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Short communication

The changes in the neuronal PC12 and the intestinal epithelial Caco-2 cells during the coculture. The functional analysis using an *in vitro* coculture system

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Abstract

The interaction between intestinal epithelial cells and peripheral neuronal cells were examined using an *in vitro* coculture system. Two cell lines, Caco-2 and PC12, were used for this experiment as an intestinal epithelial and enteric neuronal cell model, respectively. By coculturing with fully differentiated Caco-2 cells, the neurite outgrowth was induced in PC12 cells. This neurite outgrowth in PC12 was blocked by anti-nerve growth factor (NGF) polyclonal antibodies, suggesting that the neurite outgrowth in PC12 during the coculture with Caco-2 cells was due to NGF secreted from Caco-2 cells. On the other hand, coculturing with fully differentiated PC12 cells induced the decrease of transepithelial electrical resistance in Caco-2 cell monolayers. The permeability of lucifer yellow also significantly increased, suggesting that the barrier function and paracellular permeability of Caco-2 monolayers were altered by coculturing with PC12 cells. The present study suggests that this *in vitro* coculture system is a good model for the functional analysis of interaction among intestinal epithelial cells with different cell types.

Abbreviations: DMEM – Dulbecco's modified Eagle's medium; FCS – fetal calf serum; HS – horse serum; IEC – intestinal epithelial cells; NEAA – non-essential amino acids; NGF – nerve growth factor; PCR – polymerase chain reaction; TER – transepithelial electrical resistance; LY – lucifer yellow

Introduction

The function of intestinal epithelial cells (IEC) has been extensively investigated and it has been revealed that IEC have various important functions, such as the absorption of water, ions and nutrients presented in the lumen and formation of a barrier for separating the luminal content from underlying intestinal and vascular fluids.

For functional analyses of IEC, a number of studies have been performed using various vehicles such as everted sacs, brush-border membrane vesicles, isolated mucosal cells and intestinal loops (Barr and Riegelman, 1970; Windmueller and Spaeth, 1975; Osiecka et al., 1985). Cultured cell lines which have similar morphological characteristics to normal enterocytes have also been established and utilized. The use of cultured cell lines has an advantage: i.e., the function of only IEC can be studied without being affected by other types of cell. In the intestinal cell lines, Caco-2 derived from human colon adenocarcinoma has been most frequently used as a good model for studying the function of IEC (Hidalgo et al., 1989), because Caco-2 has spontaneous differentiation

ability and exhibits various enterocytic characteristics including brush-border enzyme and nutrient transporter activities (Matsumoto et al., 1990; Nicklin et al., 1992). Since Caco-2 can be cultured on semipermeable membrane and can form monolayers with tight junctions (Hidalgo et al., 1989; Hughson et al., 1990), this cell line provides a good model for studying the barrier function of intestinal enterocytes. By using this cell line, we have previously reported that the activity of a nutrient transporter and the barrier function are regulated by changing culture conditions or by food substances (Hashimoto et al., 1997; Satsu et al., 1997, 1999).

In the animal intestine, however, there exists different types of cells such as enteric neuronal cells, intestinal epithelial lymphocytes and fibroblasts beneath the monolayer of IEC. It is therefore assumed that the cross-talk among different cells including IEC exists in the intestinal tissue. To investigate the interaction between IEC and other types of cells, we tried to establish an *in vitro* coculture system. The coculture system has recently been used for analyzing the interaction between different types of cells. For example, a coculture system using astrocytes and endothelial cells has been reported as an *in vitro* blood-brain barrier model (Dehouck et al., 1994; Fillebeen et al., 1999). However, a coculture system for the enterocyte has not yet been developed.

In the present study, we established an *in vitro* coculture system and studied the interaction between IEC and a neuronal cell. Two cell lines were selected for this study: the Caco-2 cell as a model for IEC and rat pheochromocytoma cell line PC12 as a model for the neuronal cell. PC12 cells are known to be differentiated into sympathetic neuron-like cells when exposed to NGF (Greene et al., 1976) and have been most widely used for the analysis of neuronal cells.

Materials and methods

Materials

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, USA) and the PC12 cell line was obtained from Nippon Human Science (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). Fetal calf serum (FCS), horse serum (HS), L-glutamine, and penicillin-streptomycin (10,000 U ml^{-1} and 10 mg ml^{-1} in 0.9% sodium chloride, respectively) were from Gibco (Gaithersburg, MD, USA), and non-essential amino acids (NEAA) was from Cosmobio (Tokyo, Japan). Murine nerve growth factor (2.5S), polyclonal anti-human NGF antibody and a NGF-enzyme-linked immunosorbent assay (NGF-ELISA) kits were all purchased from Promega (Tokyo, Japan). Lucifer yellow CH (LY) was purchased from Sigma (St. Louis, MO, USA). All the other chemicals used were of reagent grade.

Cell culture

Caco-2 cells were cultured in 78.5-cm^2 plastic dishes with a culture medium consisting of DMEM, 10% FCS, 1% NEAA, 2% glutamine, 100 U ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin and an appropriate amount of sodium bicarbonate (Caco-2 medium). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air, the culture medium being renewed on alternate days. After they had reached confluence, the cells were sub-cultured (1:2) after trypsinization with 0.1% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS). All the cells used in this study were between passages 32 and 60.

PC12 cells were cultured in 78.5-cm^2 plastic dishes with a culture medium consisting of DMEM, 10% HS, 5% FCS, 100 U ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin and an appropriate amount of sodium bicarbonate to adjust pH 7.4 (PC12 medium). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air, the culture medium being renewed on alternate days.

TER measurement

The tight junction permeability of the Caco-2 cell monolayer was evaluated by measuring the transepithelial electrical resistance (TER) value. TER is thought to be well correlated with the change in paracellular permeability of the cell monolayer (Madara et al., 1988). The monolayer cells were rinsed with Hank's balanced salt solution (HBSS) and then incubated with HBSS at 37 °C. After 30 min, the TER value was measured with a Millicell-ERS instrument (Millipore, Bedford, MA, USA).

In vitro coculture experiments

Caco-2 cells were cultured in 12-well cell culture inserts with semipermeable support membrane

(Corning-Costar, Bedford, USA) that had been precoated with collagen type-I. The cell density was 1×10^5 cells/insert. One-half milliliter of a medium was placed in the cell culture insert (apical side), and 1.5 ml of the same medium was placed in the lower plates (basolateral side). PC12 cells were cultured in 12-well culture plate coated with collagen type-I at a density of 25,000 cells/well. More detailed procedures of the coculture experiments were described in Results.

Detection of NGF mRNA expressed in Caco-2 cells

Total RNA was extracted from the 14 days-cultured Caco-2 cells (single culturing) with an Isogen RNA isolation kit (Nippon Gene, Tokyo) and poly(A)$^+$ RNA was purified by affinity chromatography on oligo(dT)-cellulose (Pharmacia, USA). Poly(A)$^+$ RNA was reverse-transcribed with random hexamers using a first-strand cDNA synthesis kit (Pharmacia) and the polymerase chain reaction (PCR) was performed with Taq DNA polymerase.

Transport studies

The Caco-2 monolayers on Costar-transwell were washed with HBSS at both apical and basal side. A lucifer yellow (LY) solution (400 μg ml^{-1} in HBSS) was added to the apical side, and the plate was then incubated at 37 °C. After 60 min of incubation, HBSS was collected from the basal side, and the concentration of LY was determined by measuring the fluorescence intensity of each solution with an F-4000 spectrophotofluorometer (Hitachi, Japan). The excitation-emission wavelengths were 430–540 nm.

Results

Figure 1 shows the schematic representation of the *in vitro* coculture system used in this study. In this system, two different types of cells do not contact directly each other. Therefore the cross-talk between the two types of cells would be performed via soluble factors, such as cytokines, secreted from each cell line.

As a preliminary experiment, we first tested the effect of the culture medium used for PC12 (PC12 medium) on the viability and functions of Caco-2 cells, because the composition of the PC12 medium was different from that of the Caco-2 medium. The viability and functions of Caco-2 cells were not affected by changing the basolateral medium of a Caco-2 cell monolayer to the PC12 medium and by culturing the cells for further 7 days (data not shown). The PC12 medium was therefore used as a basolateral medium of Caco-2 cell monolayers during the co-culture period (Figure 1).

The effect of coculturing with Caco-2 cells on the differentiation of PC12 was then studied. The Caco-2 monolayers for the coculture experiments were used after 14 days of culture. PC12 cells were seeded to a 12-well culture plate coated with collagen type-I at a density of 25,000 cells/well, and the cells were used after 1 day of culture. In the coculture experiment, Caco-2 cells cultured on a cell culture insert for 14 days were placed on PC12 cells cultured on a plate. After starting coculture, the PC12 cells were examined by using an inverted phase-contrast microscope every day.

By culturing under a monolayer of Caco-2 which had been precultured for 14 days, PC12 cells gradually grew and differentiated into neuronal cells. After 5 days of coculture, the neurite outgrowth of PC12 cells has been fully induced (Figure 2B), whereas that was scarcely observed in control cells (Figure 2A). This neurite outgrowth-stimulatory effect of Caco-2 cells was not different between the Caco-2 cells precultured for 14 days and 21 days (data not shown). The extension of neurites induced under a coculture condition was similar to that induced by NGF (50 ng ml^{-1}) (Figure 2C). Varilek et al. (1995) have reported that intestinal epithelial cells can synthesize NGF. It is therefore likely that this phenomenon was due to NGF synthesized and secreted from Caco-2 cells. The expression of NGF mRNA in Caco-2 was then examined. The presence of mRNA coding NGF was detected in Caco-2 cells (single culturing) by reverse transcription PCR (RT-PCR), and the RT-PCR resulted in a single band matching in size with primers used (data not shown). The amount of immunoreactive NGF in the basolateral medium of Caco-2 was also measured by using an NGF-enzyme-linked immunosorbent assay (NGF-ELISA) kit according to the manufacturer's instruction. The amount of NGF was 3.82 ± 0.6 pg ml^{-1} in the basolateral medium of Caco-2 after 14 days culture. These results indicated that Caco-2 expressed NGF mRNA and secreted NGF to the basolateral medium.

Then polyclonal anti-NGF antibody was added to the medium (1:100 dilution) and cultured for 5 days. Figure 2D demonstrates that the neurite outgrowth of PC12 induced by coculturing with Caco-2 cells (Figure 2B) was markedly inhibited by the addition of

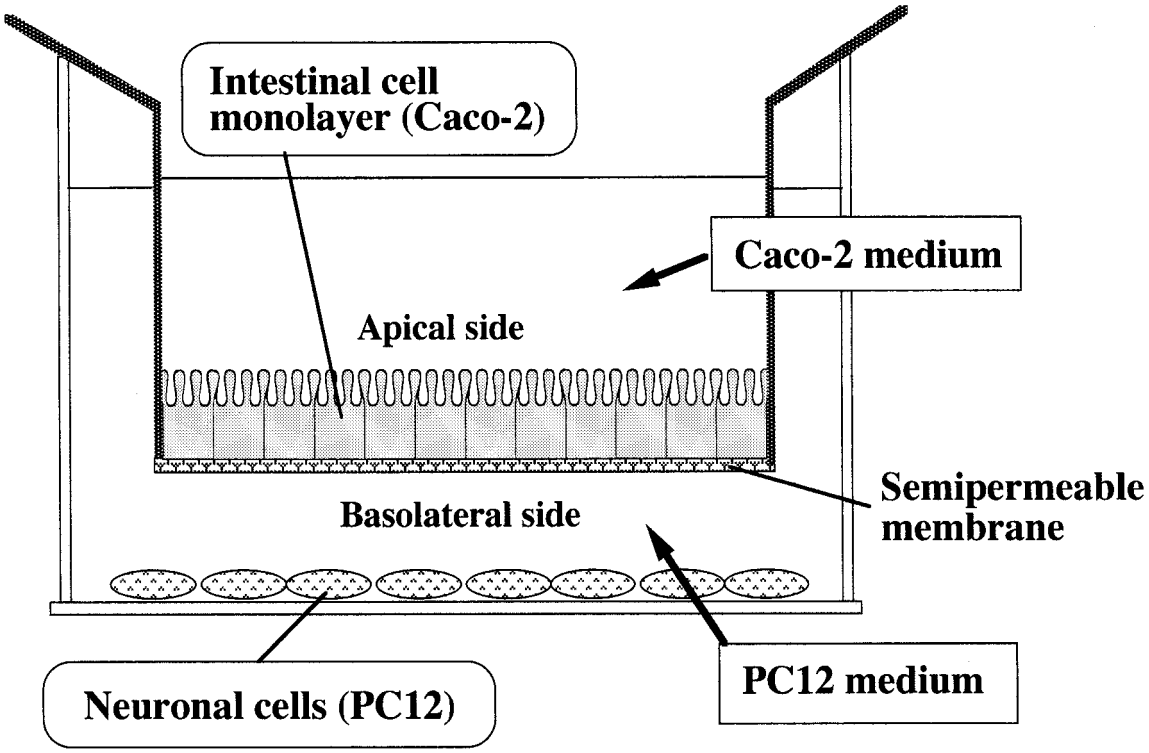

Figure 1. Schematic representation of the *in vitro* coculture system. Caco-2 cells were cultured on 12-well cell culture inserts with semipermeable support membrane precoated with collagen type-I and formed a monolayer of non-overlapping and contact-inhibited cells. PC12 cells were cultured on 12 well culture plates and cell culture inserts on which Caco-2 were cultured were placed in these wells.

antibody, indicating that the neurite extension of PC12 was mainly due to NGF secreted from Caco-2 cells.

The effect of PC12 cells on the differentiation of Caco-2 cells was also examined by using this coculture system. Caco-2 cells were seeded on a cell culture insert at a density of 1×10^5 cells/culture insert. After 1 day of seedling, the culture insert with Caco-2 cells was placed on fully differentiated PC12 cells which had been cultured on a 12-well culture plate for 7 days in the presence of NGF (50 ng ml^{-1}). In control Caco-2 cells, the basolateral medium was changed to the PC12 medium containing NGF (50 ng ml^{-1}). After coculturing with PC12 or culturing with the PC12 medium for 3, 7, 11 and 15 days, the culture inserts (Caco-2 cell monolayers) were removed from the culture plates. The TER value and the activities of sucrase and alkaline phosphatase in the Caco-2 cell monolayers were then measured.

As shown in Figure 3, the TER value of Caco-2 cell monolayers cocultured with PC12 gradually and significantly decreased comparing with the value of the control Caco-2 monolayer. On the other hand, neither the activity of sucrase nor that of alkaline phosphatase was changed by coculturing with PC12 (data not shown). These results suggest that the coculture with PC12 does not modulate the overall differentiation of Caco-2 cells but specifically alters the TER of Caco-2 cell monolayers. Changes in the cell monolayer permeability were also examined by using such leakage markers as LY (Mr 457.24). As shown in Figure 4, the LY permeability in Caco-2 cell monolayers cocultured with PC12 was extremely high compared with the value of control monolayers, in good agreement with the TER decrease caused by the coculture with PC12 (Figure 3).

Discussion

The present study demonstrated that differentiation of PC12 cells was induced by coculturing with Caco-2 cells and that NGF secreted from Caco-2 cells would be responsible for this change. It was also observed that the tight junction of Caco-2 monolayers was modulated by coculturing with PC12.

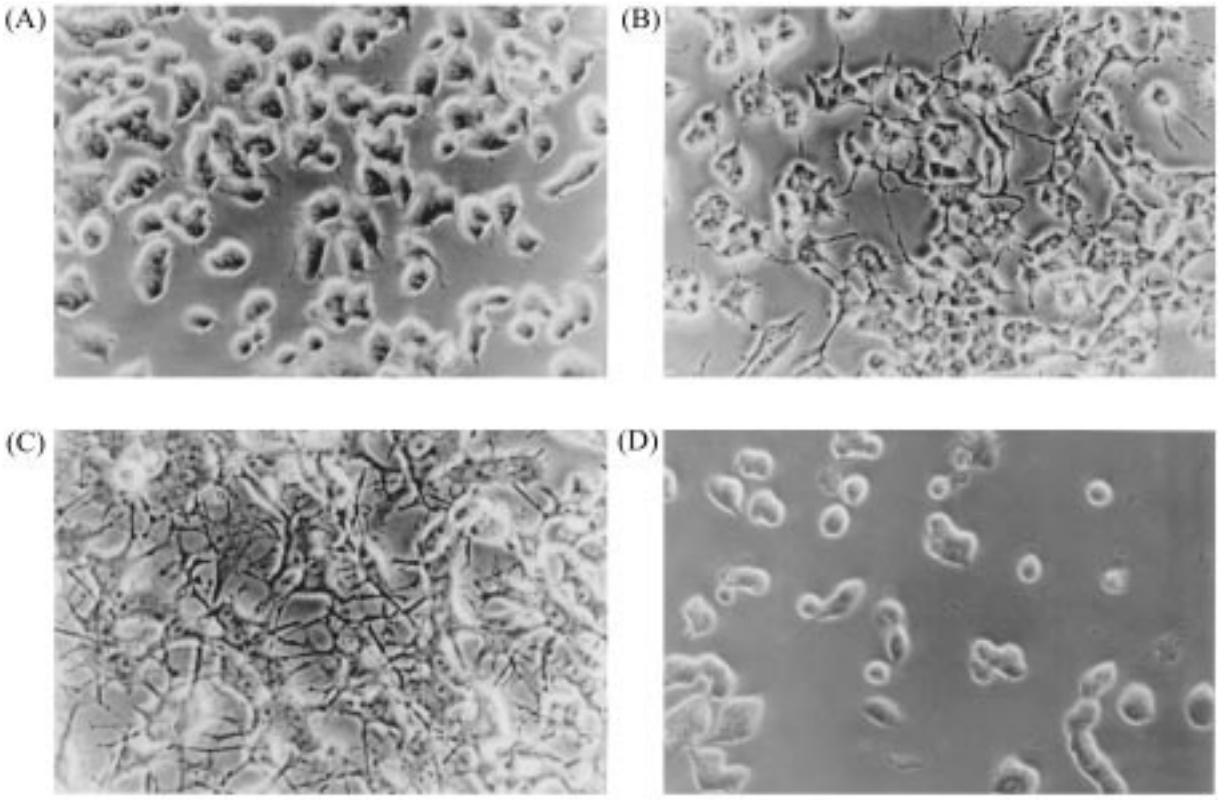

Figure 2. The morphology of PC12 cells cultured in various condition. After 6 days of coculture, PC12 cells cultured with (B) or without (A) Caco-2 cell monolayers were examined and photographed. Nerve growth factor (NGF) (C; 50 ng ml^{-1}) or anti-NGF antibody (D; 1:100 dilution) was added to the basolateral medium and the wells were examined and photographed after 6 days.

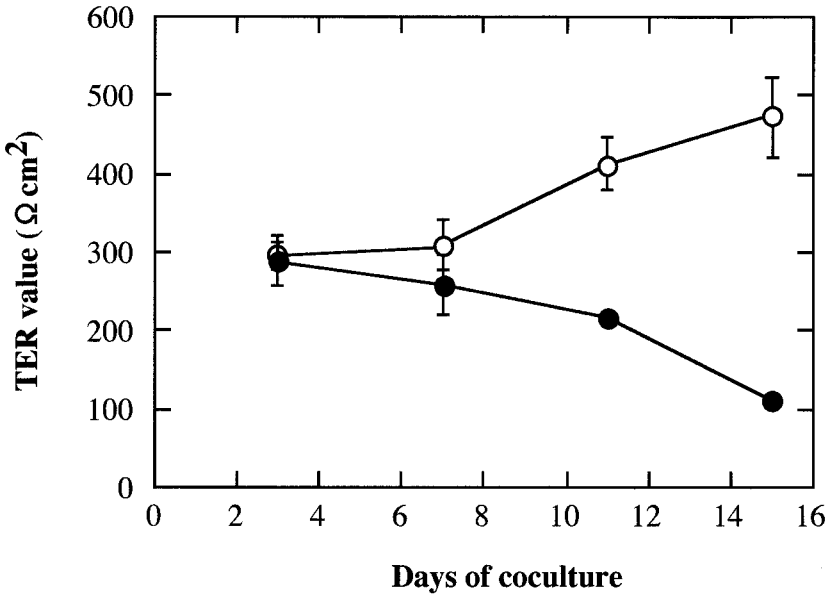

Figure 3. Changes in the TER value of Caco-2 monolayers by coculturing with PC12. After culturing Caco-2 cells for 3, 7, 11 and 15 days with (●) or without (○) PC12 cells, the TER value of the Caco-2 cell monolayers was measured. Values represent the mean ± S.E. (n = 6).

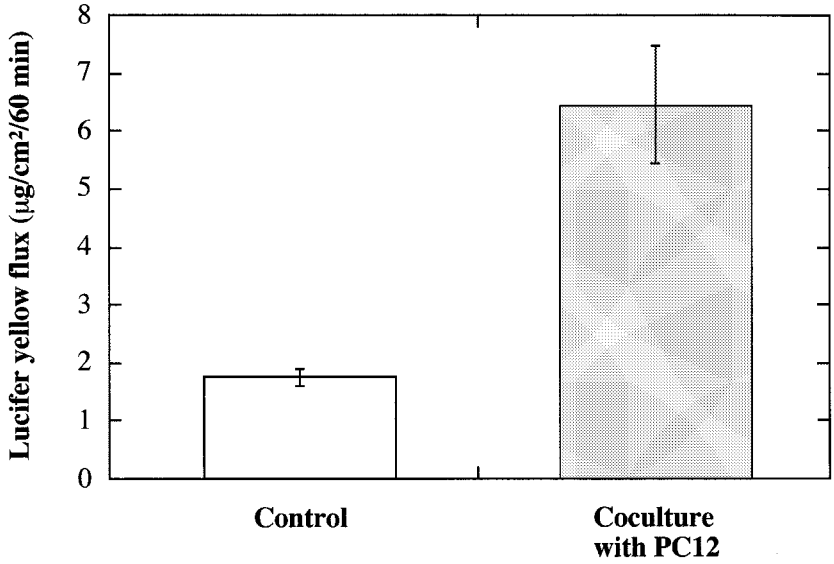

Figure 4. Permeability for lucifer yellow in Caco-2 cell monolayers cultured with or without PC12 cells. After 14 days of coculture with differentiated PC12 cells, the permeability for LY in Caco-2 cell monolayers was measured. Values represent the mean ± S.E. (n = 6).

Varilek et al. (1995) have reported that NGF is constitutively expressed and is secreted to the basolateral medium in Caco-2 cells. However, it is also known that PC12 can express NGF under certain conditions (Gill et al., 1998). Therefore, the possibility that Caco-2-derived factors enhanced the secretion of NGF by PC12, thereby inducing the neurite extension of PC12, cannot be ruled out. In the present study, however, we did not examine the autocrine secretion of NGF by PC12 cells under the coculture condition, because NGF secreted from Caco-2 and PC12 (if any) can not be distinguished by NGF-ELISA. Assessing the expression level of NGF in PC12 cells cocultured with Caco-2 by RT-PCR would be necessary to examine this possibility. Kumano et al. (2000) reported that the secretion of NGF in PC12 was induced by the addition of simvastatin, a HMG-CoA reductase inhibitor, resulting in the neurite outgrowth of PC12. However, they observed that the auticrine secretion of NGF induced by simvastatin treatment was accompanied by the apoptosis of PC12. Gill et al. (1998) reported that the neurite outgrowth from PC12 cells was induced when the cells were cultured in serum-free, NGF-free medium conditions. These findings suggest that the neurite outgrowth in PC12 cells by autocrine NGF is an exceptional phenomenon and may be not feasible in the present study. It is more likely that the neurite outgrowth of PC12 cocultured with Caco-2 was due to NGF secreted from Caco-2 monolayers, not from PC12 itself.

Although NGF from Caco-2 cell monolayers is likely to be responsible for the neurite outgrowth of PC12 cells, the amount of NGF in the Caco-2 culture medium determined by NGF-ELISA was markedly lower than that predicted from the degree of neurite outgrowth of PC12 cocultured with Caco-2. The apparent difference in the levels of NGF determined by NGF-ELISA and by bioassay using PC12 has also been observed by several investigators (Thoenen and Edgar, 1985; Assouline et al., 1987; Murphy et al., 1997), although the reasons for this difference remain unknown. One possibility is that NGF in the culture medium was degraded by proteases during the immunoassay. The addition of protease inhibitors to the culture medium may therefore be useful to determine the NGF level more accurately. It is also possible that another unknown factor produced by Caco-2 cells enhanced the activity of NGF in a cooperative manner.

An unexpected result obtained in this study was the modulation of tight junction in Caco-2 cells by the coculture with PC12 (Figures 3 and 4). Tight junction is known to have dynamic structure and is affected by various factors including cytokines and cellular constituents (Lewis et al., 1995). It is likely that an unknown soluble substance secreted from PC12 was involved in this phenomenon. PC12 cells were reported to secrete such neurotransmitters as dopamine

(Joseph et al., 1995), and they may modulate the tight junction in Caco-2 monolayers. Isolation and identification of such factors that modulate the tight junction and change the TER value of Caco-2 cell monolayers are now in progress.

In this study, by using an *in vitro* coculture system, we have observed two phenomena: One is the induction of neurite outgrowth of PC12 cells by coculturing with Caco-2 cells. The other is the modulation of tight junction of Caco-2 cell monolayers by coculturing with PC12 cells. This *in vitro* coculture system would be useful to study in detail the interaction or cross-talk between intestinal epithelial cells and nerve cells. Furthermore, it is expected that functional analyses of mutual interactions and regulations of cells present in the intestinal epithelium which consists of a variety of functional cells will be proceeded by using these coculture system.

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