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-\(\beta\) 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...
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). D-2-3(2-aminoephospho)noxy)ethane-N,N,N'-N'-tetraacetic acid was purchased from Dojindo Co. (Tokyo, Japan). Thapsigargin, 1,4-diazobicyclo[2.2.2]octane and probenecid were purchased from Sigma Co. (Tokyo, Japan). Indomethacin, N-acetyl cysteine, and superoxide dismutase (SOD) were purchased from Wako Co. (Tokyo, Japan). Cellcoxi 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\( \times 10^{5}\) 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 (MTT) assay (22).

**DNA Microarray Analysis.** Total RNA was extracted from the cells using a RNeasy kit according to the manufacturer's protocols. Samples (5 \( \mu \)g RNA) were separated by agarose gel electrophoresis. For real-time RT-PCR, synthesized cDNA was amplified by PCR and labeled with \([\alpha^{32P}]dCTP\) (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).

**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.

**Measurement of the Intracellular Ca\(^{2+}\) Concentration.** [Ca\(^{2+}\)]\(_i\), was monitored according to Tamiya (24). Cells were washed with assay buffer containing 115 \( \mu \)M NaCl, 5.4 \( \mu \)M KCl, 1.8 \( \mu \)M CaCl\(_2\), 0.8 \( \mu \)M MgCl\(_2\), 20 \( \mu \)M HEPES, and 13.8 \( \mu \)M glucose. Cells were then incubated with 4 \( \mu \)M fluo-3/AM in the assay buffer containing 0.1% bovine serum albumin, 0.04% Pluronic F127, and 2 \( \mu \)M fluo-3. Fluoro-3 fluorescence of cells in a water-jacketed cuvette (1.6 \( \times 10^{5}\) 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 (\( F_{\text{max}} \) and \( F_{\text{min}} \)) were obtained by adding 10 \( \mu \)M ionomycin and 10 \( \mu \)M ionomycin plus 5 \( \mu \)M EGTA (in Ca\(^{2+}\)-free medium), respectively. [Ca\(^{2+}\)]\(_i\) was calculated according to the following equation: 

\[
[Ca^{2+}]_i = K_d (F_{\text{max}} - F_{\text{min}}) / (F_{\text{max}} - F),
\]

where \( K_d \) is the apparent dissociation constant (400 \( \mu \)M) 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 \( \times 10^{5}\) per dish) were suspended in 0.3% agarose or 0.3% Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. The suspension was layered over 0.5% agarose or 0.8% agarose medium base layer in 35 mm culture dishes (Iwaki, Chiba, Japan). After 10 days, cells were stained with crystal violet and colonies were counted using an inverted microscope.

**Induction of Claudin-4 by NSAIDs.** Synthesis of siRNAs were purchased from Qiagen. The target DNA sequence of claudin-4 is CCCGCACAGACAAGCCTACAG, AGCACTGTGTTGGCGTACAG. For RT-PCR: claudin-4, TCGTTGCGCTCAGAGACCT, CAGAGCTTCAAGGTTGGAAG; occludin, TCCATGGGCAAAGTGAATGA; claudin-4, CATCTGGCCTTGTTCGACCAG; COX-1, CTGGCTCGAATTCCACT, CACCTGGCAACGTGCTTCTC; COX-2, CCACCAAATCAATGCTGC, CACACAGAAACAGACTC; and actin, GGACTTCGAGCAAGAGATGG, AGCACTGTGTTGGCGTACAG. For PCR cloning: claudin-4, CGGATATCCCTGACAATGCGCTTATGAGGCT, GCCCTAGATTACAGTGTTCGGAGAC.
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. 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.
Northern blot analysis (Fig. 1) confirmed claudin-4 mRNA or claudin-4 protein was induced by indomethacin. In addition, the induction by indomethacin of claudin-1, claudin-4, and occludin mRNA, respectively, in 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.

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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession no.</th>
<th>Function</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin-1</td>
<td>AF115546</td>
<td>Tight junction</td>
<td>2.00</td>
</tr>
<tr>
<td>Claudin-4</td>
<td>AK026651</td>
<td>Tight junction</td>
<td>2.54</td>
</tr>
<tr>
<td>Occludin</td>
<td>U49184</td>
<td>Tight junction</td>
<td>2.24</td>
</tr>
<tr>
<td>Tissue factor pathway inhibitor 2</td>
<td>NM_000628</td>
<td>Blood coagulation</td>
<td>2.46</td>
</tr>
<tr>
<td>Zinedin</td>
<td>AF212940</td>
<td>Calmodulin binding protein</td>
<td>2.01</td>
</tr>
<tr>
<td>Arginine-rich protein</td>
<td>AA582041</td>
<td>Carcinogenicity</td>
<td>2.00</td>
</tr>
<tr>
<td>Human urokinase-type plasminogen receptor, exon 7</td>
<td>U09937</td>
<td>Cell surface plasminogen activation</td>
<td>2.81</td>
</tr>
<tr>
<td>Chromomob homologue 4 (Drosophila Pcl class)</td>
<td>AF013956</td>
<td>Cellular memory system</td>
<td>2.30</td>
</tr>
<tr>
<td>Human low-density lipoprotein receptor gene, exon 18</td>
<td>L00352</td>
<td>Cholesterol homeostasis</td>
<td>3.43</td>
</tr>
<tr>
<td>Low-density lipoprotein receptor (familial hypercholesterolemia)</td>
<td>NM_000527</td>
<td>Cholesterol homeostasis</td>
<td>2.75</td>
</tr>
<tr>
<td>Claudin-4</td>
<td>AK094624</td>
<td>Cytoskeleton</td>
<td>2.58</td>
</tr>
<tr>
<td>Keratin 8</td>
<td>A979392</td>
<td>Cytoskeleton</td>
<td>2.37</td>
</tr>
<tr>
<td>Immediate early response 3</td>
<td>A022951</td>
<td>Differentiation</td>
<td>2.36</td>
</tr>
<tr>
<td>Prostate differentiation factor</td>
<td>A090584</td>
<td>Differentiation</td>
<td>2.00</td>
</tr>
<tr>
<td>Procollagen-proline</td>
<td>J00783</td>
<td>Disulfide isomerase/oxidoreductase</td>
<td>2.50</td>
</tr>
<tr>
<td>Glucosidase β, acid (includes glucosylceramidase)</td>
<td>AF023268</td>
<td>Glucocerebrosidase</td>
<td>2.01</td>
</tr>
<tr>
<td>Tumor necrosis factor–induced protein 6</td>
<td>M33165</td>
<td>Hyaluronan binding protein family</td>
<td>2.14</td>
</tr>
<tr>
<td>Basigin</td>
<td>X64364</td>
<td>Immunoglobulin superfamily</td>
<td>2.15</td>
</tr>
<tr>
<td>Solute carrier family 7</td>
<td>M80244</td>
<td>γ-Amino acid transporter</td>
<td>3.00</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>M11233</td>
<td>Lyosomal protease</td>
<td>2.40</td>
</tr>
<tr>
<td>Pim-1 oncogene</td>
<td>M24779</td>
<td>Protein kinase</td>
<td>2.52</td>
</tr>
<tr>
<td>Cytochrome c oxidase subunit VIII</td>
<td>J04823</td>
<td>Respiratory</td>
<td>2.90</td>
</tr>
<tr>
<td>3,4-Dihydroxy-L-phenylalanine (aromatic l-amino acid decarboxylase)</td>
<td>M76180</td>
<td>Synthesis of dopamine and serotonin</td>
<td>2.27</td>
</tr>
</tbody>
</table>

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.
by indomethacin, PGE\(_2\) (0.1-10 \mu\text{mol/L}) did not affect the level of claudin-4 in the presence and absence of indomethacin (Fig. 2A). We determined previously the level of PGE\(_2\) in the culture medium of AGS cells to be \(\sim 10\ \text{nmol/L}\) (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-\(\gamma\) (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-\(\gamma\) 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). These results suggest that neither reactive oxygen species nor extracellular signal-regulated kinase is responsible for the induction of claudin-4 by indomethacin.

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

Some drugs that are known to increase \([\text{Ca}^{2+}]_i\), 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. 3A-C, in addition to increasing \([\text{Ca}^{2+}]_i\), 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. 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 \([\text{Ca}^{2+}]_i\).

**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

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**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.
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 transfectant control (Fig. 4D), which is consistent with previous results obtained using pancreatic cancer cells (19). We compared the extent of inhibition of colony formation in soft agar with the degree of claudin-4 overproduction in these clones and found a close correlation between the two (Fig. 4A and D).

We also examined the effect of indomethacin on colony formation of AGS cells in soft agar. Because a long incubation period (10 days) was required for this assay, relatively low concentrations of indomethacin were used. As shown in Fig. 4E, indomethacin (100 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. 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 transfectant 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 inhibited the expression of claudin-4 in AGS cells.
induction of claudin-4. 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 \([\text{Ca}^{2+}]\), based on the following observations: (a) NSAIDs increased \([\text{Ca}^{2+}]\), and induced claudin-4 simultaneously; (b) thapsigargin and ionomycin increased \([\text{Ca}^{2+}]\), and induced claudin-4, and (c) the intracellular \(\text{Ca}^{2+}\) chelator \([1,2\text{-bis(2-aminophenoxy)ethane-N,N,N',N' tetraacetic acid}]\) attenuated the indomethacin-dependent induction of claudin-4. As for the mechanism for the increase in \([\text{Ca}^{2+}]\), by NSAIDs, both inhibition of sarcoplasmic/endoplasmic reticulum \(\text{Ca}^{2+}\) ATPase (endoplasmic reticulum–located \(\text{Ca}^{2+}\) pump that is responsible for accumulation of \(\text{Ca}^{2+}\) in the endoplasmic reticulum) and stimulation of the influx of extracellular \(\text{Ca}^{2+}\) 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 \([\text{Ca}^{2+}]\), suggesting that NSAIDs permeabilize membranes and stimulate the influx of extracellular \(\text{Ca}^{2+}\) (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.

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-\(\alpha\), transforming growth factor-\(\alpha\), and interleukin-1 disrupt tight junctions, whereas transforming growth factor-\(\beta\), interleukin-10, and PGE₂ are known to stimulate the function of tight junctions (41).
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