-4 dependent production of PPAR- γ ligands in macrophages by 12/15-lipoxygenase. *Nature* 1999;400:378-82.
37. Kusahara H, Komatsu H, Sumichika H, Sugahara K. Reactive oxygen species are involved in the apoptosis induced by nonsteroidal anti-inflammatory drugs in cultured gastric cells. *Eur J Pharmacol* 1999;383:331-7.
38. Elder DJ, Halton DE, Playle LC, Paraskeva C. The MEK/ERK pathway mediates COX-2-selective NSAID-induced apoptosis and induced COX-2 protein expression in colorectal carcinoma cells. *Int J Cancer* 2002;99:323-7.
39. Tepperman BL, Soper BD. Effect of extracellular Ca²⁺ on indomethacin-induced injury to rabbit dispersed gastric mucosal cells. *Can J Physiol Pharmacol* 1994;72:63-9.
40. Wang JL, Lin KL, Chen JS, et al. Effect of celecoxib on Ca²⁺ movement and cell proliferation in human osteoblasts. *Biochem Pharmacol* 2004;67:1123-30.
41. Sawada N, Murata M, Kikuchi K, et al. Tight junctions and human diseases. *Med Electron Microsc* 2003;36:147-56.
42. Tomisato W, Tanaka K, Katsu T, et al. Membrane permeabilization by non-steroidal anti-inflammatory drugs. *Biochem Biophys Res Commun* 2004;323:1032-9.

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

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