Nucleotides enhance the secretion of interleukin 7 from primary-cultured murine intestinal epithelial cells

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Abstract

Our previous studies showed that dietary nucleotides fed to mice enhanced the secretion of interleukin 7 (IL-7) and transforming growth factor β (TGF- β) from intestinal epithelial cells (IECs). To explore whether nucleotides influence IECs directly to enhance the secretion of the cytokines or not, the effects of nucleotides added *in vitro* on the cytokine secretion from primary-cultured murine IECs were examined. When the mixture of nucleotide 5'-monophosphates (CMP, GMP, IMP, and UMP) or individual nucleotide 5'-monophosphates were added to the primary culture of IECs derived from BALB/c mice, the secretion of IL-7, but not that of TGF- β , was increased significantly. Addition of nucleotides to the culture did not alter the number of the IECs. Secretion of IL-6 and granulocyte-macrophage colony-stimulating factor, which are known to be secreted from IECs, was not enhanced by the addition of nucleotides. These results demonstrate that nucleotides can affect IECs directly to enhance the secretion of IL-7, and suggest that the increased secretion of TGF- β from IECs by dietary nucleotides was due to indirect effects of the nucleotides, which may affect intestinal microflora or cells other than IECs that in turn influence the cytokine secretion of IECs.

Abbreviations: ELISA, enzyme linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; IEC, intestinal epithelial cell; IEL intraepithelial lymphocyte; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

Introduction

Orally ingested foods are digested in the gastrointestinal tract and finally absorbed by intestinal epithelial cells (IECs). IECs are multifunctional cells with the ability to not only absorb nutrients but also to secrete cytokines and to present antigens (Hershberg and Mayer, 2000; Stadnyk, 1994). Therefore, IECs are considered to play an important role in the gastrointestinal immune system. IECs regulate differentiation and development of intestinal intraepithelial lymphocytes (IELs) and Peyer's patches by secreting cytokines including interleukin 7 (IL-7). In IL-7 gene

knockout (IL-7^{-/-}) mice (Laky et al., 1998) or IL-7 receptor gene knockout mice (Maki et al., 1996), no IEL with $\gamma\delta$ cell receptors (TCR) developed. Furthermore, limited Peyer's patch formation occurred in IL-7^{-/-} mice, but transgene-directed IL-7 expression by intestinal epithelial cells increased the size and number of Peyer's patches formed and restored TCR $\gamma\delta^+$ IELs (Laky et al., 2000).

The nucleotides we focused on in this study has been reported to have various physiological functions when ingested, such as enhancement of immune responses (Carver et al., 1991; Jyonouchi et al., 1993), protection from bacterial infection (Kulkarni et al.,

Figure 1. Effect of the nucleotide mixture on secretion of IL-7 from IECs and IELs. Freshly isolated IECs (A), highly purified IECs (B) or IELs (C) were cultured at 2×10^5 cells/well for 24 h with or without $50 \mu\text{g ml}^{-1}$ nucleotide mixture (NT mix). The culture supernatants were concentrated by ultrafiltration and the concentrations of IL-7 were determined by means of ELISA. Broken lines indicate the detection limit. The values under the detection limit may not represent the correct values. * $p < 0.05$.

1986), skewed induction of Th1-type immune responses (Jyonouchi et al., 2001; Nagafuchi et al., 1997, 2000a; Sudo et al., 2000), and increase of enzymatic activity of IECs (Ortega et al., 1995; Sato et al., 1999; Tanaka et al., 1996). Our previous findings showed that dietary nucleotides increase secretion of IL-7 and transforming growth factor β (TGF- β) from IECs, and increase the proportion of TCR $\gamma\delta^+$ IELs (Nagafuchi et al., 2000b, 2003). However, it is still unclear how dietary nucleotides increase secre-

tion of IL-7 and TGF- β from IECs. There are two possible pathways: one is that the nucleotides affect IECs directly, and the other is that the nucleotides affect intestinal microflora or cells other than IECs that can then affect functions of IECs. Thus, in this study, we aimed to clarify how dietary nucleotides increase the secretion of IL-7 and TGF- β from IECs and to investigate the effects of nucleotides on secretion of other cytokines from IECs *in vitro*.

Materials and methods

Mice and reagents

Female BALB/c mice (6–9 weeks old, SPF) were purchased from Japan CLEA (Tokyo, Japan). The experiments were performed in accordance with the Guidelines for Animal Experiments of the Faculty of Agriculture, The University of Tokyo, and the law (No. 105) and notification (No. 6) of the Japanese Government.

Nucleotide 5'-monophosphates (CMP, GMP, IMP, and UMP) were purchased from Yamasa Co. (Choshi, Japan). As in our previous studies (Nagafuchi et al., 2000a,b), the nucleotide mixture was prepared in a proportion of 162:57:110:71 (CMP:GMP:IMP:UMP) in weight, which corresponds to those in human milk at 12 weeks of lactation (Janas and Picciano, 1982).

Preparation of IECs and IELs

Small intestines were removed from BALB/c mice and flushed with Hanks' balanced salt solution (HBSS) (Gibco BRL, Gaithersburg, MD, U.S.A.). The small intestines were turned inside-out with the aid of polyethylene tubing (Hibiki, Tokyo, Japan). Each inverted intestine was cut into four fragments and the fragments were placed in a 50-ml conical tube (Nunc, Roskilde, Denmark) containing 45 ml of HBSS including 5% fetal calf serum (FCS) (Gibco BRL). The tube was stirred at 37 °C for 30 min followed by vigorous shaking vertically 15 times. This process was performed twice, and the resulting supernatants were passed through a glass-wool column to remove cell debris. A discontinuous Percoll density gradient (27, 40 and 70%) (Amersham Pharmacia, Uppsala, Sweden) was then used to separate the cells. The cells that layered between the 27 and 40% interface were collected as IECs and the cells that layered between the 40 and 70% interface were collected as IELs. Highly purified IECs were then obtained by flow cytometry (FACS Vantage, Becton Dickinson, Franklin Lakes, NJ, U.S.A.) according to cell size and granularity.

Cell culture for cytokine assay

IECs and IELs were resuspended in F-12 Nutrient Mixture (Gibco BRL) containing 200 ng ml⁻¹ cholera toxin (Sigma, St. Louis, MO, U.S.A.), 2 µg ml⁻¹ hydrocortisone (Sigma), 30 µg ml⁻¹ insulin (Sigma), 100 µg ml⁻¹ transferrin (Wako Pure Chemicals, Osaka, Japan), 20 ng ml⁻¹ epidermal growth factor

(EGF) (Becton Dickinson), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. Cells (2 × 10⁵ cells/well) were then cultured in 96-well plates (Becton Dickinson) with or without nucleotides for 24 h at 37 °C, under a humidified atmosphere containing 5% CO₂ and 95% air. After incubation, culture supernatants were collected by centrifugation and stored at -20 °C. Some supernatants were concentrated by ultrafiltration using an ultrafilter (Ultrafree-MC 5000 NMWL Filter Unit, Millipore, Bedford, MA, U.S.A.). Supernatants used to determine the concentration of total TGF-β were treated with acid according to the manufacturer's instructions (Promega, Madison, WI, U.S.A.).

Cytokine assay

The concentrations of IL-1β, IL-6, IL-7, IL-10, TGF-β, tumor necrosis factor α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the culture supernatants were determined by means of enzyme-linked immunosorbent assays (ELISA). The antibodies used for detecting IL-7 were mouse monoclonal anti-human/mouse IL-7 (Genzyme, Cambridge, MA, U.S.A.) and goat polyclonal anti-mouse IL-7 (Genzyme). For other cytokines, the TGF-β, EmaxTM ImmunoAssay System (Promega) and ELISA kits for IL-1β, IL-6, IL-10, TNF-α and GM-CSF (Endogen, Woburn, MA, U.S.A.) were used.

Statistical analysis

The results were expressed as means with standard errors. The statistical significance of the data was evaluated by Student's *t*-test. Differences with a *p* value of <0.05 were considered statistically significant.

Results

Effect of nucleotides on secretion of IL-7 from IECs

IECs were freshly isolated from intestines of adult mice and cultured with or without the nucleotide mixture. When the nucleotide mixture was added to the culture, secretion of IL-7 was significantly increased (Figure 1a). Increased secretion of IL-7 was observed also when highly purified IECs were used (Figure 1b). However, IL-7 was not detected in the culture supernatant of IELs, which can contaminate the IEC fraction, either with or without nucleotide (Figure 1c). These results clearly show that nucleotides affect IECs directly to increase the secretion of IL-7.

Figure 2. Effect of individual nucleotide 5'-monophosphates on the secretion of IL-7 from IECs. Freshly isolated IECs (2×10^5 cells) were cultured for 24 h with or without $50 \mu\text{g ml}^{-1}$ nucleotides mixture (NT mix) or individual nucleotide 5'-monophosphate (CMP, GMP, IMP, and UMP). The culture supernatants were concentrated by ultrafiltration and the concentration of IL-7 were determined by means of ELISA. A broken line indicates the detection limit. * $p < 0.05$. ** $p < 0.01$.

Figure 3. Dose dependent increase of the IL-7 secretion from IECs by GMP. Freshly isolated IECs (2×10^5 cells) were cultured for 24 h with or without the indicated concentrations of GMP. The culture supernatants were concentrated by ultrafiltration and the concentrations of IL-7 were determined by means of ELISA. A broken line indicates the detection limit.

Next, we examined the effect of the individual nucleotide 5'-monophosphate, i.e. CMP, GMP, IMP or UMP, on IL-7 secretion. Each individual nucleotide 5'-monophosphate increased the secretion of IL-7 from IECs (Figure 2). The efficiency of each individual nucleotide 5'-monophosphate and the nucleotide mixture is considered to be comparable judging from the results of several experiments. We then focused on

one of the nucleotide 5'-monophosphates, GMP, to test for dose dependency of the increase of IL-7 secretion on the nucleotide concentration. When 50 or $500 \mu\text{g ml}^{-1}$ GMP was added to the culture, the concentration of IL-7 in the culture supernatants was increased compared to the medium control in a dose depending manner (Figure 3).

It is possible that the increased secretion of IL-7 in the culture was due to an increased number of IECs in the presence of nucleotides. Thus, we examined the number of IECs when the culture supernatants were collected. There was no difference in cell number between the wells with GMP-added and control wells (data not shown), excluding this possibility.

Effect of nucleotides on secretion of TGF- β from IECs

When nucleotides were administered to mice orally, the secretion of TGF- β from IECs was also increased (Nagafuchi et al., 2003). Therefore, we next examined the effect of nucleotides on TGF- β_1 secretion from IECs *in vitro*. There are two different forms of TGF- β_1 a bioactive form and a latent form, so we determined the amounts of bioactive TGF- β_1 and total TGF- β_1 in the culture supernatants of IECs. As shown in Figures 4a and b, the amounts of bioactive TGF- β_1 and total TGF- β_1 in the culture supernatants were not significantly changed by adding individual nucleotide 5'-monophosphates or the nucleotide mixture to the culture. These data suggest that the increased secretion of TGF- β from IECs isolated from mice fed nucleotides was not due to the direct effect of nucleotides on IECs but due to indirect effects.

Effect of nucleotides on secretion of other cytokines from IECs

We next examined the effects of nucleotides on the secretion of other cytokines that are known to be secreted from IECs (Galliaerde et al., 1995; Jung et al., 1995; Panja et al., 1995). As shown in Table I, secretion of IL-6 and GM-CSF from IECs was not changed by adding nucleotides. IL-1 β , IL-10 and TNF- α were not detected by means of ELISA.

Discussion

In the present study, we demonstrate that nucleotides added to primary cultures of IECs increased the secretion of IL-7 from IECs without inducing their proliferation. This indicates that nucleotides enhance the

Figure 4. Effect of nucleotides on the secretion of TGF-β₁ from IECs. Freshly isolated IECs (2×10^5 cells) were cultured for 24 h with or without $50 \mu\text{g ml}^{-1}$ nucleotides mixture (NT mix) or individual nucleotide 5'-monophosphate (CMP, GMP, IMP, and UMP). The concentrations of bioactive form of TGF-β₁ (a) and those of total TGF-β₁ in acid-treated supernatants (b) were determined by means of ELISA.

Table 1. Effect of nucleotides on cytokine secretion from IECs

	Concentration of cytokine (pg ml^{-1})	
	Control	+ $50 \mu\text{g ml}^{-1}$ GMP
IL-1β ^a	N.D. ^d	N.D.
IL-6	21.4±1.9	22.4±1.9
IL-10 ^b	N.D.	N.D.
GM-CSF	7.1±1.3	8.3±3.0
TNF-α ^c	N.D.	N.D.

Freshly isolated IECs (2×10^5 cells) were cultured for 24 h with or without $50 \mu\text{g ml}^{-1}$ GMP and then culture supernatants were collected. The concentrations of IL-7 in the culture supernatants were determined by means of ELISA. Data are shown as means ± SE.

^{a-c} Detection Limit for each cytokine was 15.6 pg ml^{-1} for IL-1β, 111 pg ml^{-1} for IL-10, and 50 pg ml^{-1} for TNF-α.

^d N.D. = Not detected.

secretion of IL-7 by affecting IECs directly. Although our previous study showed that secretion of TGF-β from IECs was also increased by feeding dietary nucleotides to mice (Nagafuchi et al., 2003), our present results showed that the secretion of TGF-β from IECs was not increased by adding nucleotides to the culture of IECs *in vitro*. Moreover, increased secretion of other cytokines examined here was not observed when nucleotides were added to the culture. Thus, it is suggested that the direct effect of nucleotides on the increase of cytokine secretion from IECs is specific to IL-7 secretion, or at least that IL-7 secretion from

IECs is more sensitive to the addition of nucleotides than secretion of other cytokines.

The observation that secretion of TGF-β from IECs was not enhanced by adding nucleotides to the culture, indicates that the increased secretion of TGF-β from IECs derived from mice fed nucleotides was caused by indirect effects of dietary nucleotides on IECs. The possible targets of dietary nucleotides are intestinal microflora and cells other than IECs that can influence the cytokine secretion of IECs, e.g. IELs, lamina propria lymphocytes, intestinal mesenchymal cells, etc. Intestinal microflora are known to have a significant impact on intestinal immunity and thus on systemic immunity. For example, in germ-free mice, the development of IELs is seriously impaired (Umesaki and Setoyama, 2000) and the induction of oral tolerance is abrogated (Sudo et al., 1997). It was reported that *Bifidobacteria*, one of the indigenous bacteria in the gut, require nucleic acids and their related substances for optimal growth *in vitro* (Tanaka and Mutai, 1980). Thus, dietary nucleotides may affect the growth or activation of microflora in the gut, which in turn may influence the cytokine secretion of IECs. However, Sudo et al. (2000) recently showed that dietary nucleotides had no effect on the composition of intestinal microflora of mice associated with human infant gut microflora. Since there is little information about the effects of nucleotides on intestinal microflora *in vivo*, further investigation is needed. Our previous finding that dietary nucleotides increased the cytokine production of IELs (Nagafuchi et al., 2003) suggests

that nucleotides can affect IELs directly and/or indirectly. Since IECs and IELs regulate the differentiation of each other (Beagley and Husband, 1998), dietary nucleotides may affect IELs directly, which in turn may up-regulate the cytokine secretion of IECs. Although we showed that nucleotides can affect IECs directly to enhance IL-7 secretion, this finding does not exclude the possibility of indirect effects of nucleotides on IL-7 secretion.

Nucleotides are now emerging as a ubiquitous family of extracellular signaling molecules. Nucleotides are recognized by a specific class of plasma membrane receptors called purinergic P2 receptors, which are further divided into 2 subfamilies, P2Y and P2X (Di Virgilio et al., 2001). P2Y receptors are G-protein-coupled 7-membrane-spanning receptors and P2X receptors are ligand-gated ion channels. These receptors recognize primarily ATP, ADP, UTP and UDP, but some of them recognize other nucleotide mono-, di- and tri-phosphates as well (Van der Wijk et al., 1999). P2 receptors have a widespread tissue distribution, and some P2Y receptors are known to be expressed in IECs (Cressman et al., 1999). Therefore, it is possible that nucleotides exert their effects on the cytokine secretion from IECs through the P2 receptors. In the present study, we examined only nucleotide mono-phosphates because we intended to reproduce, in an *in vitro* system, the results obtained in the *in vivo* study in which the mixture of nucleotide mono-phosphates were fed to mice. More intense effects might be observed if ATP, ADP, UTP or UDP were used as stimulants.

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