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 μM (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 μM) 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 ~10 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.

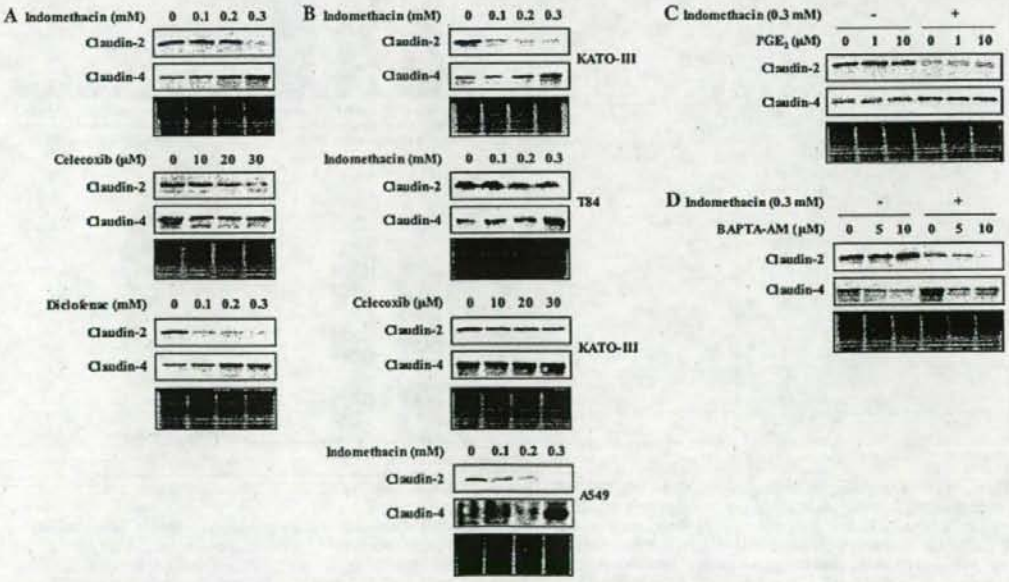

Fig. 1. Suppression of expression of claudin-2 by NSAIDs. AGS (A, C and D) and T84, KATO-III or A549 (B) cells were incubated with the indicated concentrations of each NSAID for 24 h in the presence of the indicated concentrations of PGE_2 (C) or BAPTA-AM (D). Membrane fractions (2.5 μg protein) were prepared and analyzed by immunoblotting with an antibody against claudin-2 or claudin-4. For loading control, the gels were stained with silver.

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 claudin-2 expression 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

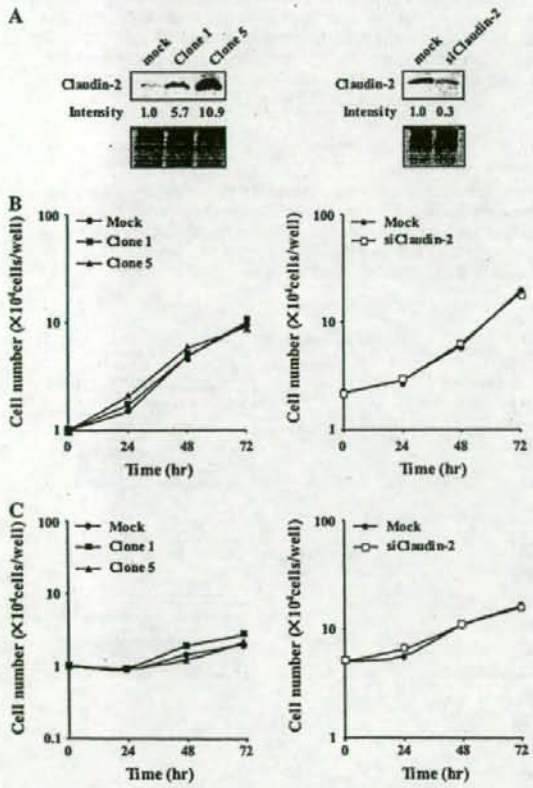

Fig. 2. Effect of alterations in claudin-2 expression on cell growth. The extent of expression of claudin-2 in each clone [stable transfectant of AGS cell with claudin-2 expression plasmid (clones 1 and 5) or plasmid expressing siRNA for claudin-2 (siClaudin-2) and each mock transfectant control] was estimated by immunoblotting experiments as described in the legend of Figure 1. For loading control, the gels were stained with silver (A). Cells of each clone were cultured for the indicated periods in the absence (B) or presence (C) of 0.2 mM indomethacin and cell numbers were determined by direct cell counting (B and C).

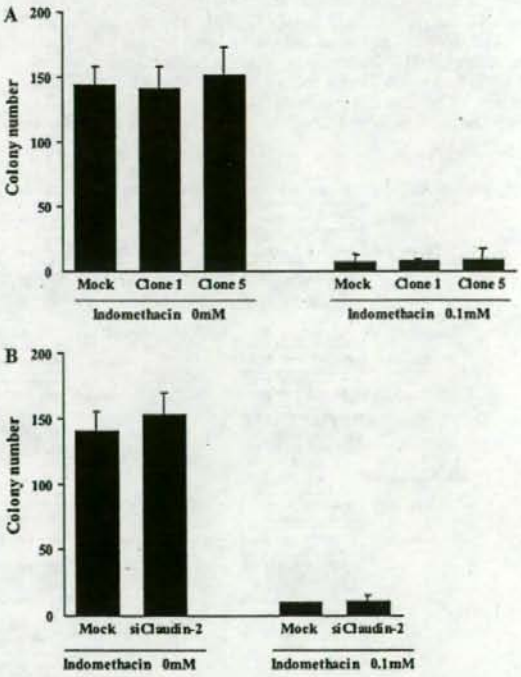

Fig. 3. Effect of alteration in claudin-2 expression on induction of anoikis. AGS cells stably transfected with claudin-2 expression plasmid (clones 1 and 5) or plasmid expressing siRNA for claudin-2 (siClaudin-2) and mock transfectant control AGS cells were cultured on agarose plates for 7 days in the absence or presence of 0.1 mM indomethacin and the viable cell numbers (number of colonies after incubation under normal conditions for 3 days) were counted. Values are mean ± SEM (n = 3).

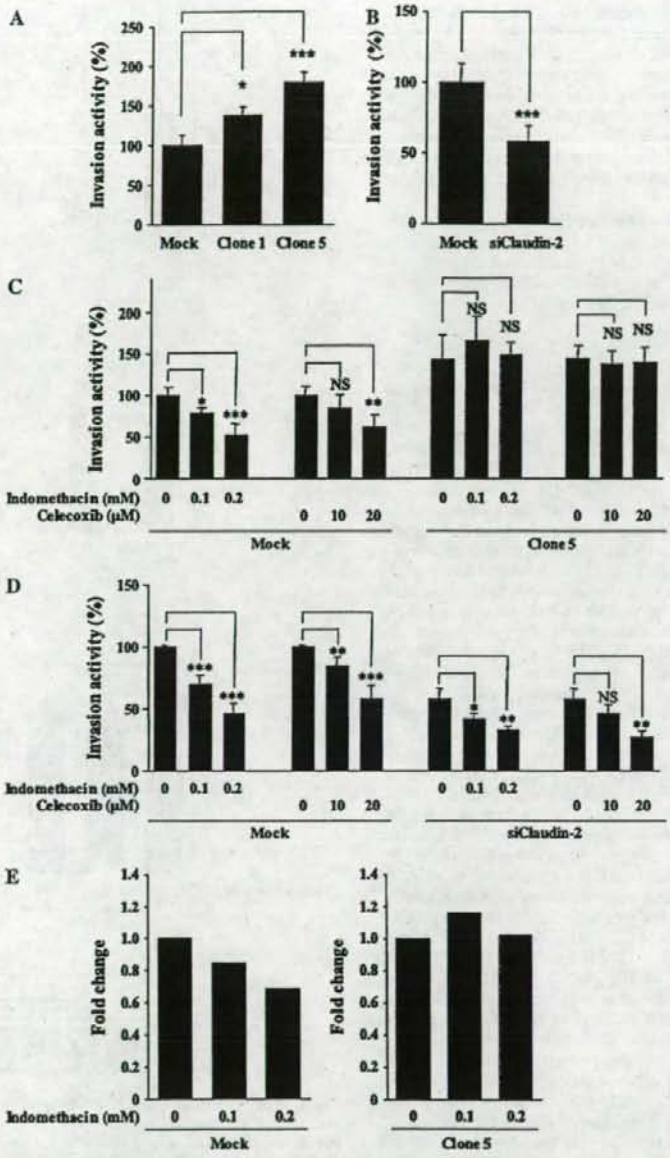

Fig. 4. Effect of alteration in claudin-2 expression on cell invasion activity. AGS cells stably transfected with claudin-2 expression plasmid or plasmid expressing siRNA for claudin-2 (siClaudin-2) and mock transfectant control AGS cells were cultured on matrigel-coated transwells for 24 h in the presence (**C, D**) or absence (**A, B**) of the indicated concentrations of indomethacin or celecoxib. Cell invasion activity was measured as described in the Materials and Methods and is expressed relative to the control. Values are mean ± SEM ($n = 3$), $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, NS, not significant (**A–D**). The stable transfectant of the claudin-2 expression plasmid (clone 5) and the mock transfectant control cells were cultured for 24 h in the presence of the indicated concentrations of indomethacin. The mRNA expression of *claudin-2* was estimated by real-time reverse transcription–PCR as described in the legend of Table 1 (**E**).

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

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 extracellar 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 this 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

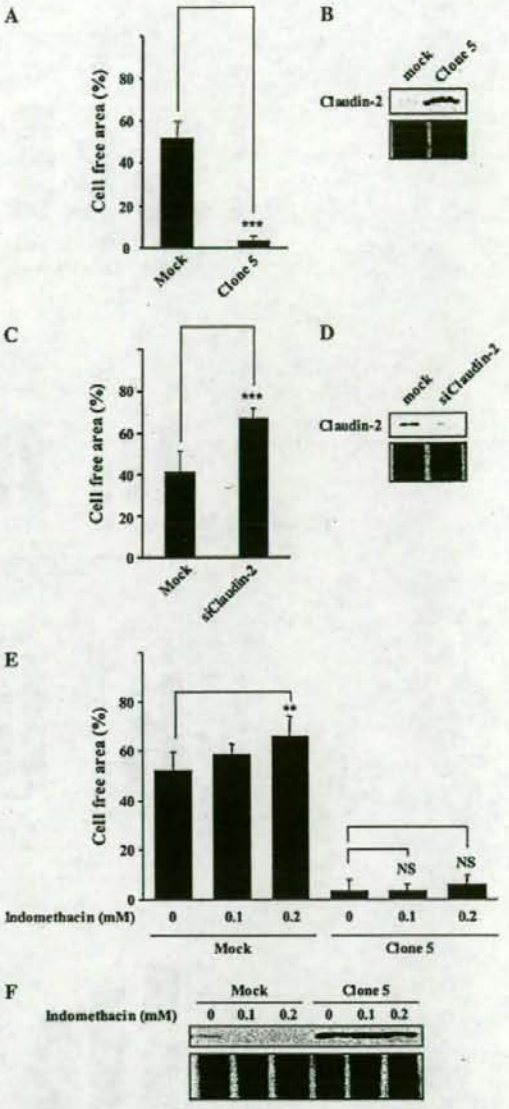

Fig. 5. Effect of alterations in claudin-2 expression on cell migration activity. AGS cells stably transfected with claudin-2 expression plasmid (clone 5) or plasmid expressing siRNA for claudin-2 (siClaudin-2) and mock transfectant control AGS cells were wounded and cultured for 24 h in the presence (**E**, **F**) or absence (**A**–**D**) of the indicated concentrations of indomethacin. The cell-free area was measured after a 24 h incubation and expressed relative to the area before the incubation. Values are mean ± SEM ($n = 3$), *** $P < 0.001$; ** $P < 0.01$; NS, not significant (**A**, **C** and **E**). The extent of expression of claudin-2 in each clone under the conditions was estimated by immunoblotting experiments as described in the legend of Figure 1. For loading control, the gels were stained with silver (**B**, **D** and **F**).

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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Expression and function of TETRAN, a new type of membrane transporter

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ABSTRACT

In contrast to transport across basolateral membranes, the mechanism governing transport of organic anions across the luminal membranes of proximal tubules has remained unclear. We recently found Tetracycline transporter-like protein (TETRAN), a human ortholog of yeast Tpo1p that can transport anionic Non-steroidal anti-inflammatory drugs (NSAIDs). In this study, we examine the expression and function of TETRAN. *TETRAN* mRNA is expressed in various human tissues, including kidney. When overexpressed in cultured cells, TETRAN was predominantly localized on cytoplasmic membranes. Immunohistochemical analysis of human and mouse kidney tissue showed that TETRAN was expressed at the luminal membranes of proximal tubules. Overexpression of TETRAN in cultured cells facilitated the uptake of organic anions such as indomethacin (a NSAID) and fluorescein. The results suggest that TETRAN is a novel human organic anion transporter, and that it serves as a transporter for some NSAIDs and various other organic anions at the final excretion step.

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin are a useful family of therapeutics. However, NSAID use is associated with gastrointestinal and renal complications [1,2]. We recently reported that not only a cyclooxygenase (COX)-dependent decrease in the gastric mucosal level of prostaglandins (PGs), but also COX-independent gastric mucosal cell death induced by NSAIDs is required for formation of NSAID-induced gastric lesions [3]. As for NSAID-induced renal complications, while renal COX-inhibition (PG decrease) is thought to be involved by decreasing renal blood flow, a COX-independent and as yet unknown mechanism is also thought to be involved in producing this side effect [4].

The kidney plays an important role in elimination of toxic organic anions and cations from the body, which is a major determinant of the pharmacokinetics of clinically used drugs. The renal proximal tubule is the primary site at which various organic anions and cations are taken up from the blood across the basolateral membranes and excreted to the urine across the luminal, brush border membranes. Both of these types of transport are believed to be mediated mainly by membrane transporters [5–7]. Compared to the transport mechanism for basolateral membranes, the mechanism governing transport across the luminal membranes of proximal tubules is poorly understood at present [6,7]. For example, recent studies have revealed that the organic anion transporters (OAT)1-3 play a central role in the transport of organic anions from the blood, across basolateral membranes to the proximal tubule [8,9]. On the other hand, although some transporters with organic anion transporting activity, such as OAT4, and organic anion transporting peptide (OATP)1 have been reported to be expressed on the luminal membranes of proximal tubules [10–14], it is not clear that these transporters contribute to the transport of organic anions from proximal tubule cells into the urine. Thus, other types of as yet unknown transporters are thought to exist on the luminal membranes of proximal tubules and to contribute to the transport of organic anions [8,9]. Most of NSAIDs are organic anions and thus, the molecular mechanism governing membrane transport of NSAIDs across the luminal membranes of proximal tubules has not been defined. Further to this, no transporter has directly been

Abbreviations: ABC, ATP binding cassette; COX, cyclooxygenase; MATE, multidrug and toxin extrusion; MFS, major facilitator super-family; MRP, multidrug resistance-associated protein; NPT, sodium-dependent inorganic phosphate transporter; NSAID, non-steroidal anti-inflammatory drug; OAT, organic anion transporter; PAH, *p*-aminohippuric acid; TEA, tetraethylammonium bromide; TETRAN, tetracycline transporter-like protein.

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shown to transport NSAIDs. Identification of transporters involved in the excretion of NSAIDs into urine may be important for understanding the pharmacokinetics of NSAIDs and the mechanisms governing their renal side-effects.

A useful strategy for identifying new types of transporters in humans, is to search for homologs and orthologs of yeast and bacterial transporters. For example, we recently identified a human ortholog of the bacterial multidrug and toxin extrusion (MATE) family, which confers multidrug resistance on bacteria. This ortholog, MATE1, is a new type of human transporter involved in the transport of organic cations across the luminal membranes of proximal tubules, in other words, in the final step of excretion of organic cations into the urine [15,16]. On the other hand, we previously screened yeast (*Saccharomyces cerevisiae*) genes for those whose overexpression confers resistance to indomethacin, and identified the *TPO1* gene, which belongs to the major facilitator super-family (MFS) of transporters. Like the MATE family proteins, Tpo1p confers multidrug resistance on yeast [17]. By carrying out a BLAST search, we identified a human ortholog of Tpo1p, tetracycline transporter-like protein (TETRAN), which was predicted to be a drug transporter based on its amino acid sequence similarity to a tetracycline transporter in *Escherichia coli*. TETRAN shows no homology to other human transporters. Thus, TETRAN might be a new type of membrane transporter involved in the transport of NSAIDs; however, its expression and function have not yet been examined. In this study, we examined the function of TETRAN. Our results suggest that TETRAN can transport not only some NSAIDs but organic anions in general. Furthermore, analysis of the expression of TETRAN in cells and tissues suggests that TETRAN is involved in the transport of organic anions across the luminal membranes of proximal tubules.

Materials and methods

Chemicals. [α-^{32}P]dCTP (6000 Ci/mmol) and [^{3}H]indomethacin (5 Ci/mmol) were purchased from Amersham Bioscience and American Radiolabeled Chemicals Inc., respectively. Nylon membrane displaying mRNA from human tissues (Human 12-Lane Multiple Tissue Northern Blot I and Human 12-Lane Multiple Tissue Northern Blot III) was from BD Biosciences. Antibodies against Myc and actin were purchased from Santa Cruz Biotechnology Inc. A polyclonal antibody against TETRAN was prepared in rabbits by repeated injections of GST-fusion polypeptides encoding the N-terminal region of TETRAN (MGWGGGGGCTPRPPIHQQPPERR).

Localization of TETRAN. Immunocytochemical analysis of TETRAN was performed as described previously [18] with some modifications. Cells were cultured on 8-well Lab-Tek II glass slides (Nunc). Samples were incubated with each antibody and then with Alexa Fluor 594 goat anti-rabbit IgG. After mounting with VECTASHIELD, samples were inspected using fluorescence microscopy (Olympus BX51).

Indirect immunofluorescence microscopy analysis and the HRP-DAB methods were performed as described previously [15,16]. The immunoreactivity was detected using either an Olympus BX60 microscope or an Olympus FV300 confocal laser microscope.

Immunoblotting and Northern blotting analyses. Whole cell extracts were prepared as described previously [19]. Brush border membranes were prepared from mouse kidney as described previously [20]. The protein concentrations of samples were determined by the Bradford method [21]. Samples were applied to 10% SDS–PAGE gels and subjected to electrophoresis, and proteins were then immunoblotted with their respective antibody. Northern blotting analysis was done as described previously [22].

Transport assay. Uptake experiments were performed as described previously [23] with some modifications. HEK293 cells were seeded in 24-well plates at a density of 1×10^5 cells/well and cultured for 2 days. After pre-incubation with transport assay medium at 37 °C for 30 min, cells were incubated in transport assay medium containing either fluorescein or [^{3}H]indomethacin at 37 °C. The fluorescence was read using a fluorometer or cells were trapped on nitrocellulose filters and the radioactivity was measured in a liquid scintillation counter.

Statistical analysis. All values are expressed as means ± standard deviation (SD). Two-way analysis of variance (ANOVA), 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

Expression of TETRAN in tissues and cells

Expression of *TETRAN* mRNA in various human tissues was examined by Northern blotting analysis. The size of the detected mRNA in bases, relative to sizing standards was similar to that reported previously in cultured cells [22] (Fig. 1). High levels of expression of *TETRAN* mRNA were observed in various tissues including the heart, kidney, and prostate (Fig. 1).

For detection of TETRAN by immunoblotting and immunostaining, we established HEK293 cells stably expressing Myc-tagged TETRAN and prepared a rabbit polyclonal antibody against the N-terminal region of TETRAN. Antibody against Myc or TETRAN identified a band with a molecular weight similar to the expected weight (about 50 kDa) in cells expressing Myc-tagged TETRAN but not in mock control cells (Fig. 2A and B). This established that the antibodies would be suitable for immunoblotting.

Among the TETRAN-expressing tissues shown in Fig. 1, we focused on the kidney and examined the location of TETRAN expression in this tissue by immunohistochemical analysis. Sections were prepared from mouse kidney and subjected to immunohistochemical analysis with antibody against TETRAN. Identification of each component of kidney (such as glomeruli, proximal convoluted tu-

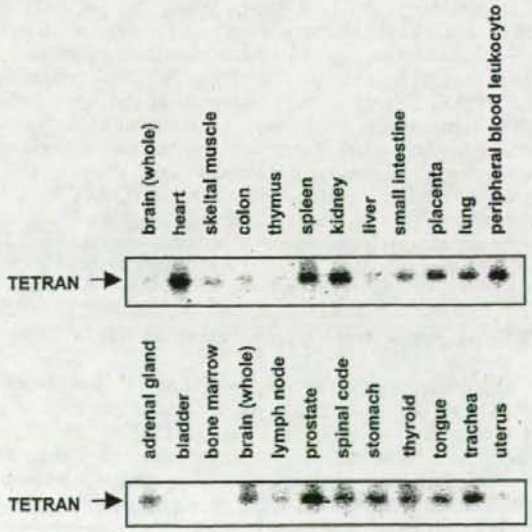

Fig. 1. mRNA expression of TETRAN in various human tissues. The mRNA expression of TETRAN was examined by Northern blotting analysis using commercially available nylon membranes with mRNAs from various human tissues.

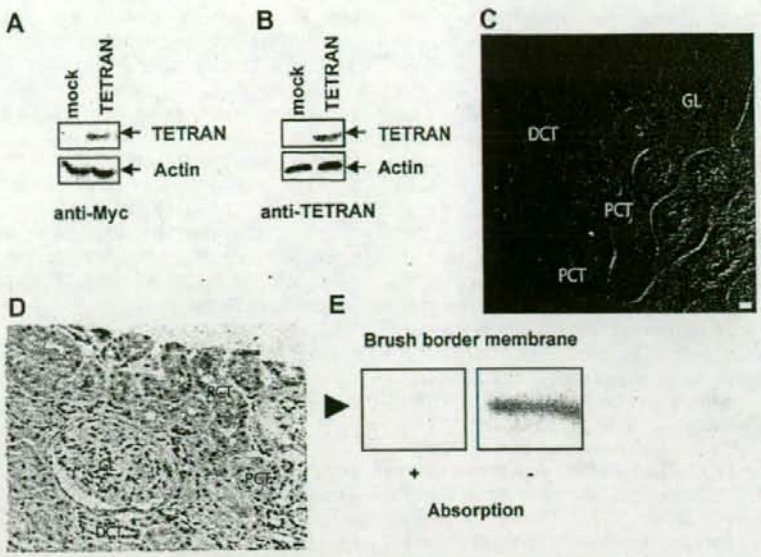

Fig. 2. Expression of TETRAN in mouse and human kidneys. Whole cell extracts were prepared from HEK293 cells stably expressing Myc-tagged TETRAN or mock control cells (A,B). Samples were analyzed by immunoblotting with an antibody against Myc (A) or TETRAN (B). In mouse kidney, mouse TETRAN was observed in the luminal membrane of renal tubules by indirect immunofluorescence microscopy (C). For human samples, immunohistochemical analysis was performed using the HRP-DAB method (D). GL, glomeruli; PCT, proximal convoluted tubule; DCT, distal convoluted tubule. Bar = 10 μm (C,D). Brush border membranes were prepared from mouse kidney and were analyzed by immunoblotting with an antibody against TETRAN before and after the absorption by N-terminal fragments of TETRAN (E).

bule, and distal convoluted tubule) was done based on results from hematoxylin and eosin staining of sections (data not shown). As shown in Fig. 2C, strong immunoreactivity was observed in the luminal membranes (brush border membranes) of proximal convoluted tubules but not of distal convoluted tubules. We also performed immunohistochemical analysis on human kidney sections. As shown in Fig. 2D, TETRAN expression was observed in the luminal membranes of proximal convoluted tubules but not in other parts of the sections. We also prepared brush border membranes from mouse kidney and looked for the presence of TETRAN by immunoblotting. The band corresponding to mouse TETRAN (52 kDa) was detected in the brush border membrane fractions and this band disappeared after the absorption of antibody by the N-terminal fragments of TETRAN (Fig. 2E). These results show that TETRAN is predominately expressed at the luminal membranes (brush border membranes) of proximal tubules.

Observations using fluorescent microscopy showed that although green fluorescent protein (GFP) alone showed a diffuse, evenly distributed signal in cells, while GFP-TETRAN seemed to be localized on the cytoplasmic membranes (data not shown). Indirect immunofluorescence assay with antibody against Myc or TETRAN also suggested that TETRAN is predominantly localized at the cytoplasmic membranes (data not shown).

TETRAN-dependent uptake of organic anions and NSAIDs

Using fluorescein, a fluorescent organic anion, we examined the effect of TETRAN expression on the uptake of organic anions into HEK293 cells. As shown in Fig. 3A and B, TETRAN-expressing cells have greater fluorescein uptake than mock control cells for all of the attempted incubation periods and fluorescein concentrations. Based on the results shown in Fig. 3B, we calculated the Km and Vmax values to be 13 μM and 75 pmol/mg/min, respectively. On the other hand, the uptake of a fluorescent organic cation (rhodamine 123) was indistinguishable between TETRAN-expressing cells and mock control cells (data not shown).

Next we examined the energy source of TETRAN. Since the amino acid sequence of TETRAN suggests that TETRAN does not use ATP hydrolysis for transport [22], we assumed that TETRAN used proton motive force (sum of membrane potential and of transmembrane pH gradient). Thus, we examined the effects of valinomycin (a dissipator of membrane potential) or nigericin (a dissipator of transmembrane pH gradient) on TETRAN-mediated uptake of fluorescein. We also examined the effects of depletion of Na^+ (or K^+) from the assay medium and alteration in assay medium pH on TETRAN-dependent indomethacin uptake. The results were similar to those obtained for indomethacin uptake (see below).

We also tested whether TETRAN is involved in efflux of fluorescein. After incorporation of fluorescein into cells, the medium was changed to one without fluorescein and the decrease in the amount of fluorescein in cells was measured. To incorporate approximately equal amounts of fluorescein, the incorporation reaction was done with a lower concentration of fluorescein in TETRAN-expressing cells than in mock control cells. As shown in Fig. 3C, a significant decrease in the amount of fluorescein in cells, in other words efflux of fluorescein, was observed in TETRAN-expressing cells but not in mock control cells, suggesting that TETRAN can mediate fluorescein efflux.

We compared the uptake of radiolabeled indomethacin by TETRAN-expressing cells to that of mock control cells. As shown in Fig. 4A, TETRAN-expressing cells showed higher indomethacin uptake activity than mock control cells. Analysis of the dose–response profile of TETRAN-dependent indomethacin uptake (Fig. 4B) gave a Km and Vmax of 3.6 μM and 18 pmol/mg/min, respectively. Both valinomycin and nigericin significantly inhibited the uptake of indomethacin into TETRAN-expressing cells but not into mock control cells (Fig. 4C). Furthermore, simultaneous treatment of TETRAN-expressing cells with valinomycin and nigericin caused a greater level of inhibition than individual treatment (Fig. 4C), suggesting that the transport activity of TETRAN depends on the proton motive force. In medium lacking Na^+ or K^+ TETRAN-dependent uptake

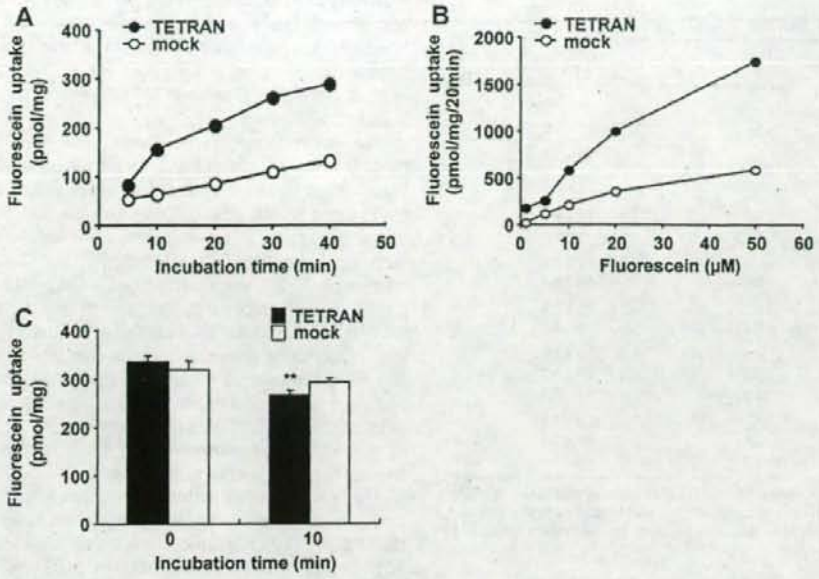

Fig. 3. TETRAN-dependent uptake of fluorescein. HEK293 cells stably expressing Myc-tagged TETRAN or mock control cells were cultured in the presence of 1 μM (A) or the indicated concentrations (B) of fluorescein for the indicated periods (A) or 20 min (B). Cells expressing TETRAN or control cells were cultured for 10 min with 5 or 20 μM of fluorescein, respectively. The medium was then changed and the cells were cultured for a further 10 min (C). The amounts of fluorescein incorporated into cells were determined by fluorometer. Values are means ± SD. ($n = 3$). $^{**}P < 0.01$.

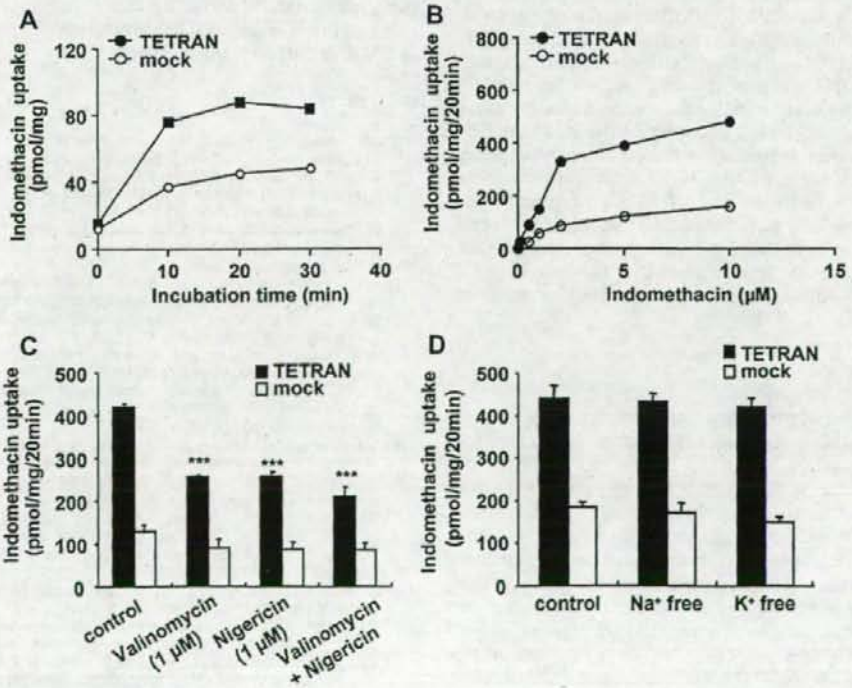

Fig. 4. TETRAN-dependent uptake of indomethacin. HEK293 cells stably expressing Myc-tagged TETRAN or mock control cells were cultured in the presence of 1 μM (A), 10 μM (C,D) or the indicated concentrations (B) of indomethacin for the indicated periods (A) or 20 min (B–D). The transport assay was performed in assay buffer containing valinomycin (a dissipator of membrane potential) or nigericin (a dissipator of transmembrane pH gradient) (C), without Na^+ or K^+ (D). The amounts of indomethacin incorporated into cells were determined by liquid scintillation counter. Values are means ± SD ($n = 3$). $^{***}P < 0.001$.

Table 1
cis-Inhibition of indomethacin transport in TETRAN

Compounds	mM	Transport, % of control % ± SD
Control		100 ± 3.8
PAH	0.5	51 ± 1.8
TEA	0.1	100 ± 3.6
Probenecid	1.0	76 ± 3.4
Diclofenac	0.1	74 ± 2.3***
	0.2	54 ± 1.6***
Mefenamic acid	0.2	63 ± 2.1***
	0.4	48 ± 3.2***
Etodolac	0.2	58 ± 2.5***
	0.4	43 ± 1.7***
Aspirin	0.4	98 ± 5.1
	0.8	98 ± 3.7
	1.0	91 ± 5.2
Acetoaminophen	0.4	100 ± 3.8
	0.8	98 ± 4.7
	1.0	94 ± 3.5

Inhibitory effects of various chemicals on TETRAN-dependent uptake of indomethacin. Indomethacin (10 μM) uptake for 20 min was examined in the presence of the indicated concentrations of each compound, as described in the legend of Fig. 3. Values are means ± SD ($n = 3$). ***$P < 0.001$.

of indomethacin was similar to that in control medium (Fig. 4D), suggesting that the transport activity of TETRAN is independent of Na^+ or K^+. We also examined the effect of the medium pH on the transport activity of TETRAN. TETRAN-dependent uptake of indomethacin was lower in medium with higher pH (tested from pH 6.0 to 8.0) (data not shown).

In order to identify substrates for TETRAN-mediated transport, we examined the inhibitory effects of various chemicals on TETRAN-dependent indomethacin uptake. PAH showed a strong inhibitory effect (Table 1). On the other hand, tetraethylammonium (TEA), a representative organic cation, showed no inhibitory effect on indomethacin uptake (Table 1), supporting the idea that TETRAN is an organic anion transporter. A similar competition assay using various NSAIDs suggested that TETRAN can transport some but not all NSAIDs. Of the NSAIDs and NSAID-related drugs tested, diclofenac, mefenamic acid and etodolac, but not aspirin or acetaminophen, significantly inhibited the TETRAN-dependent uptake of indomethacin (Table 1). This suggests that the former group and latter group of NSAIDs are or are not, respectively, transported by TETRAN.

Discussion

For the elimination of toxic chemicals from the body through the urine, both organic anions and cations must be transported from the blood across the basolateral membranes and excreted to the urine across the luminal membranes of proximal tubules. Transporters located on these membranes play a major role in the process [5–7]. Compared to transporters involved in transport of chemicals across basolateral membranes, transporters responsible for transport across the luminal membranes are poorly understood [5–7]. Using a strategy of searching for homologs (or orthologs) of bacterial transporters, we recently found a human homolog of the MATE family, MATE1. MATE1 is localized on the luminal membranes of tubules (both proximal and distal tubules) and possesses organic cation transport activity [15]. Furthermore, we identified TETRAN, as a human ortholog of a yeast transporter (Tpo1p) that transports various toxic chemicals [22]. In this study, we have found that TETRAN is localized on the luminal membranes of proximal tubules and possesses organic anion transport activity. The results suggest that TETRAN is a new type of organic anion transporter involved in transport across the luminal membranes of proximal tubules, because TETRAN shows no homology to other human transporters [22].

The environment around the luminal membranes of proximal tubules *in vivo* is unique and its reproduction *in vitro* is difficult. Thus, although we detected very weak efflux activity of TETRAN for organic anions (Fig. 3C), we assume that TETRAN is involved in the final step of excretion of organic anions into the urine, the step that cannot be fully attributed to previously reported transporters [5–7]. In other words, coordinately with other transporters (OAT4, sodium-dependent inorganic phosphate transporter (NPT)1 OATP1 and NRP2), TETRAN may play an important role in the final step of excretion of organic anions. It is also possible that TETRAN is involved in re-absorption of organic anions from the urine to the proximal tubule. The experiments using valinomycin and nigericin suggest that TETRAN uses the proton motive force for the transport of organic anions. However, the exact mechanism for TETRAN-dependent transport of organic anions is unclear at present.

Using radiolabeled indomethacin, we have shown that TETRAN can transport indomethacin. Competition assays not only confirm that TETRAN is an organic anion transporter but also suggest that TETRAN can transport some other NSAIDs (diclofenac, mefenamic acid, and etodolac). Examination of the ability or inability of TETRAN to transport other NSAIDs is required if a structure–function relationship for TETRAN-dependent transport of NSAIDs is to be identified. As was described earlier, major side-effects of NSAIDs include renal dysfunction and nephrotoxicity [2]. If TETRAN is involved in the final step of excretion of NSAIDs into the urine, alterations in the activity of TETRAN should affect the renal side-effects of NSAIDs. Furthermore, it is also possible that NSAIDs competitively inhibit the TETRAN-dependent excretion of toxic organic anions into the urine and that this mechanism is involved in the onset of NSAID-induced renal side-effects.

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