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Nucleotide and Nucleoside Supplementation May Morphologically Promote the Differentiation of Human Caco-2 Cells

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Summary The effects of nucleotide and nucleoside supplementation on the formation of tight junctions and the expression of microvilli, as indexes of morphological differentiation were studied by using a human colon adenocarcinoma cell line (Caco-2 cells). The formation of tight junctions and the expression of microvilli were evaluated by measuring the transepithelial electrical resistance (TEER) and observing the cell surface under electron microscopic analysis, respectively. To clarify the nutritional significance of human milk nucleotides, we used a nucleotide mixture (and a corresponding nucleoside mixture) with a composition similar to that found in human milk. Nucleotides had no effect on TEER, but nucleosides markedly promoted the increase of TEER. When alkaline phosphatase activity in the brush border membrane was enhanced by the addition of triiodothyronine (TIT), nucleotides also promoted the increase of TEER. Cytidine and CMP predominant in the mixture influenced the increase of TEER materially. Furthermore, an electron microphotograph of the cell surface showed that nucleosides contributed to the expression of microvilli. Thus the results presented in this study suggest that nucleotide and nucleoside supplementation may enhance the morphological differentiation of Caco-2 cells.

Key Words nucleotide, nucleoside, tight junction, Caco-2 cell, microvillus

Interest has recently increased in the nutritional properties of nucleotides ubiquitously occurring in most tissues because they have been reported to have some possible biological effects (*1*). The nucleotides and their metabolites are currently under investigation with regard to their effects on the immune function (*2*, *3*), lipid metabolism (*4*, *5*), and cerebral function (*6*). These studies suggest that an exogenous supply of nucleotides may be essential in cells having a rapid turnover rate and a limited capacity for the de novo synthesis of nucleotides.

On the other hand, intestinal cells are prompt in turnover and require a large amount of nucleotides for DNA and RNA synthesis (*7*). It therefore seems indispensable for these cells to be adequately supplied with nucleotides. Some studies have shown that nucleotides may be beneficial to the function of enterocytes in rats (*8*, *9*). Incidentally, nucleotide supplementation promotes the proliferation and differentiation of intestinal cells (*10*). Bueno et al. have also reported that nucleotides promote histological and ultrastructural recovery from intestinal damage in rats after diarrhea induced by lactose (*11*). Furthermore, it is well known that human milk contains large quantities of nucleotides compared with bovine milk (*7*, *12*). Despite these investigations, the significance of human milk nucleotides in morphological changes of tight junctions (TJ) and microvilli has not yet been assessed at the cellular level.

Caco-2 cells, a cell line derived from a human colonic adenocarcinoma, spontaneously differentiate into mature enterocyte-like cells that exhibit many of the morphological and functional characteristics of enterocytes when they reach confluence. These cells form microvilli and TJ and express brush-border membrane enzymes such as maltase, sucrase, aminopeptidase, or alkaline phosphatase (*13–16*). Therefore Caco-2 cells are frequently used as a model of intestinal cells for the study of differentiation, transport function, physiology, pharmacology, and bacterial pathogenesis (*10*, *13–19*). We have investigated the significance of nucleotides occurring in human milk also with the aid of these cells. This study deals with the effectiveness of nucleotides in the differentiation of Caco-2 cells from the viewpoints of the enhancement of transepithelial electrical resistance (TEER) as an index of TJ formation and the expression of microvilli.

MATERIALS AND METHODS

Chemicals and reagents. All nucleotides and nucleosides were purchased from Yamasa Co., Ltd. (Chiba, Japan). The nucleotide mixture contained CMP, UMP, IMP, AMP, and GMP in the proportion of 10 : 1 : 1 : 1 : 1 in weight, based on the proportion of human milk nucleotides (*12*). The nucleoside mixture contained cytidine, uridine, inosine, adenosine, and guanosine in the proportion of 10 : 1 : 1 : 1 : 1 in weight. Cosmedium 001, a serum-free medium, was obtained from Cosmobio (Tokyo, Japan). Fetal calf serum (FCS), glutamine, and MITO were purchased from Dainihon

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Seiyaku (Osaka, Japan), Nissui (Tokyo), and Becton Dickinson Labware (Lincoln Park, NJ, USA), respectively. Triiodothyronine was from Sigma (St. Louis, MO). All other reagents were purchased from Wako Pure Chemicals (Osaka).

Caco-2 cells culture. Caco-2 cell line (ATCC No. HTB37) was obtained from Dainihon Seiyaku. Cells were routinely cultured in DMEM containing 10 mM HEPES, 50 μg/mL transferrin, 10 μg/mL insulin, 0.6 mg/mL glutamine, 7% FCS, 10 μg/mL nonessential amino acids (NEAA), and 3.7 mg/mL $NaHCO_3$ at 37°C under a humidified atmosphere of 5% CO_2 and 95% air.

Measurement of TEER. TEER was measured according to the method of Hashimoto and Shimizu (*20*). Caco-2 cells grown to confluent monolayers in medium containing FCS were trypsinized with a 0.25% trypsin solution containing 0.02% EDTA and 0.9% NaCl in 0.01 M phosphate buffer (pH 7.2, PBS). The cells were then suspended in Cosmedium and seeded at a cell density of 1×10^5 cells/mL in Millicell CM (Millipore, Molsheim, France) precoated with collagen. A Millicell electrical resistance system (Millipore) was used to record TEER, which was corrected for fluid resistance.

The precoating of Millicell CM with collagen was done as follows. After mixing one part of 0.3% tissue culture collagen solution (pH 3.0, Funakoshi, Tokyo, Japan) with three parts of 60% ethanol solution, 50 μL of the mixture was dropped on the membrane and allowed to dry for 4 h. Cosmedium 001 containing 0.1% MITO and 0.6 mg/mL glutamine with or without nucleotides was then added, and TEER was measured every 2 or 3 d.

Assay of alkaline phosphatase activity. After Caco-2 cells were cultured by using Matrigel™-precoated plates (Collaborative Research Inc., Bedford, MA), the activity of alkaline phosphatase was measured with a test kit (Wako Pure Chemicals) and expressed as nmol/min/mg of protein. Protein was determined according to a modification by Peterson (*21*) of Lowry's method with bovine serum albumin (Sigma) as the standard, after the cells were harvested and solubilized by sodium dodecyl sulfate (SDS).

Electron microscopy of Caco-2 cell. Caco-2 cells were seeded onto a glass plate (5 mm×5 mm) in petri dishes and cultured for 2 wk in medium with or without nucleotides. The cell monolayer grown on the glass plate was washed three times with PBS and fixed with 2.5% glutaraldehyde (Wako, Tokyo) in PBS overnight at room temperature and postfixed in 0.5% OsO_4 (Wako). After dehydration through an ethanol, the cells were lyophilized. Electron micrographs of the cells coated with platina were then taken with a Hitachi S-800 scanning electron microscope operated at 5 kV.

Statistical analysis. Data were obtained as the means±SD and statistically analyzed by Tukey's multiple comparison test following one-way ANOVA. Differences were considered significant at $p<0.05$.

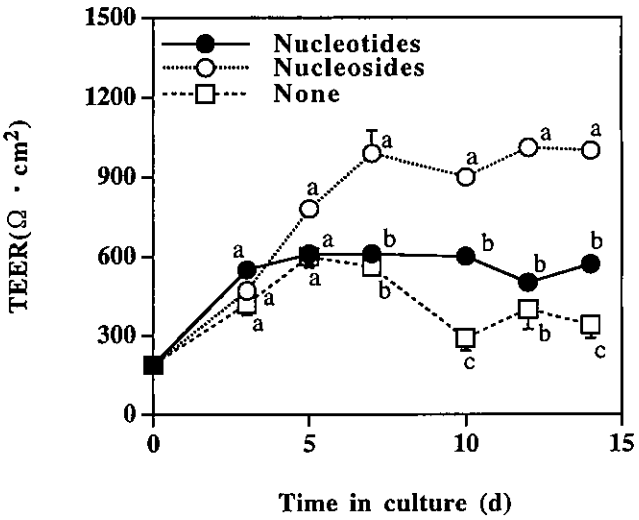

Fig. 1. Effects of nucleotides or nucleosides on the TEER of Caco-2 cell monolayers. Values are the means±SD of three or four determinations. Data at each timepoint marked with different letters are significantly different ($p<0.05$). The final concentration of the nucleotide or nucleoside mixture or of cytidine was 10 μg/mL.

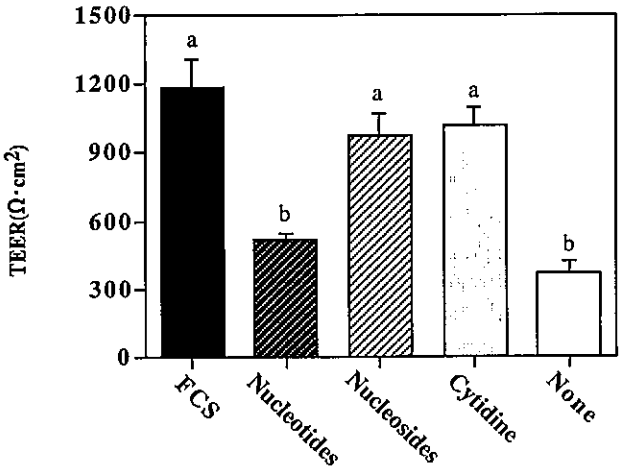

Fig. 2. Effects of nucleotides, nucleosides, or cytidine on the TEER of Caco-2 cell monolayers cultured for 14 d. Values are the means±SD of three or four determinations. Different letters above the error bar denote significant differences ($p<0.05$). The final concentration of the nucleotide or nucleoside mixture or of cytidine was 10 μg/mL.

RESULTS

Effects of nucleotides and nucleosides on the TEER of Caco-2 cell monolayers

Nucleotides at a final concentration of 10 μg/mL in medium had no effect on TEER until 10 d (Fig. 1). After the addition of nucleosides, the TEER gradually increased during cultivation and at 7 d reached a plateau (Fig. 1). The addition of cytidine alone, which is the main component of the nucleoside mixture, elevated the TEER to the same extent as observed in the presence of the nucleoside mixture (Fig. 2).

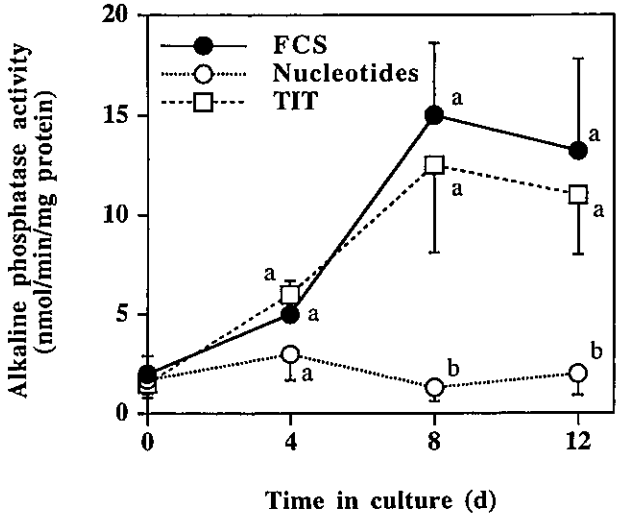

Fig. 3. Effects of nucleotides, TIT, or FCS on alkaline phosphatase activity. Values are the means±SD of three or four determinations. Data at each timepoint marked with different letters are significantly different ($p<0.05$). The final concentration is as follows: nucleotide mixture, 10 μg/mL; TIT, 50 ng/mL; FCS, 7%.

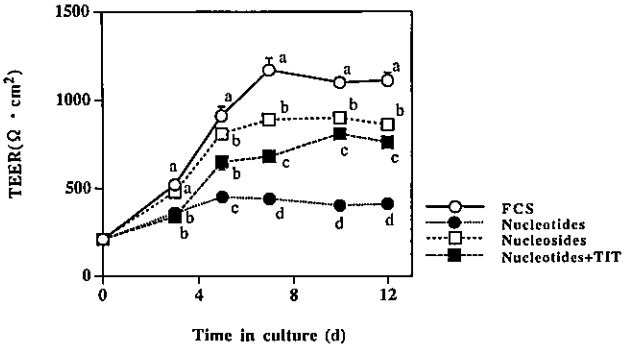

Fig. 4. Effects of nucleotides and TIT on the TEER of Caco-2 cell monolayers. Values are the means±SD of three or four determinations. Data at each timepoint marked with different letters are significantly different ($p<0.05$). The final concentration of the nucleotide or nucleoside mixtures was 10 μg/mL, that of TIT was 50 ng/mL.

Alkaline phosphatase activity of Caco-2 cells

Changes in the alkaline phosphatase activity of Caco-2 cells are illustrated in Fig. 3. The addition of nucleotides was not associated with any increase in the enzyme activity of Caco-2 cells cultured under serum-free conditions. On the other hand, when the cells were cultured in medium supplemented with FCS or triiodothyronine (TIT), the enzyme activity gradually increased from d 4 to d 8 and remained at a high level thereafter.

Effects of nucleotides and TIT on the TEER

The effect of nucleotides and TIT on the TEER of Caco-2 cell monolayers are illustrated in Fig. 4. The TEER of Caco-2 cells in the presence of both nucleotides and TIT significantly increased. However, nucleosides had a more elevating effect on TEER compared with nucleotides and TIT. TIT alone did not influence the TEER

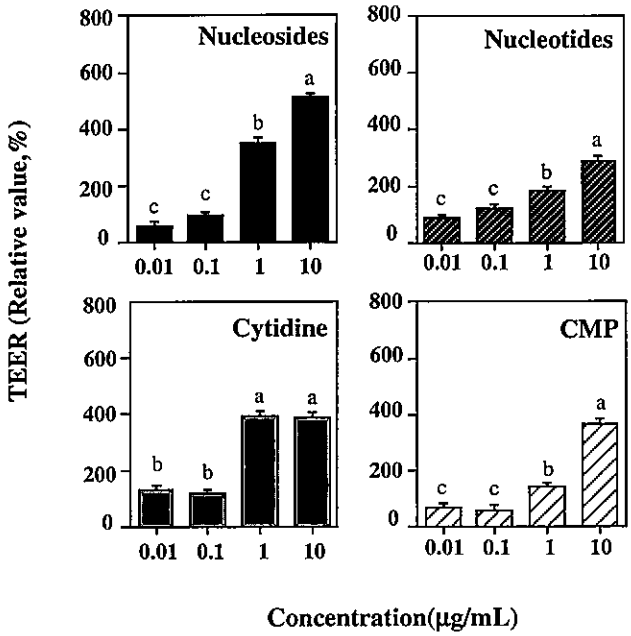

Fig. 5. Effects of nucleotide or nucleoside concentrations on the changes in TEER of Caco-2 cell monolayer cultured for 12 d. Values are the means±SD of three determinations. Different letters above the error bar denote significant differences ($p<0.05$). Cultures containing nucleotide mixture and CMP also contained 50 ng/mL TIT.

(data not shown).

Effects of nucleotides and nucleosides at different concentrations on the TEER

The effects of different concentrations of the nucleotide or nucleoside mixtures on the TEER of Caco-2 cell monolayers are illustrated in Fig. 5. TIT was added in culture with nucleotides or CMP alone. TEER was expressed in % as a relative value of that in the control (nucleotide unsupplementation). A significantly increased TEER was observed in the presence of nucleoside mixture or cytidine alone at 1.0 μg/mL. Similarly, a significant difference was detected at 1.0 μg/mL of nucleotide mixture or CMP alone. However, nucleosides were more effective in TEER than nucleotides were. At concentrations of less than 0.1 μg/mL, neither nucleosides nor nucleotides had any effect on the TEER.

Effects of nucleosides on the expression of microvilli

The cells on 14 d after culture in the presence of nucleosides showed well-developed microvilli compared with the control (nucleoside unsupplementation) (Fig. 6). Nucleosides promoted not only the increase of TEER, but also the expression of microvilli, taken as an index of morphological differentiation of Caco-2 cells.

DISCUSSION

In this study, we showed that nucleotide supplementation had no effect on TEER, but nucleoside supplementation promoted its increase. As shown in Fig. 3, Caco-2 cells expressed no alkaline phosphatase in serum-free medium regardless of the presence or absence of nucleotides. It was therefore supposed that exogenous nucleotides had no influence on the formation

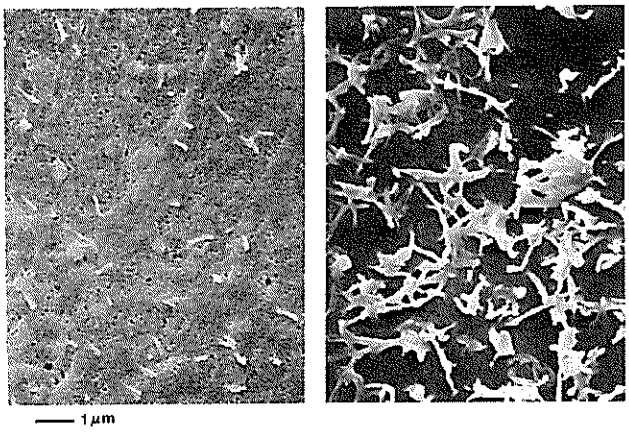

Fig. 6. Scanning electron micrograph of a Caco-2 cell monolayer cultured in medium containing nucleosides for 14 d. The final concentration of the nucleoside mixture was 10 μg/mL.

of TJ because the cells could not sufficiently use nucleotides. The addition of TIT, however, known to induce the expression of alkaline phosphatase (*22*), promoted the increase of TEER in the presence of nucleotides. Accordingly, these results suggest that nucleosides generated by the hydrolysis of nucleotides may be important in the TJ formation of Caco-2 cells.

The effects of both nucleotides in the presence of TIT and nucleosides on TEER were dose-dependent. Although both nucleosides and nucleotides induced a significant increase of TEER at the concentration of 1.0 μg/mL, nucleoside had a more elevating effect on TEER than nucleotides did. These results suggest that nucleosides may be more efficient than nucleotides. It is unclear, however, whether the alkaline phosphatase activity of Caco-2 cells cultured in medium containing TIT is sufficient to convert nucleotides to nucleosides. On the other hand, nucleotides contained in human milk are also absorbed from the intestine after being digested to nucleosides by alkaline phosphatase (*7*). Because this enzyme has been found in the intestine of fetus, it is likely that newborn infants are capable of dephosphorylating nucleotides to some extent (*23*). It remains obscure, however, whether the digestive process of nucleotides in newborn infants is sufficient to form TJ. Further study is needed to clarify these points.

In this experiment, we used a nucleotide mixture similar to human milk in nucleotide composition to assess its nutritional significance. We have previously showed that human milk nucleotides range in concentration from 5 to 11 μg/mL (*12*). Their concentration used in this study is the same as in human milk. Moreover, it is characteristic of human milk that CMP predominates and other nucleotides are in minor amounts (*12*). In our previous study (*24*), it was proved that CMP promoted the proliferation and differentiation of IEC-6 cells. Tanaka et al. (*25*) also reported that CMP had no effect on the proliferation of human fetal small intestine in organ cultures, despite its promotion of cell differentiation. In this connection, we indicated that CMP (and cytidine) actually elevated TEER, responsible for TJ formation. Certainly, CMP serves as a most important factor in cell proliferation and/or differentiation. On the assumption that human milk has an optimal proportion of nucleotides, it seems quite reasonable to think that CMP predominant in human milk nucleotide plays a significant role in the development of the intestine of infants.

The tight junction formed between intestinal epithelial cells has a dynamic structure that resists the passage of macromolecules, although water and ions can easily pass through (*26*, *27*). Therefore the barrier formed by TJ prevents the penetration of proteins and antigens across the space between intestinal cells (paracellular route) (*27*). It is highly probable that nucleotides in milk obstruct the intestinal absorption of proteins or antigens via the formation of TJ. This hypothesis is supported by previous reports that breast-fed infants show lower intestinal permeability by the paracellular route, in comparison with formula-fed infants (nucleotide unsupplemented formula) (*28*, *29*).

Moreover, we demonstrated that nucleosides, which are in the form preferable for intestinal absorption, promote the expression of microvilli making further improvement in digestion and in the absorption of nutrients. Cosgrove et al. (*30*) reported that catch-up growth in small for gestational aged infants, whose intestinal mucosa was damaged by intrauterine malnutrition, was improved by the feeding of nucleotide supplemented formula. This improved growth may be due to the trophic effect of nucleotides, resulting from the improvement of digestive and absorptive capacity by the formation of microvilli.

Thus our findings indicated that nucleotides and nucleosides may promote the morphological differentiation of Caco-2 cells, including the formation of TJ and microvilli, even though the effect of nucleotides depended on alkaline phosphatase. Their significance in infants and their mechanism of action need to be further investigated.

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