Regular Article

**Megalin/Cubilin-mediated Uptake of FITC-labeled IgG by OK Kidney Epithelial Cells**

Junya NAGAI, Koya SATO, Ryoko YUMOTO and Mikihisa TAKANO*

Department of Pharmaceutics and Therapeutics, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

**Summary:** In this paper, we characterize the uptake mechanism of fluorescein isothiocyanate-labeled human immunoglobulin G (FITC-hIgG) in opossum kidney (OK) epithelial cells, which have been shown to express megalin and cubilin. Confocal immunofluorescence microscopy showed the punctate expression of the neonatal Fc receptor FcRn in the cytoplasm, but not on the cell surface membrane. Temperature- and energy-dependent uptake of FITC-hIgG was observed at pH 7.4 but not at pH 6.0, indicating that the internalization of FITC-hIgG might not be due to FcRn, which has a binding affinity for IgG under acidic conditions. Under physiological pH conditions, human and bovine serum ¥-globulin decreased FITC-hIgG uptake in a concentration-dependent manner. In addition, FITC-hIgG uptake was inhibited by various megalin and/or cubilin ligands including albumin, cytochrome c, transferrin and gentamicin. Endosomal acidification inhibitors (bafilomycin A1 and chloroquine) significantly decreased the uptake of FITC-hIgG. Clathrin-dependent endocytosis inhibitors (phenylarsine oxide and chlorpromazine) decreased FITC-hIgG uptake. Potassium depletion and hypertonicity, conditions known to inhibit clathrin-dependent endocytosis, also decreased FITC-hIgG uptake. In contrast, caveolin-dependent endocytosis inhibitors (nystatin and methyl-¢-cyclodextrin) did not decrease, but rather increased the uptake of FITC-hIgG. These observations suggest that the internalization of FITC-hIgG in OK cells might be, at least in part, due to megalin/cubilin-mediated, clathrin-dependent endocytosis.

**Keywords:** IgG; receptor-mediated endocytosis; megalin; cubilin; FcRn; renal proximal tubule; OK kidney epithelial cell line

**Introduction**

Immunoglobulin G (IgG) is a monomeric molecule of approximately 150 kDa that predominates in secondary or memory immune response against infectious organisms. At the molecular level, IgG is composed of two light chains and two heavy chains and has two sites for binding to specific antigens (Fab sites). The non-antigen-binding site, composed of portions of the two heavy chains, is called the Fc region. IgG is actively transported across the placenta and provides passive immunity to the newborn infant until the neonate’s own immune system matures. Normally, IgG constitutes approximately 80% of total serum immunoglobulins in humans. The normal serum concentration of IgG, the second most abundant protein in the plasma after albumin, is 7–15 mg/mL.1)

In the glomerulus, circulating proteins are filtered to varying degrees depending on their molecular sizes and charges.2–4) Permeation across the glomerular filtration barrier of low-molecular-weight proteins such as ¥-microglobulin (12 kDa), ø1-microglobulin (26 kDa) and retinol-binding protein (21 kDa) are almost completely unrestricted in normal individuals. In contrast, the glomerular filtration of high-molecular-weight proteins such as IgG (150 kDa) and ø2-macroglobulin (720 kDa) is highly restricted. However, high- and intermediate-molecular-weight plasma proteins such as albumin (67 kDa), transferrin (80 kDa) and IgG appear in the ultrafiltrate when glomerular proteinuria is

---

Received: March 19, 2011, Accepted: June 7, 2011
J-STAGE Advance Published Date: June 28, 2011, doi:10.2133/dmpk.DMPK-11-RG-022

*To whom correspondence should be addressed: Mikihisa TAKANO, Department of Pharmaceutics and Therapeutics, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan. Tel. +81-82-257-5315, Fax. +81-82-257-5319, E-mail: takanom@hiroshima-u.ac.jp

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan.
caused by a defect in the glomerular filtration barrier, comprising of the endothelium, the glomerular basement membrane and the podocyte filtration slit membrane.  

A variety of compounds, including albumin, vitamin-binding proteins, hemoglobin, β2-microglobulin, lysozyme, cytochrome c and gentamicin, are taken up by receptor-mediated endocytosis in the renal proximal tubular cells.1–9 The two endocytic receptors megalin and cubilin, which are abundantly expressed in the apical membrane of the renal proximal tubular cells, cooperatively function and act as scavenger receptors for filtered proteins, peptides and drugs.7,10–12

Although IgG is not detected or only marginally detected in the proximal tubules of normal kidney, IgG has been found to be concentrated at the base of brush-border membranes and within cytoplasm in the proximal tubular epithelial cells in remnant kidneys after 5/6 renal mass ablation.13 In addition, in patients with glomerular diseases, renal proximal tubules are reported to reabsorb urinary IgG in a cationic preferential manner.14 Recently, Motoyoshi et al. revealed that IgG as well as albumin preferentially accumulated in proximal tubule cells expressing megalin, compared to megalin-deficient proximal tubular cells, when megalin knockout mosaic mice were treated with an immunotoxin, leading to massive non-selective proteinuria.15

The purpose of this study was to characterize the uptake of IgG in opossum kidney (OK) epithelial cells, an established cell line which has similar properties to the renal proximal tubular cells. OK cells are shown to express megalin and cubilin,23,24 and are often employed as an in vitro model system to study the receptor-mediated endocytosis in renal proximal tubular epithelial cells.23–27

**Materials and Methods**

**Materials:** Fluorescein isothiocyanate-labeled IgG from human serum (FITC-hIgG), albumin from human serum, phenylarsine oxide, nystatin, chloroquine and bafilomycin A1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cytochrome c from horse heart, 2,4-dinitrophenol, γ-globulin from bovine serum, gentamicin sulfate, polyvinyl B sulfate and chlorpromazine were purchased from Nacalai Tesque (Kyoto, Japan). 2-Deoxy-d-glucose was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Sodium azide was purchased from Katayama Chemical Industries Co., Ltd. γ-Globulin from human serum and methyl-β-cyclodextrin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used in the experiments were of the highest purity available.

**Cell culture:** Opossum kidney (OK) cells were cultured in medium 199 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Biological Industries Ltd., Kibbutz Be’er Ha’Emek, Israel) without antibiotics in an atmosphere of 5% CO2-95% air at 37°C, and were subcultured every 5–7 days using 0.02% EDTA and 0.05% trypsin. OK cells were used between passage 87 and 111. The medium was replaced every 2 days, and the cells were used for the experiments on 5–7 days after seeding.

**Western blot analysis:** For Western blot analysis of FcRn in OK cells, whole cell lysate from OK cells was prepared at 7 days after seeding. After removal of the culture medium, the cells were washed and collected in the preparation buffer (150 mM NaCl, 10 mM Tris, 5 mM EDTA, pH 7.4) with a cell scraper. The cell suspension was centrifuged at 10,000 rpm at 4°C for 5 min. The supernatant was discarded, and the cell pellets were washed and resuspended in the preparation buffer including 1% (v/v) Triton X-100, 0.1% (w/v) SDS and 1% (w/v) deoxycholic acid and centrifuged at 10,000 rpm at 4°C for 5 min. The supernatant was used as a sample for immunoblotting. The sample (50 μg protein per lane) was subjected to SDS-polyacrylamide gel electrophoresis with 12% polyacrylamide gel, and the proteins were transferred for 50 min to a polyvinylidene difluoride membrane at 4°C. The membrane was blocked in 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20.5 mM Tris, pH 7.4) containing 0.05% Tween 20 (TBS-T) overnight at 4°C. The membrane was washed three times in TBS-T and was incubated with the anti-FcRn (K-12) goat antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA).
speciﬁc incubated at 37°C for 2 h. The membrane was washed three times in TBS-T and was incubated with horseradish peroxidase-conjugated anti-goat donkey antibody (Santa Cruz Biotechnology Inc.) (1:20,000 dilution) at 37°C for 2 h. Then, the membrane was washed three times in TBS-T and visualized with enhanced chemiluminescence (ECL-Plus, GE Healthcare UK Ltd., Buckinghamshire, England).

**Immunofluorescence:** OK cells were grown on 35-mm glass-bottom culture dishes for 3 days. The cells were washed three times in Dulbecco’s phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM NaH2PO4, 1.5 mM KH2PO4, pH 7.4) (PBS) and ﬁxed with 2% paraformaldehyde in PBS for 3 min. The cells were washed three times in PBS and then permeabilized with 0.1% Triton X-100 in PBS for 10 min. After the cells were blocked with 0.1% non-fat dry milk in PBS for 60 min, the cells were incubated with the anti-FcRn (K-12) goat antibody (1:10 dilution) at room temperature for 60 min. The cells were washed three times in PBS and then Alexa Fluor488-conjugated donkey anti-goat IgG antibody (Molecular Probes, Inc, Eugene, OR, USA) (1:100) was added, and the cells were incubated at room temperature for 60 min. After the cells were washed three times in PBS and then mounted in Vectashield H-1000 (Vector Laboratories Inc., Burlingame, CA, USA), immunofluorescent signals were viewed using a confocal laser scanning ﬂuorescence microscope (LSM510 invert, Carl Zeiss, Jena, Germany).28

**Binding and uptake studies:** Binding and uptake of FITC-hIgG were measured in OK cells grown on 12-well plates. Briefly, experiments were performed in PBS supplemented with 1 mM CaCl2, 0.5 mM MgCl2 and 5 mM D-glucose [PBS(G)]. After removal of the culture medium, each well was washed and preincubated with PBS(G) adjusted to the pH value stated. Then, PBS(G) containing FITC-hIgG was added to each well and the cells were incubated at 37°C (cell association) or 4°C (binding) for a speciﬁed period. At the end of the incubation, the incubation buffer was aspirated and the wells were rinsed rapidly three times with 1 mL of ice-cold PBS with 1 mM CaCl2 and 0.5 mM MgCl2 [PBS(+)]. The cells were scraped with a cell scraper into 0.4 mL of ice-cold PBS(+) and the wells were rinsed again with 0.4 mL of ice-cold PBS(+) to improve the recovery of the cells. The cells were centrifuged at 4°C for 3 min at 10,000 rpm and the supernatant was aspirated. The cell pellet was resuspended gently in 0.4 mL of ice-cold PBS(+) and centrifuged at 4°C for 5 min at 10,000 rpm. The pellet was solubilized in 0.7 mL of PBS containing 0.1% Triton X-100 at room temperature for 30 min and centrifuged for 5 min at 10,000 rpm. The supernatant was used for ﬂuorescence and protein assay. The ﬂuorescence in the lysate was measured by using a F-2700 ﬂuorescence spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan) at an excitation wavelength of 500 nm and an emission wavelength of 520 nm. To correct for the intrinsic ﬂuorescence of the cells, cells incubated without FITC-hIgG were prepared as described above, and then the ﬂuorescence intensity values from the cell lysate were subtracted from those of cells incubated with FITC-hIgG. Protein content was analyzed by the Bradford method with bovine serum albumin as a standard.29 The accumulation of the ﬂuorescent probe was normalized for the protein content of the cells in each well.

The half-maximal inhibitory concentration (IC50) values of competitors were determined by the following Hill equation:

\[
A = \frac{100}{1 + ([I]/IC50)^n}
\]

where \([I]\) is the concentration of the competitor, \(A\) is the percentage of FITC-IgG association in the presence of a competitor to that in the absence of a competitor, and \(n\) is the Hill coefficient. The KaleidaGraph program (Version 3.08, Synergy Software, PA, USA) was used for the curve-ﬁtting. The IC50 value was assessed from the curve-ﬁtting to the above-mentioned equation.

**Cell treatment:** To examine the effects of inhibitors on FITC-hIgG uptake, OK cells were preincubated with PBS(G) in the absence or presence of the inhibitors at 37°C. The pretreatment conditions for each inhibitor was as follows: sodium azide (10 mM) plus 2-deoxy-D-glucose (5 mM) for 30 min, 2,4-dinitrophenol (1 mM) for 30 min, bafilomycin A1 (100 nM with 0.1% DMSO) for 30 min, chloroquine (50 μM) for 30 min, phenylarsine oxide (1–5 μM with 0.5% DMSO) for 10 min, chlorpromazine (10–50 μM) for 30 min, nystatin (3–100 μM with 0.5% DMSO) for 30 min, and methyl-β-cyclodextrin (1–10 mM) for 30 min. After preincubation, cells were washed three times and were incubated with 0.4 mL of PBS(G) containing FITC-hIgG in the presence of the inhibitors (except for phenylarsine oxide, which was added only during the preincubation) at 37°C for 120 min. The control cells were treated with the same concentration of DMSO in each experiment. The effects of potassium depletion and hypertonicity on uptake were examined as described previously.30 Briefly, the buffer used for potassium depletion contained 140 mM NaCl, 20 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, 1 mg/mL D-glucose (pH 7.4) (HEPES buffer). Control cells were incubated with HEPES buffer supplemented with 10 mM KCl. HEPES buffer supplemented with 450 mM sucrose and 10 mM KCl was used as the hypertonic medium.

**Preparation of Fc and Fab fractions from γ-globulin:** To produce Fab and Fc fragments, human γ-globulin was digested with papain (1:20 w/w papain/protein) at 37°C for 4 h in 10 mM phosphate buffer (pH 7.4) containing 20 mM l-cysteine and 20 mM EDTA. Fc fragments were separated from Fab fragments by a HiTrap rProtein A FF afﬁnity column (GE Healthcare). The solution containing each fragment was dialyzed using a cellulose membrane at 4°C for 24 h and was concentrated by freeze drying.
Confocal laser scanning microscopy of FITC-hIgG uptake: OK cells were cultured in 35-mm glass-bottom dishes for 5 days. The cells were incubated with FITC-hIgG (500 µg/mL) at 37°C for 60 min. At 30 min before the end of the incubation, LysoTracker Red DND-99 (75 nM) (Invitrogen) was added to the incubation buffer containing FITC-hIgG. After being washed with ice-cold PBS (+) three times, the cells were fixed with 4% paraformaldehyde for 30 min and were viewed using a confocal laser scanning fluorescent microscope (LSM510 invert, Carl Zeiss).

Statistical analysis: All experiments were conducted in triplicate, and the data are presented as means ± S.E. Differences were evaluated by Student’s t-test or one-way analysis of variance with Tukey-Kramer’s test for post hoc analysis. A p value of less than 0.05 was considered statistically significant.

Results

Expression and cellular localization of FcRn in OK cells: We first examined the expression of FcRn in OK cells by immunoblotting. With goat polyclonal antibody raised against a peptide mapping within an internal region of human FcRn, a protein with the apparent molecular mass of 50 kDa was detected on the cell lysate from OK cells (Fig. 1A).

Next, the cellular localization of FcRn in OK cells was examined by immunofluorescence confocal microscopy. In the non-permeabilized OK cells, faint punctate staining was observed in the cytoplasm, but not on the cell surface membrane (data not shown). When OK cells were permeabilized with 0.1% Triton X-100, the localization of FcRn was observed in the cytoplasm (Fig. 1B). In addition, a diffuse staining of the nucleus was detected (Fig. 1B). When the permeabilized cells were incubated in the absence of primary antibody, there was no staining in the cells (data not shown). We examined the immunocytochemical localization of megalin and cubilin in the OK cells employed in this study by confocal laser scanning microscopy. As shown in Supplementary Figure 1, megalin and cubilin appeared to be expressed in the plasma membrane of the OK cells.

pH-Dependent binding and uptake of FITC-hIgG in OK cells: The extracellular pH dependence of binding and association of FITC-hIgG was evaluated by incubating with incubation buffer in the pH range 6.0–7.4 at 4°C and 37°C, respectively (Fig. 2). The binding of FITC-hIgG to the membrane surface of OK cells tended to increase with a decrease in pH of the incubation buffer, but there were no significant differences among the three incubation pHs tested. In contrast, the association of FITC-hIgG increased according to an increase in pH of the incubation buffer. The association of FITC-hIgG at pH 6.7 and pH 7.4 was significantly higher than that at pH 6.0. When the amounts of FITC-hIgG at each pH were compared between binding (4°C) and association (37°C), significant differences were observed at pH 6.7 and pH 7.4, but not at pH 6.0. Therefore, the following uptake studies of FITC-hIgG were performed under physiological pH conditions (pH 7.4).

Localization of FITC-hIgG taken up by OK cells: Intracellular localization of FITC-hIgG was examined by confocal laser scanning microscopy (Fig. 3). When OK cells were incubated with FITC-hIgG at 37°C for 60 min, the cells exhibited a punctate fluorescent pattern. The green fluorescence of FITC-hIgG was partly colocalized with LysoTracker Red, a marker for late endosomes and
lysosomes with red fluorescence, as indicated by the yellow color in the merged image. In contrast, there was little internalization of FITC-IgG in OK cells when the cells were incubated at 4°C. Furthermore, FITC-IgG internalization at 37°C was not detected in the presence of an excess of human γ-globulin. Thus, these images indicate that FITC-hIgG binds to the plasma membrane of OK cells under physiological pH conditions, and is partly internalized into acidic intracellular compartments such as lysosomes.

Effects of metabolic inhibitors on FITC-hIgG uptake in OK cells: To investigate whether the uptake process of FITC-hIgG in OK cells is ATP-dependent, the effects of metabolic inhibitors were examined. The metabolic inhibitors employed in this study were as follows: sodium azide, which inhibits oxidative energy metabolism; 2-deoxyglucose, a competitive inhibitor of glycolysis; and 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. As shown in Figure 4A, a mixture of metabolic inhibitors (sodium azide and 2-deoxyglucose) and 2,4-dinitrophenol dramatically decreased the association of FITC-hIgG, but not the binding of FITC-hIgG. These observations revealed that FITC-hIgG was taken up by OK cells in an ATP-dependent manner.

Effects of endosomal acidification inhibitors on FITC-hIgG uptake in OK cells: The effects of bafilomycin A₁ and chloroquine, endosomal acidification inhibitors, on FITC-hIgG association were examined. Bafilomycin A₁ prevents endosomal acidification by inhibiting vacuolar-type H⁺-ATPase, which actively pumps H⁺ into the endosomal compartments. Chloroquine is a lysosomotropic agent which has a buffering capacity preventing endosomal
acidification. As shown in Figure 4B, baflomycin A₁ significantly decreased the association of FITC-hlgG in OK cells, and chloroquine also decreased FITC-hlgG association, although the p value (p = 0.052) was slightly higher than the level of significance.

Effects of γ-globulin, Fab and Fc fragments on FITC-hlgG uptake in OK cells: To investigate the specificity of the uptake of FITC-hlgG in OK cells, the effects of serum γ-globulin from human and bovine serum were examined (Figs. 5A and 5B). Both human and bovine serum
γ-globulin inhibited FITC-hIgG association in OK cells in a concentration-dependent manner. The IC_{50} values of human and bovine serum γ-globulin were 0.48 and 0.93 mg/mL, respectively. Next, we compared the effects of Fab and Fc fragments of human IgG on FITC-hIgG association in OK cells (Fig. 5C). Both fragments decreased the association of FITC-hIgG with increasing concentrations in the incubation buffer, and the IC_{50} value of Fab fragment (0.77 mg/mL) was 4.4-fold greater than that of Fc fragment (0.17 mg/mL).

**Effects of human and bovine serum albumin on FITC-hIgG uptake in OK cells:** Serum albumin is suggested to have no effect on IgG binding to FcRn. In contrast, megalin/cubilin-mediated uptake of albumin in OK cells is known to be inhibited by non-specific hIgG.

Therefore, we examined the effects of human and bovine albumin on FITC-hIgG association. As shown in Figures 5D and 5E, both human and bovine serum albumin efficiently decreased the association of FITC-hIgG in a concentration-dependent manner with the IC_{50} values of 0.15 and 0.19 mg/L, respectively.

**Effects of various megalin/cubilin ligands on FITC-hIgG association in OK cells:** The effects of various megalin and/or cubilin ligands on FITC-hIgG association were investigated. Cytochrome c, transferrin and gentamicin inhibited the association of FITC-hIgG in a concentration-dependent manner with IC_{50} values of 0.30, 0.75 and 0.22 mg/mL, respectively (Figs. 6A–6C). In addition, polymyxin B was found to have a very strong inhibitory potency, and the uptake was decreased to 17.1% of control by 0.1 mg/mL polymyxin B (Fig. 6D). Furthermore, the effect of retinol-binding protein, a specific ligand for megalin, was examined. Retinol-binding protein at a concentration of 0.38 mg/mL significantly inhibited FITC-hIgG association, which resulted in a reduction to 40.9% of the control value (data not shown).

**Effects of endocytosis inhibitors on FITC-hIgG uptake in OK cells:** We characterized the molecular mechanisms underlying the endocytic pathway of FITC-hIgG in OK cells. First, the effects of inhibitors of clathrin-dependent endocytosis on FITC-hIgG association were
examined. The inhibitors of clathrin-dependent endocytosis employed in this study were phenylarsine oxide, an inhibitor of clathrin-coat formation, and chlorpromazine, a cationic amphiphilic drug that induces a redistribution of clathrin-coated pit component AP-2 to endosomes. As shown in Figures 7A and 7B, both clathrin-dependent endocytosis inhibitors decreased the association of FITC-hIgG in a concentration-dependent manner. Furthermore, we examined the effects of potassium depletion and hypertonicity, which inhibit clathrin-coated pit formation, on FITC-hIgG association. As shown in Figure 7C, depletion of intracellular potassium and sucrose-induced hypertonicity significantly decreased the association of FITC-hIgG. Thus, these observations indicate that FITC-hIgG uptake by OK cells is, at least in part, mediated by clathrin-dependent endocytosis.

Next, we examined the effects of inhibitors of caveolin-dependent endocytosis on FITC-hIgG association. Nystatin is a cholesterol-binding drug that inhibits caveolin-mediated endocytosis, and methyl-β-cyclodextrin is a cholesterol-sequestering drug that disrupts caveolae integrity. Interestingly, with increasing concentrations of these inhibitors, the association of FITC-hIgG was stimulated at low concentrations, and then decreased to the control level at higher concentrations (Fig. 8). Thus, FITC-hIgG uptake in OK cells was biphasically modulated by these inhibitors of caveolin-dependent endocytosis, although the precise mechanisms remain unknown.

Discussion

The OK cell line, derived from the American opossum kidney, expresses a variety of proximal tubular cell properties such as transport systems of hexoses, amino acids, phosphate and organic anions/cations and responsiveness to parathyroid hormone. In addition, OK cells
In addition, endosomal acidification might be essential for the internalization of FITC-hIgG in OK cells.40 In this study, the expression of FcRn in OK cells was revealed by immunoblotting (Fig. 1A). Furthermore, confocal immunofluorescent microscopy showed that FcRn is present as punctate spots in the cytoplasm, whereas little staining on the plasma membrane was observed (Fig. 1B). FcRn binds IgG under acidic pH conditions (below 6.5).19 Therefore, no or little expression of FcRn on the cell membrane surface might explain no significant increase in the binding of FITC-hIgG with a decrease in pH of the incubation buffer (Fig. 2). Concerning the pH dependence of the association of FITC-hIgG, the pattern in OK cells was quite different from that in Caco-2 human intestinal epithelial cells expressing FcRn.38 In the previous report by our laboratory, both association and binding of FITC-hlgG in Caco-2 cells were shown to dramatically increase with a decrease in the pH of the incubation buffer, suggesting that FcRn might be involved in the internalization of FITC-hlgG in the intestine. In addition, endosomal acidification inhibitors baflomycin A1 and chloroquine significantly increased FITC-hlgG association in Caco-2 cells, possibly due to a decrease in FcRn-mediated recycling and/or transcytosis of FITC-hlgG.38 In contrast, the association of FITC-hlgG in OK cells was inhibited by these endosomal acidification inhibitors (Fig. 4B). Megalin and the low-density lipoprotein (LDL) receptor contain epidermal growth factor (EGF)-like repeats and YWTD spacer regions, which are responsible for pH-dependent release of ligands in endosomal compartments.71 The acid-dependent dissociation of ligands from the receptor is considered essential for receptor recycling. Therefore, impairment of endosomal acidification might reduce the recycling of megalin to the cell surface membrane, resulting in a decrease in megalin-mediated endocytosis. In another paper, we showed that baflomycin A1 inhibited the endocytic uptake of FITC-albumin in OK cells.39 Thus, endosomal acidification might be essential for the internalization of IgG as well as albumin in OK cells.

No involvement of FcRn in the binding and internalization of FITC-IgG was observed in OK cells, in contrast with the findings obtained in human renal proximal tubular cells (RPTECs).21 The inconsistency between these studies remains to be clarified, but it might be due to the differences in cellular localization of FcRn between the two culture cells. Cell surface expression of FcRn was detected in RPTECs,21 but not in OK cells in the present study. In addition, it might be due to species differences in binding affinity of human IgG to FcRn expressed in these two cell lines, since species diversity in IgG-FcRn interactions has been reported.40 However, at present, it is unknown whether human IgG binds to FcRn expressed in OK cells.

In order to characterize the ligand specificity of a receptor that is responsible for uptake of FITC-hlgG in OK cells, we examined the effects of various compounds on FITC-hlgG association. Figures 5A and 5B showed that γ-globulin from human and bovine serum inhibited the association of FITC-hlgG in a concentration-dependent manner. Thus, it is likely that the receptor(s) that might be involved in the internalization of FITC-hlgG in OK cells recognize IgGs from some species. Furthermore, we found that both Fab and Fc fragments decreased the association of FITC-hlgG in OK cells (Fig. 5C), but the IC50 value of the Fab fragment (0.77 mg/mL) was 4.4-fold higher than that of the Fc fragment (0.17 mg/mL). When the IC50 values of human γ-globulin, Fab and Fc fragments were expressed on a molar basis, the apparent IC50 values were 3.2, 15.4 and 3.5 µM, respectively. Thus, the IC50 value of Fc fragment was almost

---

Fig. 8. The effects of caveolin-dependent inhibitors on FITC-IgG association in OK cells
After preincubation of the cells for 30 min in the absence (control) or presence of various concentrations of nystatin and methyl-β-cyclodextrin, the cells were incubated with the buffer (pH 7.4) including FITC-IgG (50 µg/mL) in the absence (control) or presence of various concentrations of nystatin (A) and methyl-β-cyclodextrin (B) at 37°C for 2h. Each column represents the mean ± S.E. of three determinations. *p < 0.05, significantly different from the value of control.
the same as that of human γ-globulin, indicating that the receptor(s) responsible for FITC-hlgG uptake might preferentially recognize the Fc region of FITC-hlgG. On the other hand, the Fab fragment also showed an inhibitory effect on the association of FITC-IgG in OK cells. Immunoglobulin light chains, known as Bence-Jones proteins, have been reported to be internalized by megalin/cubilin-mediated endocytosis in the renal proximal tubular cells. Since the Fab region consists of a common heavy chain and different light chains, the inhibitory effect of the Fab fragment on FITC-hlgG in OK cells might occur by binding competition between their light chains.

Albumin is shown to bind to megalin, cubilin and FcRn. Like γ-globulins, albumin from human and bovine serum inhibited the association of FITC-hlgG in OK cells with IC50 values of 0.15 and 0.19 mg/mL, respectively (Figs. 5D and 5E). When the IC50 values of albumin from human and bovine serum were expressed on a molar basis, the apparent IC50 values were 2.3 and 2.9 µM, respectively. Among the various compounds tested in this study, human serum albumin was the most potent competitor for FITC-hlgG association in OK cells. Albumin and IgG, bind to FcRn at low pH but not at physiological pH; however, IgG and albumin bind to distinct sites on FcRn. In this study, the effect of albumin on FITC-hlgG association was investigated at pH 7.4, and albumin was found to competitively inhibit the association of FITC-hlgG. Therefore, the inhibitory effect of albumin on FITC-hlgG association in OK cells might suggest the involvement of receptor(s) other than FcRn in the internalization of FITC-hlgG in OK cells. Furthermore, the strong inhibitory potency of albumin against FITC-hlgG association might support the above-mentioned hypothesis that FITC-hlgG is internalized by preferential binding of its Fc region, rather than its Fab region, to the receptor(s) because Batuman et al. reported that albumin did not compete with the binding of immunoglobulin light chains on primary cultures of rat renal proximal tubular cells.

In addition to albumin, various ligands of megalin and/or cubilin, such as cytochrome c, transferrin, retinol binding protein, gentamicin and polymyxin B, significantly inhibited the association of FITC-hlgG in OK cells (Fig. 6). The competitively inhibitory effects of these megalin/cubilin ligands on FITC-hlgG association might suggest the involvement of megalin/cubilin in the uptake pathway of FITC-hlgG in OK cells. In the renal proximal tubule, megalin and cubilin are co-localized throughout the endocytic apparatus including the clathrin-coated pits, indicating that megalin/cubilin-mediated endocytosis is clathrin-dependent. As shown in Figures 7A and 7B, two typical inhibitors of clathrin-dependent endocytosis significantly decreased the association of FITC-hlgG in OK cells. In addition, potassium depletion and hypertonicity, which inhibit clathrin-coated pit formation, markedly decreased the association of FITC-hlgG in OK cells (Fig. 7C). Taken together, these results strongly suggested that megalin/cubilin-mediated, clathrin-dependent endocytosis plays an important role in FITC-hlgG uptake in OK cells.

Not only clathrin-dependent endocytosis but also alternative pathways including caveolin-dependent endocytosis have been implicated in the internalization of proteins and peptides into cells. Caveolae are flask-shaped, cholesterol/sphingolipid-rich invaginated microdomains of the cell surface characterized by the presence of structural proteins such as caveolin-1. In this study, the effects of nystatin and methyl-β-cyclodextrin, which are sterol-binding drugs that sequester cholesterol, on FITC-hlgG association were investigated in OK cells. The two inhibitors for caveolin-dependent endocytosis did not decrease, but rather significantly increased the association of FITC-hlgG in OK cells (Fig. 8). In a previous report with rat primary cultured alveolar type-I-like epithelial cells, we also observed that methyl-β-cyclodextrin significantly increased FITC-albumin uptake, which is inhibited by inhibitors of clathrin-dependent endocytosis. Although the reason why nystatin and methyl-β-cyclodextrin stimulated the uptake of FITC-hlgG in OK cells remains to be clarified, possible changes in membrane fluidity following cholesterol sequestration by these sterol-binding drugs might modulate the uptake activity via clathrin-dependent endocytosis.

In conclusion, we characterized the uptake mechanisms of FITC-hlgG in OK cells. The internalization of FITC-hlgG in OK cells was suggested to be, at least in part, due to megalin/cubilin-mediated, clathrin-dependent pathway, but not to FcRn-mediated pathway.

Acknowledgment: We thank the Analysis Center of Life Science, Natural Science Center for Basic Research and Development, Hiroshima University, for the use of their facilities.

References


41) Batuman, V., Verroust, P. J., Navar, G. L., Kaysen, J. H., Goda, F. O., Campbell, W. C., Simon, E., Pontillon, F., Lyles, M., Bruno, J. and Hammond, T. G.: Myeloma light chains are ligands...


