New model for studying the migration of immune cells into intestinal epithelial cell monolayers

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Received 7 July 2003; accepted in revised form 29 October 2003

Key words: Caco-2, cell migration, chemokine, intestinal epithelium, THP-1

Abstract

A novel cell culture system was constructed to analyze the direct interaction between intestinal epithelial cells and immune cells. Human intestinal epithelial Caco-2 cells were monolayer-cultured on the under side of a permeable membrane (12 μ m pore size) in a Millicell insert. Integrated monolayers of Caco-2 cells had formed after 12 days of culture. Human monocyte/macrophage-like THP-1 cells were then added to the upper chamber of the insert, and their migration into the Caco-2 cell monolayers was observed by confocal laser scanning microscopy, after staining the cells with specific antibodies. When MCP-1, a β -chemokine, was added to the apical side of the monolayer, a greater number of THP-1 cells migrated into the Caco-2 cell monolayers. This cell culture system will be useful for studying the behavior of macrophages in the intestinal epithelial cell monolayers at the initial stage of an intestinal immune reaction.

Abbreviations: DCs – dendritic cells; EPEC – enteropathogenic *Escherichia coli*; F-actin – filamentous actin; FCS – fetal calf serum; LTA – lipoteichoic acid; NEAA – non-essential amino acids; PGN – peptidoglycan; PMA – phorbol myristate acetate; TcdA – TcdA-10463; TcdB – TcdB-10463; TER – transepithelial electrical resistance; TLR – toll-like receptor; TJ – tight junction

Introduction

The intestinal epithelium serves as a barrier against enteric bacteria. However, some pathogenic bacteria invade by crossing the intestinal epithelium after damaging the tight junctional barrier and cause inflammation. For example, the anaerobic bacterium, *Clostridium difficile*, which is the etiological agent of pseudomembranous colitis, secretes two toxins, TcdA-10463 (TcdA) and TcdB-10463 (TcdB). TcdA and TcdB alter the distribution of ZO-1, a major component of the tight junction (TJ), and F-actin, inducing an increase in the paracellular permeability of the intestinal epithelial

cell monolayers (Nusrat et al. 2001). Enteropathogenic *Escherichia coli* (EPEC) induces dephosphorylation of occludin, another TJ component; dephosphorylated occludin becomes dissociated from TJ which also reduces the intestinal epithelial barrier functions (Simonovic et al. 2000).

An innate immune response is induced against infection by microbial pathogens. A number of bacterial components, including lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA) and lipopeptides, stimulate the innate immune system. The toll-like receptor (TLR) family in the intestinal epithelial cells provides pathogen recognition (Takeda et al. 2003), and intestinal

epithelial cells secrete chemokines in response to bacterial components. These chemokines induce the migration of immune cells from beneath the epithelial cell monolayers.

Immune cells have been reported to influence intestinal epithelia. Dendritic cells (DCs) are migratory, and phagocytic cells have the capacity for sampling and presenting antigens. It has been reported that DCs express tight junction proteins such as occludin, claudin-1, E-cadherin and β -catenin (Rescigno et al. 2001). This suggests that DCs infiltrate between intestinal epithelia without influencing the intestinal barrier function, and sample the bacteria present in the intestinal tract prior to bacterial infection (Rescigno et al. 2001). T-lymphocytes have also been reported to express occludin by a treatment of phorbol myristate acetate (PMA) (Alexander et al. 1998). Alexander et al. (1998) have suggested that the lymphocyte expression of occludin might facilitate both the binding and extravasation of T-lymphocytes across the endothelial and epithelial layers. Martin et al. (1998) have reported that gap-junctional communication between macrophages and intestinal epithelial cells is a means by which inflammatory cells might regulate intestinal epithelial cell functions. Thus, epithelial cells and immune cells, including macrophages, seem to have direct interactions. However, there are few experimental methods by which to study the direct interactions between immune cells and intestinal epithelial cell monolayers at cellular and molecular levels. We will propose from the results of this study, a novel method by which to investigate direct interactions between the intestinal epithelial cells and macrophages by using human cell lines.

Materials and methods

Materials

The human colon adenocarcinoma cell line, Caco-2, was obtained from American Type Culture Collection (Rockville, MD, USA), and the human monocytic leukemia cell line, THP-1, was obtained from Human Science Research Resources Bank (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and Hanks' balanced salt solution (HBSS) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Nonessential

amino acids (NEAA), penicillin–streptomycin (10,000 U ml⁻¹ and 10 μ g ml⁻¹ in 0.9% sodium chloride, respectively), and phosphate buffered saline (PBS) were all purchased from Gibco (Gaithersburg, MD, USA). Fetal calf serum (FCS) from Asahi Techno Glass (Chiba, Japan), and collagen type I from Nitta Gelatin (Osaka, Japan). Plastic dishes and plates were purchased from Corning-Costar (Cambridge, MA, USA) and Millicells of 12 mm diameter with a 12- μ m pore polycarbonate membrane were purchased from Millipore (Billerica, MA, USA). Human recombinant MCP-1 was purchased from Pepro Tech (London, UK), and human recombinant IL-8 from Cosmo Bio (Tokyo, Japan).

Cell culture

Caco-2 cells were cultured in DMEM supplemented with 10% FCS, 1% NEAA, 20 U of penicillin ml⁻¹ and 20 μ g of streptomycin ml⁻¹. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. The monolayers became confluent 6–7 days after seeding at 1.0×10^5 cells per 100-mm dish, and the cells were passaged by a treatment with 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid in PBS. All the cells used in this study were between passages 40 and 60.

THP-1 cells were cultured in DMEM supplemented with 10% FCS, 20 U of penicillin ml⁻¹ and 20 μ g of streptomycin ml⁻¹. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air.

Transepithelial electrical resistance (TER) measurement

The under side of the membrane of a Millicell insert was coated with 6% collagen (type I) in 0.02 N HCl. After 30 min, the membrane was washed twice with PBS. Caco-2 cells were seeded at a density of 2.5×10^5 cells cm⁻² on the coated membrane of the Millicell insert that had been put upside-down in a well. The cells were cultured while changing the culture medium every 1 or 2 days. The integrity of the cell monolayers was evaluated during the culture by measuring the TER value with Millicell-ERS equipment (Millipore). The cells were gently rinsed twice with

HBSS and equilibrated in the same solution for 30 min at 37 °C before the measurement.

Chemotaxis assay

Millicell inserts with polycarbonate membranes (12 μm pore size) were set in a microplate well in the normal attitude. Chemokines (MCP-1 and IL-8) were dissolved at 50 ng ml^{-1} in HBSS and added to the lower chamber of the Millicell inserts. THP-1 cells (1.0×10^6 cells ml^{-1}) were added to the inserts and allowed to migrate through the membrane. After incubating for an adequate time, the Millicell inserts were removed, and the migrated cells in the lower chamber were counted by using haemocytometer (Erma).

Immunofluorescence staining of the cell monolayers

Millicell membranes with cell monolayers were gently rinsed with PBS, treated with 0.2% Triton X-100 for 2 min, and then washed three times with PBS. The washed membranes were fixed with 4% paraformaldehyde for 30 min. The membranes were then washed three times with PBS, treated with 0.05% Triton X-100 to increase the membrane permeability for 5 min, washed five times with PBS and dried. The dried membranes were blocked in 3% skim milk (Snow Brand, Japan) for 2 h and then incubated with the primary antibody for 2 h. The membranes were next washed three times with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and blocked with 3% skim milk for 2 h. They were finally treated with the FITC- (or TRITC-) conjugated secondary antibody for 1 h. Immunofluorescence staining was visualized with a Fluoview FV500 confocal laser scanning microscope (Olympus, Japan).

Results

Upside-down culture of the Caco-2 cell monolayers on a Millicell membrane

It was necessary in this study to culture THP-1 cells on the basal side of the Caco-2 cell monolayers so that direct interaction between the two cells was attained. A Millicell membrane with a pore size of 12 μm was selected for this purpose,

Figure 1. A co-culture system used in this study.

this pore size being sufficiently large for THP-1 cells to pass through the membrane. Caco-2 cells were seeded on the under side of the Millicell insert (Figure 1) and cultured to form monolayers. The TER values of the cell monolayers were measured after 4, 9, 12, and 17 days of culture. The TER value of the cell monolayers increased with increasing culture period, reaching about 500 $\Omega \cdot \text{cm}^2$ after 17 days of culture (Figure 2). This result indicated that integrated Caco-2 cell monolayers had formed on the under side of the Millicell membrane with a 12- μm pore size.

Chemotaxis assay

A chemotaxis assay was conducted to investigate whether THP-1 cells can migrate across the Millicell membrane. THP-1 cells were added to the upper chamber of the Millicell insert, and then IL-8 (α -chemokine) or MCP-1 (β -chemokine) was

Figure 2. Change in the transepithelial electrical resistance during the formation of Caco-2 cell monolayers. TER values were measured with a Millicell-ERS (Millipore). Each value is the mean $\pm$ SD ($n = 3$).

Figure 3. Effect of chemokines on the chemotaxis of THP-1 cells. THP-1 cells were added to the upper chamber and incubated with (a) 50 ng ml⁻¹ of IL-8 and (b) 50 ng ml⁻¹ of MCP-1 added to the lower chamber. Each value is the mean $\pm$ SD ($n = 3$). #, *, **Significantly different from the control value (without chemokines) by a *t*-test (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$).

added to the lower chamber. The number of cells that migrated across the membrane was counted after incubating the insert for an appropriate time. The chemotactic migration of THP-1 cells were stimulated by adding 50 ng ml⁻¹ of MCP-1 to the lower chamber, indicating that THP-1 cells were able to pass through the 12 μ m-pore membrane. No chemotaxis of THP-1 cells was apparent when they were stimulated by IL-8 (Figure 3). It is known that the chemotaxis of THP-1 is stimulated by β -chemokine (Imada et al. 1998). The present results suggest that this culture system would be suitable for evaluating THP-1 cell migration.

Immunofluorescence staining of the cells

Detection by immunofluorescence staining was applied to distinguish between the Caco-2 and

THP-1 cells. E-cadherin and CD18 were selected as the marker proteins to separately stain Caco-2 and THP-1 (Figure 4). When Caco-2 and THP-1 were exposed to the anti-E-cadherin antibody, only the Caco-2 cells were stained (Figure 4a), whereas the anti-CD18 antibody only stained the THP-1 cells (Figure 4b).

Co-culture and immunofluorescence

A Millicell insert with Caco-2 cell monolayers on the under side of the membrane was set in a microplate well, and THP-1 cells were cultured on the upper side of the membrane. After co-culturing for 24 h, the THP-1 cells that had migrated into the Caco-2 cell monolayers were observed by immunofluorescence multi-staining. Compared with the control, a larger number of THP-1 cells had migrated into the Caco-2 cell monolayers when 50 ng ml⁻¹ of MCP-1 had been added to the underside (apical side) of the membrane (Figure 5a, c). A 200 μ g ml⁻¹ amount of saponin added to the apical side also increased the number of migrated THP-1 cells (Figure 5b), while MCP-1 and saponin added together had the greatest increasing effect (Figure 5d). Although THP-1 cells migrated between Caco-2 cell monolayers apparently increased by adding MCP-1 and/or saponin, the present study was not able to show quantitative data because counting the number of THP-1 cells penetrated the Caco-2 cell monolayers by a microscopical means is still difficult. A new method for quantitative analysis should be developed for this purpose.

Discussion

Human intestinal epithelial Caco-2 cells and monocytic THP-1 cells were co-cultured across a porous membrane filter. Caco-2 cell monolayers were cultured on the underside of the membrane filter, a basal membrane being attached to the filter, while THP-1 cells were put on the upper side of the membrane filter (Figure 1). Since the membrane pore size (12 μ m) was large enough for THP-1 cells to migrate across the membrane filter, it was possible to observe direct interactions between the two types of cells. The addition of

Figure 4. Selective staining of Caco-2 cells and THP-1 cells by the specific fluorescent antibodies. (a) Caco-2 and THP-1 cells were immunostained by the rabbit anti-E-cadherin antibody (primary antibody) at $10 \mu\text{g ml}^{-1}$ and FITC-conjugated anti-rabbit IgG (secondary antibody) at $60 \mu\text{g ml}^{-1}$. (b) Caco-2 and THP-1 cells were immunostained by the mouse anti-CD18 antibody (primary antibody) at $10 \mu\text{g ml}^{-1}$ and TRITC-conjugated anti-mouse IgG (secondary antibody) at 1:64 dilution. Bar: $50 \mu\text{m}$.

chemokines such as MCP-1 to the apical side of the Caco-2 cell monolayers markedly increased the number of THP-1 cells that migrated into the Caco-2 monolayers, suggesting that this system could be used to study the interaction between the cells. MCP-1 has been reported to induce release of arachidonic acid in monocytes (Locati et al. 1994). Stimulation of the chemotaxis of monocytes by arachidonic acid has also been reported (Kreuzer et al. 1996). In the case of polymorphonuclear leukocytes (PMN), apically released chemokines play an important role in the final step of PMN transmigration across the epithelial monolayer to the intestinal lumen (McCormick et al. 1998). Considering these findings, it is likely that the addition of MCP-1 to the apical side of the Caco-2 induced the chemotaxis of THP-1 cells

and their migration into the Caco-2 cell monolayers.

It is known that immune cells express certain surface proteins during migration into the epithelial cell monolayers. T-lymphocytes have been reported to have the ability to express occludin, a major tight junction protein, which might facilitate both the binding and extravasation of T-lymphocytes across the endothelial and epithelial layers (Alexander et al. 1998). DCs have also been reported to express tight junction proteins such as occludin, claudin-1, E-cadherin and β -catenin (Rescigno et al. 2001). Kucharzik and Williams (2003) have recently reported that neutrophils transmigrated across epithelial cells, in which membrane glycoprotein CD47 or neutrophil β_2 integrin CD11b/CD18 played an important role.

Figure 5. Confocal laser scanning microscopic observations of THP-1 cells that had migrated into the Caco-2 cell monolayers. Caco-2 and THP-1 cells that had been co-cultured for 24 h were immunostained. (a) Control; (b) treated with saponin at $200 \mu\text{g ml}^{-1}$; (c) treated with MCP-1 at 50 ng ml^{-1} ; (d) treated with saponin at $200 \mu\text{g ml}^{-1}$ and MCP-1 at 50 ng ml^{-1} . Saponin and MCP-1 were all added from the apical side. Upper panel: CD18 of THP-1 cells stained (red) by the TRITC-conjugated antibody. Lower panel: Z-stage picture of co-cultured Caco-2 and THP-1 cells. CD18 of THP-1 cells was stained (red) by the TRITC-conjugated antibody. E-cadherin of Caco-2 cells was stained (green) by the FITC-conjugated antibody.

These results suggest that THP-1 cells would also express adhesive molecules like TJ-related proteins. We found, in preliminary experiments that two TJ proteins, occludin and ZO-2, were expressed in THP-1 cells (unpublished data). ZO-2 in THP-1 cells migrated from the cytoplasm to cell membrane in response to stimulation by LPS (data not shown), suggesting that occludin and ZO-2 would play a role in cell-cell interactions and the

subsequent migration of THP-1 between the Caco-2 cell monolayers. The significance of the expression of these TJ proteins in the migratory characteristics of THP-1 cells will be the subject of a future study.

Chemokines are likely to play an important role in the migration of immune cells. The addition of MCP-1 to the apical side of the Caco-2 cell monolayers markedly enhanced the THP-1

migration (Figure 5). Kim et al. (2002) have reported that intestinal epithelial cells secreted CXC and CC chemokines, including IL-8 and growth-related oncogene (GRO)- α . The secretion of MCP-1 in response to *Clostridium difficile* Toxin A was also observed (Kim et al. 2002). Kucharzik et al. (2002–2003) have indicated that the basolateral secretion of chemokines allowed facile neutrophil adhesion to the intestinal epithelial cells and transmigration. The present findings suggest that monocyte/macrophage cells can migrate between intestinal epithelial cell monolayers at the initial stage of an intestinal inflammatory reaction. MCP-1 added to the apical side of the Caco-2 monolayers induced an increase in the number of THP-1 cells adhering to the basal side of the Caco-2 monolayers, suggesting that chemokines secreted to the apical side also play an important role in immune cell transmigration. The toll-like receptor (TLR) family serves for pathogen recognition. Since Caco-2 cells express some of the molecules of the TLR family (Melmed et al. 2003), Caco-2 cells would secrete chemokines in response to bacterial components. The secretion of such cytokines as IL-8 and MCP-1 to the apical and basal sides of the Caco-2 cell monolayers has been reported (Kim et al. 2002; Akhtar et al. 2003). Our co-culture system will be useful for analyzing the responses of intestinal epithelial cells to external factors, including bacterial components, in terms of the induction of immune cell migration via the chemokines.

Another factor that could affect immune cell migration into the epithelium is the tightness or leakiness of the epithelial cell monolayers. Persidsky et al. (2000) have reported that immune-activated brain mononuclear phagocytes down-regulated tight junction proteins and special polarized transport systems in brain microvascular endothelial cells. Boven et al. (2000) have also reported that monocyte infiltration was highly associated with loss of the tight junction protein, ZO-1, in brain endothelial cell monolayers. Schmitz et al. (2002) have recently found that HIV-infected macrophages produced increased amounts of cytokines in co-culture with peripheral blood mononuclear cells (PBMC), the culture supernatant inducing a TER decrease in human intestinal HT-29 cells. These findings suggest that a reduction of the intestinal epithelial barrier function would facilitate the migration of

immune cells. Saponin is known to dramatically increase the permeability of Caco-2 cell monolayers (Narai et al. 1997). Although the cytotoxicity of saponin was not apparent by an LDH assay, the TER value of the Caco-2 cell monolayers was rapidly decreased by a treatment with 200 $\mu\text{g ml}^{-1}$ of saponin (data not shown). In the present study, treatment of the Caco-2 cell monolayers with saponin induced increased migration of THP-1 cells. The co-culture system proposed in this paper will also be useful to evaluate the cell-migration modulatory activity of various factors including cytokines and dietary substances.

Acknowledgement

This paper was partly supported by the Grant-in-Aid for Creative Scientific Research, Japan Society for the Promotion of Science (13GS0015).

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