NSAIDs suppress the expression of claudin-2 to promote invasion activity of cancer cells

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Non-steroidal anti-inflammatory drugs (NSAIDs) show chemopreventive effects; however, the precise molecular mechanism of these effects is still unclear. On the other hand, the expression of proteins that form tight junctions (TJs) (such as claudins) in clinically isolated tumors is frequently altered relative to normal tissue. We previously reported that NSAIDs upregulate the expression of claudin-4 and that this upregulation contributes to NSAID-dependent inhibition of both migration activity and anchorage-independent growth of cancer cells. In the current study, we have systematically examined the effects of various NSAIDs on the expression of various TJ proteins and have found that NSAIDs specifically and drastically inhibit the expression of claudin-2. Overexpression or suppression of claudin-2 expression caused stimulation or inhibition, respectively, of the invasion and migration activity of cancer cells. Furthermore, NSAIDs inhibited the invasion and migration activity of cancer cells and this inhibition was suppressed by overexpression of claudin-2. In contrast, neither cell growth nor apoptosis induced by lack of anchorage of cancer cells was affected by overexpression or suppression of expression of claudin-2. These results suggest that inhibition of claudin-2 expression by NSAIDs contributes to NSAID-dependent inhibition of invasion of cancer cells in vitro and that it may be involved in the chemopreventive effects of NSAIDs by inhibiting metastasis in vivo.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are useful drugs for alleviating pain, fever and inflammation. The anti-inflammatory action of NSAIDs is mediated via their inhibitory effect on cyclooxygenase (COX) activity and synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. A range of epidemiological studies have revealed that prolonged NSAID use reduces the risk of cancer, whereas preclinical and clinical studies have indicated that some NSAIDs are effective in the treatment and prevention of cancer (1). Because the antitumor activity of NSAIDs is potent and the safety of longtime use of NSAIDs has been proven by a vast amount of clinical data, NSAIDs have attracted considerable attention as a new type of antitumor drug. In the USA, the Food and Drug Administration has approved the use of celecoxib, an NSAID, for the treatment of familial adenomatous polyposis.

PGs, such as PGE2, inhibit apoptosis of cancer cells and stimulate growth and invasion of cancer cells and angiogenesis (2–4). Furthermore, overexpression of COX-2 (a subtype of COX) in various tumor cells and tissues has been reported (5,6). Therefore, it was believed that the antitumor effect of NSAIDs is mediated only through the inhibition of COX. However, several lines of evidence suggest that the antitumor effect of NSAIDs also involves a COX-independent mechanism (7,8). In order to reveal the nature of this COX-independent mechanism, we systematically searched for genes whose expression is upregulated by indomethacin (an NSAID) in a COX-independent manner in human gastric carcinoma (AGS) cells. This study has revealed that expression of some genes encoding tight junction (TJ) proteins (claudin-4, claudin-1 and occludin) is induced by indomethacin (9).

TJs are the most apical intercellular structures in epithelial and endothelial cells and create a physiological barrier separating the apical and basolateral spaces. TJs contain transmembrane proteins such as occludin and the claudins, whose C-terminal regions interact with cytosolic proteins, such as zonula occludens-1 (10,11). TJs have attracted considerable attention in relation to tumor progression because a loss of TJ structure and function is frequently observed in epithelium-derived cancers (12). This loss of TJ structure and function is thought to promote cancer progression through allowing constitutive accessibility of cancers to nutrients and growth factors (13) and other mechanisms. Furthermore, alteration of expression of the proteins that constitute TJs, especially claudins has been shown to modulate various cell functions related to tumor progression in vitro (12,13). For example, we recently reported that overexpression of claudin-4 or suppression of expression of claudin-4 caused a decrease or an increase, respectively, in the anchorage-independent growth and migration activity of AGS cells (9). Studies from other groups have also shown that alterations to the expression of claudins (claudin-1, -3, -4, -5) affect the invasion and migration activities and anchorage-independent growth of cancer cells (14–18). However, the effect of expression of other claudins, including claudin-2, on cell functions related to cancer progression has remained unknown. Among the claudins, claudin-2 is unique because its expression increases the paracellular permeability of some molecules in other words claudin-2 decreases the function of TJs (19,20) and its messenger RNA (mRNA) expression is regulated by various mechanisms (21–23).

Based on the observations described above, it is reasonable to hypothesize that NSAIDs achieve their antitumor activity by altering the expression of TJ proteins. In fact, we previously reported that induction of expression of claudin-4 by NSAIDs contributes to NSAID-dependent inhibition of both migration and anchorage-independent growth of cancer cells (9). In this study, we examined the effects of various NSAIDs on the expression of various TJ proteins in AGS cells and found that NSAIDs inhibit the expression of claudin-2 specifically and extensively. Experiments using an overexpression plasmid and small interfering RNA (siRNA) revealed that expression of claudin-2 increases the invasion and migration activity of AGS cells and suggests that NSAID-dependent inhibition of the invasion and migration activity of AGS cells is due to the NSAID-dependent downregulation of claudin-2 expression. We suggest that NSAIDs positively or negatively affect the expression of claudins, depending on the claudin species, and that this action plays an important role in conferring the chemopreventive effect of NSAIDs through inhibition of metastasis.

Materials and methods

Chemicals and media

Ham F12, Dulbecco’s modified Eagle’s medium and RPMI1640 medium were obtained from Nissui Pharmaceutical Co (Tokyo, Japan). HilyMax and 1,2-bis(2-aminophenoxy)ethane-N,N,N’-tetraacetic acid (BAPTA-AM) were obtained from Dojindo Co (Kumamoto, Japan). Fetal bovine serum (FBS), fibronectin, G418, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide and diclofenac were obtained from Sigma Co (Tokyo, Japan). Indomethacin was from Wako Co (Osaka, Japan). Celecoxib was obtained from LKT Laboratories (St. Paul, Minnesota). The plasmid psiRNA-h7SKneo was from InvivoGen (San Diego, California) and pcDNA3.1(TM2000) were from In vitrogen (Tokyo, Japan). The RNeasy kit was obtained from Qiagen (Tokyo, Japan), first-strand complementary DNA (cDNA) synthesis kit was from GE Healthcare (Tokyo, Japan) and IQ SYBR Green Supermix was from Bio-Rad (Tokyo, Japan). Matrigel was from BD Biosciences
(Tokyo, Japan) and the 24-well transwells were from Costar. Antibody against claudin-2 was from Zymed (San Francisco, California) and those against claudin-4 and actin were from Santa Cruz Biotechnology (Sata Cruz, California).

Cell culture and stimulation or suppression of claudin-2 expression

AGS and KATO-III cells (human carcinoma cell lines derived from stomach) were cultured in RPMI1640 medium containing 10% FBS and T-84 (human colonic adenocarcinoma cell line) and A549 (human lung adenocarcinoma) cells were cultured in Ham F12/Dulbecco’s modified Eagle’s medium containing 5% FBS. Cells (2 × 10^5 cells per well in a 24-well plate) were cultured for 24 h and then used in the experiments. Cell viability was determined by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide method as described previously (24).

A full-length human CLDN2 cDNA was polymerase chain reaction (PCR) amplified from AGS cell cDNA and cloned into pcDNA3.1(+) to create the plasmid for overexpression of claudin-2.

We used the siRNA sequence of 5’-ACCTCGAGATCTAGGCAGCTC-TACTTCAGAGAGTGAAGTCCGATGATCTT-3’ as annealed oligonucleotides for repressing claudin-2 expression. A DNA fragment encoding this siRNA was cloned into a psiRNA-h7SKneo to create the plasmid for expression of this siRNA.

Transfection of AGS cells with plasmids was carried out using Lipofectamine (TM2000) or HilyMax according to the manufacturer’s protocols. The stable transfectants expressing claudin-2 or siRNA for claudin-2 were selected by immunoblotting analysis. Positive clones were maintained in the presence of 300 µg/ml G418.

Real-time reverse transcription–PCR

Total RNA was extracted using an RNAex kit according to the manufacturer’s protocol. Samples (2.5 µg RNA) were reverse transcribed using a first-strand cDNA synthesis kit according to the manufacturer’s instructions. Synthesized cDNA was used in real-time reverse transcription–PCR [Chromo 4 instrument (Bio-Rad)] experiments using iQ SYBR GREEN Supermix and analyzed with Opticon Monitor Software according to the manufacturer’s instructions. The real-time PCR cycle conditions were 2 min at 50°C, followed by 10 min at 90°C and finally 45 cycles of 95°C for 30 s and 63°C for 60 s. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, the actin cDNA was used as an internal standard.

Immunoblotting analysis

Membrane fractions were prepared as described previously (25). The protein concentration of the sample was determined by the Bradford method. Samples were applied to 12% polyacrylamide gels containing sodium dodecyl sulfate, and the protein concentration was determined by the Bradford method. Samples were then subjected to real-time reverse transcription–PCR using a specific primer set for each gene. The protein concentrations were normalized to actin expression and expressed relative to the control sample (i.e., without NSAID control). We performed real-time RT–PCR experiments twice on each cDNA and showed the mean of them.

### Table I. Alteration of mRNA expression of TJ-related genes by NSAIDs

<table>
<thead>
<tr>
<th></th>
<th>IND</th>
<th>CEL</th>
<th>DIC</th>
<th>ASP</th>
<th>IBU</th>
<th>NIM</th>
</tr>
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<tbody>
<tr>
<td><strong>CLDN1</strong></td>
<td>1.43</td>
<td>1.64</td>
<td>1.43</td>
<td>1.24</td>
<td>2.16</td>
<td>1.75</td>
</tr>
<tr>
<td><strong>CLDN2</strong></td>
<td>0.01</td>
<td>0.48</td>
<td>0.12</td>
<td>0.01</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>CLDN3</strong></td>
<td>0.47</td>
<td>1.78</td>
<td>0.99</td>
<td>0.51</td>
<td>0.59</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>CLDN4</strong></td>
<td>2.37</td>
<td>1.51</td>
<td>2.45</td>
<td>3.95</td>
<td>4.42</td>
<td>2.89</td>
</tr>
<tr>
<td><strong>CLDN5</strong></td>
<td>0.57</td>
<td>0.76</td>
<td>0.19</td>
<td>0.18</td>
<td>0.47</td>
<td>1.92</td>
</tr>
<tr>
<td><strong>CLDN6</strong></td>
<td>0.73</td>
<td>0.86</td>
<td>0.98</td>
<td>1.00</td>
<td>0.74</td>
<td>0.86</td>
</tr>
<tr>
<td><strong>CLDN7</strong></td>
<td>1.45</td>
<td>1.75</td>
<td>1.42</td>
<td>1.26</td>
<td>2.03</td>
<td>1.37</td>
</tr>
<tr>
<td>Occludin</td>
<td>1.35</td>
<td>1.06</td>
<td>1.32</td>
<td>1.53</td>
<td>1.83</td>
<td>1.01</td>
</tr>
<tr>
<td>Zonula occludens-1</td>
<td>2.10</td>
<td>1.86</td>
<td>1.77</td>
<td>0.86</td>
<td>1.64</td>
<td>1.53</td>
</tr>
</tbody>
</table>

AGS cells were incubated with 0.35 mM indomethacin (IND), 30 µM celecoxib (CEL), 0.2 mM diclofenac (DIC), 5 mM aspirin (ASP), 1 mM ibuprofen (IBU) or 0.18 mM nimesulid (NIM) for 24 h and total RNA was extracted. Samples were subjected to real-time reverse transcription–PCR using a specific primer set for each gene. Values were normalized to actin expression and expressed relative to the control sample (i.e., without NSAID control). We performed real-time RT–PCR experiments twice on each cDNA and showed the mean of them.

### Suppression of claudin-2 expression by NSAIDs

We used a real-time reverse transcription–PCR technique to examine the effect of various NSAIDs on expression of claudins and other TJ proteins in AGS cells. First, we examined the effect of various NSAIDs on cell viability and determined the concentration of each NSAID required to reduce cell viability to 70% (0.35 mM indomethacin, 30 µM celecoxib, 0.2 mM diclofenac, 5 mM aspirin, 1 mM ibuprofen and 0.18 mM nimesulid). We next examined the effects of these concentrations of NSAIDs on mRNA expression of various TJ proteins (Table I). The mRNA expression of CLDN4 was clearly upregulated by all NSAIDs tested and that of CLDN1 and occludin was slightly upregulated, as described previously (9). Of all the proteins tested, a characteristic feature in response to the NSAIDs was identified for claudin-2: all of the NSAIDs tested suppressed the mRNA expression of claudin-2 and the extent of the suppression was more distinct than for other claudins (Table I). The mRNA expression of CLDN7 and zonula occludens or CLDN5 and CLDN6 showed a tendency to be increased or decreased, respectively, by NSAIDs. However, for these genes, the changes in expression were not observed in response to some NSAIDs and the extent of the changes were not as distinct as was observed for CLDN4 and CLDN2 (Table I).

### Invasion assay

The invasion assay was done as described previously (27), with some modifications. Serum-free RPMI1640 medium containing 5 mg/ml matrigel was applied to the upper chamber of a 24-well transwell and incubated at 37°C for 4 h. Cell suspension was applied to the matrigel and the lower chamber of the transwell was filled with culture media containing 10% FBS and 5 µg/ml fibronectin. The plate was incubated at 37°C for 24 h. Cells were removed from the upper surface of the membrane and the lower surface of the membrane was stained for 10 min with 0.5% crystal violet in 25% methanol, rinsed with distilled water and air-dried overnight. The crystal violet was then extracted with 0.1 M sodium citrate in 50% ethanol and the absorbance was measured at 585 nm.

### Cell migration assays

In vitro wound healing assays were used to assess cell migration as described previously (9,28). Confluent AGS cells on a 24-well plate were used for this assay. Two linear wounds were made with a 200 µl pipette tip. The cell-free area was measured before and after a 24 h incubation (healing step) by use of Scion Image software (Scion Corporation, Frederick, Maryland).

### Statistical analysis

All values are expressed as the mean ± SEM. Two-way analysis of variance, followed by the Tukey test or the Student’s t-test for unpaired results, was used to evaluate differences between more than three groups or between two groups, respectively. Differences were considered to be significant for values of P < 0.05.

### Results

Effect of NSAIDs on expression of claudins

We used a real-time reverse transcription–PCR technique to examine the effect of various NSAIDs on expression of claudins and other TJ proteins in AGS cells. First, we examined the effect of various NSAIDs on cell viability and determined the concentration of each NSAID required to reduce cell viability to 70% (0.35 mM indomethacin, 30 µM celecoxib, 0.2 mM diclofenac, 5 mM aspirin, 1 mM ibuprofen and 0.18 mM nimesulid). We next examined the effects of these concentrations of NSAIDs on mRNA expression of various TJ proteins (Table I). The mRNA expression of CLDN4 was clearly upregulated by all NSAIDs tested and that of CLDN1 and occludin was slightly upregulated, as described previously (9). Of all the proteins tested, a characteristic feature in response to the NSAIDs was identified for claudin-2: all of the NSAIDs tested suppressed the mRNA expression of claudin-2 and the extent of the suppression was more distinct than for other claudins (Table I). The mRNA expression of CLDN7 and zonula occludens or CLDN3, CLDN5 and CLDN6 showed a tendency to be increased or decreased, respectively, by NSAIDs. However, for these genes, the changes in expression were not observed in response to some NSAIDs and the extent of the changes were not as distinct as was observed for CLDN4 and CLDN2 (Table I). COX exists as two subtypes, COX-1 and COX-2.
and nimesulid are COX-2-specific NSAIDs and thus, the results in Table I suggest that NSAIDs suppress the expression of \( CLDN2 \) mRNA irrespective of their COX-2 specificity.

The effect of NSAIDs on the expression of claudin-2 was also examined at the protein level by immunoblotting. Indomethacin, celecoxib and diclofenac not only increased the amount of claudin-4 but also decreased the amount of claudin-2 in the membrane fraction (Figure 1A). As for claudin-4, we have shown previously similar results using whole-cell extract (9). A decrease in the amount of claudin-2 was observed with 0.1 mM diclofenac, which did not significantly affect the cell viability (data not shown), suggesting that this decrease is not a result of cell damage. Similar results were obtained with whole-cell extracts (data not shown), showing that the NSAID-induced decrease in the amount of claudin-2 in the membrane fraction is not due to an alteration of its subcellular localization. Combining the results in Table I and Figure 1A, we consider that NSAIDs decrease the amount of claudin-2 through downregulating their transcription.

We also examined the effect of NSAIDs on the expression of claudin-2 and claudin-4 in other types of cells: KATO-III and T-84 which are cell lines derived from gastric cancer and A549 is from lung cancer. As shown in Figure 1B, indomethacin decreased or increased the amount of claudin-2 or claudin-4, respectively, in the membrane fraction of these cell lines (Figure 1B). Furthermore, we showed that celecoxib altered the amount of claudin-2 and claudin-4 even in KATO-III cells. It has been reported that both COX-1 and COX-2 mRNAs are expressed in AGS cells, whereas COX-2 mRNA expression is very low in KATO-III cells (29,30), and we confirmed these phenotypes by real-time reverse transcription–PCR analysis (data not shown). Therefore, a COX-2-specific NSAID, celecoxib, altered the amount of claudin-2 and claudin-4 even in cells lacking COX-2 expression (Figure 1B). Furthermore, whereas indomethacin inhibited both COX-1 and COX-2 at a concentration of \(<1\mu M\) (31), altered expression of the claudins required higher concentrations (Figure 1). These findings suggest that NSAIDs affect the expression of claudin-2 and claudin-4 independently of COX inhibition. For claudin-4, we have shown previously results similar to those in Figure 1B using whole-cell extracts (9).

**Mechanism for suppression of claudin-2 expression by indomethacin**
For further confirmation that NSAIDs suppress expression of claudin-2 independently of COX inhibition, we examined the effect of PGE\(_2\), a major PG in gastric mucosa, on the suppression of claudin-2 expression by indomethacin. PGE\(_2\) (1 or 10 \(\mu M\)) did not affect the level of claudin-2 in either the presence or absence of indomethacin (Figure 1C). Also, PGE\(_2\) did not affect the level of claudin-4, as described previously (9). We previously determined the level of PGE\(_2\) in the culture medium of AGS cells to be \(\sim10\ \text{nm}\) (32). Therefore, inhibition of PGE\(_2\) synthesis by indomethacin does not seem to be involved in the suppression of expression of claudin-2 by indomethacin.

We recently reported that NSAIDs, due to their membrane-permeabilizing activity, increase the intracellular Ca\(^{2+}\) level by stimulating Ca\(^{2+}\) influx across the cytoplasmic membrane (33,34). Furthermore, we reported that this Ca\(^{2+}\) increase is responsible for the induction of expression of claudin-4 by NSAIDs: we showed that an intracellular Ca\(^{2+}\) chelator (BAPTA-AM) inhibited the NSAID-induced expression of claudin-4 (9). We have confirmed this result and found that BAPTA-AM did not affect the suppression of expression of claudin-2 by indomethacin (Figure 1D). These results suggest that suppression of expression of claudin-2 by indomethacin is not mediated through an increase in the intracellular Ca\(^{2+}\) level.

**Role of NSAID-induced suppression of claudin-2 expression in the antitumor effect in vitro**
In order to understand the role of suppression of claudin-2 expression in the chemopreventive effect of NSAIDs, we examined the effect of claudin-2 expression on various cell functions related to cancer progression, such as cell growth, anoikis (apoptosis induced by lack of cell-matrix interaction), and invasion and migration activity.

**Fig. 1.** Suppression of expression of claudin-2 by NSAIDs. AGS (A, C and D) and T84, KATO-III or A549 (B) cells were incubated with the indicated concentrations of each NSAID for 24 h in the presence of the indicated concentrations of PGE\(_2\) (C) or BAPTA-AM (D). Membrane fractions (2.5 \(\mu g\) protein) were prepared and analyzed by immunoblotting with an antibody against claudin-2 or claudin-4. For loading control, the gels were stained with silver.

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We constructed stable transfectants of AGS cells that continuously overexpress claudin-2. Moderate and high levels of claudin-2 expression in clone 1 and clone 5, respectively, were confirmed by immunoblotting analysis (Figure 2A). We also constructed stable transfectants of AGS cells that continuously express siRNA to suppress the basal expression of claudin-2 (siClaudin-2) (Figure 2A).

Figure 2B shows the growth curve of each clone. Growth of claudin-2-overexpressing clones (clones 1 and 5) and a claudin-2-downregulating clone (siClaudin-2) was indistinguishable from that of each mock transfectant control, demonstrating that expression of claudin-2 does not affect the growth of AGS cells. We also examined the effect of alteration in the expression of claudin-2 on cell growth in the presence of indomethacin. Indomethacin (0.2 mM) inhibited the cell growth and alteration in the expression of claudin-2 did not affect this inhibition (Figure 2C). Therefore, suppression of claudin-2 expression by NSAIDs does not seem to be involved in inhibition of cell growth by NSAIDs.

We also examined the effect of variation in the expression of claudin-2 on induction of anoikis. As shown in Figure 3A and B, the number of colonies (viable cell number), after incubation under conditions where there was a lack of cell–matrix interaction, was indistinguishable between claudin-2-overexpressing or claudin-2-downregulating clone and each mock transfectant control. In the presence of 0.1 mM indomethacin, induction of anoikis was clearly inhibited and this inhibition was not affected by alteration in the expression of claudin-2 (Figure 3A and B). This suggests that the expression of claudin-2 does not affect the induction of anoikis in AGS cells.

The effect of expression of claudin-2 on invasion activity of AGS cells was examined by use of a matrigel transwell assay. As shown in Figure 4A, the claudin-2-overexpressing clone showed significantly greater cell invasion activity than the mock transfectant control. This effect was more apparent in clone 5 than in clone 1 (Figure 4A), being well correlated to the extent of overexpression of claudin-2 (Figure 2A). On the other hand, the claudin-2-downregulating clone showed less cell invasion activity than the mock transfectant control (Figure 4B). These results suggest that expression of claudin-2 stimulates the cell invasion activity of AGS cells.

We also examined the effect of indomethacin or celecoxib on cell invasion activity. Indomethacin or celecoxib dose dependently inhibited the cell invasion activity and this inhibitory effect was not observed in the claudin-2-overexpressing clone (Figure 4C). Treatment with indomethacin or celecoxib resulted in lower cell invasion activity in claudin-2-downregulating clone (Figure 4D). The concentrations of indomethacin or celecoxib used in Figure 4C and D did not affect the growth of AGS cells (data not shown). Treatment with these concentrations of indomethacin suppressed the mRNA expression of claudin-2 genes.
CLDN2 in the mock transfectant control but not in the claudin-2 overexpressing clone (Figure 4E). Based on these results, we consider that NSAIDs inhibit cell invasion activity through suppression of claudin-2 expression.

The results in Figure 4 suggest that the cell migration activity of AGS cells is affected by expression of claudin-2 and we tested this hypothesis using wound healing assays; we measured the cell-free area 24 h after making a wound. Since neither alterations to
Suppression of claudin-2 expression by NSAIDs

claudin-2 expression nor addition of indomethacin (<0.2 mM) affected the growth of AGS cells (Figure 2B and data not shown), the less cell-free area means the higher cell migration activity. As shown in Figure 5A, claudin-2 overexpressing cells showed significantly higher cell migration activity than the mock transfectant control. On the other hand, the claudin-2 downregulating clone showed lower cell migration activity than the mock transfectant control (Figure 5C). These results suggest that expression of claudin-2 stimulates the cell migration activity of AGS cells, which is opposite to what was observed for claudin-4, the expression of which was shown to inhibit the cell migration activity of AGS cells (9).

As shown in Figure 5E, indomethacin inhibited the migration activity of AGS cells and this inhibitory effect was not observed in claudin-2-overexpressing cells. All of these results support the hypothesis that the suppression of claudin-2 expression by indomethacin is involved in the inhibition of cell migration activity.

Discussion

At least 24 types of claudins are known to exist (11), and among them claudin-2 is unique because it is the only claudin whose expression has been shown to make TJs leaky (19,20). Results of the current study reveal that in its response to NSAIDs, claudin-2 is also unique: all of the NSAIDs tested clearly and specifically suppressed the expression of claudin-2.

We have shown previously that induction of claudin-4 expression by NSAIDs is mediated by an increase in the intracellular Ca\(^{2+}\) level: thapsigargin and ionomycin which are known to increase the intracellular Ca\(^{2+}\) level, induced expression of claudin-4 and the intracellular Ca\(^{2+}\) chelator (BAPTA-AM) attenuated the NSAID-dependent induction of claudin-4 expression (9). This Ca\(^{2+}\) increase is caused by stimulation of an influx of extracellular Ca\(^{2+}\) due to the membrane-permeabilizing activity of NSAIDs (33–35). However, results of this study show that downregulation of expression of claudin-2 by NSAIDs is not mediated by the same mechanism. BAPTA-AM did not affect this downregulation. Thus, the mechanism governing NSAID-dependent inhibition of claudin-2 expression is unclear at present.

Although a number of recent papers have shown the effects of expression of various claudins on the invasion and migration activities of cancer cells (14–16,18), the effects of claudin-2 expression have remained unknown. In this study, we have shown that overexpression of claudin-2 or suppression of claudin-2 expression increases or decreases, respectively, the invasion and migration activities of AGS cells. Cell invasion activity mainly depends on cell migration activity and on degradation of the extracellular matrix, which is mainly mediated by matrix metalloproteinases (MMPs). Since claudin-2 expression upregulated the expression of MMPs (MMP1, MMP2 and MMP9) and stimulated MMP activity (data not shown), we believe that the claudin-2-dependent increase in cell invasion activity is due to stimulation of both cell migration activity and MMP activity. Results of this study show that expression of claudin-2 contributes to the stimulation of invasion activity of cancer cells, and thus expression of claudin-2 may contribute to tumor metastasis in vivo. It has been reported that claudin-2 expression is frequently upregulated in clinically isolated colorectal and gastric tumors, compared with normal tissues (36). It has also been reported that the extent of claudin-2 expression correlates with the degree of progression of cancer (37). Thus, the results of the current study suggest that upregulation of claudin-2 in cancers contributes to cancer progression.

We have previously reported, using the same cells and assay system, that expression of claudin-4 inhibited cell migration activity and anchorage-independent growth of cancer cells (9). Here, we have shown that expression of claudin-2 does not affect the induction of anoikis (apoptosis induced by lack of cell–matrix interaction). Thus, it seems that depending on the claudin species, claudins positively or negatively affect the progression of cancer through various mechanisms. The mechanism whereby expression of claudin-2 stimulates the migration activity of AGS cells remains unknown. One possibility is that the negative effect of claudin-2 on the function of TJs is responsible for this phenotype. Another possibility is that claudin-2 achieves this effect via a TJ-independent mechanism, as is the case is that the negative effect of claudin-2 on the function of TJs is responsible for this phenotype. Another possibility is that claudin-2 achieves this effect via a TJ-independent mechanism, as is the case
for claudin-1 that was reported to translocate into the nucleus, affect cancer-related gene expression and stimulate cell invasion activity (15).

NSAIDs seem to achieve their chemopreventive effect through various mechanisms, not only by stimulation of apoptosis, cell growth suppression and inhibition of angiogenesis but also by inhibition of metastasis, in which cell invasion and migration activities play an important role (38,39). In this study, we suggest that NSAID-dependent inhibition of invasion and migration activities involves suppression of claudin-2 expression by NSAIDs: NSAID-dependent suppression of migration and invasion activities was not observed in the claudin-2-overexpressing clone. As described above, we have previously suggested that NSAID-dependent inhibition of migration activity involves induction of claudin-4 expression by NSAIDs (9). Thus, these alterations in claudin expression seem to be involved in NSAID-dependent suppression of migration and invasion activities (suppression of metastasis). Screening of NSAIDs to identify molecules that potently induce claudin-4 expression and suppress claudin-2 expression may be useful for obtaining more potent NSAIDs for cancer treatment.

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References


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