B vehicle DSS + SC (5)

+ Ce (10) + SC (5) + Ce (10) + IND (1)

+ dm-PGE2 (0.25) + SC (5) + Ce (10)

+ dm-PGE2 (0.25) + IND (1)

C

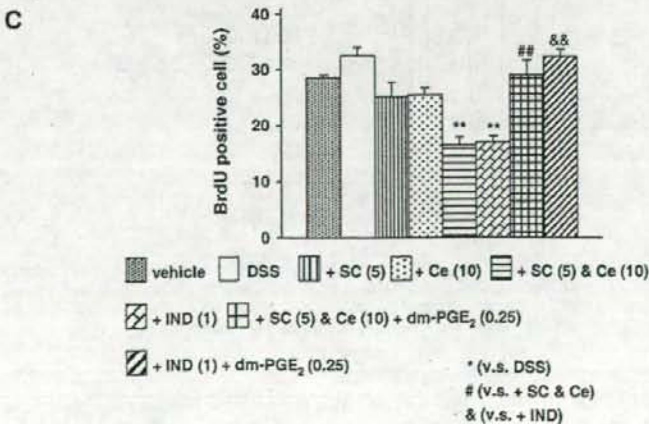

Fig. 6 (*continued*).

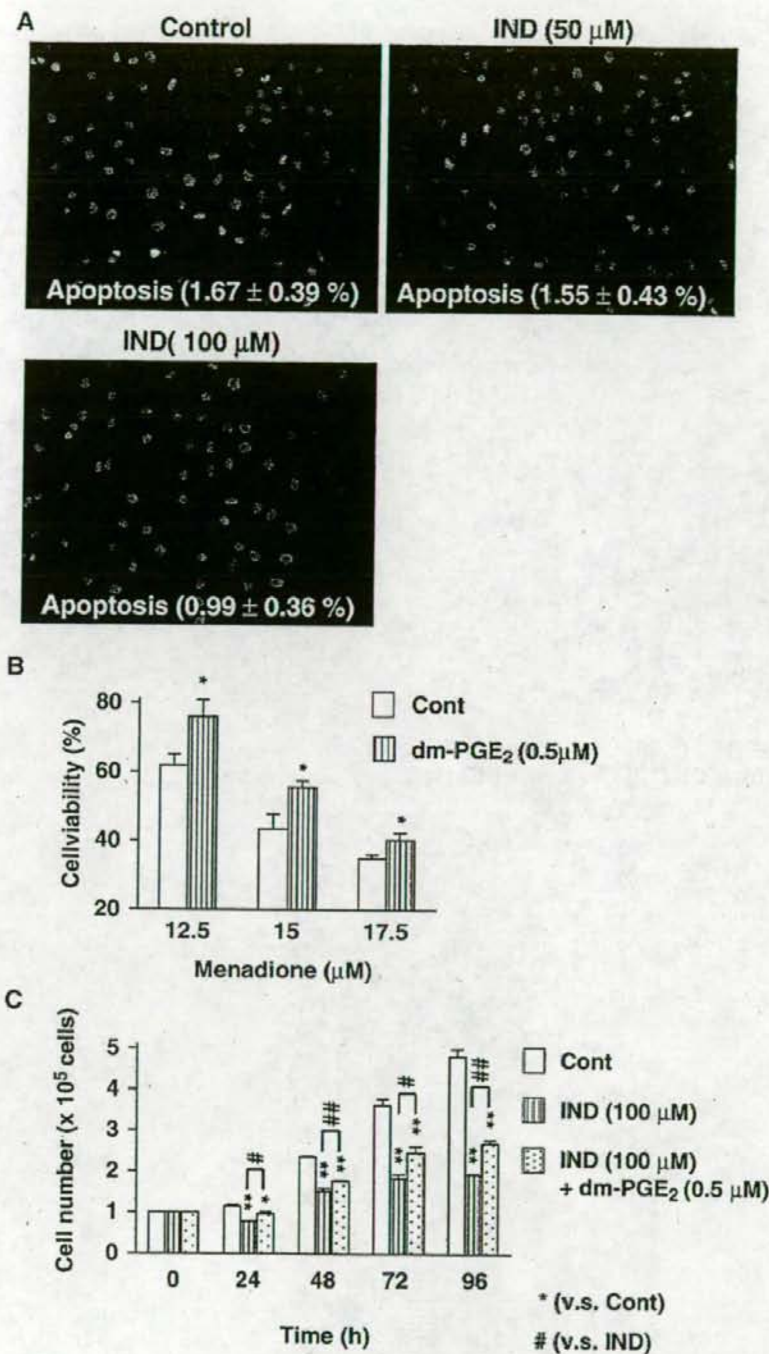

Fig. 7. Effect of indomethacin and dm-PGE_2 on apoptosis and cell growth *in vitro*. IEC6 cells were incubated with the indicated concentrations of indomethacin for 24 h, stained with Hoechst 33342 and the relative number of Hoechst 33342-positive cells was determined. Magnification of all photomicrographs is ×100 (A). Cells were pre-incubated with the indicated concentration of dm-PGE_2 for 1 h and further incubated with the indicated concentrations of menadione (B) or 100 μM indomethacin (C) for the indicated periods in the presence of the same concentrations of dm-PGE_2 as in the pre-incubation step. Cell viability was determined by MTT assay (B). Total viable cell numbers were counted (C). Values shown are mean±S.E.M. (n=3). **(or ##)P<0.01; *(or #)P<0.05.

we used half of the highest dose of SC-560 or celecoxib that was used in the other experiments (single NSAID administration). It may therefore be concluded that the effects of SC-560 and celecoxib are synergistic. These results suggest that non-selective NSAIDs, rather than COX-1- or COX-2-selective inhibitors, exacerbate the development of DSS-induced colitis and inflammatory bowel disease. This idea is supported by the majority of

previous clinical and animal studies, although some results are inconsistent with this idea (Bonner, 2001; El Miedany et al., 2006; El-Medany et al., 2005; Evans et al., 1997; Felder et al., 2000; Mahadevan et al., 2002; Martin et al., 2005; Okayama et al., 2007; Reuter et al., 1996; Yamada et al., 1993). The importance of COX-1 and COX-2 in protecting against inflammatory bowel disease and inflammatory bowel disease-related experimental colitis was also suggested by other studies (Okayama et al., 2007). For example, both COX-1- and COX-2 deficient mice exhibited a phenotype of sensitivity to DSS-induced colitis (Morteau et al., 2000). Furthermore, rapid development of colitis in IL-10-deficient mice (without DSS) was observed in response to administration of both COX-1- and COX-2-selective inhibitors (Berg et al., 2002).

As for the primary mechanism for the exacerbation of DSS-induced colitis by NSAIDs (SC-560/celecoxib or indomethacin), we conclude that the intestinal level of PGE_2 plays a major role, based on the following observations. In DSS-treated mice, administration of a combination of SC-560 and celecoxib or of indomethacin dramatically decreased the intestinal level of PGE_2, however, administration of SC-560 or celecoxib alone did not greatly decrease the level of PGE_2. Simultaneous administration of dm-PGE_2 suppressed the exacerbation of DSS-induced colitis by NSAIDs. These results also suggest that PGE_2 synthesis at intestinal tissues involves both COX-1 and COX-2. Other evidences are consistent with PGE_2 playing an important role in the protection of intestinal tissues against DSS-induced colitis. For example, administration of PGE_2 suppressed the development of DSS-induced colitis, and PGE_2 has been reported to be involved in the regeneration of epithelial crypts after DSS-induced damage (Cohn et al., 1997). It is well known that COX-2 is up-regulated and that the level of PGE_2 is increased in the intestinal tissues of inflammatory bowel disease patients relative to unaffected individuals (Singer et al., 1998). The results of the current study suggest that these responses ameliorate the development of inflammatory bowel disease.

Pro-inflammatory cytokines and cell adhesion molecules positively contribute to the progression of inflammatory bowel disease and colitis in animal models of inflammatory bowel disease. Pro-inflammatory cytokines activate, and thereby stimulate the release of reactive oxygen species from, leukocytes and cell adhesion molecules are essential for recruitment of blood circulating leukocytes into inflamed intestinal tissues (Danese et al., 2005; Kinoshita et al., 2006). However, in this study, we have shown that administration of PGE_2 does not significantly affect the mRNA levels of these proteins. This suggests that the expression of pro-inflammatory cytokines and cell adhesion molecules is not involved in the suppression of NSAID-dependent exacerbation of DSS-induced colitis by PGE_2. However, we assume that this is due to our experimental conditions and that pro-inflammatory cytokines and cell adhesion molecules are involved in the protective effects of PGE_2 against inflammatory bowel disease and inflammatory bowel disease-related experimental colitis, because a number of previous studies have clearly shown that PGE_2 inhibits the production of pro-inflammatory cytokines (particularly TNF-α and cell adhesion molecules (Kabashima et al., 2002; Kunkel et al., 1988).

Invasion of pathogenic bacteria across the intestinal mucosa is responsible for the development of inflammatory bowel disease. Therefore, the decrease in the intestinal mucosal cell number due to stimulation of apoptosis and inhibition of cell proliferation stimulates the development of inflammatory bowel disease (Kabashima et al., 2002; Tessner et al., 1998). In this study, we have shown that in DSS-treated mice the level of apoptosis and epithelial cell growth at the intestinal mucosa is stimulated and inhibited, respectively, by administration of NSAIDs (SC-560/celecoxib or indomethacin) and that this alteration is suppressed by simultaneous administration of PGE_2. This suggests that the alterations in the levels of intestinal mucosal cell growth and apoptosis are involved in the exacerbation of DSS-induced colitis by NSAIDs and its suppression by PGE_2. In accordance with this idea, it has been reported that PGE_2 stimulates intestinal epithelial growth (Tessner et al., 1998). We have also shown that indomethacin inhibited the growth of cultured intestinal cells and that addition of exogenous dm-PGE_2 recovered this cell growth, suggesting that NSAIDs affect cell growth partially through decreasing the level of PGE_2. We could not detect apoptosis induced by indomethacin *in vitro*. Based on our previous results with gastric mucosal cells (Tomisato et al., 2001), this is probably due to the low concentrations of indomethacin used. Higher concentrations of indomethacin may induce apoptosis in the intestinal cells; however, such high concentrations may not be achieved *in vivo*. Alternatively, we consider that reactive oxygen species released from activated leukocytes are responsible for the increased apoptosis in the presence of NSAIDs (SC-560/celecoxib or indomethacin) *in vivo* and for its suppression by PGE_2, because we have shown that PGE_2 protects intestinal cells from menadione-induced apoptosis. We have also demonstrated that NSAIDs (SC-560/celecoxib or indomethacin) inhibit the mRNA expression of mucin proteins and that PGE_2 suppresses this inhibition both in *vivo* and *in vitro*. These results suggest that NSAIDs directly affect the expression of mucin proteins through decreasing the level of PGE_2 and that this is involved in the exacerbation of DSS-induced colitis by NSAIDs and its suppression by PGE_2. The protective role of mucin proteins against DSS-induced colitis was genetically confirmed recently, using *muc2*-deficient mice (Van der Sluis et al., 2006).

In accordance with previous results (Cuzzocrea et al., 2001), we have shown that administration of celecoxib ameliorates the progression of DSS-induced colitis, suggesting that celecoxib is beneficial for the treatment of inflammatory bowel disease. In fact, some previous clinical studies support this idea (El Miedany et al., 2006; El-Medany et al., 2005). Our results also show that celecoxib, under conditions where it was observed to have a protective effect against DSS-induced colitis, did not significantly affect the intestinal level of PGE_2. Furthermore, we recently found that some other COX-2 selective inhibitors do not ameliorate DSS-induced colitis (Tanaka K. et al. unpublished results). These results suggest that a mechanism other than COX inhibition is also involved in conferring the protective effect of celecoxib against DSS-induced colitis. It was reported that celecoxib has various COX-independent effects on cells, such as induction of the endoplasmic reticulum (ER) stress response, inactivation of phosphatidylinositol 3-kinase (PI3K)/3-phosphoinositide dependent kinase-1 (PDK1)/Akt and inactivation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (Ding et al., 2005; Tsutsumi et al., 2004; Zhang et al., 2004). Identification of a COX-independent mechanism that confers the protective effect of celecoxib against DSS-induced colitis will be important for application of this drug in the clinical treatment of inflammatory bowel disease.

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Effect of Claudin Expression on Paracellular Permeability, Migration and Invasion of Colonic Cancer Cells

Masaya TAKEHARA, Tomoko NISHIMURA, Shinji MIMA, Tatsuya HOSHINO and Tohru MIZUSHIMA*

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan

* To whom correspondence should be addressed. E-mail: mizu@gpo.kumamoto-u.ac.jp

Abstract

Alteration in the expression of claudins, consisting of tight junctions (TJs), has been reported in various clinically isolated tumors. Claudins play an important role not only in the intercellular barrier function of TJs but also in migration and invasiveness of cancer cells. However, the use of different types of cells and different claudins in these studies has complicated the picture. In this study, we systematically examined the effect of claudin (claudin-1, -2, -3, -4 and -15) overexpression on the paracellular permeability, migration and invasiveness of Caco-2 colonic cancer cells. Overexpression of claudin-4 or claudin-2 increased or decreased, respectively, paracellular permeability. Overexpression of claudin-4 specifically stimulated the invasive activity of the Caco-2 cells. Furthermore, activation of matrix metalloproteinase (MMP)-2 and MMP-9 were observed in the claudin-4-overexpressing cells, suggesting that the invasive activity was stimulated through an increase in MMP activity. Overexpression of claudin-2 or claudin-3 and -4 stimulated or inhibited, respectively, the migration activity of the Caco-2 cells. Immunostaining analysis revealed that each of the overexpressed claudins localized at TJs under the conditions used to evaluate paracellular permeability. In contrast, they localized mainly in intracellular compartments under experimental conditions designed to assess cell invasion and migration. Overall, the results of this study show that the effect exerted by the claudins on the intercellular barrier function of TJs, as well as on cell migration and invasive activity, differs depending on the particular claudin species. Furthermore, the subcellular localization of the claudins varies according to the culture conditions.

Key words: tight junction, claudin, invasion, permeability, cancer.

INTRODUCTION

Tight junctions (TJs), the most apical intercellular structures in epithelial and endothelial cells, create a physiological intercellular barrier separating the apical and basolateral spaces, as well as regulating the paracellular permeability of various solutes. They also act as a divide between the apical and basolateral membranes, thereby maintaining cell polarity. TJs contain transmembrane proteins such as claudins, occludin and junctional adhesion molecules. The C-terminal regions of these proteins interact with cytosolic proteins, such as zonula occludens (ZO)-1, -2 and -3, which are linked to the actin cytoskeleton and are potentially involved in signal transduction.[1-6] Among these transmembrane proteins, the claudin family of proteins (claudin-1 to -24) play a major role in maintaining the intercellular barrier.[7,8]

Given that a loss of TJ structure and function is frequently observed in epithelium-derived cancers [9-12], TJs have attracted considerable attention in relation to this disease. The loss of TJ structure and function is thought to promote cancer cell proliferation by allowing constitutive accessibility of cancers to nutrients and growth factors. As TJs function as a barrier against cancer cell invasion, loss of TJ structure and function could also stimulate the metastasis of tumors.[11,13-15]

Alteration in the expression of the constituent proteins of TJs, in particular claudins, is frequently observed in tumors clinically isolated from various types of tissues, including colon, breast, pancreas, prostate, uterus and ovary.[9-12,16-20] It was initially believed that these alterations in expression affect cancer development only through the modulation of the barrier function of TJs. However, a number of recent studies suggest that the expression of certain claudins modulates the invasiveness and

migration of cancer cells through various mechanisms.[9,11] For example, we recently reported that overexpression of claudin-4 or claudin-2 causes a decrease or an increase, respectively, in the migration activity of gastric carcinoma (AGS) cells.[21,22] Studies from other groups have also shown that claudin overexpression (claudin-1, 3, 4, 5) can affect the invasiveness and migration of various types of cancer cells.[16,23-26]

Thus, an alteration in claudin expression appears to play a role in the progression of tumors, both by modulating the barrier function of TJs and by altering the migration and invasiveness of the cancer cells. However, the overall relationship between claudin expression and these cell functions has not been fully elucidated, partly due to the different types of cells and different cell culture conditions (i.e. cell density) used in the various studies. For example, although we showed that overexpression of claudin-4 decreases cell migration activity in AGS cells, other groups have reported that the overexpression stimulates cell invasion and migration in human ovarian cancer cells [25], but inhibits the invasiveness of pancreatic cancer cells.[16] The relationship between the barrier function of TJs and cell migration and invasion also remains unclear, as these two functions were not investigated simultaneously in most studies. Furthermore, the subcellular localization of overexpressed claudins is still open to debate; some reports have demonstrated their localization at TJs whereas others have described their localization in intracellular component.[24,27-29] In this study, we selected Caco-2 cells (human carcinoma cell line derived from colon) for investigation of these issues, as functional TJs can be formed in these cells, and assay systems for their invasion and migration activities have been established.[30,31] Our results reveal that the TJ intercellular barrier function, as well as cell migration and invasion, are affected differently, depending on the claudin species being overexpressed. We also found that

subcellular localization of claudins alters according to the culture conditions.

MATERIALS AND METHODS

Chemicals and media

Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co. Fetal bovine serum (FBS), fibronectin and G418 were purchased from Sigma, non-essential amino acids (NEAAs) from BioWhittaker, and lipofectamine (TM2000) and pcDNA3.1(-) from Invitrogen. The RNeasy kit was obtained from Qiagen, the first-strand cDNA synthesis kit came from GE Healthcare and iQ SYBR Green Supermix was from Bio-Rad. Matrigel was purchased from BD Biosciences and the 24-well transwells were from Costar. Antibodies against claudin-1, claudin-2, claudin-3, claudin-15 and ZO-1 were from Zymed and those against claudin-4, occludin and actin were from Santa Cruz Biotechnology. Fluorescein isothiocyanate-dextran (4 kDa; FD4) was obtained from Fluka Biochemika.

Cell culture and plasmid construction for overexpression of claudins

Caco-2 cells were cultured in DMEM containing 10% FBS.

Full-length human *claudin-1, -3* and *-15* cDNAs were PCR-amplified, using genome prepared from Caco-2 cells, and cloned into pcDNA3.1(-) to create the plasmid for overexpression of each claudin. The construction of the overexpression plasmids for claudin-2 and claudin-4 was as previously described [21,22].

Transfection of Caco-2 cells with plasmids was carried out using Lipofectamine (TM2000) according to the manufacturer's protocols. The stable transfectants

expressing each claudin were selected by immunoblotting analysis. Positive clones were maintained in the presence of 400 μg/ml G418.

Gelatin zymography

The proteolytic activity of matrix metalloproteinase (MMP)-2 and -9 was assessed by SDS-PAGE using zymogram gels containing 0.1% (w/v) gelatin, as described previously [32]. The culture medium was concentrated and the protein concentration was determined according to the Bradford method.[33] Following electrophoresis at 4°C, the gels were washed with 2.5% Triton X-100 for 1 h at 37°C and incubated with zymogram development buffer for 2 days at 37°C. Bands were visualized by staining with Coomassie Brilliant Blue.

Real-time RT-PCR

Total RNA was extracted using an RNeasy 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 RT-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, *actin* cDNA was used as an internal standard.

Immunoblotting analysis

Whole cell extracts were prepared as described previously.[21] The protein concentration of the sample was determined by the Bradford method.[33] Samples were applied to 12% polyacrylamide gels containing SDS, subjected to electrophoresis, and proteins then immunoblotted with each antibody.

Cell invasion assay

The cell invasion activity was measured by transwell matrigel invasion assay as described previously,[34] with some modifications. Serum-free 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. The cell suspension was applied to the matrigel and the lower chamber was filled with medium containing 10% FBS and 5 μg/ml fibronectin. The plate was incubated at 37°C for 48 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 assay

Cells in serum-free medium were applied to the upper chamber of the transwell and the lower chamber was filled with medium containing 10% FBS and 5 μg/ml fibronectin. The plate was incubated at 37°C for 48 h, and migrated cell were assessed as described for cell invasion assay.

Immunofluorescence microscopy

Caco-2 cells were grown in the Lab-Tek II chamber slide system (Nalge Nunc International). Cells were fixed in ice-cold methanol or acetone for 20 min and blocked in PBS containing 3% bovine serum albumin (BSA) for 30 min. The samples were then incubated with each primary antibody. After washing, samples were incubated with the respective secondary antibody conjugated with Alexa Fluor 594 or Alexa Fluor 488 (Molecular Probes). Images were captured on a confocal laser-scanning fluorescence microscope (FLUOVIEW FV500-IX-UV, Olympus).

Measurement of transepithelial resistance (TER)

Caco-2 cells were seeded at an initial density of 4.3 x 10^5 cells/cm^2 in the upper chamber of transwells. The cells were incubated at 37°C for 7 days, with a change of medium every second day. TER was measured using an epithelial voltohmmeter (Millipore). The results were expressed as the measured resistance in Ohms multiplied by the area of the filter (0.33 cm^2).

Permeability assay for FITC-dextran

We determined the permeability of Caco-2 cells by measuring transepithelial passage of FD4. The cells were seeded in the upper chamber of a 24-well transwell and incubated at 37°C for 7 days. FD4 (5 mg/ml) was added to the upper chamber. Aliquots were withdrawn from the lower chambers after 4 h and measured for fluorescence at 520 nm with excitation at 485 nm. An apparent permeability coefficient (P_{app}) was calculated as described previously.[35)]

Statistical analysis

All values are expressed as the mean ± standard deviation (S.D.). 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

Overexpression of claudins and their subcellualr localization

Among the claudins, we selected claudin-1, -2, -3, -4, and -15 for study based on the fact that their expression has been linked to tumor progression, as well as the availability of their corresponding antibodies. We then examined the effect of overexpression of these claudins on both the intercellular barrier function of TJs, and on cell migration and invasion. This was achieved by constructing stable transfectants of Caco-2 cells that continuously overexpress each claudin. As shown in Fig, 1A, we first confirmed the overexpression of each claudin by immunoblotting analysis.

We then examined the subcellular localization of the overexpressed claudins by immunostaining. As shown in Fig. 1B, each of the claudins localized at the cell surface (see *XY* image). Co-immunostaining assay for claudin and ZO-1 or occludin revealed good correspondence in their localization. This co-localization was also observed in panels of the *XZ* image (Fig. 1B). Such strong immunostaining for claudin was not observed in mock transfectant control cells (data not shown). The results presented in Fig. 1B suggest that each overexpressed claudin localizes at TJs.

We used cells at high density for experiments shown in Fig. 1B, as was also the case for the experiments illustrated in Fig. 2. However, as a lower density of cells (migrating and growing cells) is used in the invasion and migration assays (see Figs. 3 and 4), we also monitored the localization of each overexpressed claudin in cells cultured at low density. As shown in Fig. 1C, in this situation the claudins did not localize at the cell surface, but instead were found throughout the intracellular

compartments. It therefore seems that the overexpressed claudins only gradually localized at the cell surface (TJs) in response to increasing cell density.

Effect of overexpression of claudins on the barrier function of TJs

We examined the effect of overexpression of each claudin on the intercellular barrier function of TJs by examining the TER and permeability of FD4. TER is a measure of ion flux, mainly reflecting the ion flux across the TJs.[6] The TER in the mock transfectant control was 160 Ohm·cm^2 (Fig. 2A), which is similar to the value previously reported [30]. Overexpression of claudin-4 dramatically increased the TER, whereas overexpression of claudin-3 resulted in a similar but less pronounced effect (Fig. 2A). In contrast, overexpression of claudin-1, -2 and -15 produced a slight but significant decrease in the TER (Fig. 2A).

As shown in Fig. 2B, overexpression of claudin-4 or claudin-2 significantly decreased or increased, respectively, FD4 permeability, whereas overexpression of the other claudins had no significant effect (Fig. 2B). These results suggest that claudin overexpression can either positively or negatively affect the barrier function of TJs in Caco-2 cells, depending on the particular claudin species. In particular, overexpression of claudin-4 or claudin-2 seems to increase or decrease, respectively, the intercellular barrier function of TJs.

Effect of overexpression of claudins on cell invasion

Figure 3A shows the growth curve of each clone. The growth of each of the

claudin-overexpressing clones was indistinguishable from that of the mock transfectant control, demonstrating that the claudins did not affect the growth of the Caco-2 cells.

The effect of overexpression of each claudin on cell invasiveness was then examined using the transwell matrigel gel invasion assay. As shown in Fig. 3B, the claudin-4-overexpressing clone showed significantly greater cell invasion activity than the mock transfectant control. In contrast, clones overexpressing the other claudins produced similar results to the control (Fig. 3B), highlighting the specificity of the claudin-4 response.

Mechanism for alteration of cell invasion activity by overexpression of claudin-4

Cell migration is an important factor in determining cell invasiveness. We therefore examined the effect of overexpression of each claudin on cell migration, using the transwell chamber assay. As shown in Fig. 4A, claudin-2-overexpressing cells showed significantly greater cell migration activity than the mock transfectant control cells, whereas the claudin-3- or claudin-4-overexpressing cells showed less. These results reflect those previously observed in AGS cells.[21,22]

It has been reported that dynamic F-actin restructuring, in other words the formation of actin stress fibers, occurs in migrating cells and that this plays an important role in migration.[36] We used an immunostaining technique to examine the effect of overexpression of each claudin on F-actin architecture. A wound healing assay was used to obtain migrating cells, with the emergence of actin stress fibers being assessed 48 h after making the wound. As shown in Fig. 4B, typical actin stress fibers were observed in claudin-2-overexpressing cells. However, such a response was not

observed in either the control cells or in those expressing the other claudins (Fig. 4B). These results suggest that overexpression of claudin-2 stimulates the formation of actin stress fibers, leading to the greater migration activity of these cells.

We next examined the localization of each overexpressed claudin in the wound healing cells. As shown in Fig. 4C (upper panel), not only claudin-2 but also the other claudins were absent from the cell surface on the wounded side, but were present on the surface elsewhere. Distal to the wound, however, each of the claudins was found at the cell surface on all sides of the cell (Fig. 4C, lower panel). These results suggest that claudins generally translocate from the cell surface to the intracellular compartments at the site where cell migration occurs.

The results illustrated in Fig. 4A suggest that the higher invasive activity of cells expressing claudin-4 cannot be explained by its effect on cell migration. MMPs, especially MMP-2 and MMP-9, play an important role in cell invasion[37,38)] and some claudins have been reported to modulate the activity of MMPs.[24,26)] We therefore examined the effect of overexpression of each claudin on MMP-2 and MMP-9 activity using gelatin zymography. MMPs are proteolytically activated from pro-MMPs and both pro-MMPs and mature MMPs can be detected using this technique [39)]. The band intensity of MMP-2, indicative of MMP-2 activity, was higher in cells expressing claudin-4 than in mock transfectant control cells (Fig. 5A). Similar results were obtained for MMP-9 and pro-MMP-9 (Fig. 5A). In contrast, expression of the other claudins (claudin-1, -2, -3, -15) did not affect so clearly the intensity of these bands (Fig. 5A). These results suggest that the expression of claudin-4 specifically increases MMP-2 and MMP-9 activity, and that this may be responsible for the claudin-4-mediated stimulation of cell invasion.

Finally, mRNA expression of *MMP-2* and *MMP-9* in cells expressing each claudin was examined by real-time RT-PCR. As shown in Fig. 5B, the mRNA expression of both genes was up-regulated in cells expressing claudin-4 but not in those expressing the other claudins, suggesting that the higher activity of MMP-2 and MMP-9 in claudin-4-expressing cells is at least partly due to their higher expression.