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Interleukin-6 Facilitates *Escherichia coli* Transcytosis in the Caco-2 Cell Monolayer System

Makoto Usami¹, Emi Matsushita¹, Miho Domori², Yumiko Inoue², Akinori Miki¹, Nobumasa Kataoka¹, Yoshiki Tabuchi¹, and Mikio Sekita²

Aim: Bacterial translocation is a major concern as a cause developing sepsis or multiple organ failure under systemic inflammatory response with hypercytokinemia aroused from surgical stress. However, direct effect of interleukin 6 (IL-6) on bacterial translocation has rarely been reported. In this study, the effect of IL-6 on enterocyte with enteronon-invasive *E. coli* interaction was evaluated in the intestinal epithelial cells.

Methods: Direct effect of IL-6 on matured villus enterocytes, confluent Caco-2 cells on porous filter with double chamber system (Transwell), was evaluated by measuring permeability from apical (intestinal lumen side) to basolateral chamber (blood vessel side). Viable *E. coli* c-25 permeability, fluorescein sulfonic acid permeability, and transepithelial electrical resistance, LDH secretion, and ultrastructural analysis were performed under 100 ng/ml of IL-6 in the basolateral chamber medium.

Results: IL-6 directly stimulated *E. coli* passage across Caco-2 monolayer cells by transcellular route with intact nuclear and cytoplasmic structure. Paracellular permeability change and cellular injury were not observed. It is suggested that IL-6 stimulates enterocytes from basolateral side, affects enterocyte-*E. coli* interaction, and increases *E. coli* transcytosis.

Conclusions: IL-6 directly facilitates *E. coli* transcytosis in monolayer intestinal cells.

**Key Words**
bacterial translocation, Caco-2 cell, IL-6, *Escherichia coli*, transcellular pathway.

**Introduction**

An important function of the intestinal mucosa is to act as a local defense barrier in preventing bacteria and endotoxin normally contained within the lumen of the gut from escaping and spreading to distant sites. Under certain circumstances, however, bacteria and endotoxin are capable of crossing this gut mucosal barrier and spreading to the mesenteric lymph nodes and systemic organs, a process termed bacterial translocation. In recent years it has become increasingly clear that the gastrointestinal tract and its contents, including bacteria and their products, may influence the outcome of injured and critically ill patients. Systemic inflammatory responses with elevated proinflammatory cytokine levels are produced by various surgical injuries. Despite abundant clinical and experimental evidence associating severe trauma, sepsis, and multiple organ failure with elevated cytokine levels and altered intestinal function, the role of proinflammatory cytokines on intestinal integrity has received little attention.
Recent investigations have suggested a relationship between high levels of interleukin-6 (IL-6), one of proinflammatory cytokines highly elevated in patients under surgical stress, and poor outcome after major surgical procedure. We reported the positive correlation between blood IL-6 levels and intensity of metabolic disturbances in patients after major gastrointestinal surgery. Moreover, Hack et al reported high levels of IL-6 in patients with septic shock, and that the IL-6 level on admission appeared to have a prognostic significance. Gennari et al have shown that IL-6 plays a major role in the intensity of translocation of Escherichia coli (E. coli) from the intestine following burn injury in mice model. From these results, correlation between IL-6 and intestinal integrity has postulated.

The purpose of this study was to assess the direct effect of IL-6 on enterocyte integrity and/or bacterial translocation. To address this issue, we used the Caco-2 cell line grown on permeable supports as an experimental model to determine whether bacteria were able to cross the epithelial cell monolayer. Caco-2 cells, derived from transformed human colonic carcinoma cells, have been characterized extensively. They have been shown to be highly polarized with a well-formed brush border, express several differentiated markers typical of adult enterocytes and behave like small intestine. Using Caco-2 monolayer system, E. coli C25 permeability, fluorescein sulfonic acid (FS) permeability, and transepithelial electrical resistance (TEER) were assessed to compare their levels in basolateral chambers (blood vessel side) with levels in apical chambers (intestinal lumen side) under IL-6 stimulation in basolateral chambers (blood vessel side).

Materials and Methods

Caco-2 cells (Nihon Seiyaku Co., Japan) were maintained at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% fetal calf serum (FCS), 1% L-glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and sodium bicarbonate (24 mM), in an atmosphere of 5% CO2 and 100% humidity. All culture media were obtained from Dainippon Seiyaku Co., Japan.

Growth medium was placed twice a week and cell viability was assessed using 0.4% trypan blue solution. Confluent monolayers growing in 75 cm2 tissue culture flasks were harvested with a solution of 0.25% trypsin–2.65 mM EDTA in PBS (Gibco) and seeded at a density of 6.25 x 105 cells/cm2 in Transwell inserts (Costar), 12 mm in diameter (surface area 1.0 cm2), and polycarbonate filters with 0.4 μm pore size (Fig 1, 2, some of ultrastructural analysis was done using 3.0 μm pore size). In vitro stimulation with pro-inflammatory cytokines...
were performed by the addition of human recombinant IL-6 (R&D Systems) in the basolateral chambers during 24 hours, concentrations indicated in the results, 10 days after seeding. TEER were examined to evaluate structure integrity of the Caco-2 cell monolayer on day 8 and 10 postseeding with an electrical resistance system (EVOM-6; World Precision Instruments), equipped with a pair of STX-2 electrode. Probes were placed at the apical and basolateral chamber of three point of the insert and resistance was measured with the voltohmmeter. The relationship of TEER values to Caco-2 monolayer integrity has been investigated by Hidalgos and found to correlate with the presence of well-formed tight junctions\(^9\). The Caco-2 cells were used in experiments only after the TEER had risen above 130 ohms·cm\(^2\)\(^1\(^0\). Because the Transwell membranes are opaque, it is impossible to evaluate the monolayers directly with phase-contrast microscopy. TEER value of Transwell plate was increased within 12% of the value in the CO\(_2\) incubator until 1 hour after medium change due to increase in medium pH\(^1\(^1\).\)

Five hundred μg/ml of FS (Molecular Probes) dissolved in the medium without FCS were loaded 200 μl into the apical compartment of the Transwell chambers during 3 hours after removal of 200 μl of culture medium. The concentrations of the FS in the apical and basolateral compartments were assayed after dilution in PBS for fluorescence using a fluorescence spectrophotometer (RF 540, Shimazu, Japan) at an excitation wave length of 492 nm (slit width 2 nm) and an emission wave length of 515 nm (slit width 10 nm)\(^1\(^1\).\) The FS permeability of monolayers was expressed as percent ratio of concentration in the basolateral chambers versus concentration in the apical chamber. FS has small molecular weight, 478, but is not permeable of the cellular membrane in physiological pH due to its lipophobicity. FS passes through tight junction space\(^1\(^1\),\(^1\(^2\).\) It is confirmed in the preliminary experiments that during the first 3 hours, one forth of the FS passed in 24 hours was appeared in the basolateral component and the concentration was within the measurable range.

Ten ml of heart-infusion broth (Nissui) was precultured during 24 hours at 37 °C from one platinum needle of bacteria (Fig 3, E. coli C 25, kind gift from Dr. Kikuji Itoh, Tokyo University) from nutrient agar (Nissui) kept at 4 °C\(^1\(^3\).\). An inoculum of 0.5 ml of heart-infusion broth containing 10\(^7\) colony forming units

**Figure 2.** Light micrograph of confluent Caco-2 cells grown on Transwell polycarbonate filters, 0.4 μm pore size, after 10 days culture (toluidine blue staining, x 1000).
(cfu)/ml was added to the apical side of the Caco-2 cell monolayer after removal of culture media. E. coli C25 does not have enteroinvasive character and reflects the indigenous intestinal flora. Bacterial translocation was then measured by quantitatively culturing samples of medium (100 µl) obtained from the basal chamber at 3 hours after the bacteria were placed in the apical chamber. The number of viable translocated E. coli C25 was quantitated by pour plate assay with heart infusion agar (Eiken Kagaku) incubated aerobically at 37°C for 24 hours. E. coli C25 is the streptomycin resistant strain and used for the bacterial translocation study. Preliminary experiment did not show the difference of the parameters measured in this experiment between E. coli C25 in heart infusion broth and E. coli C25 in culture medium.

To assess cell membrane integrity, release of the cytosolic enzyme lactate dehydrogenase (LDH) was measured from Caco-2 cells grown on 12-mm Transwell membranes. LDH activity in media samples from the basolateral compartments was determined spectrophotometrically using a single reagent system (LDH–Cytotoxic Test, Wako) on a ELISA reader (Benchmark Microplate Reader, Biorad). Medium LDH levels were expressed as percent levels in control wells. Preliminary experiments were performed to compare culture medium in control wells with total cellular LDH by solubilizing the monolayer with 1.0 ml of 0.1% Triton X–100 in PBS followed by centrifugation and assaying the supernatant.

Correlation among TEER, FS permeability, and medium LDH concentration were evaluated in a preliminary time-course study.

Cell proliferation was evaluated using the bromodeoxyuridine (BrdU) assay kit (Boehringer Mannheim) following the manufacture’s manual. 6.25 × 10⁵ Caco-2 cells/wells (0.1 ml) were seeded in 96 wells plates, medium with or without IL-6 was changed after 24 hours, then BrdU labeling solution was added during 2 hours after 24 hours.

Electron microscopy for ultrastructural study, Caco-2 cell monolayers on Transwell membranes were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 hours at room temperature. Specimens were rinsed four times in 10% glucose in 0.1 M sodium cacodylate buffer and post-fixed in 0.5% OsO₄ in 0.05 M sodium cacodylate buffer. After dehydration through a graded series of ethanol (35%, 50%, 70%, 96%, and 4 times 100%, 10 min each), intact membranes were embedded in resin (6.3 ml of Epon 812, 3.7 ml of Araldite 502, 13.9 ml of DDSA, 0.3 ml of DMP–30, Nissin EM) by first infiltration in a 1:1 mixture of ethanol and resin for 1 hour, a 100% resin mixture for one day. Resin was polymerized at 60°C for 2 days. Ultrathin sections cut with a diamond knife
were negatively stained with uranyl acetate-lead citrate and examined under a transmission electron microscope operated at 80 kV (JEM-1220, JEOL)\(^4\). The semithin sections stained with 1% toluidine blue were examined by standard light microscope.

**Statistical analysis**

Data were expressed as mean ± standard deviation (SD) and Student’s \(t\) test was employed to compare mean value from two groups. Chi square test was employed to compare incidence. The statistically significance was assured when \(p\) value was less than 0.05.

**Results**

Caco-2 cell monolayer cultures

As shown in Figures 2, Caco-2 cells grown on permeable supports in bicameral chambers under control condition formed polarized monolayers of columnner epithelial cells. Cells had long, densely packed microvilli in the apical side and well developed junctional complexes, including tight junctions and many desmosomes localized among the length of the interdigitating lateral membranes (Fig 4)\(^4\).

TEER levels were 165 ± 21 ohms·cm\(^2\) in 10 day old cells indicating enough integrity of this monolayer system\(^9\). The Caco-2 monolayers showed low permeability to FS with only 5.5% of the small molecular dye crossing the monolayer during the 3 hours test period. Correlation between TEER and FS permeability indicated negative relationship with statistical significance (Fig 5, \(p < 0.01\)) in the preliminary time course study. Released LDH level in the medium was under 7% of total cellular LDH. Statistically significant negative relationship between TEER and LDH levels were observed.

Bacterial transcytosis across Caco-2 cell monolayer

The ability of *E. coli* C-25 to translocate was assessed by counting the viable bacteria in the media of basolateral chamber. The total numbers translocated are shown in Fig 6. In 10 day old cells, 1% of the *E. coli* C-25 inoculum in the apical chamber was recovered from the basolateral chamber. Ultrastructural analysis of bacterial transcytosis indicated that *E. coli* C-25 were seen in membrane-bound vacuoles, leaving the nuclear and cytoplasmic materials intact (Fig 4). Cell membrane and the tight junctions were also unaffected\(^4\). This enterocyte-invasive *E. coli* C-25 passed Caco-2 cell monolayer via transcellular route, but not via paracellular route. *E. coli* C-25 in heart-infusion broth did not show direct injury on Caco-2 cell monolayer in the parameters evaluated in this study. Percent increase of LDH concentration in basolateral components with *E. coli* was under 14 %, decrease of TEER and FS were under 10% and 5% of the control, respectively, in preliminary experiments.

Effect of IL-6 in the basolateral chamber on Caco-2 cell monolayer

FS permeability, TEER, and LDH concentration did not change with 100 ng/ml of IL-6 in the basolateral chamber (Table). DNA synthesis depressed to 91.3 ± 0.5% of control value by the same amount of IL-6 without statistical difference in 96 well microplate culture. No remarkable change in ultrastructure of Caco-2 monolayer cells was observed.
Figure 4. Transmission electron micrograph of the Caco-2 cell monolayer and E. coli C-25 incubated with culture medium. Cells possess well-developed, long, densely packed microvilli in A (× 5000) and cell-cell junctional complex showing zonula occludens and desmosomes in C (× 8000). E. coli C-25 were observed in the apical chamber in adjacent to microvilli in B (× 6000) and cytosol of Caco-2 cells without disturbance of nuclear and cytoplasmic materials in D (× 10000).

Figure 5. Correlation among TEER, FS permeability, media LDH concentration.
TEER - FS : Y = 29.372 - 0.078 × X; R = 0.753, p < 0.001
TEER - LDH : Y = 0.589 - 0.001 × X; R = 0.779, p < 0.001
with IL-6 in this study. Average viable E. coli counts of basolateral chamber under IL-6 stimulation was $3.7 \pm 7.6 \times 10^5$ cfu/ml, ranging from 0 to $30.0 \times 10^5$ cfu/ml, in comparison with $1.1 \pm 5.0 \times 10^5$ cfu/ml, ranging from 0 to $2.8 \times 10^5$ cfu/ml, without IL-6 ($p<0.01$). The incidence of wells showing over 100 cfu/ml of viable E. coli with addition of IL-6 was 56.7% vs. 33.3% without IL-6, and those over 500 cfu/ml with IL-6 was 50.0% vs. 6.7% without ($p<0.01$). These data indicated that E. coli translocation across Caco-2 cell monolayer increased under IL-6 stimulation from basolateral side.

**Discussion**

Our in vitro data indicated that IL-6 directly stimulated E. coli passage across Caco-2 monolayer cells by transcellular route with intact nuclear and cytoplasmic structure. No effect in TEER and FS permeability showed that paracellular permeability was not affected by IL-6. The increase in E. coli transcytosis in Caco-2 cells stimulated by IL-6 could not attributed to a general effect on cell number or cellular membrane injury, because inhibition in DNA synthesis measured by BrdU assay was minimal and cellular injury assessed by medium LDH release did not occurred. Addition of IL-6 from basolateral (blood vessel side) chamber of Caco-2 monolayer cells represents an *in vitro* model of hypercytokinemia or local inflammation induced by immune cells in the submucosal tissue. And E. coli transcytosis from apical (lumen side) to basolateral (blood vessel side) chamber suggests bacterial intestinal translocation to regional lymphnodes or systemic circulation. This direct effect on enterocyte was IL-6 specific, and other pro-inflammatory cytokines studied in our series of studies, both IL-1β and TNF α did not show statistically significant effect on E. coli translocation (preparing for other paper). Gennari et al's *in vivo* experiment in mice indicated that IL-6 plays major role in E. coli translocation from the intestine following burn injury and host's ability to kill translocated organism by anti-IL-6 antibody administration. Our observations support their result in enterocyte level.

Established mechanisms of IL-6 are various aspects of the immune responses, acute-phase reaction, hematopoiesis, endocrine responses, and nervous system function. IL-6 can enhance the immune response by acting as a co-stimu-
lutor for T cell proliferation and inducing enhanced antibody secretion by plasma cells. At the intestinal mucosa, IL-6 appears to be a very important cytokine in the mucosal immune response as it can preferentially enhance Ig A secretion by mucosal B cells.

However, several articles supported relationship between enterocytes and IL-6. Caco-2 monolayer cells possess IL-6 receptor in both the apical and basolateral membranes, with dissociation constants of 9.9 and 11.2 nM, respectively \(^{16, 17}\). Several reports indicate direct effect of IL-6 on intestinal epithelial cells in increased acute phase protein response, decreased apolipoprotein B and triacylglycerol secretion\(^{8}\), and downregulated sucrase-isomaltase\(^{9}\), matured microvilli enzyme, gene expression\(^{20}\). Molmenti et al demonstrated that IL-6, at a concentration of 100 ng/ml, caused the expression of several acute phase proteins; \(\alpha^1\)-antitrypsin, transferrin, \(\alpha^1\)-antichymotrypsin, complement factor B, complement proteins C3 and C4, serum amyloid A, acute phase reactant fibrinogen, and ceruloplasmin. Also reports indicating enterocytes as inflammatory cells are accumulating recently. IL-6 secretion from enterocyte has been reported by exposure to \(E. coli\) in Caco-2 monolayer cells\(^{16}\), but is not supported in other report\(^{21}\). Secretion of IL-6 to the apical surface could allow the rapid dissemination of an inflammatory signal via the lumen of the intestine. Such acute phase protein response of Caco-2 cells induced by IL-6 might effect on bacteria–enterocyte interaction. Further investigation is required to clarify the mechanism of IL-6 on \(E. coli\) transcytosis and bacteria–enterocyte interaction using this Caco-2 monolayer cells.

\(E. coli\) C-25 used in this study does not have enteroinvasive character and ultrastructural change observed in this model was minimal in comparison with Panigrahi et al's report indicating severe structural changes by \(Salmonella typhimurium\) in Caco-2 model\(^{14}\). Less translocation with porous membrane of Transwell itself without Caco-2 monolayer cells in our preliminary experiments is in accordance with the report that formalin–fixed bacteria does not translocate. The mechanism responsible for this observation in combination with IL-6 action should be clarified.

The Caco-2 cells represent an excellent in vitro model for some functions of differentiated villus enterocytes and form a tight monolayer allowing a clear distinction between apical and basolateral pathway. Caco-2 cells are human cells and already identified to have numerous proteins and mature enterocyte–linked functions of normal intestinal epithelium reviewed by Meunier et al and suitable research tools for intestinal absorption and intestinal metabolism\(^{17}\).

In conclusion, we have presented evidence that a novel proinflammatory response of IL-6 on Caco-2 cells to bacterial–enterocyte interaction and an increased \(E. coli\) transcytosis with intact tight junction permeability.

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IL-6 on *E. coli* Transcytosis in Enterocyte Monolayer

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