Effects of endocytosis inhibitors on internalization of human IgG by Caco-2 human intestinal epithelial cells

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ABSTRACT

Aims: The purpose of this study was to characterize the internalization mechanism of human IgG into the epithelial cells of human small intestine, employing human intestinal epithelial cell line Caco-2 as an in vitro model system.

Main methods: Real-time PCR analysis and uptake studies of fluorescein isothiocyanate-labeled IgG (FITC-IgG) from human serum were performed using Caco-2 cells.

Key findings: Real-time PCR analysis showed that mRNA level of the neonatal Fc receptor (FcRn) was increased during the differentiation process in Caco-2 cells. The binding of FITC-labeled human IgG to the membrane surface of Caco-2 cells increased with a decrease in pH of incubation buffer. The uptake of FITC-IgG was also stimulated at acidic pH and was time-dependent. The binding and uptake of FITC-IgG at pH 6.0 was partially, but significantly, decreased by human γ-globulin in a concentration-dependent manner. A mixture of metabolic inhibitors (sodium azide and 2-deoxyglucose) significantly inhibited the uptake, but not the binding, of FITC-IgG. In addition, endosomal acidification inhibitors such as bafilomycin A1 and chloroquine significantly increased the accumulation of FITC-IgG. Clathrin-dependent endocytosis inhibitors (phenylarsine oxide and chlorpromazine) and caveolin-dependent endocytosis inhibitors (nystatin and indomethacin) did not decrease the uptake of FITC-IgG at pH 6.0. In contrast, macropinocytosis inhibitors such as cytochalasin B and 5-(N-ethyl-N-isopropyl) amiloride significantly decreased the uptake of FITC-IgG at pH 6.0.

Significance: The internalization of human IgG in human intestine might be, at least in part, due to FcRn-mediated endocytosis, which could occur by a process other than clathrin- and caveolin-dependent mechanisms.
Introduction

The major histocompatibility complex class I-related Fc receptor FcRn, the cognate receptor for IgG, plays an important role in IgG transport in various tissues (Roopenian and Akilesh, 2007). Initially, the receptor was identified in the neonatal rat small intestine, where it mediates the postnatal transfer of IgG from colostrum and milk (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985). The intestinal uptake of maternal IgG is followed by apical-to-basolateral transcytosis and release into the circulation. In rodent intestine, FcRn is suggested to be developmentally regulated since its functional expression at birth is markedly down-regulated after weaning (Martín et al., 1997; Gill et al., 1999).

FcRn is a heterodimer consisting of a transmembrane $\alpha$-chain noncovalently associated with $\beta_2$-microglobulin. The Fc fragment of IgG binds to FcRn at acidic pH (below 6.5) and weak or no binding at neutral pH, which is consistent with the presence of histidine residues within IgG responsible for FcRn binding (Raghavan et al., 1995; Vaughn and Björkman, 1998). It is assumed that binding of IgG to FcRn can occur at the cell surface exposed to an acidic environment such as the apical plasma membrane of enterocytes.

In humans, FcRn is expressed in intestinal epithelial cells in both the fetus and adult (Israel et al., 1997; Shah et al., 2003; Yoshida et al., 2006). Israel et al. (1997) observed that the human small intestinal epithelial cells were diffusely and strongly stained in the apical region with an antibody to human FcRn. Dickinson et al. (1999) reported that villous enterocytes of adult human small intestine showed delicate linear staining in the region of the apical membrane. In addition, a punctuate staining in crypt enterocytes was observed in the apical membrane and in the apical cytoplasm below the apical membrane. Like human small intestinal enterocytes, FcRn
was observed to be expressed in the polarized human intestinal Caco-2 and T84 epithelial cell lines. Furthermore, it is shown that FcRn transports IgG across T84 monolayers by receptor-mediated bidirectional transcytosis (Dickinson et al., 1999).

The first cellular event in transcytosis consists of the internalization of molecules by endocytosis, via a selective receptor-mediated process or by bulk uptake at cell surface domain (Schaere et al., 1991). The pathway for FcRn-mediated transcytosis of IgG has been actively examined in cultured epithelial cells, the human placenta and fetal enterocytes. On the other hand, information concerning the internalization process of IgG into the cells is limited. Wu and Simister (2001) found that the uptake of $^{125}$I-Fc was markedly decreased by alanine replacement of both Trp-311 and the dileucine motif in the cytoplasmic domain of rat FcRn. Thus, it is likely that IgG bound to FcRn under acidic environments is internalized by endocytosis. However, the molecular mechanisms underlying the internalization process of IgG still remain to be fully clarified. Therefore, the present study was undertaken to characterize IgG internalization including its binding and uptake processes in human enterocytes using Caco-2 cells, which exhibit well-developed microvilli and a polarized distribution of intestinal brush-border enzyme.
Materials and methods

Materials

FITC-labeled IgG from human serum (FITC-IgG), FITC-labeled dextran with a molecular mass 145 kDa (FITC-dextran), phenylarsine oxide, nystatin, indomethacin, 5-(N-ethyl-N-isopropyl) amiloride, bafilomycin A₁ and chloroquine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium azide and chlorpromazine hydrochloride were purchased from Nacalai Tesque (Kyoto, Japan). 2-deoxy-D-glucose was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Cytochalasin B was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used in the experiments were commercial products of the highest purity available.

Cell culture

Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine, 100 units/mL penicillin G and 100 μg/mL streptomycin in an atmosphere of 5% CO₂-95% air at 37°C, and were subcultured every 7 days using 0.02% EDTA and 0.05% trypsin, as described previously (Takano et al., 1998). The cells were used between passages 30 and 46.

Conventional RT-PCR

Total RNA was extracted from Caco-2 cells using MagExtractor RNA kit (TOYOBO Co., LTD., Osaka, Japan). RT-PCR was performed using a ReverTra Dash RT-PCR kit (TOYOBO Co., LTD.). The primer pair of FcRn sense and antisense (sense: 5’-
CTCTCCCTCCTGTACCACCTTACC-3’; antisense: 5’-
ATAGCAGGAAGGTAGCTCCTTGT-3’) was specific for a 457-bp fragment of FcRn transcripts. The primer pair of multidrug resistance-related protein 2 (MRP2) sense and antisense (sense: 5’-ACACCAACCAGAAATGTGTC-3’; antisense: 5’-CCAGGCCTTCCAAATCTC-3’) was specific for a 659-bp fragment of MRP2 transcripts. The primer pair of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense and antisense (sense: 5’-
GCAGGGGGGAGCCAAAAGGG-3’; antisense: 5’-TGCCAGCCCCAGCGTCAAAG-3’) was specific for a 567-bp fragment of GAPDH transcripts. The PCR conditions consisted of an initial denaturation at 94°C for 1 min, followed by amplification for each cycle number (FcRn, 20 cycles for FcRn, 24 cycles for MRP2, 19 cycles for GAPDH) of 60 sec at 95°C (denaturation), 60 sec at each annealing temperature (69 °C for FcRn, 62 °C for MRP2, 69°C for GAPDH), and 60 sec at 72°C (extension). The PCR products were separated by electrophoresis in a 2.0% agarose gel and stained with ethidium bromide.

Real-time PCR

Real-time PCR was performed using SYBR Green Realtime PCR Master Mix (TOYOBO Co., LTD.). Total RNA was extracted using MagExtractor RNA kit (TOYOBO Co., LTD.) from Caco-2 cells cultured for 7, 14 and 21 days after seeding. Total RNA (0.1 μg) was reverse transcribed with oligo(dT) primers using a ReverTra Dash RT-PCR kit (TOYOBO Co., LTD.). The primer pair of FcRn sense and antisense (sense: 5’-CAGGGTGGAGCTGGAATCTC-3’; antisense: 5’-CGCTGCCGTGAGTAGCAA-3’) was specific for an 83-bp fragment of FcRn transcripts. The primer pair of GAPDH sense and antisense (sense: 5’-
CCACCCATGGCAAATTC-3’; antisense: 5’-TGGGATTTCCATTGATGACAA-3’) was
specific for a 69-bp fragment of GAPDH transcripts. The PCR conditions consisted of an initial
denaturation at 95°C for 1 min, followed by amplification for 45 cycles of 5 sec at 95°C, 5 sec at
60°C, and 15 sec at 72°C. The threshold cycle (Ct) values for FcRn mRNA and GAPDH mRNA
were determined using the second derivative maximum method. After amplification, melt
analysis was performed by heating the reaction mixture from 65°C to 95°C. Melt curves were
transformed to the negative first-derivative melting curves ([-dF/dt] vs temperature). The
negative first-derivative peaks were used to identify specific PCR products because the peak is
characteristic of the PCR product melt temperature.

**Binding and uptake studies**

Binding and uptake of FITC-IgG and FITC-dextran were measured in Caco-2 cells
attached to the 12-well plates. Briefly, fresh medium was replaced every 2 days, and the cells
were used on the 20-22 days after seeding. Experiments were performed in Dulbecco’s
phosphate-buffered saline (PBS buffer containing in mM, 137 NaCl, 3 KCl, 8 Na₂HPO₄, 1.5
KH₂PO₄, 0.1 CaCl₂ and 0.5 MgCl₂) supplemented with 5 mM D-glucose (PBS(G) buffer). After
removal of the culture medium, each well was washed and preincubated with PBS(G) buffer.
Then, PBS(G) buffer containing FITC-IgG or FITC-dextran was added to each well and the cells
were incubated at 37°C (cell association) or 4°C (binding) for a specified period. Gel filtration
chromatography confirmed that no free-FITC was present in the solution preparations of FITC-
IgG and FITC-dextran. At the end of the incubation, the wells were rinsed rapidly three times
with 1 mL of ice-cold PBS buffer at the same pH as the incubation buffer. The cells were
scraped with rubber policeman into 0.3 mL of the ice-cold PBS buffer and the wells were washed
again with 0.3 mL of the ice-cold PBS buffer to improve the recovery of the cells. The cells were
centrifuged at 4°C for 5 min at 10,000 rpm and the supernatant was aspirated. The cell pellet was
resuspended gently in 0.3 mL of the ice-cold PBS buffer and centrifuged again. The pellet was homogenized in PBS buffer (pH 7.4) containing Triton X-100 (0.1% v/v), and the homogenate was used for fluorescence and protein assays. The fluorescence in the homogenate was measured by using a microplate fluorometer (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. In each uptake or binding study, the cells which were incubated under the same conditions except without FITC-IgG or FITC-dextran were prepared. The fluorescence intensity values from the cell homogenate were subtracted from those of the cells which were incubated with FITC-IgG or FITC-dextran. Protein content was analyzed by Bradford method with bovine serum albumin as a standard (Bradford, 1976). The accumulations of these fluorescent probes were normalized for the protein content of the cells in each well.

*Cell treatment*

Stock solutions of bafilomycin A₁ and cytochalasin B were prepared in dimethyl sulfoxide (DMSO) at concentrations of 100 μM and 20 mM, respectively. Other inhibitors were dissolved on the day of each experiment. Sodium azide, 2-deoxy-D-glucose and chloroquine were prepared in PBS(G) buffer. Phenylarsine oxide, nystatin and 5-(N-ethyl-N-isopropyl)amiloride were dissolved in DMSO. The final concentration of DMSO was less than 0.5%. The control cells were treated with the same concentrations of DMSO in each experiment. To study the effects of these inhibitors, the cells were incubated in the presence of inhibitors for 30 min except 10 min for phenylarsine oxide. Thereafter, the cells were washed three times, and were incubated for 2 h with PBS(G) buffer containing FITC-IgG or FITC-dextran with or without each inhibitor.
Statistical analysis

Statistically significant differences were determined by Student’s $t$-test, or one way analysis of variance (ANOVA) with the Tukey-Kramer’s test or the Dunnett’s test for post hoc analysis. A $p$ value less than 0.05 was considered statistically significant.
Results

Expression of FcRn mRNA in Caco-2 cells

First, the expression of mRNA for FcRn in Caco-2 cells employed in this study was analyzed by RT-PCR analysis. In addition, the mRNA expression of GAPDH and MRP2 was used as an internal control and a marker which is abundantly expressed in the intestine (Mottino et al., 2000; Van Aubel, 2000), respectively. As shown in Fig. 1, PCR amplification with reverse transcription of total RNA from Caco-2 cells (21 days after seeding) gave a product from each mRNA tested in this study. No band was detected when the total RNA from Caco-2 cells was subjected to PCR without reverse transcription.

Effect of days in culture on the mRNA levels of FcRn

It is reported that Caco-2 cells, after confluence, undergo an enterocytic differentiation, which is characterized by morphological polarity with junctional complexes and a typical brush border expressing hydrolases such as sucrase-isomaltase (Pinto et al., 1983). To investigate whether differentiation affects the mRNA level of FcRn in Caco-2 cells, FcRn mRNA levels was quantified by real-time PCR relative to the level of GAPDH mRNA as an internal control. The real-time PCR analysis was performed for the mRNAs isolated from preconfluent, confluent and postconfluent Caco-2 cells, which were cultured for 7, 14 and 21 days after seeding, respectively. The results showed a significant increase in mRNA levels of FcRn during the differentiation process in Caco-2 cells (Fig. 2). Therefore, the following binding and uptake assays were performed by employing Caco-2 cells cultured for 20-22 days after seeding.
As the assays were performed at 4°C and 37°C, the terms “binding of FITC-IgG” and “cell association of FITC-IgG” were used to refer to the amounts of “FITC-IgG bound to the membrane surface” and “bound and internalized FITC-IgG”, respectively. By gel-permeation chromatography, no detectable degradation product of FITC-IgG was observed in homogenates from the cells incubated with FITC-IgG at 37°C for 180 min (data not shown).

**pH-dependent binding and uptake of FITC-IgG**

The binding of FITC-IgG (200 μg/mL) to the membrane surface of Caco-2 cells increased with a decrease in pH of the incubation buffer (Fig. 3A). There was a significant difference when the binding of FITC-IgG at pH 6.0 was compared to that at pH 7.4. The binding of FITC-dextran (200 μg/mL) with a molecular size of 145 kDa was also examined at pH 7.4 or pH 6.0. The amounts of FITC-dextran binding were significantly lower than those of FITC-IgG under both pH conditions. The similar pattern was obtained when the effect of pH on cell association of FITC-IgG was examined (Fig. 3B). The amount of cell association of FITC-IgG was 8.8-fold higher than that of FITC-dextran at pH 6.0, while it was 2.1-fold as high as at pH 7.4.

**Effect of incubation time on FITC-IgG uptake**

When FITC-IgG was incubated with Caco-2 cells at 37°C at pH 7.4 or pH 6.0, the amount of cell association of FITC-IgG increased with time at either pH (Fig. 4). The amount of the cell association of FITC-IgG at pH 6.0 was significantly higher than that at pH 7.4 at all time points examined. The longer incubation time resulted in the greater differences in the amount of cell association of FITC-IgG between pH 7.4 and pH 6.0 (Fig. 4).
Effect of human $\gamma$-globulin on binding and uptake of FITC-IgG

To examine the specificity of the binding and uptake of FITC-IgG in Caco-2 cells, the effect of human $\gamma$-globulin on these processes was investigated. Human $\gamma$-globulin significantly inhibited the binding and cell association of FITC-IgG in a concentration-dependent manner (Fig. 5). However, the inhibition was not complete; the decreased amounts of the binding and association of FITC-IgG were 19.5% and 21.4% of control, respectively, even in the presence of 10 mg/mL human $\gamma$-globulin.

Effect of metabolic inhibitor mixture on FITC-IgG uptake

As shown in Fig. 3, the amount of FITC-IgG association at pH 6.0 at 37°C was clearly higher than the binding estimated at 4°C. Therefore, the uptake process would be temperature-dependent. We then examined whether the uptake process at pH 6.0 is ATP-dependent or not. As shown in Fig. 6, a mixture of metabolic inhibitors (sodium azide and 2-deoxyglucose) significantly inhibited the cell association of FITC-IgG, but not the binding of FITC-IgG. This observation reveals that FITC-IgG is internalized in an ATP-dependent manner.

Effects of endosomal acidification inhibitors on FITC-IgG uptake

It has been hypothesized that endosomal acidification plays an important role in FcRn-mediated recycling of IgG, leading to prevention from degradation in lysosome, and/or in FcRn-mediated bidirectional transcytosis. Therefore, we examined the effects of bafilomycin A$_1$ and chloroquine, endosomal acidification inhibitors (Sasaki et al., 2001; Antohe et al., 2001), on uptake of FITC-IgG in Caco-2 cells. Figure 7 shows that these endosomal acidification inhibitors significantly increased the amount of cell association of FITC-IgG, possibly following a decrease
in FcRn-mediated recycling and/or transcytosis of FITC-IgG. Similar results were observed when the effects of these inhibitors on FITC-IgG were examined at pH 7.4 (data not shown).

**Effects of various endocytosis inhibitors on FITC-IgG uptake**

To characterize the molecular mechanisms underlying the endocytic pathway of FITC-IgG in Caco-2 cells, we examined the effects of inhibitors for clathrin-dependent endocytosis, caveolin-dependent endocytosis and macropinocytosis on FITC-IgG uptake at pH 6.0. First, the effects of phenylarsine oxide and chlorpromazine, inhibitors of clathrin-dependent endocytosis, on FITC-IgG uptake were investigated. There were no significant changes in the cell association and binding of FITC-IgG in the cells treated with phenylarsine oxide at a concentration range of 1-10 μM (Fig. 8). Since the treatment with 30 μM phenylarsine oxide at 37°C induced cell detachment, a higher concentration was not evaluated. Chlorpromazine (50 μM) significantly increased the cell association of FITC-IgG (206.3 ± 9.9 % of control, n=3), without affecting the binding of FITC-IgG. In addition, we examined the effect of potassium depletion, which leads to a decrease in clathrin-dependent endocytosis (Hansen et al., 1993). Depletion of intracellular potassium by incubation with potassium-free buffer also significantly increased the cell association of FITC-IgG (133.7 ± 11.9 % of control, n=3).

Next, the effect of nystatin, an inhibitor of caveolin-dependent endocytosis, was examined. Figure 9 shows that nystatin at 30 μM had no effect on the cell association and binding of FITC-IgG at pH 6.0. Cell detachment was observed when the cells were treated with 50 μM nystatin. Indomethacin (100 and 300 μM), another inhibitor for caveolin-dependent endocytosis, also did not significantly affect the cell association and binding of FITC-IgG (data not shown).
Furthermore, the involvement of macropinocytosis in FITC-IgG uptake was investigated. For this purpose, cytochalasin B and 5-(N-ethyl-N-isopropyl) amiloride were used in this study since cytochalasin and amiloride analogues inhibit membrane ruffling and macropinocytosis. Cytochalasin B decreased the cell association of FITC-IgG in a concentration-dependent manner, and its inhibitory effect was significant at 40 μM or higher (Fig. 10). In contrast, there was no significant effect of cytochalasin B on the binding of FITC-IgG, except at a concentration of 80 μM. Like cytochalasin B, 5-(N-ethyl-N-isopropyl) amiloride (EIPA) reduced the cell-association of FITC-IgG in a concentration-dependent manner (Fig. 11). In contrast, EIPA had no significant effect on the cell-association of FITC-dextran (Fig. 11). Therefore, it is unlikely that the effect of EIPA on the cell association of FITC-IgG results from decreased fluid-phase endocytosis.
Discussion

The human FcRn cDNA and predicted amino acid sequences are relatively similar to that of rat FcRn with 69% nucleotide identity and 65% predicted amino acid identity (Story et al., 1994). The mRNA of human FcRn is detected in many tissues including adult small intestine. Ober et al. (2001) showed that human and mouse FcRn have different binding affinities for IgGs from different species; mouse FcRn binds IgGs from various species with high affinity, while human FcRn binds only human, guinea pig and rabbit IgG, but not mouse and rat IgG. In addition, intestinal expression level of rodent FcRn declines rapidly after weaning, while human FcRn is expressed in intestinal epithelial cells in both the fetus and adult. Therefore, Caco-2 cell line, which is of human origin, and FITC-labeled IgG from human serum were employed in this study, in order to characterize the mechanism of the internalization of human IgG in the human intestine.

FcRn is expressed in villous as well as crypt enterocytes of normal adult human small intestine (Israel et al., 1997; Dickinson et al., 1999). Caco-2 cells are well-known to form a confluent monolayer under standard culture conditions, and undergo spontaneous enterocytic differentiation in culture and mimic the crypt to villous maturation observed in vivo. There are several reports showing the mRNA and protein expression of FcRn in Caco-2 cells (Dickinson et al., 1999; Claypool et al., 2004). However, the differentiation-dependent regulation of FcRn in Caco-2 cells has not been reported yet. In this study, we found that the level of FcRn mRNA in confluent Caco-2 cells was increased by about 3-fold, as compared to preconfluent Caco-2 cells. Nabokina et al. (2005) showed that the mRNA levels of human thiamin transporter-1 and -2 (the products of the \( SLC19A2 \) and \( SLC19A3 \) genes, respectively) increased with differentiation from
pre-confluence to post-confluence in Caco-2 cells. Furthermore, they showed that a NF1 binding site and a SP1/GC-box binding site are involved in differentiation-dependent activation of the \textit{SLC19A2} and the \textit{SLC19A3} promoters. Mikulska and Simister (2000) showed that the presence of binding sites for several transcription factors including Sp1 and AP1 in the promoter region of the human FcRn gene. Therefore, these transcription factors might play an important role in the observed differentiation-dependent regulation of FcRn mRNA in Caco-2 cells, though further studies are needed to elucidate the molecular mechanisms underlying the up-regulation of FcRn mRNA during differentiation.

In accordance with the reported binding property of FcRn to IgG, the pH-dependent binding of FITC-IgG to the membrane surface of Caco-2 cells was observed in this study. The binding activity of FITC-IgG at pH 6.0 was significantly greater than that of FITC-dextran (145 kDa), while it was 2.2-fold at pH 7.4. This observation might indicate an involvement of FcRn in FITC-IgG binding to the plasma membrane of Caco-2 cells. On the other hand, the binding of FITC-IgG to the membrane surface of Caco-2 cells was not completely inhibited by unlabeled human \(\gamma\)-globulin even at a concentration of 10 mg/mL (ca. 70 \(\mu\)M), which is much higher than the binding affinities of human IgG\(_1\) for soluble human FcRn (10 nM and 1923 nM) (Bitonti et al., 2004). In contrast, Ellinger et al. (2001) showed that the plasma membrane binding in BeWo cells expressing human FcRn consisted of two different IgG binding systems with association constants of \(1.0 \times 10^6\) M\(^{-1}\) and \(6.9 \times 10^2\) M\(^{-1}\), which were explained as a low-affinity binding site and nonspecific binding, respectively. Therefore, the binding affinities of IgG to purified soluble FcRn might have a tendency to be higher than those to FcRn expressed in the plasma membrane of culture cells.
Like the binding property, the cell association of FITC-IgG in Caco-2 cells was pH-dependent. The uptake ratio of FITC-IgG to FITC-dextran at pH 6.0 was 8.8-fold, indicating that FITC-IgG is more efficiently taken up into the cells than FITC-dextran, a fluid-phase endocytosis marker. In addition, the uptake of FITC-IgG in Caco-2 cells was affected by depletion of ATP, which is essential for internalization by endocytosis. Since the luminal pH values of the human small intestine are shown to be around 6.0 (Ashford, 2002), IgG can bind to FcRn expressed on the luminal membrane of the enterocytes. Therefore, as shown in neonatal rodent intestine, receptor (FcRn)-mediated endocytosis might be, at least in part, responsible for the internalization of IgG from the mucosal side of the human small intestine.

Endosomal acidification is essential for the stability of IgG/FcRn complexes in the early endosome because IgG binds FcRn with high affinity at acidic pH but not at neutral pH. IgG bound to FcRn is either recycled back to the cell surface or transcytosed to the other side of the cell membrane, whereas IgG which dissociated from FcRn by disruption of endosomal acidification might be delivered to the lysosomes, resulting in the accumulated IgG and its degraded products. In this study, disruption of endosomal acidification by bafilomycin A1 and chloroquine significantly increased the accumulation of FITC-IgG in Caco-2 cells. These observations are consistent with the results that chloroquine treatment induced the heavy accumulation of IgG/FcRn complexes in the lysosome-like structures of human placental endothelial cells (Antohe et al., 2001). In addition, Dickinson et al. (1999) showed that the apical-to-basal transport of IgG-biotin across the human intestinal T84 cells was completely inhibited by bafilomycin A1 treatment. Taken together, interaction between IgG and FcRn in an acidic endosomal compartment might be crucial for the intracellular routing of IgG and FcRn, which is involved in recycling and transcytosis pathways. Contrary to these results, Goebl et al. (2008) recently reported that bafilomycin A1 had no effect on internalization of Fc at neutral pH.
in human endothelial cells expressing GFP fusion proteins of FcRn. In addition, they showed that internalization of Fc in the cells expressing GFP-FcRn was not affected by the extracellular medium pH. We observed that bafilomycin A₁ significantly increased FITC-IgG accumulation at pH 7.4 as well as at pH 6.0. The reason for the inconsistencies between these results is not clear at present, but it might be due to the differences in experimental conditions including the culture cells employed.

Earlier electron microscopic studies revealed that IgG was observed in coated pits and coated vesicles in the new born rat intestine and the syncytiotrophoblast of human term placenta (Abrahamson and Rodewald, 1981; Leach et al., 1990). In addition, recently, He et al. (2008) showed that a coated vesicle pinching off from a coated pit was involved in the first steps of FcRn-mediated IgG transport in neonatal rat intestine. However, surprisingly, the internalization of FITC-IgG at pH 6.0 in Caco-2 cells was not inhibited by phenylarsine oxide, a typical inhibitor of clathrin-dependent endocytosis (Takano et al., 2004; Yumoto et al., 2006). Chlorpromazine, another inhibitor of clathrin-dependent endocytosis (Yumoto et al., 2006), rather increased the cell association of FITC-IgG. Furthermore, potassium depletion, which induces the disappearance of clathrin-coated pits from the plasma membrane (Hansen et al., 1993; Yumoto et al., 2006), also had a tendency to increase the cell association of FITC-IgG. Several endocytic pathways that do not use clathrin, including caveolin-dependent endocytosis, have been reported (Conner and Schmid et al., 2003; Mayor and Pagano, 2007). However, no effects of nystatin and indomethacin, inhibitors of caveolin-dependent endocytosis (Ikehata et al., 2008), on the cell association of FITC-IgG were observed. Thus, clathrin- and caveolin-independent endocytosis seems to be involved in the internalization of FITC-IgG in Caco-2 cells. We observed that cytochalasin B and 5-(N-ethyl-N-isopropyl) amiloride, which inhibit macropinocytosis (Kee et al., 2004; Tagawa et al., 2008), significantly decreased the cell association of FITC-IgG. However,
these observations alone might not be enough to suggest an involvement of macropinocytosis in IgG internalization in Caco-2 cells since cytochalasin and amiloride analogues potently affect various endocytic processes by inhibiting actin polymerization and transmembrane Na\(^+\) transport systems, respectively.

In conclusion, the internalization of FITC-labeled human IgG in human enterocyte-like Caco-2 cells was pH, temperature and ATP-dependent. In addition, endosomal acidification inhibitors increased the accumulation of FITC-IgG. Clathrin-dependent and caveolin-dependent endocytosis inhibitors had no significant inhibitory effect on the internalization of FITC-IgG. Macropinocytosis inhibitors decreased the uptake of FITC-IgG. These observations suggest that the internalization of human IgG in human intestine might be due to FcRn-mediated, clathrin- and caveolin-independent endocytosis, and macropinocytosis may partly be involved in the process.
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References


Figure legends

**Fig. 1.** RT-PCR analysis of FcRn mRNA in Caco-2 cells. Total RNA (0.2 μg) from Caco-2 cells was reverse-transcribed and first-strand cDNA synthesized was amplified with a set of specific primers described in Materials and methods. The PCR products with (+) or without (-) reverse transcription were separated by electrophoresis through a 2% agarose gels and stained with ethidium bromide. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and multidrug resistance-related protein 2 (MRP2) were used as an internal control and a marker which is abundantly expressed in the intestine. M indicates size marker.

**Fig. 2.** Expression level of FcRn mRNA in preconfluent, confluent and postconfluent Caco-2 cells. Real-time PCR was performed using FcRn gene-specific primers and total RNA (0.1 μg) from preconfluent (day 7), confluent (day 14) and postconfluent (day 21) Caco-2 cells. Real-time PCR was performed as described in Materials and methods. Data were expressed as the ratio of threshold cycle (Ct) values from the FcRn gene and the GAPDH gene. Each symbol represents the mean ± S.E. of three monolayers. *p<0.05, significantly different from the value of each substrate at pH 7.4.

**Fig. 3.** The pH dependence of binding and association of FITC-IgG and FITC-dextran by Caco-2 cells. (A): Binding of 200 μg/mL FITC-IgG (open circle) or 200 μg/mL FITC-dextran (closed circle) was measured for 2 h at 4°C at various pH values. (B): Association of 200 μg/mL FITC-IgG (open circle) or 200 μg/mL FITC-dextran (closed circle) was measured for 2 h at 37°C at various pH values. Each symbol represents the mean ± S.E. of three monolayers. *p<0.05,
significantly different from the value of each substrate at pH 7.4. †p<0.05, significantly different from the value of FITC-IgG at each pH

**Fig. 4.** Time courses of FITC-IgG association at pH 7.4 and pH 6.0 by Caco-2 cells. Association of FITC-IgG (200 µg/mL) by Caco-2 cells was measured for 30 min, 1 h and 3 h at 37°C at pH 7.4 (open circle) or pH 6.0 (closed circle). Each symbol represents the mean ± S.E. of three monolayers. *p<0.05, significantly different from the value at pH 7.4.

**Fig. 5.** The effect of human γ-globulin on binding and association of FITC-IgG by Caco-2 cells. The binding (A) and association (B) of FITC-IgG (200 µg/mL) at pH 6.0 in the absence (control) or presence of various concentrations of human γ-globulin were measured for 120 min at 4°C and 37°C, respectively. Each symbol represents the mean ± S.E. of three monolayers. *p<0.05, significantly different from each control.

**Fig. 6.** The effect of sodium azide plus 2-deoxy-D-glucose on FITC-IgG binding and association by Caco-2 cells. After preincubation of the cells for 30 min in the absence (control) or presence of 10 mM sodium azide (NaN₃) plus 5 mM 2-deoxy-D-glucose, the cells were incubated with the buffer (pH 6.0) including FITC-IgG (200 µg/mL) in the absence or presence of these metabolic inhibitors for 2 h at 37°C (association) or 4°C (binding). Each column represents the mean ± S.E. of three monolayers. *p<0.05, significantly different from each control.

**Fig. 7.** The effects of bafilomycin A₁ and chloroquine on FITC-IgG association by Caco-2 cells. After preincubation of the cells for 30 min in the absence (control) or presence of 100 nM bafilomycin A₁ or 50 µM chloroquine, the cells were incubated with the buffer (pH 6.0)
including FITC-IgG (200 µg/mL) in the absence of each inhibitor for 2 h at 37°C. Each column represents the mean ± S.E. of three monolayers. *$p<0.05$, significantly different from the value of control.

**Fig. 8.** The effect of phenylarsine oxide on FITC-IgG association and binding by Caco-2 cells. After preincubation of the cells for 10 min in the absence (control) or presence of various concentrations of phenylarsine oxide, the cells were incubated with the buffer (pH 6.0) including FITC-IgG (200 µg/mL) in the absence or presence of phenylarsine oxide for 2 h at 37°C (association, open circle) or 4°C (binding, closed circle). Each symbol represents the mean ± S.E. of three monolayers.

**Fig. 9.** The effect of nystatin on FITC-IgG association and binding by Caco-2 cells. After preincubation of the cells for 30 min in the absence (control) or presence of 30 µM nystatin, the cells were incubated with the buffer (pH 6.0) including FITC-IgG (200 µg/mL) in the absence or presence of nystatin for 2 h at 37°C (association) or 4°C (binding). Each column represents the mean ± S.E. of three monolayers.

**Fig. 10.** The effect of cytochalasin B on FITC-IgG association and binding by Caco-2 cells. After preincubation of the cells for 30 min in the absence (control) or presence of various concentrations of cytochalasin B, the cells were incubated with the buffer (pH 6.0) including FITC-IgG (200 µg/mL) in the absence or presence of cytochalasin B for 2 h at 37°C (association, open circle) or 4°C (binding, closed circle). Each symbol represents the mean ± S.E. of three monolayers. *$p<0.05$, significantly different from each control.
Fig. 11. The effect of 5-(N-ethyl-N-isopropyl) amiloride (EIPA) on association of FITC-IgG and FITC-dextran by Caco-2 cells. After preincubation of the cells for 30 min in the absence (control) or presence of various concentrations of EIPA, the cells were incubated with the buffer (pH 6.0) including 200 μg/mL FITC-IgG (open circle) or 200 μg/mL FITC-dextran (closed circle) in the absence or presence of EIPA for 2 h at 37°C. Each symbol represents the mean ± S.E. of three monolayers. *p<0.05, significantly different from each control.
Fig. 1
Fig. 2
Fig. 3

(A) FITC-substrate binding (ng/mg protein/2 h)

(B) FITC-substrate association (ng/mg protein/2 h)
Fig. 4

FITC-IgG association (ng/mg protein) vs. Incubation time (min)

- pH 7.4
- pH 6.0
Fig. 5

(A) FITC-IgG binding (ng/mg protein/2 h)

(B) FITC-IgG association (ng/mg protein/2 h)

γ-globulin concn. (mg/mL)
Fig. 6

Association Binding

FITC-IgG association or binding (ng/mg protein/2 h)

Control NaN3 +2DOG

NaN3 +2DOG
Fig. 7

FITC-IgG association (ng/mg protein/2 h)

Control  Bafilomycin A1  Chloroquine

*
Fig. 8

Phenylarsine oxide concn. (μM) vs. FITC-IgG association or binding (ng/mg protein/2 h)

![Graph showing association and binding vs. concentration of Phenylarsine oxide]

- Association
- Binding
Fig. 9

FITC-IgG association or binding (ng/mg protein/2 h)

Control  Nystatin  Control  Nystatin

Association

Binding
Fig. 10

FITC-IgG association or binding (ng/mg protein/2 h) vs. Cytochalasin B concn. (μM)

- Association
- Binding

* denotes significant difference.
Fig. 11

![Graph showing FITC-substrate association](image)