

Platelet Derived Endothelial Cell Growth Factor/Thymidine Phosphorylase Enhanced Human IgE Production

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ABSTRACT

Background: Angiogenesis is one pathogenesis of allergic airway disease.

Methods: A potent angiogenic factor is platelet-derived endothelial cell growth factor (PD-ECGF), also known as thymidine phosphorylase (TP) in the field of cancer-associated research. Vascular endothelial growth factor (VEGF) is another representative angiogenic factor. Both factors were added to the culture system of human peripheral blood mononuclear cells (PBMC) with IL-4 and anti-CD40 monoclonal antibody (mAb). Total IgE levels in the supernatants and signal transduction of stimulated PBMC were evaluated.

Results: Addition of PD-ECGF enhances *in vitro* IgE production by PBMC in the presence of IL-4 and anti-CD40 mAb, but VEGF does not enhance IgE production. Although PD-ECGF catalyzes the reversible phosphorylation of thymidine to 2-deoxy-D-ribose-1-phosphate (2DDR), treatment of 2DDR has no effect on IgE production by human PBMC. Both IL-4 and anti-CD40 mAb induce PD-ECGF by human PBMC. Thymidine phosphorylase inhibitor (TPI), 5-chloro-6-[1-(2-iminopyrrolidinyl) methyl] uracil hydrochloride reduce IgE production via blocking of STAT6-phosphorylation.

Conclusions: Taken together, these results suggest TP involvement in the enhancement of IgE production and suggest that TPI is a novel strategy against IgE-related allergic disease.

KEY WORDS

IgE, platelet-derived endothelial cell growth factor (PD-ECGF), thymidine phosphorylase, thymidine phosphorylase inhibitor (TPI), VEGF

INTRODUCTION

Neovascularization in exaggerated Th2 inflammation and airway remodeling are cornerstones in the pathogenesis of allergic airway disease.¹ Increased vessel number, vessel size, and vascular surface area contribute to the severity of airway obstruction and airway hyper-responsiveness in patients with asthma.² In allergic rhinitis, increased vessel number and permeability are also important features of nasal mucosa.^{3,4}

Vascular endothelial growth factor (VEGF) and

platelet-derived endothelial cell growth factor (PD-ECGF) are representative critical regulators of angiogenesis. In the pathogenesis of allergic rhinitis, VEGF plays an important role: it increases vascular permeability in a mouse model of allergic rhinitis.⁴ An elegant VEGF-transgenic mouse model demonstrated that VEGF enhances respiratory antigen sensitization, augments antigen-induced Th2 inflammation, increases the accumulation and activation of dendritic cells, and serves a key role in antigen-induced Th2 inflammation and cytokine elaboration.⁵ The expression level of VEGF in nasal biopsies of pa-

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tients with allergic rhinitis is about three times higher than that of control, as determined using microarray analysis.⁶ Immunohistochemical analyses have revealed that VEGF expression in endothelial cells of nasal mucosa in patients with allergic rhinitis is significantly higher than that in controls.^{7,8} Allergen challenge to the nasal cavity increases the nasal fluid VEGF concentration in patients with allergic rhinitis.⁶

As an endothelial mitogen, PD-ECGF was isolated from platelets.⁹ Expression of PD-ECGF is elevated in many solid tumors and in chronically inflamed tissues.^{10,11} Exaggerated PD-ECGF is associated with nasal symptoms in allergic rhinitis.³ In fact, PD-ECGF also has thymidine phosphorylase (TP) activity: TP catalyzes the reversible conversion of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate (2DDR), which is a powerful protein-glycating agent that generates oxygen radicals. Oxygen radicals increase the angiogenic factors VEGF and IL-8.¹² Consequently, 2DDR is necessary for the angiogenic effect of TP. The level of TP activity in nasal mucosa from patients with allergic rhinitis was significantly higher than that from normal subjects.¹³

In fact, IgE is a crucial molecule for allergic rhinitis. Cross-linking of allergen-specific IgE bound to the high affinity IgE receptor on the surface of mast cells with multivalent allergens engenders the release of both preformed and newly generated mediators, and manifests nasal allergic symptoms. Although angiogenic factors play an important role in allergic disease,¹⁴ the effect of angiogenic factor on IgE production remains unclear. In this study, we investigated whether two representative angiogenic factors (VEGF and PD-ECGF) enhance *in vitro* IgE production by human PBMC. Additionally, the effect of anti-angiogenic factor on IgE production was examined.

METHODS

REAGENTS

Human IL-4 was a gift from Ono Pharmaceutical (Osaka, Japan). Anti-CD40 mAb G28-5 and anti-human IgE mAbs (CIA-E-7.12 and CIA-E-4.15) were gifts from Dr. A. Saxon, University of California, Los Angeles, CA, USA. Anti-human PD-ECGF mAb was a gift from Dr. S. B. Fox, Christchurch School of Medicine, Christchurch, New Zealand. An inhibitor of TP (TPI), 5-chloro-6-[1-(2-iminopyrrolidinyl) methyl] uracil hydrochloride ($K_i = 2 \times 10^{-8}$ M) was a gift received from Taiho Pharmaceutical (Tokyo, Japan). The following reagents were obtained commercially: alkaline phosphatase-labeled goat anti-human IgE (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), recombinant PD-ECGF (Leinco Technologies, St. Louis, MO, USA), 2DDR (Wako Pure Chemical Industries, Osaka, Japan), VEGF (R&D Systems, Minneapolis, MN, USA), mouse anti-human IgG (Sigma Chemical, St. Louis, MO, USA), mouse

anti-human IgA (Sigma Chemical), alkaline phosphatase-labeled goat anti-human IgG Fab (Tago, Burlingame, CA, USA), alkaline phosphatase-labeled goat anti-human IgA (Tago), anti-PD-ECGF (R&D Systems), anti-STAT6 (R&D Systems), anti-phospho-STAT6 (Y641) (R&D Systems), and anti- β -actin (Sigma Chemical).

CELLS AND CELL CULTURES

Peripheral blood mononuclear cells (PBMC) were isolated from healthy non-atopic volunteers using Ficoll-Hypaque centrifugation. The PBMC (1×10^6 cells/ml) were cultured in complete medium prepared from RPMI supplemented with 2 mM of glutamine, 100 units/ml of penicillin and 100 μ g/ml of streptomycin in the presence of IL-4 (100 units/ml) and anti-CD40 mAb (0.1 μ g/ml) for 7, 10, 14, and 21 days, which are optimal conditions for IgE production.^{15,16} The IgD⁺ B cells were purified from PBMC using naive B Cell Isolation Kit II in a MACS system (Miltenyi Biotec, Tokyo, Japan). Isolated B cells were cultured in the same conditions as those for PBMC.

Ig MEASUREMENTS

Total IgE, IgA and IgG levels in the supernatants of stimulated cells were measured using ELISA, as described previously.^{15,16} Briefly, microtiter plates were coated overnight at 4°C with mAb of two types to anti-human IgE (CIA-E-7.12 and CIA-E-4.15), or anti-human IgA, or anti-human IgG. After the wells were blocked with 0.1% gelatin for at least 1 hr, 100 μ l of culture supernatants were plated in duplicate wells. Then the plates were incubated for 2 hr at room temperature. After washing, alkaline phosphatase-labeled IgE or IgA or IgG was added for detection. Absorbance at 405 nm was read using an ELISA reader (Bio-Tek Instruments, Burlingame, CA, USA). The sensitivity of the assay for the IgE subclasses was 0.1 ng/ml.

RNA EXTRACTION, RT-PCR, REAL-TIME PCR STRATEGY

Total mRNA was obtained from stimulated and non-stimulated human PBMC using a total RNA isolation kit (NucleoSpinTM RNA II; Machery-Nagel, Duren, Germany). Total RNA (0.5 μ g) was reverse-transcribed to cDNA using a first-strand cDNA synthesis kit (GE Healthcare Japan, Tokyo, Japan) under conditions recommended by the manufacturer.

For this study, PCR assays were conducted in 50- μ l reactions containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl₂, 5% DMSO, 50 pM primer/reaction, and 2.5 U of *Taq* polymerase. For detection of PD-ECGF, PCR was conducted using 40 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min. Primers for PD-ECGF were 5'-GCTTCGTGGCCGCTGTGGTG-3' and 5'-TCTGCTCTGGGCTCTGGATGA-3'.^{17,18} Primers for the ϵ -germline transcript were 5'-A

GGCTCCACTGCCCGGCACAGAAATA-3' and 5'-GGACAAGTCCACGTCCATGA-3.¹⁹

The respective amplifications of suppressors of cytokine signaling 1 (SOCS1), SOCS3, SOCS5 and β 2-microglobulin-cDNA were performed in a MicroAmp optical 96-well reaction plate (Applied Biosystems, Branchburg, NJ, USA). All TaqMan probe-primer combinations assay product and Applied Biosystems products were used for this study. Real-time PCR was conducted under conditions recommended by the manufacturer. The threshold cycle number (Ct) was determined using sequence detector software (ver. 1.1, Applied Biosystems) and transformed using Ct methods, as described by the manufacturer, with β 2-microglobulin as the calibrator gene.

WESTERN BLOT

The PBMC were boiled with electrophoresis sample buffer for 3 min and separated using 12.5% SDS-PAGE. The separated proteins were transferred electrophoretically to polyvinylidene difluoride membranes at the same protein concentration per lane. The membranes were blocked at room temperature for 1 h in pH 7.4 PBS with 10% BSA. They were then probed with Ab, as described previously.^{20,21} Band quantities of PD-ECGF expression of PBMC were analyzed from findings of immunoblotting using a Bio Image System (Genomic Solutions, Ann Arbor, MI, USA).

IMMUNOPRECIPITATION

Cells were collected by centrifugation and lysed in triton buffer. The lysate was clarified and incubated with excess protein A-Sepharose 4B. The clarified sample was immunoprecipitated with Ab and protein A-Sepharose 4B at 4°C.^{20,21} The immune complexes were then processed for immunoblotting as described above.

STATISTICS

Data were expressed as mean $\pm$ SEM. The statistical significance of effects on Ig production was determined using the Wilcoxon signed rank test.

RESULTS

PD-ECGF ENHANCED IgE PRODUCTION, BUT NOT VEGF

The basal level of IgE from PBMC for 10 days culture without IL-4 and anti-CD40 mAb was under the limit of ELISA (<0.1 ng/ml). The culture of PBMC with IL-4 and anti-CD40 mAb induced IgE for 10 days (119.4 ± 8.1 ng/ml). Then PBMC was cultured for 10 days with IL-4 and anti-CD40 mAb in various concentrations of recombinant VEGF and recombinant PD-ECGF. As Figure 1 shows, VEGF did not induce enhancement of IgE production significantly in any of seven independent preparations of PBMC. Actually, VEGF did not enhance IgE production between day 7

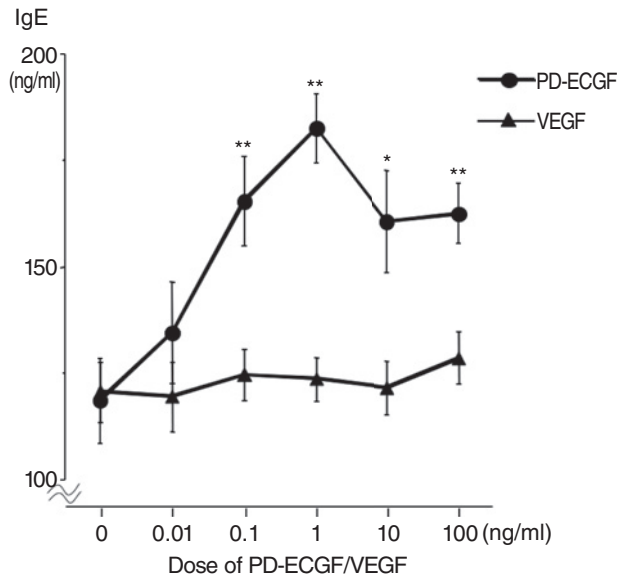


Fig. 1 Effects of VEGF and PD-ECGF on IgE production by PBMC. Human PBMC from non-atopic healthy donors ($n = 7$) was cultured with IL-4 and anti-CD40 mAb for 10 days with various concentrations of VEGF or PD-ECGF. Data were expressed as mean $\pm$ SEM (* $P < 0.05$; ** $P < 0.01$ compared with IgE values for stimulation with IL-4 plus anti-CD40 mAb in the absence of PD-ECGF).

and day 21 (data not shown).

The PD-ECGF enhanced the production of IgE induced by IL-4 plus anti-CD40 mAb in a bell-shaped pattern (Fig. 1). The maximum effect of PD-ECGF on increased IgE production was obtained at concentrations of 1 ng/ml in seven independent preparations of PBMC. The PD-ECGF (1 ng/ml) enhanced production of IgE (154.2% compared to control, $P < 0.01$) by IL-4 plus anti-CD40 mAb without affecting production of IgA (98.5% compared to control). The PD-ECGF (1 ng/ml) also enhanced production of IgG (140.9% compared to control, $P < 0.05$) by IL-4 plus anti-CD40 mAb. Neither VEGF nor PD-ECGF showed any effect on the viability or cell number of PBMC, as shown by trypan blue dye exclusion (data not shown). Actually, PD-ECGF had no effect on cell proliferation of the PBMC cells by deterrence of MTT assay (data not shown).

When purified B cells were cultured with IL-4 and anti-CD40 mAb in the presence of PD-ECGF (1 ng/ml), production of IgE was slightly greater than that in the absence of PD-ECGF (Fig. 2). However, no significant difference was found between the two groups. VEGF did not enhance IgE production by IL-4 plus anti-CD40 mAb in purified B cells (Fig. 2).

2DDR HAD NO EFFECT OF IgE PRODUCTION

The PD-ECGF has thymidine phosphorylase, which catalyzes the reversible phosphorolysis of thymidine

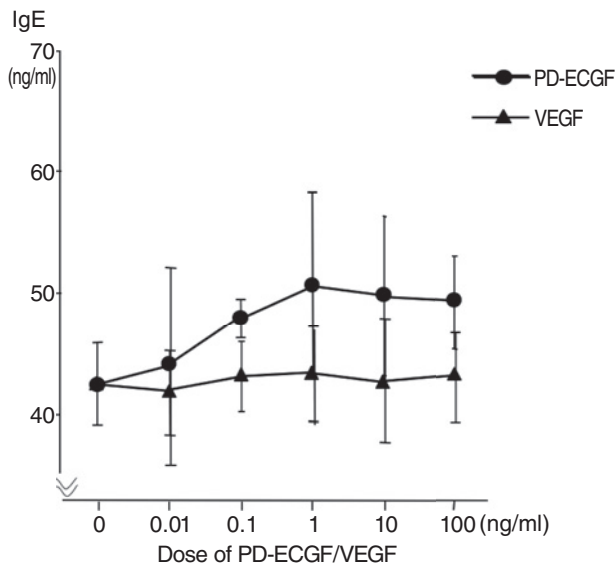


Fig. 2 Effect of VEGF and PD-ECGF on IgE production by B cells. Human purified B from non-atopic healthy donors ($n = 5$) was cultured with IL-4 and anti-CD40 mAb for 10 days with various concentrations of VEGF or PD-ECGF. Data were expressed as mean $\pm$ SEM.

to 2DDR, but which itself has angiogenic activity.¹² We added various concentrations of 2DDR to the culture system to confirm that angiogenic activity is involved with IgE production enhancement by human PBMC. Each addition of 2DDR (0.01-100 ng/ml) showed no effect on IgE production induced by IL-4 plus anti-CD40 mAb (98.3-105.0% compared to control), which suggests that the angiogenic activity caused no direct enhancement of IgE production.

CELL ACTIVATION

It is assumed that PD-ECGF and TP are identical. Tumor-infiltrating macrophages frequently show overexpression of TP, which has been reported to indicate a potential role in increased tumor activity.¹⁰ The level of TP activity in nasal mucosa from patients with allergic rhinitis was significantly higher than that from normal subjects.¹³ To investigate the mechanism of PD-ECGF mediated enhancement of IgE production by human PBMC, the expression of TP in human PBMC was examined. As Figure 3a shows, stimulation of IL-4 and anti-CD40 mAb induced messenger RNA (mRNA) expression of PD-ECGF in all of three independent PBMC. Non-stimulated PBMC showed no expression of PD-ECGF mRNA. The AF-10, B cell lines were used as a positive control expressing PD-ECGF mRNA. Non-stimulated purified B cells showed no expression of PD-ECGF. Stimulation of IL-4 and anti-CD40 mAb also induces a weak band of PD-ECGF expression in purified B cells (data not shown).

Western blot analysis also showed that both IL-4

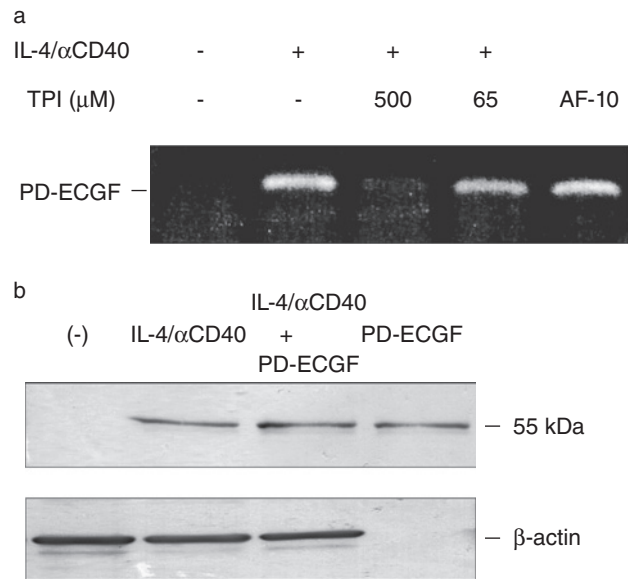


Fig. 3 PD-ECGF expression by PBMC. **a)** PBMC from non-atopic healthy donors was cultured with or without IL-4 and anti-CD40 mAb for 24 hr and RT-PCR was conducted. With AF-10 cells, the B cell line was the positive control for PD-ECGF mRNA expression. **b)** PBMC from non-atopic healthy donors were cultured with or without IL-4 and anti-CD40 mAb for 3 days. Then PD-ECGF protein expression was examined using Western blotting. The PD-ECGF added to the culture system and the culture medium were used as positive controls.

and anti-CD40 mAb induced PD-ECGF protein expression in PBMC (Fig. 3b). Spontaneous PD-ECGF protein expression was not shown in PBMC. A culture system with added PD-ECGF was used as a positive control.

PD-ECGF DID NOT AFFECT IgE CLASS SWITCHING

The Ig isotype switching is preceded by germ-line transcription from the heavy chain locus that will subsequently undergo switch recombination.¹⁹ To assess the induction of ϵ -germline transcript by PD-ECGF, a RT-PCR strategy was undertaken with IgD⁺ B cells. Stimulation of IL-4 induced ϵ -germline transcript by IgD⁺ B cells. PD-ECGF did not induce ϵ -germline transcription by IgD⁺ B cells (data not shown). This result demonstrates that PD-ECGF is not a switch factor for IgE.

THYMIDINE PHOSPHORYLASE INHIBITOR (TPI)

An extremely potent TPI²² was generated in Japan. Figure 3 portrays that TPI inhibited mRNA expression of PD-ECGF. Actually, TPI has been shown to inhibit TP completely *in vitro* with a K_i value of 20 μ M without affecting other enzymes.²³ As Figure 4 shows, the addition of TPI reduced IgE production by

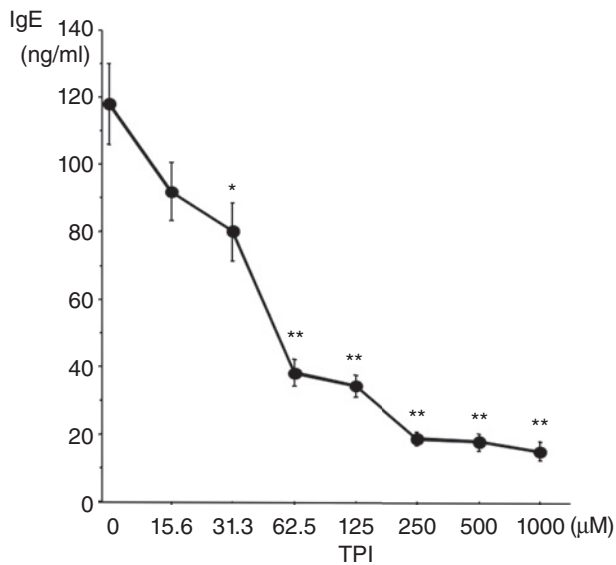


Fig. 4 Effect of TPI on IgE production. Human PBMC from non-atopic healthy donors ($n = 6$) was cultured with IL-4 and anti-CD40 mAb for 10 days with various concentrations of TPI. * $p < 0.05$; ** $p < 0.01$ compared with IgE values for stimulation with IL-4 plus anti-CD40 mAb in the absence of TPI.

PBMC stimulated with IL-4 and anti-CD40 mAb dose-dependently. In all six dependent experiments, at least 31.3 μM TPI significantly inhibited IgE production by PBMC (67.8% compared to control, $P < 0.05$) without affecting production of IgA (89.5% compared to control). The same concentration of TPI also inhibited IgG production by PBMC (70.5% compared to control, $P < 0.05$). Cell viability was unaffected by the treatment of 500 μM TPI (data not shown).

INHIBITION MECHANISM OF TPI ON IgE PRODUCTION

The signal transducer and activator of transcription 6 (STAT6) is an obligatory transcription factor in IL-4 signaling. To analyze whether STAT6 plays a role in regulating IgE production in response to TPI, antiphosphotyrosine immunoprecipitates of cell lysates from B cells treated with or without TPI were subjected to immunoblotting with anti STAT6 Ab. Stimulation of IL-4 and anti-CD40 triggered rapid and sustained phosphorylation of STAT6 (Fig. 5). Addition of PD-ECGF enhanced phosphorylation of STAT6. However, the exposure of IL-4 and anti-CD40 stimulated B cells to TPI blocked the phosphorylation of STAT6, but not expression of STAT6.

DISCUSSION

Results of this study demonstrate that PD-ECGF/thymidine phosphorylase (TP) enhanced IgE production *in vitro* by PBMC stimulated with IL-4 plus anti-CD40 mAb. The angiogenic factors 2DDR (a degradation product of thymidine generated by TP enzy-

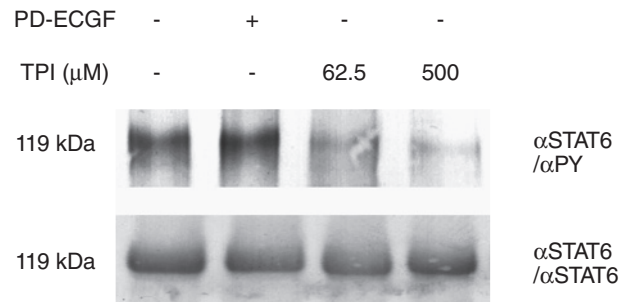


Fig. 5 TPI blocked STAT6 phosphorylation of B cells. Human purified B cells were stimulated with IL-4 and anti-CD40 in the absence or presence of PD-ECGF/TPI for 3 hr and harvested. The sample was immunoprecipitated using anti-phosphotyrosine Ab and blotted with anti-STAT6 Ab. The same amount of lysate as that before immunoprecipitation was applied to each lane and blotted with anti-STAT6 Ab.

matic activity) and VEGF showed no effect on IgE production. Stimulation with IL-4 plus anti-CD40 mAb induced mRNA and the protein of TP in PBMC. The treatment of TPI inhibited the IgE production *in vitro* by PBMC that had been stimulated with IL-4 plus anti-CD40 mAb. The exposure of TPI blocked phosphorylation of STAT6, which was induced by IL-4 and anti-CD40 mAb stimulation. These results suggest that TP is an influential factor on IgE production *in vitro* by a human peripheral blood system.

The PD-ECGF/TP was found in platelet lysate. It is reportedly a classic growth factor that binds its cell receptor to exert angiogenic activity.⁹ In carcinoma cell lines, TP inhibits hypoxia-induced apoptosis via blocking of the activation of p38 mitogen-activated protein kinase (MAPK) but not C-Jun N-terminal kinase (JNK).²⁴ However, p38 MAPK is necessary for CD40-mediated class switching to IgE in human B cells.²⁵ Reportedly, B lymphocyte stimulator activates p38 MAPK in human Ig class switch recombination.²⁶ For that reason, we inferred at the outset of this research that TP might be a candidate of IgE class switching inhibitor via blocking of p38MAPK. Nevertheless, results show that TP enhanced IgE production by PBMC.

IL-4 binds the IL-4 receptor (IL-4R) α subunit. The IL-4R α chain, which is phosphorylated by Jak3, binds to STAT6 via interaction between the phosphotyrosine motif and Src homology 2 domain of STAT6.²⁷ Subsequently, STAT6 itself is phosphorylated by Jak1 and homodimerizes. The dimeric form translocates to the nucleus where it binds to IL-4 response elements, initiating transcription of IgE gene.²⁸ Consequently, STAT 6 is necessary for IgE class switch recombination. The TPI blocked IL-4 plus anti-CD40 induced STAT6 phosphorylation in B cells. Suppressor of cytokine signaling (SOCS) 5, which is expressed predominantly in Th1 cell, inhibits the IL-4-mediated

STAT6 activation.²⁹ Furthermore, IFN γ induces SOCS1 in a STAT1-dependent manner,³⁰ SOCS1 inhibits STAT6 phosphorylation, and TPI did not induce SOCS5 and SOCS1 in B cells in our experiments (data not shown). Therefore, nucleic acid metabolism in cells or unknown factors might be involved in suppression of STAT6-phosphorylation. Further studies must be undertaken to address this important point.

Earlier studies showed TP expression to be upregulated by various inflammatory cytokines of monocyte and macrophage origin such as IL-1 β , TNF α , and IFN γ . This inflammation-mediated upregulation of TP might provide favorable conditions for angiogenesis and malignancy of human tumors.³¹ The infection of nasal mucosa by viruses and bacteria releases IL-1 β and TNF α from epithelial cells, fibroblasts, and basal cells. Reportedly, several cells of mucosal epithelium have high TP in normal tissues such as fibroblast and basal cells.³² Consequently, the released IL-1 β and TNF α might enhance TP-production of monocytes and macrophages, fibroblasts, and basal cells by the autocrine system. Increased TP might enhance IgE production to antigens in nasal mucosa because we demonstrated local IgE class switching and local IgE syntheses in human allergic nasal mucosa.¹⁵ This speculation might engender clinical worsening of allergic symptoms by mild virus infection in patients with allergic rhinitis.

Certainly, PD-ECGF/TP is not a class switch factor for IgE because PD-ECGF did not induce ϵ -germline transcript. Perhaps PD-ECGF/TP reinforces the signals of IL-4 plus anti-CD40 mAb. We demonstrated in an earlier study that IL-4 plus anti-CD40 mAb induces Ig isotype switch for μ to γ 1, γ 3, and γ 4.³³ The result that PD-ECGF enhanced IgG production by IL-4 plus anti-CD40 mAb is consistent with that result. When PD-ECGF is added to the PBMC culture system in the absence of IL-4 plus anti-CD40 mAb, IgG production was not increased (data not shown).

Although VEGF has been demonstrated to serve an important role in the pathogenesis of allergic rhinitis⁴, VEGF itself apparently has no effect on IgE production *in vitro* in this experiment. Clinical data also show a nonsignificant correlation between serum IgE and sputum VEGF in asthmatic children.³⁴

As a potent antiangiogenic factor, endostatin prevented the development of asthma in one study using a mouse model.³⁵ Treatment with endostatin inhibits the airway hyperresponsiveness, pulmonary allergic inflammation, production of antigen-specific IgE, and lung inflammatory mediators in VEGF-dependent and VEGF-independent mechanisms. Antiangiogenesis therapy by antiangiogenic medicine reportedly reduces asthma symptoms in patients with rheumatoid arthritis and moderately severe asthma.³⁶ A new anti-tumor drug, TAS102, is combined for TPI and cytotoxic pyrimidine analog for patients with cancer. Cur-

rently, TAS102 is being evaluated in phase I studies as an oral formulation.³⁷ Results of this study and evidence from the literature suggest that TPI is an effective new method of inducing protection against IgE-related allergic disease.

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