TABLE 2

Frequencies of Thromboxane A2 Receptor 795TT Genotype and Serum IgE Concentrations in Patients with Atopic Dermatitis

	IgE (IU/ml)	*n*	TT	Frequency of TT (%)
Control		178	64	36.0
Atopic dermatitis total		360	139	38.6
	<500	93	37	39.8
	>500	264	102	38.6
	>1000	228	91	39.9
	>2000	199	77	38.7
	>3000	175	74	42.3
	>4000	155	65	41.9
	>5000	128	58	45.3
	>6000	110	55	50.0
	>7000	100	52	52.0
	>8000	89	52	58.4
	>9000	80	45	56.3
	>10000	72	42	58.3

enrichment for the genetic variant of TXA2 receptor amongst the AD population ($P = 0.549$). However, the frequency of individuals homozygous for TXA2 receptor 795T allele was significantly increased in AD patients with very high IgE concentrations (IgE >8000 IU/ml; Odds ratio = 2.50, 95% CI: 1.49–4.22, $P = 0.0004$). Patients with extensive AD showed a borderline association with TXA2 receptor 795T homozygosity ($P = 0.050$). Neither AD patients with asthma nor those with rhinitis showed a significant association with this variant of TXA2 receptor ($P = 0.470$ and 0.307, respectively). There was no significant association of the TXA2 receptor variant with AD patients who showed positive house dust mite specific IgE titre ($P = 0.674$). The distribution of TXA2 receptor genotypes did not differ between controls and asthmatics without concomitant AD ($P = 0.529$). Allergic rhinitis patients without concomitant AD did not show an association with this variant ($P = 0.406$).

Frequencies of homozygosity for TXA2 receptor 795T genotype increased steadily according to the elevation of serum total IgE concentrations in patients with atopic dermatitis (Table 2).

Details of the genotype frequencies of IL-4 and IL-4Rα were described elsewhere (7). AD patients who had TXA2 receptor TT genotype and IL-4Rα Ile/Ile genotype showed very high IgE concentrations (>8000 IU/ml) more frequently than patients with TXA2 receptor CC or CT genotype and IL-4Rα Val/Val or Val/Ile genotype (Odds ratio 7.56 [95% CI 2.79–20.50], $P = 0.00005$). AD patients with TXA2 receptor TT and IL-4 CT or TT genotype did not show very high IgE concentrations compared to patients with TXA2 receptor CC or CT and IL-4 CC genotype (Table 3).

AD patients with TXA2 receptor TT genotype and IL-4Rα Ile/Ile genotype showed extensive disease compared to patients with TXA2 receptor CC or CT genotype (Odds ratio 3.36 [95% CI 1.38–8.20], $P = 0.007$) (Table 4).

DISCUSSION

We have shown that a genetic variant of TXA2 receptor is associated with AD with very high serum IgE levels, whereas there are no significant association with AD in general. The results of our study suggest that this variant of TXA2 receptor plays little role in the susceptibility of AD in general. On the other hand, our results indicate that this variant has a strong effect on elevation of serum total IgE levels, particularly in a very high range of serum IgE levels (>8000 IU/ml), in patients with AD. Interestingly, the asthmatics or the rhinitis patients without concomitant AD seldom showed the extreme values greater than 8000 IU/ml in our population, suggesting that the effect of this variant might be specific to AD. The mechanism underlying such a great elevation of IgE levels in AD remains unclear. The prevalence of TXA2 receptor 795TT genotype increased steadily according to the elevation of IgE levels in patients with AD, and it is difficult to define a "borderline" for extreme values. Although patients with AD often show elevated IgE productivity, there are some patients without any evidence of elevated IgE productivity. Thus the pathogenic role of high IgE in AD remains to be established. Most AD patients with the extreme values greater than 8000 IU/ml showed extensive disease, in which >70% body

TABLE 3

Total Serum IgE Concentration of Patients with Atopic Dermatitis and Genetic Variants of TXA2 Receptor, IL-4, and IL-4Rα

				IgE (IU/ml)			
TXA2 receptor genotype	IL-4 genotype	IL-4Rα genotype	*n*	<8000	>8000	Odds ratio (95% CI)	*P*
CC + CT	CC		6	4 (67%)	2 (33%)		
TT	CT + TT		56	31 (55%)	25 (45%)	1.61 (0.27–9.54)	0.468
CC + CT		Val/Val + Val/Ile	65	56 (86%)	9 (14%)		
TT		Ile/Ile	31	14 (45%)	17 (55%)	7.56 (2.79–20.50)	0.00005

TABLE 4

Extent of Atopic Dermatitis and Genetic Variants of TXA2 Receptor and IL-4Rα

TXA2 receptor genotype	IL-4Rα genotype	*n*	Extensive*	Odds ratio (95% CI)	*P*
CC + CT	Val/Val + Val/Ile	65	19 (29%)		
TT	Ile/Ile	31	18 (58%)	3.36 (1.38–8.20)	0.007

* >70% of body surface was affected.

surface was affected, in this Japanese population. Therefore, there is a possibility that other factors, like the extent and the duration of the disease, which affect the elevation of IgE levels relate to this variant of TXA2 receptor.

Although Unoki *et al.* reported an association between a genetic variant of TXA2 receptor and bronchial asthma (11), we could not find a significant association between TXA2 receptor 795C/T polymorphism and asthma. Patients with bronchial asthma often have histories of AD (14), and asthmatics in the previous study might have included a large proportion of patients who had AD. Further studies with appropriate classification of phenotypes are needed.

IL-4Rα plays an important role in the initiation of isotype switching to IgE in human B cells, and functional assays in both human and murine B cell lines revealed that the Ile50 variant of IL-4Rα upregulates IgE synthesis (18). In contrast, TXA2 receptor is expressed within the immune system on monocytes and it has been reported that TXA2 receptor induces the production of the immunosuppressive cytokine transforming growth factor β1 and modulates T cell activation (19). In our previous study the IL-4Rα Ile50 variant did not itself show a strong association with elevation of IgE levels in AD (7). However, our data here suggest a strong interaction between TXA2 receptor TT and IL-4Rα Ile/Ile genotypes. It is possible that the genetic variant of IL-4Rα acts as an inducer of the IgE synthesis and the variant of TXA2 receptor synergizes with IL-4Rα as a disease-modifying gene in chronic inflammation of AD and extreme elevation of IgE. Since the severity of AD is highly correlated with the levels of serum IgE and severe chronic cases show the most extreme values (9), it may be possible to utilize the genetic variants of TXA2 receptor and IL-4Rα as predictors of high IgE levels, and of severe chronic AD. The population we studied included few mild cases of AD and a study using another population including mild cases with a clear definition for severity of AD is now needed. Our data suggest that genetic variants of the receptors for TXA2 and IL-4 may directly associate with severity and course of AD, and may ultimately be useful in the prediction and the management of severe chronic cases of AD.

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Expression of receptor activator of NF-kappa B ligand and osteoprotegerin in culture of human periodontal ligament cells

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The receptor activator of NF-kappa B ligand (RANKL) and its decoy receptor, osteoprotegerin (OPG), are the important proteins implicated in osteoclastogenesis. In this study, we investigated the expressions of RANKL and OPG in cultured human periodontal ligament (PDL) cells and their roles in osteoclastogenesis. Northern blotting revealed that the OPG mRNA was down-regulated remarkably by application of 10^{-8} M one-alpha, 25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and 10^{-7} M dexamethasone (Dex). In contrast, RANKL mRNA was up-regulated by the same treatment. Western blotting demonstrated decrease of OPG by the application of 1,25-$(OH)_2D_3$ and Dex. Tartrate-resistant acid phosphatase-positive multinuclear cells were markedly induced when the PDL cells were cocultured with mouse bone marrow cells in the presence of an anti-OPG antibody together with 1,25-$(OH)_2D_3$ and Dex. These results indicate that PDL cells synthesize both RANKL and OPG and that inactivation of OPG may play a key role in the differentiation of osteoclasts.

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Osteoclasts, the multinucleated cells that resorb bone, develop from hematopoietic cells of the monocyte/macrophage lineage (1–3). It has been demonstrated that osteoblasts or stromal cells play a role in osteoclast differentiation, and interaction of these cells with osteoclast precursors is important for the osteoclastogenesis (1–3). Recently, the receptor activator of NF-kappa B (RANK) ligand (RANKL) (4) [also known as osteoprotegerin (OPG) ligand (OPGL) (5, 6), osteoclast differentiation factor (ODF) (7) or tumor necrosis factor (TNF)-related activation-induced cytokine (8, 9)] and OPG (10) [also known as osteoclastogenesis inhibitory factor (11, 12)] were identified. RANKL, a polypeptide ligand for OPG, is a member of the TNF family of cytokines and promotes differentiation and activation of osteoclasts (5–7). It was also indicated that the effect of RANKL was not species restricted (6). OPG is a member of TNF receptor superfamily and was shown to suppress bone resorption by inhibiting osteoclastogenesis (10–12). RANKL and OPG are considered to act as positive and negative regulators of osteoclast differentiation, respectively (2, 3, 5–7, 10–12).

The periodontal ligament (PDL) is situated between the alveolar bone and the cementum of the tooth root (13–15). Recent reports showed that PDL cells expressed OPG mRNA (16) and produced OPG protein *in vitro* (17). The secreted OPG had an ability to inhibit osteoclast differentiation in mouse bone marrow cultures (17). In this context, human PDL cells are likely to prevent resorption of hard tissues such as bone and tooth root

through the OPG-dependent mechanism. There is no report of RANKL expression in PDL cells. However, osteoblasts are known to express RANKL (2, 3, 5–7, 18–20) as well as OPG, and PDL cells show functional similarity in hard tissue formation to osteoblasts (14, 15). It is an open question whether the expressions of RANKL and OPG in PDL cells are altered when the PDL cells are treated with bone-resorbing factors such as one-alpha, 25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and Dexamethasone (Dex).

Here we have examined expressions of RANKL and OPG mRNAs in human PDL cells and aimed to clarify the relation of OPG production and osteoclast formation *in vitro*. We employed RT-PCR for the detection of RANKL and OPG mRNAs in the PDL cells cultured in the presence or absence of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex. The amount of these mRNAs was analyzed by Northern blotting, and OPG protein produced by the PDL cells was analyzed by Western blotting. Furthermore, induction of osteoclasts that can be identified as tartrate-resistant acid phosphatase (TRAP)-positive cells (1–3, 18–26) was investigated in cocultures of mouse bone marrow cells and human PDL cells. We also investigated whether the TRAP-positive cells induced in the cocultures could resorb mineralized tissues by using scanning electron microscopy (SEM).

Materials and methods

Cell culture

PDL cells were obtained from the middle third of the root surface of healthy human premolars ($n = 20$, donors' age: 10 to 12 years old) as described previously (27). Informed consent was obtained from all of the donors before tooth extraction, which was carried out during the course of orthodontic treatment in our hospital. PDL tissues were cut into pieces using a surgical blade and digested by collagenase (2 mg/ml) at 37°C for 30 min. Then the tissues were washed by Dulbecco's phosphate-buffered saline and placed on culture dishes and maintained in alpha-modified minimum essential medium (alpha-MEM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL). Fibroblastic cells outgrew from the PDL tissues 8 used as PDL cells (Fig. 1). When the cells had reached confluence, they were detached with 0.2% trypsin and 0.02% EDTA 4Na in phosphate-buffered saline and subcultured in a 1 : 4 split ratio. All of the experiments were performed using cells from three to five passages cultured in alpha-MEM supplemented with 10% FBS in the absence or presence of 10^{-8} M 1,25-$(OH)_2D_3$ (Wako Pure Chemicals, Tokyo, Japan) and/or 10^{-7} M Dex (Sigma Chemical Co., St. Louis, MO, USA) for 6 d. All of the cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Fig. 1. Phase-contrast microograph of the PDL cells in passage 4. Most of the cells showed fibroblast-like appearance. Bar = 50 μm.

Isolation of total RNA

Total RNA was extracted from the cultured PDL cells with the use of ISOGEN (Nippon Gene, Tokyo, Japan) as described previously (24, 27–29). The pellet of total RNA was washed briefly with 75% ethanol, resuspended in 30 μl of DEPC-treated water, and stored at −80°C. The concentration of the total RNA was determined by measuring the optical density at 260 nm with the use of a spectrophotometer.

Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was treated with DNase I (Takara Shuzo Co. Ltd, Kyoto, Japan) for 1 h at 37°C to remove genomic DNA. Single-strand cDNA was synthesized from 2.0 μg of the treated RNA by incubating for 1 h at 42°C with SuperScript™ II Rnase H⁻ reverse transcriptase (Gibco BRL) and oligo $(dT)_{12-18}$ primer (Gibco BRL).

To amplify the DNA fragment coding the partial RANKL and OPG, primers were constructed on the basis of the sequences of RANKL and OPG (6, 10) as described previously (24). The sizes of the amplified DNA and the primers used in the present study were as follows: RANKL (605 bp, nucleotide; 918–1520), 5′-CCAGCAT CAAAATCCCAAGT-3′ (5′ primer) and 5′-CCCCTTCAGATGATCCT TC-3′ (3′ primer), OPG (575 bp, nucleotide; 362–934), 5′-TGCAGTACGT CAAGCAGGAG-3′ (5′ primer) and 5′-TGACCTCTGTGAAAACAGC-3′ (3′ primer), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (306 bp, nucleotide; 78–384), 5′-CGG AGTCAACGGATTTGGTCGTAT-3′ (5′ primer) and 5′-AGCCTTCTCCA TGGTGGTGAAGAC-3′ (3′ primer) (24, 27). The reaction mixture contained 12.5 pmol of each primer, 20 mM dATP, dGTP, dCTP, and dTTP (Perkin Elmer, Norwalk, CT, USA), 2.5 μl of 10 × PCR buffer, 0.5 U of Taq DNA polymerase (Perkin Elmer) and distilled water. The mixed solution was overlaid with 50 μl of mineral oil (Sigma), and PCR was performed for 35 cycles at 94°C for 1 min, at 56°C for 1 min, and at 72°C for 1 min in a DNA Thermal cycler (model 480; Perkin Elmer). Then, 8 μl of the PCR product was mixed with 2 μl of bromophenol blue-loaded buffer [0.25% bromophenol blue, 0.35% xylene cyanol, 40% (w/v) sucrose in water] and electrophoretically separated in a 1% (w/v) agarose gel in TAE buffer. After staining with 0.5 μg/ml ethidium bromide, bands of the PCR products were visualized with ultraviolet light and photographed with polaroid film.

Northern blot analysis

Total RNA was prepared as described above and separated on a 1.2% agarose-formaldehyde gel. The separated mRNA was transferred onto a GeneScreen Plus membrane (Dupont, Boston, MA, USA) and hybridized with [^{32}P]dCTP-labeled cDNA of RANKL (nucleotide, 918–1520), OPG (362–934) and GAPDH (78–384) as described previously (28, 29). The ^{32}P signal on the fixed membrane was analyzed by using a BAS 2000 Bio-Imaging Analyzer (Fuji Film, Tokyo, Japan).

Western blot analysis for OPG

OPG protein assay was carried out by Western blot analysis. Since OPG protein is secreted from cells into the medium in a culturing condition (10–12, 17, 24, 30, 31), OPG was extracted from the medium after 6 d of culture. PDL cells were cultured in alpha-MEM supplemented with 10% FBS in the absence or presence of 10^{-8} M 1,25-$(OH)_2D_3$ and/or 10^{-7} M Dex for 6 d. Before the collection of conditioned medium for Western blotting, medium was exchanged for 1 ml serum-free medium. After 7 h culture, we collected the conditioned medium as a sample. Then 20 μl of the conditioned media was dissolved in SDS buffer without dithiothreitol, incubated at 95°C for 5 min, and resolved electrophoretically on 10% SDS-polyacrylamide gels. Serum-free medium was used as a negative control of the conditioned medium. Proteins were electrophoretically transferred to a PVDF membrane (Immobilon Transfer Membrane; Nihon Millipore Ltd, Japan). After blocking, the membrane was incubated overnight at 4°C with a goat antihuman OPG antibody (R & D Systems, Minneapolis, MN, USA) diluted 1 : 2000 in TBST buffer (10 mM Tris HCl pH 7.0, 100 mM NaCl, 0.1% Tween20). The blots were further incubated with a rabbit antigoat secondary antibody (ZYMED Laboratories Inc., South San Francisco, USA) for 1 h at room temperature. Specific protein bands on the membrane were detected by using an enhanced AP conjugate substrate kit (BIO RAD Laboratories, California, USA) as described previously (24, 27, 30, 32).

Osteoclastogenesis *in vitro*

Bone marrow cells from femurs of 3 to 5-week-old C57BL/6 mice were inoculated at 1×10^5 cells/well onto confluent PDL cell layers in 24-well plates (Corning Glass Inc., NY, USA) as described previously (21–26, 30, 31, 33). The cultures were maintained in alpha-MEM supplemented with 10% FBS in the absence or presence of 10^{-8} M 1,25-$(OH)_2D_3$ and/or 10^{-7} M Dex for 6 d. The adherent cells were fixed with 10% formalin in phosphate-buffered saline for 10 min and treated with ethanol-acetone (50 : 50, v/v) for 1 min. TRAP staining was carried out by incubating the cells in 0.1 M sodium acetate buffer (pH 5.0) containing AS-MX phosphate (Sigma) and red violet LB salt (Tokyo Kasei Co., Tokyo, Japan) in the presence of 50 mM sodium (21–26, 30, 31). Induction of TRAP-positive multinuclear cells (MNCs) in the cocultures was further examined by treating the cells with a goat antihuman OPG antibody (1 μg/1 ml, R & D Systems Inc.) that was added to the medium containing 10^{-8} M 1,25-$(OH)_2D_3$ and/or 10^{-7} M Dex during the 6 d of culture. MC3T3-E1 cells were used as supporting cells of the osteoclast formation in coculture instead of PDL cells (24, 25).

The use of animals in this study was approved by the Animal Care and Use Committee of our university and was in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Scanning electron microscopy

To determine whether TRAP-positive MNCs could resorb dentin, we performed resorption pit assay (6, 25). After bovine dentin slices (4 mm in diameter) had been placed in 24-well plates, we cultured PDL cells and mouse bone marrow cells on the slices in the presence of 10^{-8} M 1,25-$(OH)_2D_3$, 10^{-7} M Dex and the goat antihuman OPG antibody (R & D Systems Inc.) for 6 d. Dentin slices that were denuded of attached cells were washed in distilled water, dehydrated in ethanol, and critical-point-dried with liquid CO_2. The slices were treated with 1% aqueous osmium tetroxide, mounted on conductive stubs, and then sputter coated with platinum and palladium in an ion coater (Hitachi E-1030, Hitachi, Tokyo, Japan). The specimens of the dentin slices were examined using SEM (Hitachi S-4000, Hitachi) at an accelerated voltage of 5 keV and photographed with polaroid film.

Statistical analysis

Results are expressed as means ± SD, Statistical differences between groups were determined using one-way analysis of variance followed by Bonferroni comparisons between pairs of groups, Data with a P-value < 0.01 were considered statistically significant.

Results

RANKL and OPG mRNA expression in PDL cells treated with 1,25-$(OH)_2D_3$/Dex

RANKL and OPG mRNAs were detected by RT-PCR in the PDL cells

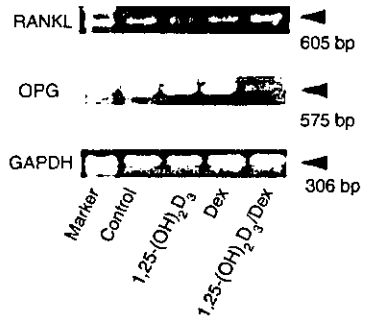

Fig. 2. RT-PCR analysis of RANKL and OPG mRNA expressions in cultures of PDL cells. RANKL (upper panel), OPG (middle panel) and GAPDH (lower panel) mRNA expressions were detected in all of the PDL cell cultures examined. PCR products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide (marker: *ø*X174/*Hae*III digest).

cultured with the control medium or the medium containing 10^{-8} M 1,25-$(OH)_2D_3$ and/or 10^{-7} M Dex. Specific bands of 605 bp and 575 bp corresponding to RANKL and OPG mRNAs, respectively, are shown in Fig. 2. The sequences of these PCR products were 100% identical with those of RANKL and OPG (6, 10, 24).

Northern blot analysis showed slight decrease of OPG mRNA in cultures of PDL cells treated with either 10^{-8} M 1,25-$(OH)_2D_3$ or 10^{-7} M Dex, The OPG expression was dramatically decreased by the combination of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex (Fig. 3). In contrast, RANKL mRNA was increased by the treatment with either 10^{-8} M 1,25-$(OH)_2D_3$ or 10^{-7} M Dex, and the level was elevated synergistically by the combination of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex.

Effect of 1,25-$(OH)_2D_3$/Dex on OPG production by PDL cells

As shown in Fig. 4A, secreted OPG was present as monomer and dimer molecules in the conditioned medium of the PDL cell culture. Both monomer and dimer OPG proteins were significantly decreased by the treatment with either 10^{-8} M 1,25-$(OH)_2D_3$ or 10^{-7} M Dex and by the cocktail of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex (Fig. 4A,B).

These data are consistent with the results of Northern blot analysis. No bands were detected in the negative control.

Osteoclastogenesis *in vitro*

Osteoclastogenesis did not occur when bone marrow cells and PDL cells were cocultured in the control medium or in the control medium supplemented with 1 μg/ml of the anti-OPG antibody for 6 d (Fig. 5A). Furthermore, in the presence of either 10^{-8} M 1,25-$(OH)_2D_3$ or 10^{-7} M Dex, the PDL cells failed to induce osteoclast formation, and the addition of the antihuman OPG antibody had no effect (Fig. 5A). However, by coadministration of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex, TRAP-positive mononuclear cells and TRAP-positive MNCs (1.3 ± 1.2/well) were induced (Fig. 5A,B). In the presence of 1 μg/ml of the antihuman OPG antibody together with 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex for 6 d, the number of TRAP-positive MNCs markedly increased (Fig. 5A,C).

MC3T3-E1 cells, one of the mouse osteoblast cell lines, were used instead of PDL cells as a positive control for supporting osteoclastogenesis. MC3T3-E1 cells did not induce TRAP-positive MNCs in the control medium but could induce many TRAP-positive MNCs by coadministration of 10^{-8} M 1,25$(OH)_2D_3$ and 10^{-7} M Dex without antihuman OPG antibody (Fig. 5D).

Resorption pit assay

TRAP-positive MNCs, which were induced in the coculture of human PDL cells and mouse bone marrow cells in the presence of 1 μg/ml of the antihuman OPG antibody together with 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex for 6 d, had the ability to resorb dentin. Resorption pits were observed on the surface of the bovine dentin by SEM (Fig. 5E).

Discussion

In this study, we demonstrated the expression of both RANKL and OPG mRNAs in cultures of human PDL cells by RT-PCR. Northern blot analysis showed that the mRNA levels were altered by the treatment with osteotropic factors such as 1,25-$(OH)_2D_3$ and Dex. Western blot analysis showed that production of OPG protein in PDL cells was suppressed by the treatment with 10^{-8} M 1,25-$(OH)_2D_3$ and/or 10^{-7} M Dex. The ability of PDL cells to support osteoclastogenesis was examined by coculturing with C57BL/6 mouse bone marrow cells, We demonstrated that the PDL cells could induce TRAP-positive MNCs from mouse osteoclast progenitor cells when bioactivity of the secreted OPG from the PDL cells was normalized by an antihuman OPG antibody in the presence of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex. SEM observation revealed that TRAP-positive MNCs differentiated from mouse osteoclast progenitor cells could resorb bovine dentin.

The recent report showed that OPG mRNA was expressed in PDL cells *in vitro and* the expression was modulated by treatments with various cytokines (16). It was also shown that OPG protein was produced by PDL cells and inhibited osteoclastogenesis in the culture of mouse bone marrow cells (17). We confirmed these properties of human PDL cells and indicated that expression of OPG mRNA and protein could be suppressd by the treatment

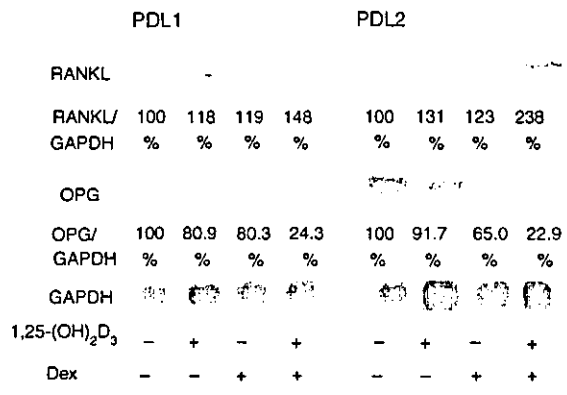

Fig. 3. Effects of osteotropic factors on the expression levels of RANKL and OPG mRNAs in cultures of the PDL cells derived from two donors. Total RNA (20 μg/lane) was analyzed by Northern blotting using probes of human RANKL, OPG and GAPDH. The PDL cells were exposed to the indicated reagents for 6 d. The mRNA levels of RANKL (upper panel), OPG (middle panel) and GAPDH (lower panel) are shown. Under each panel, the ratios of RANKL and OPG to GADPH are indicated as a percentage of the control value (set at 100%). This Northern blot is representative of three experiments.

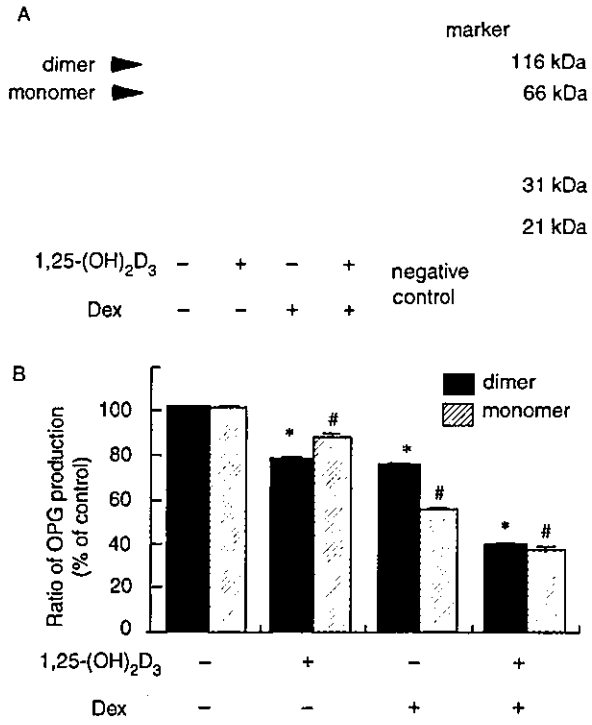

Fig. 4. Production of OPG in cultures of PDL cells. Conditioned media were collected and analyzed by Western blotting under non-reducing condition. (A) Production of OPG, which was detected as bands of both dimers and monomers, was down-regulated by the treatment with the indicated reagents. This Western blot is representative of three experiments. (B) Ratios of OPG in the conditioned media to the OPG in the normal medium (control values were normalized to 100%). Data are expressed as means ± SD of three experiments. An asterisk (*) represents significant change from the control value of dimer ($P < 0.01$). A sharp mark (#) indicates significant change from the control of monomer ($P < 0.01$).

with 1,25-$(OH)_2D_3$ and Dex. Western blotting revealed that the PDL cells produced both monomer and dimer of OPG *in vitro* (Fig. 4A). As both forms of OPG are shown to inhibit osteoclastogenesis (11), both monomer and dimer of OPG produced by the PDL cells should have an ability of inhibiting osteoclastogenesis.

It has been shown that RANKL is highly expressed in osteoblastic cells and stromal cells (2, 3, 5–7, 18–20). Since RANKL expressions in osteoblasts were modulated by treatments with 10^{-8} M 1,25-$(OH)_2D_3$ and/or 10^{-7} M Dex (6, 18, 19), we investigated whether the expression levels of RANKL mRNAs in PDL cells could be modulated by these factors. Similar to the previous findings in osteoblasts and stromal cells (6, 18, 19), RANKL mRNA in the PDL cells was up-regulated by the treatments with 10^{-8} M 1,25-$(OH)_2D_3$ and/or 10^{-7} M Dex (Fig. 3). Especially, the RANKL mRNA expression was increased synergistically by the coadministration of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex for 6 d.

As RANKL is a potent inducer of osteoblast differentiation and activation (2, 3, 5–7, 18, 19), and the PDL lies between the alveolar bone and the cementum of the tooth root (13), it is important to determine whether the RANKL protein expressed in PDL cells can induce osteoclastogenesis or not. As we described previously (24, 25), even when the bone marrow cells derived from C57BL/6 mice (cell density: 1×10^5 cells/well in 24-well plates) were cultured in the presence of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex, they did not differentiate into TRAP-positive MNCs when osteoclastogenesis-supporting cells were absent in the culture. Because C57BL/6 mouse bone marrow cells could differentiate into TRAP-positive MNCs only when they were cocultured with osteoclastogenesis-supporting cells (24, 25), we aimed to induce osteoclastogenesis as a positive control by using MC3T3-E1 cells. A large number of TRAP-positive MNCs were induced in the presence of 10^{-8} M 1,25$(OH)_2D_3$ and 10^{-7} M Dex (Fig. 5D).

Since Lacey *et al.* indicated that the effect of RANKL on the osteoclastogenesis is not species restricted (6), the ability of PDL cells to support osteoclastogenesis was examined by coculturing with C57/BL/6 mouse bone marrow cells. TRAP-positive MNCs were not induced when the bone marrow cells and the PDL cells were cocultured in the control medium or in the control medium supplemented with 1 μg/ml of the antihuman OPG antibody (Fig. 5A). Similarly, treatment of the cocultures with either 10^{-8} M 1,25-$(OH)_2D_3$ or 10^{-7} M Dex had no effect regardless of the presence of the antihuman OPG antibody. After the treatment with 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex, TRAP-positive MNCs were only sparsely observed (Fig. 5A,B), although RANKL mRNA expression was increased to 148–238% of the control level and OPG mRNA expression was apparently reduced (Fig. 3). In contrast, the number of the MNCs markedly increased when the coculture was treated with 1 μg/ml of the antihuman OPG antibody together with 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex (Fig. 5A,C). Since OPG protein was reduced but still detectable in the medium after the treatment with 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex (Fig. 4), it is most likely that further administration of the antihuman OPG antibody coculture eventually eliminated the OPG activity. These results indicate that the marked increase in TRAP-positive MNCs in the coculture was due to the activation of RANKL that occurred as a result of the neutralization of OPG. In other words, OPG secreted from the PDL cells was functionally active and inhibited osteoclastogenesis by suppressing RANKL activity *in vitro*. It was shown that TRAP-positive MNCs

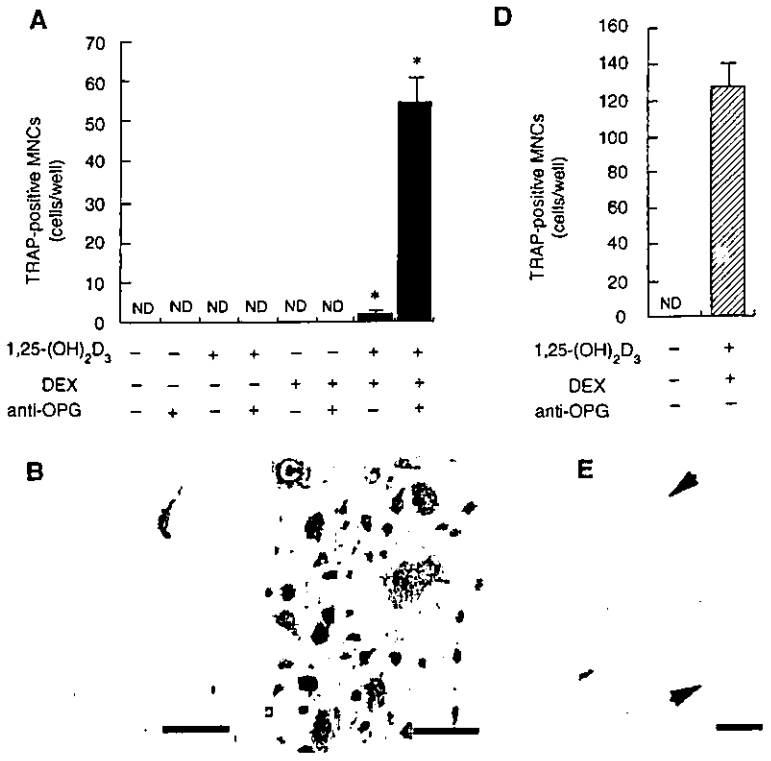

Fig. 5. PDL cells induce osteoclastogenesis *in vitro*. (A) The number of TRAP-positive MNCs that contain two or more nuclei. Data are presented as means ± SD (n = 6/column). Asterisks indicate that the difference between these values is statistically significant ($P < 0.01$). (B) A few TRAP-positive MNCs were induced by the combination of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex. Bar = 50 μm. (C) TRAP-positive MNCs were induced abundantly by the treatment with 1 μg/ml of the antihuman OPG antibody together with 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex. Bar = 50 μm. (D) The number of TRAP-positive MNCs induced when bone marrow cells and MC3T3-E1 cells were cocultured for 6 d. Data are expressed as means ± SD (n = 4/column). MC3T3-E1 cells could support osteoclastogenesis in the presence of 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex. (E) Scanning electron micrograph of resorption pits (arrowheads) on the surface of the bovine dentin. The resorption pits were formed when mouse bone marrow cells and human PDL cells were cocultured on the dentin slice in the presence of 10^{-8} M 1,25-$(OH)_2D_3$, 10^{-7} M Dex, 1 μg/ml of the antihuman OPG antibody. Bar = 50 μm.

induced *in vitro* had the ability to resorb hard tissues like dentin (1, 2, 6, 17, 21–23, 25, 26). To determine whether the TRAP-positive MNCs in the coculture of bone marrow cells and PDL cells had the ability to resorb dentin or not, we performed resorption pit assay.

After coculturing mouse bone marrow cells with PDL cells on dentin slices in the presence of 1 μg/ml antihuman OPG antibody together with 10^{-8} M 1,25-$(OH)_2D_3$ and 10^{-7} M Dex for 6 d, we confirmed resorption pits on the surface of the dentin slices by SEM (Fig. 5D). The results clearly indicate that TRAP-positive MNCs derived from mouse bone marrow cells can resorb mineralized tissues.

In this study, we demonstrated that PDL cells express both RANKL and OPG, representative local regulators of osteoclastogenesis. As indicated in Fig. 5, TRAP-positive MNCs were rarely observed when PDL cells and bone marrow cells were cocultured in the presence of 1,25-$(OH)_2D_3$ and 10^{-7} M Dex, but not the antihuman OPG antibody. This is comparable to the induction of TRAP-positive MNCs in the coculture of MC3T3-E1 cells and bone marrow cells and may explain the function of PDL cells in keeping the tooth root intact by inhibiting osteoclastogenesis in the physiological environment (17). Further study of RANKL and OPG expressions in the PDL tissue may provide an insight into the mechanism through which the alveolar bone and the tooth maintain their functions and structures.

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Differential regulation of thymus- and activation-regulated chemokine induced by IL-4, IL-13, TNF-α and IFN-γ in human keratinocyte and fibroblast

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Abstract

The CC chemokine thymus- and activation-regulated chemokine (TARC/CCL17) acts on CC chemokine receptor 4 (CCR4), which is known to be selectively expressed in Th2 cells. In order to compare the regulatory profiles of TARC production by tumor necrosis factor-α (TNF-α), IFN-γ, interleukin-4 (IL-4) and IL-13 in keratinocytes and fibroblasts, HaCaT cells, a human keratinocyte cell line, and NG1RGB cells, a human skin fibroblast cell line, were used. The expression of TARC protein was measured using enzyme-linked immunosorbent assay (ELISA), and the mRNA level was detected by reverse transcriptase polymerase chain reaction (RT-PCR). The spontaneous expression of TARC protein and mRNA levels were augmented by TNF-α and IFN-γ and were inhibited by IL-4 and IL-13 in the keratinocytes. The fibroblasts expressed the TARC protein and mRNA only in the presence of IL-4+TNF-α or IL-13+TNF-α stimulation. IFN-γ further enhanced the IL-4+TNF-α or IL-13+TNF-α-induced TARC production in the fibroblasts. Thus, TNF-α and IFN-γ -induced TARC production was differentially regulated by IL-4 and IL-13 in human keratinocytes and fibroblasts.

Keywords: Keratinocytes; Fibroblasts; Thymus-and activation-regulated chemokine; TNF-α; IFN-γ; IL-4; IL-13

1. Introduction

Chemokines, a family of low-molecular-weight proteins that induce specific types of leukocyte chemotaxis, play essential roles in regulating extravasation and tissue accumulation of lymphocytes during immune and inflammatory responses [1,2]. Thymus-and activation-regulated chemokine (TARC/CCL17) was identified by cloning the

Abbreviations: CCR4, CC chemokine receptor 4; TARC, thymus-and activation-regulated chemokine.

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D3A gene from peripheral blood mononuclear cells (PBMCs) after stimulation with PHA [3]. TARC is a basic protein with a predicted molecular weight of 8 kDa, and is produced by dendritic cells [3–5], keratinocytes [6,7] and bronchial epithelial cells [8]. TARC acts as a specific ligand for CC chemokine receptor 4 (CCR4), which is predominantly expressed on Th2-type CD4+T cells [2,3]. Thus, TARC selectively chemoattracts Th2 cells into inflammatory sites. In a murine study, a monoclonal antibody against TARC inhibited Th2-mediated bacteria-induced fulminant hepatic failure [9].

Predominant infiltration of Th2 cells has been found in allergic disorders such as atopic dermatitis and asthma [10–12]. TARC has been shown to be overproduced in keratinocytes of NC/Nga mice exhibiting atopic dermatitis-like lesions [4]. Lymphocytes that infiltrate acute or early lesional skin of atopic dermatitis are mainly Th2 cells, which produce interleukin-4 (IL-4), IL-5, IL-10 and IL-13. However, it has been reported that Th1 cells, which produce IFN-γ, increase in number in chronic lesions [13,14]. Keratinocytes also secrete various kinds of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) [15]. Thus, it would be interesting to know how these cytokines regulate the production of TARC. In this study, we examined the production of TARC in keratinocytes and fibroblasts in the presence of IL-4, IL-13 or TNF-α, IFN-γ, and we found that IL-4 and IL-13 reciprocally regulated the production of TARC in keratinocytes and in fibroblasts.

2. Materials and methods

2.1. Reagents

Dulbecco's modified eagle's medium (DMEM), penicillin–streptomycin and trypsin–EDTA were purchased from Gibco-BRL (Grand Island, NY). RITC 80-7 medium was purchased from IWAKI Glass (Chiba, Japan). Fetal bovine serum (FBS) was obtained from HyClone (Logan, USA). Recombinant human IL-4 and IL-13 were purchased from PeproTech (Rocky Hill, NJ). Recombinant human TNF-α and IFN-γ, and agarose were purchased from Sigma (Life Science Research, USA). Human TARC was obtained from R&D Systems (Minneapolis, MN). An RNA isolation reagent, Isogen, and ethidium bromide were purchased from Nippon Gene (Tokyo, Japan). Superscript™ II RNase reverse transcriptase was purchased from Gibco-BRL.

2.2. Cell culture

HaCaT cells, a spontaneously immortalized, nontumorigenic human skin keratinocyte cell line (kindly provided by Dr N.E. Fusenig, DKFZ Heidelberg), and NG1RGB cells, a normal human skin fibroblast cell line (kindly provided by Dr H. Nagamune, Tokushima University, Japan), were used for the experiments. HaCaT cells were maintained in DMEM with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml). NG1RGB cells were maintained in RITC 80-7 with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml). HaCaT cells (4×10^5 cells per well) were plated into 24-well plates, and NG1RGB cells (2×10^6 cells per well) were plated into six-well plates, respectively. They were then cultured for 24 h. Before stimulation, all of the culture medium in each well was replaced by identical formation containing 0.1% BSA in place of FBS. The cells were treated with the medium only or with various concentrations (0.1–100 ng/ml) of TNF-α, IFN-γ, IL-4 and IL-13. After incubation for the indicated periods of time, the supernatant was collected from each culture dish and centrifuged at 15 000 rpm for 1 min and then stored at −80 °C until further analysis.

2.3. TARC enzyme-linked immunosorbent assay (ELISA)

Concentrations of TARC in the supernatant were measured with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions using an ELISA reader, Labsystems Multiskan MS (DAINIPPON, Tokyo). This ELISA method enabled detection of TARC concentrations of greater than 15.6 pg/ml.

2.4. Detection of TARC by reverse transcriptase polymerase chain reaction (RT-PCR)

Human keratinocyte HaCaT cells and fibroblast NG1RGB cells were cultured and stimulated as described above, and then the cells were washed with PBS and total RNA was extracted. Extracted RNA was subjected to reverse transcription using a superscript™ II RNase reverse transcriptase kit. Transcripts of the constitutively expressed gene for β-actin served as control in each sample. The sequences of the PCR primers were as follows: TARC sense, 5′-ATGGCCCCACTGAAGATGCT-3′; TARC antisense, 5′-TGAACACCAACGGTGGAGGT-3′; and β-actin sense, 5′-CCTCGCCTTTGCCGATCCGC-3′; β-actin antisense, 5′-AGGTAGTCAGTCAGGTCCCG-3′. These primers yielded PCR products of the expected sizes of 332 bp for TARC mRNA and 598 bp for β-actin mRNA. The initial stage of the PCR protocol consisted of denaturation at 94 °C for 7 min, annealing at 58 °C for 2 min and elongation at 72 °C for 3 min. The second stage, followed by 34 cycles of TARC cDNA amplification, consisted of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and elongation at 72 °C for 2 min. For the amplification of β-actin cDNA, the cycles included one step of denaturation at 94 °C for 7 min, annealing at 56 °C for 2 min and elongation at 72 °C for 3 min, and then 29–34 cycles of the second step by the same procedure as that of the second stage of TARC cDNA PCR. The results were visualized on a 2% agarose gel, which was then stained with ethidium bromide (1 μg/ml) and examined with PrintgraphFX (ATTO, Tokyo) consisting of a charge-coupled device camera, an ultraviolet transilluminator, and an analysis program.

2.5. Statistical analysis

Data are expressed as means ± S.E.M. Statistical significance of differences was analyzed using an analysis of variance (ANOVA) method ANOVA. A P-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Production of TARC protein and mRNA expression were augmented by TNF-α and IFN-γ but were inhibited by IL-4 and IL-13 in human keratinocytes

We first studied the effects of TNF-α, IFN-γ, IL-4 and IL-13 on the production of TARC by human keratinocytes. HaCaT cells were cultured with medium only or with 0.1–100 ng/ml of TNF-α, IFN-γ, IL-4 and IL-13 for 72 h, and the concentrations of TARC in the supernatants were quantified by ELISA. TNF-α (Fig. 1A) and IFN-γ Fig. 1B) significantly augmented the spontaneous production of TARC from HaCaT cells in a dose-dependent manner. In contrast, IL-4 (Fig. 1C) and IL-13 (Fig. 1D) dose-dependently inhibited the TARC production. Time-course experiments showed that the up- or down-regulatory effects of these cytokines were time-dependent for 72 h (Fig. 2). To confirm the results, we next examined the expression levels of TARC mRNA in HaCaT cells incubated with medium only, IL-4, IL-13, TNF-α or IFN-γ for 24 h. In accordance with the protein levels, the TARC mRNA expression was enhanced by TNF-α and IFN-γ and inhibited by IL-4 and IL-13 (Fig. 3). In each experiment, the inhibitory effects of IL-4 and IL-13 on TARC production by keratinocytes were similar, but the enhancing effect of IFN-γ was stronger than that of TNF-α.

In order to determine whether similar regulatory mechanisms operate in fibroblasts, we examined the TARC production by NG1RGB cells, a human fibroblast cell line. However, NG1RGB cells did not produce detectable amounts of TARC even in the presence of TNF-α, IFN-γ, IL-4 and IL-13 (Table 1).

3.2. Reciprocal regulation by IL-4 and IL-13 of TARC production by keratinocytes and fibloblasts in the presence of TNF-α

To further elucidate the regulatory effects, we next examined the co-stimulatory response of TARC production in the keratinocytes and the fibroblasts under various combinations of these

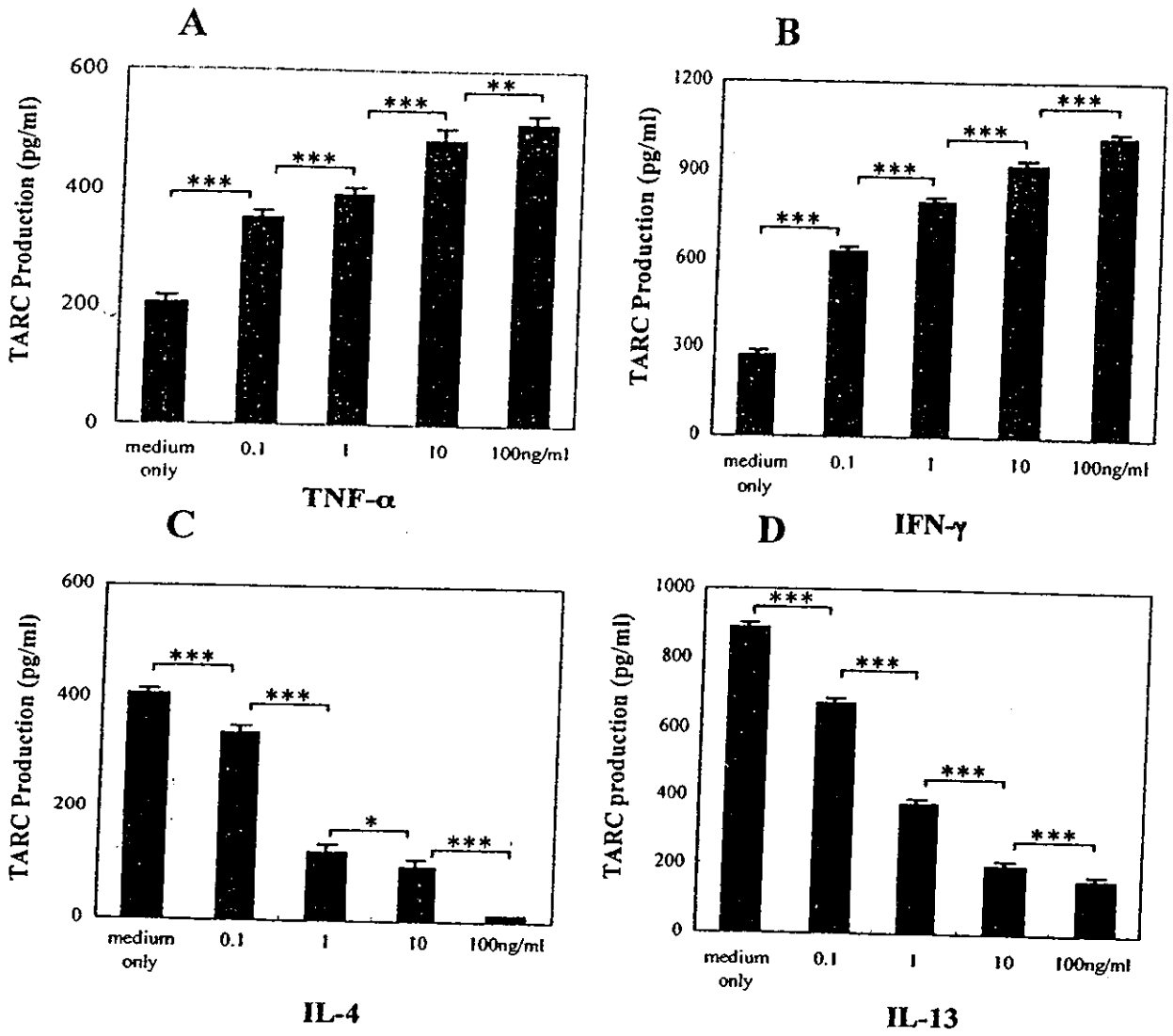

Fig. 1. Production of TARC by HaCaT keratinocytes stimulated with various concentrations of TNF-α (0–100 ng/ml), IFN-γ (0–100 ng/ml), IL-4 (0–100 ng/ml) or IL-13 (0–100 ng/ml). HaCaT cells were cultured for 72 h with the indicated concentrations of cytokines. Data are presented as means ± S.E.M. Representative data are shown ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

four cytokines. The augmenting effects of TNF-α and IFN-γ were neutralized by IL-4 and IL-13 in the keratinocytes. IFN-γ did not further enhance the up-regulatory effect of TNF-α in the keratinocytes (Table 1).

In sharp contrast, IL-4, IL-13, TNF-α and IFN-γ did not induce the TARC production in the fibroblasts. The significant production of TARC was observed only in the dual presence of IL-4 and TNF-α or IL-13 and TNF-α (Table 1). Interestingly, IFN-γ? synergistically enhanced the TARC production induced by TNF-α+IL-4 or TNF-α+IL-13 in the fibroblasts (Table 1).

To confirm the effects of TNF-α+IL-4 or TNF-α+IL-13 on TARC production by the fibroblasts, we next examined the mRNA levels of TARC in the fibroblasts stimulated with various combinations of cytokines. As shown in Fig. 4, TNF-α induced detectable mRNA accumulation for TARC only in the presence of IL-4 or IL-13. In

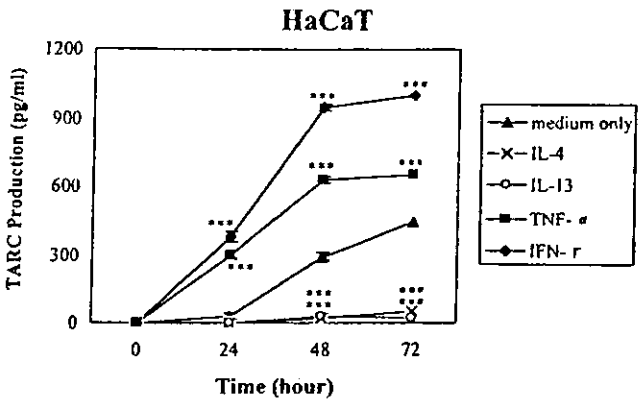

Fig. 2. Time courses of TARC production from HaCaT keratinocytes sitmulated with TNF-α (50 ng/ml), IFN-γ (100 ng/ml), IL-4 (10 ng/ml) or IL-13 (50 ng/ml) for 24, 48 or 72 h. Data are presented as means±S.E.M. Representative data are shown ($n=3$). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, compared with representative medium only.

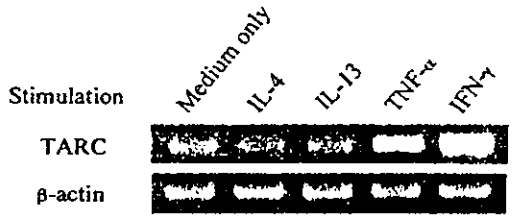

Fig. 3. Comparison of TARC mRNA levels by HaCaT keratinocytes incubated with IL-4 (10 ng/ml), IL-13 (50 ng/ml), TNF-α (50 ng/ml) or IFN-γ (100 ng/ml) for 24 h. Representative data of two independent experiments with similar results are shown.

addition, IFN-γ further enhanced the accumulation of TARC mRNA induced by TNF-α+IL-4 or TNF-α+IL-13 stimulation in the fibroblasts.

4. Discussion

We examined the production of TARC by keratinocytes and fibroblasts, two major cell populations of skin, in a mixture of proinflammatory (TNF-α), Th1 (IFN-γ) and Th2 (IL-4 and IL-13) cytokines. In keratinocytes, the synthesis of TARC was strongly enhanced by TNF-α and IFN-γ but was inhibited by IL-4 and IL-13 both in protein and in mRNA levels. In contrast, IL-4 and IL-13 induced the production of TARC protein and mRNA by fibroblasts in the presence

Table 1
Levels of TARC protein expression by human kerationcyte HaCaT cells and fibroblast NG1RGB cells

Stimulation	TARC Production (pg/ml)	
	HaCaT cells	NG1RGB cells
Medium only	291.45	0
IL-4	25.05[a]	0
IL-13	27.10[a]	0
TNF-α	628.02[a]	0
IFN-γ	946.54[a]	0
TNF-α+IFN-γ	865.19[a]	0
IL-4+TNF-α	154.06[b]	286.22[b]
IL-13+TNF-α	128.56[b]	246.45[b]
IL-4+IFN-γ	377.83[c]	0
IL-13+IFN-γ	442.24[c]	0
IL-4+TNF-α+IFN-γ	682.20[d]	797.75[d]
IL-13+TNF-α+IFN-γ	625.60[d]	755.28[d]

HaCaT cells and NG1RGB cells were incubated for 48 h with IL-4 (10 ng/ml), IL-13 (50 ng/ml), TNF-α (50 ng/ml), IFN-γ (100 ng/ml) or various combinations of these cytokines. Representative data are shown ($n=3$). [a], $P<0.001$ in comparison with Medium only; [b], $P<0.001$ with TNF-α; [c], $P<0.001$ with IFN-γ; [d], $P<0.001$ with TNF-α+IFN-γ.

of TNF-α. IFN-γ per se did not stimulate the fibroblasts to synthesize TARC protein but it synergistically augmented the IL-4 +TNF-α- or IL-13+TNF-α-induced TARC synthesis in the fibroblasts.

Various types of inflammatory cells and their cyto-chemokines play important roles in the pathomechanisms of atopic dermatitis. Many investigators have demonstrated a predominant emergence of Th2 cells both in peripheral blood and in skin lesions of patients with atopic dermatitis [16–18]. The infiltration of Th2 cells in patients with atopic dermatitis is clearly demonstrable in vivo, particularly in atopic patch test sites, with allergens. Yamada et al. reported that infiltration of IL-4-producing lymphoid cells was observed in the perivascular area of the dermis even at 2 h after application of mite allergen [19]. However, in later stages of the disease, Th1-type IFN-γ ?is produced in the lesions of atopic dermatitis [13,14]. TNF-α has been reported to be up-regulated in mast cells in the lesional skin of atopic dermatitis [20]. In the present study, we demonstrated that both human skin keratinocytes and fibroblasts are potent producers of TARC in a

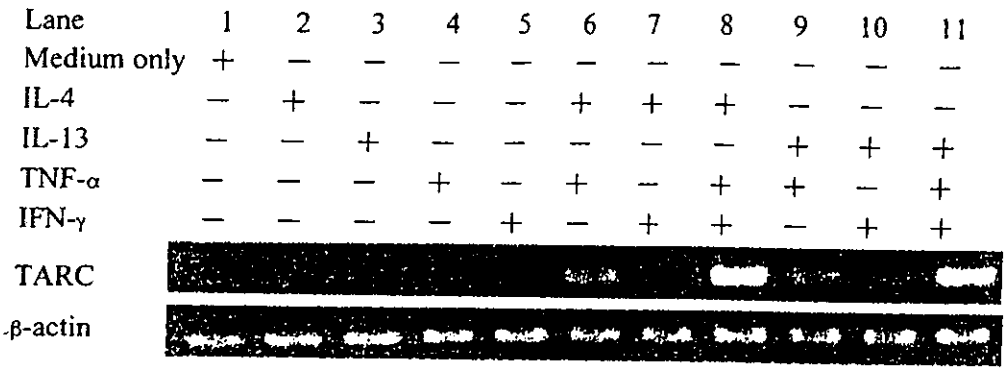

Fig. 4. Levels of TARC mRNA expression by human fibroblast NG1RGB cells incubated for 24 h with IL-4 (10 ng/ml), IL-13 (50 ng/ml), TNF-α (50 ng/ml), IFN-γ (100 ng/ml) or various combinations of these cytokines. Representative data of two independent experiments with similar results are shown.

certain combination with TNF-α, IFN-γ, IL-4 and IL-13. Vestergaard et al. reported that TARC was highly expressed in the basal epidermis of lesional skin of NC/Nga atopic dermatitis model mice [6]. Kakinuma et al. recently reported serum TARC levels of patients with atopic dermatitis were significantly higher than those of healthy control subjects and patients with psoriasis. The serum TARC levels were significantly correlated with clinical severity scores and with levels of eosinophils and serum soluble E-selectin. Kakinuma et al. also immunohistologically detected the expression of TARC in the lesional epidermis of atopic dermatitis [21].

Since TARC is a specific chemoattractant for Th2 cells, it would be interesting to know the differential regulation of TARC synthesis by keratinocytes and fibroblasts. PAM 212 cells, a murine keratinocyte cell line, produced TARC after stimulation with TNF-α or IFN-γ [6]. In the previous study, IL-10 greatly augmented the TNF-α+IFN-γ-induced TARC production by human keratinocyte HaCaT cells [22]. An obvious difference between keratinocytes and fibroblasts is their responses to IL-4 and IL-13. In our experiments, IL-4 and IL-13 inhibited the production of TARC by HaCaT cells but synergistically induced the de novo synthesis of TARC by NG1RGB fibroblasts only in the presence of TNF-α. Human corneal fibroblasts have also been shown to produce TARC only in the presence of both IL-4 and TNF-α [23]. These results indicate (1) that fibroblasts require dual stimulation with IL-4 and TNF-α for the production of TARC, (2) that TNF-α and IFN-γ act as enhancers of TARC production by keratinocytes and fibroblasts and (3) that IL-4 and IL-13 act as inhibitors of TARC production by keratinocytes but as enhancers of TARC production by fibroblasts. Sekiya et al. have recently reported the regulation of TARC production by bronchial epithelial cells. Two bronchial cell lines, A549 and BEAS-2B, reacted to produce TARC protein by dual stimulation with TNF-α+IL-4 [8]. Moreover, IFN-γ synergistically up-regulated the TNF-α+IL-4-induced TARC production, manifesting a similar regulatory pattern to that observed in the NG1RGB skin fibroblasts in the present study.

Campbell et al. reported that CCR4 and TARC were important in the recognition of skin vasculature by circulating T cells and in directing lymphocytes that were involved in systemic immunity to their target tissues [24]. It is known that the much higher level of mononuclear cell infiltration is observed in the dermis than in the epidermis of a chronic lesion of atopic dermatitis [25].

Although the precise molecular mechanisms remain unclear, the differential regulation by IL-4 and IL-13 in the TARC production by keratinocytes and fibroblasts is a very interesting evidence.

Acknowledgements

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CD40-mediated tumor necrosis factor receptor-associated factor 3 signaling upregulates IL-4-induced germline Cε transcription in a human B cell line

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Abstract

Induction of germline Cε transcription in B cells by IL-4, which is a critical initiating step for IgE class switching, is enhanced by CD40 engagement. Although signaling by CD40 is initiated by the binding of tumor necrosis factor receptor-associated factor (TRAF) family members to its cytoplasmic domain, whether those TRAF family proteins mediate enhancement of germline Cε transcription is not evident. We report here that CD40-induced TRAF3-dependent activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase 1 (MEK1) is involved in the upregulation of IL-4-driven germline Cε transcription in a human Burkitt's lymphoma B cell line, DG75. Among the six known TRAF proteins, TRAF2, 3, 5, and 6 associated with CD40 in an unstimulated state, and the levels of these four proteins were unaffected by anti-CD40 stimulation. Antisense oligodeoxynucleotide (ODN) for TRAF3 inhibited CD40-induced activation of MEK1-ERK pathway by decreasing expression of TRAF3 protein, but antisense ODNs for TRAF2, 5, and 6 were ineffective. Furthermore, CD40-mediated enhancement of IL-4-driven germline Cε transcription was inhibited by antisense ODN for TRAF3 and by a MEK1 inhibitor, PD98059. These results suggest that in DG75 cells, TRAF3-induced MEK1 activation may be involved in CD40-mediated upregulation of IL-4-driven germline Cε transcription.

Keywords: CD40; TRAF3; ERK; MEK1; IL-4; Germline Cε transcription

CD40, expressed in various cell types, including B cells, dendritic cells, follicular dendritic cells, and macrophages, is a member of the tumor necrosis factor receptor (TNFR)[1] superfamily [1]. The ligand for CD40 (i.e., CD154) is expressed on activated T cells, mast cells, and basophils and provides $CD40^+$ cells with contact-dependent signals. The function of CD40 is well characterized in B cells, where it plays an important role in regulating a vast range of B cell responses. Engagement of CD40 on B cells mediates rescue from apoptosis, upregulation of CD23 and CD56, clonal expansion, germinal center formation, and Ig class switching [1]. In particular, IgE isotype switching is initiated by IL-4 or IL-13 induction of germline Cε transcription and is subsequently brought about by CD40 ligation by CD154 or agonistic anti-CD40 antibodies [2]. Although germline Cε transcription is not inducible in general by CD40 ligation alone, CD40-mediated signaling upregulates IL-4- or IL-13-driven germline Cε transcription and is required for the DNA recombination events. However, the intracellular signaling pathways utilized by the CD40 molecule in the regulation of germline Cε transcription and switch recombination remain poorly understood.

The cytoplasmic domain of CD40, which lacks any motifs for transducing signals into B cells, has been shown to interact with four members of the six known TNFR-associated factor (TRAF) family proteins, TRAF2, 3, 5, and 6 [3–5]. The most reproducible signaling events documented in B cells following CD40 engagement is the activation of NF-κB and c-Jun N-terminal kinase (JNK). The importance of NF-κB for

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[1] *Abbreviations used:* TRAF, tumor necrosis factor receptor-associated factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK1, MAPK/ERK kinase 1; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; AS, antisense; S, sense; ODN, oligodeoxynucleotide; TNFR, tumor necrosis factor receptor; mAb, monoclonal antibody.

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IgE production is demonstrated by a generation of p50/NF-κB knockout mice that have defects in IgE class switching [6]. Moreover, several groups have documented the role of TRAFs in germline Cε transcription. Brady et al. [7] demonstrated that overexpression of TRAF6 is sufficient to activate germline Cε transcription. In addition, Leo et al. [8] reported that overexpression of TRAF2 or 6 leads to the activation of germline Cε transcription, but they also showed that dominant-negative form of TRAF2, 3, 5, or 6 suppressed germline Cε activation in response to IL-4 plus anti-CD40 antibody. In contrast, Jalukar et al. [9] showed that TRAF is not necessary for CD40-mediated NF-κB activation. Therefore, further studies are required to determine their precise contribution.

All the experiments described above analyzed the induction of germline Cε transcription in the transfectants with a reporter plasmid under the control of Cε promoter in the presence of expression vectors for wild-type or dominant-negative TRAFs. To more directly examine the role of TRAFs in germline Cε transcription, the present study employed antisense strategy for the downregulation of TRAFs and evaluated the germline Cε mRNA levels by quantitative RT-PCR. Our results demonstrate that the ligation of CD40 induces TRAF3-dependent activation of MEK1/ERK pathway and thereby contributes to the upregulation of IL-4-driven germline Cε transcription in a human Burkitt's B lymphoma cell line, DG75.

Materials and methods

Cells and reagents

The human Burkitt's lymphoma B cell line DG75 was kindly provided by Fujisaki Cell Center (Hayashibara Biochemical Laboratories, Okayama, Japan) and was maintained in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (culture medium). Recombinant human IL-4 was obtained from Genzyme (Cambridge, MA). An agonistic anti-human CD40 monoclonal antibody (mAb) (EA-5) was purchased from Ancell (Bayport, MN). Polyclonal antibodies to TRAF1, TRAF2, TRAF3, TRAF4, TRAF5, TRAF6, and ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-phospho-ERK (Thr202, Tyr204) antibody and GST–Elk-1 were obtained from New England Biolabs (Bervely, MA). PD98059 (MEK-1 inhibitor) and SB203580 (p38 MAPK inhibitor) were obtained from Calbiochem Corp. (La Jolla, CA).

Immunoprecipitation and Western blot analysis

DG75 cells were stimulated with or without anti-CD40 mAb (1 μg/ml) for the indicated times and lysed with lysis buffer containing 20 mM Tris–HCl (pH 7.4), 1% Triton X-100, 50 mM each NaCl and NaF, 5 mM EDTA, 1 mM each Na_3VO_4 and phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin. The lysates were precleared with protein A–Sepharose (Amersham Pharmacia Biotech, Buckinghamshire, UK), immunoprecipitated with the appropriate antibody, separated on SDS–PAGE, and transferred to nitrocellulose membranes. Proteins were probed with appropriate antibodies and visualized by enhanced chemiluminescence (ECL detection system, Amersham Pharmacia Biotech).

Antisense and sense oligodeoxynucleotides

Based on the human TRAF family cDNA sequences, antisense and sense phosphorothioate oligodeoxynucleotides (AS and S ODNs) were designed against nucleotides 1–21 each of TRAF2, 3, 5, and 6. They were TRAF2 AS: AGCTGCAGCCATGAGAGCTGT; TRAF2 S: ACAGCTCTCATG GCTGCAGCT; TRAF3 AS: CTT TTTACTCGACTCCATTTT; TRAF3 S: AAAATGG AGTCGAGTAAAAAG; TRAF5 AS: TGAATAAGCC ATTGTGGGGCT; TRAF5 S: AGCCCCACAATGGC TTATTCA; and TRAF6 AS: ACAGTTTAGCAGAC TCATAG T; TRAF6 S: ACTATGAGTCTGCTAAAC TGT. The AS and S ODNs were resuspended in culture medium and 48 h was allowed for DG75 cells to uptake the ODNs.

Quantitative competitive RT-PCR

Extraction of total cellular RNA and cDNA synthesis by RT were performed as described previously [10]. Levels of cDNA were quantified by means of a competitive PCR method. The competitors were constructed by using PCR mimic construction kit (Clontech Laboratories, Palo Alto, CA). Briefly, the composite primers, which have the target germline Cε or β-actin gene sequence attached to a sequence of v-erbB gene fragment, were prepared and amplified by PCR using v-erbB cDNA as a template. To assure the attachment of target-specific sequence, the second-round PCR was performed with primers corresponding to a part of target gene in the composite primers. The products were purified and used as competitors. β-Actin mimic was kindly provided by Dr. M. Ebisawa (National Sagamihara Hospital, Sagamihara, Japan) [11]. The sequences of composite primers were as follows: germline Cε, 5′-ATCCACA GGCACCAAATGGACGACCCGCAAGTGAAATCT CCTCCG-3′ and 5′-GCCAGGTCCACCACCAGACA GGTGATCTGTCAATGCAGTTTGTAG-3′; β-actin, 5′-GTACGTTGCTATCCAGGCTGCGCAAGTGAA ATCTCCTCCG-3′ and 5′-CTTAATGTCACGCACGA TTTCCTCTGTCAATGCAGCAGTTTGTAG-3′. Competitive PCR was performed using serial 10-fold