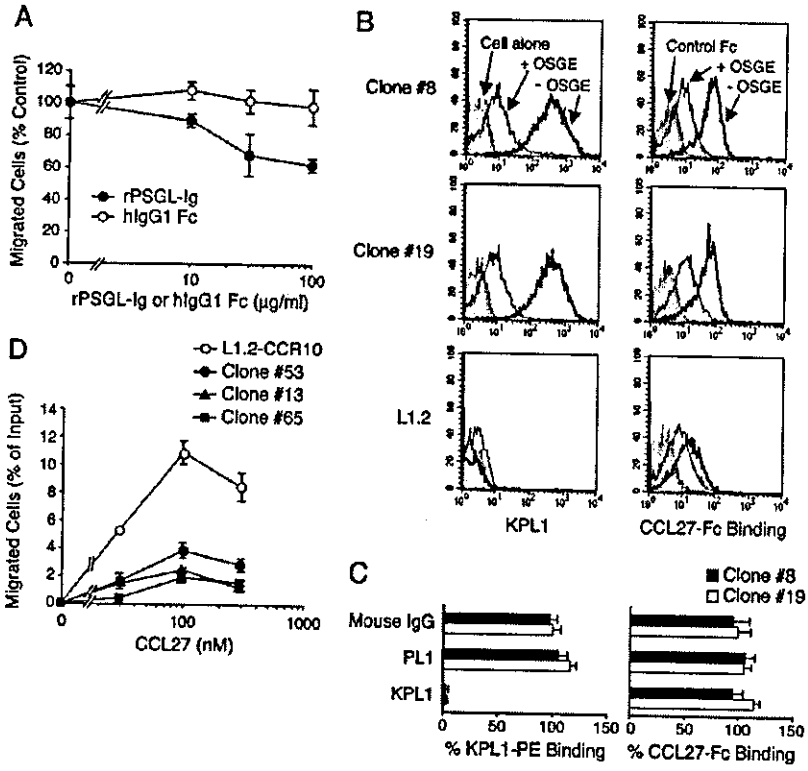

FIG. 7. **Effect of PSGL-1 on CCL27-induced chemotaxis of L1.2 cells expressing CCR10.** *A*, effect of rPSGL-Ig on the CCL27-induced chemotaxis of CCR10-expressing L1.2 cells. CCL27 at 100 nM together with rPSGL-Ig or human IgG1 Fc was placed in the lower chamber, and CCR10-expressing L1.2 cells were placed in the upper chamber of a ChemoTx apparatus. Three hours later, the number of cells that had migrated into the lower chamber was counted. Migrated cells are expressed as percentages of the cells that migrated in response to CCL27 in the absence of rPSGL-Ig and human IgG. The data are the means ± S.E. from triplicate wells. One of three similar experiments is shown. *B*, CCL27 binding to PSGL-1 expressed on the cell surface. L1.2 cells and L1.2 clones expressing human PSGL-1 (clones 8 and 19) were treated or not with OSGE, stained with anti-PSGL-1 mAb KPL-1 (*left panels*) and CCL27-Fc or control Fc (*right panels*), and analyzed by flow cytometry. Unstained cells (*left panels*) or cells stained with control Fc (*right panels*) are shown in *filled histograms*, which do not show any shift by OSGE treatment. One of three similar experiments is shown. *C*, effect of anti-PSGL-1 mAbs on CCL27-Fc binding. L1.2 clones expressing human PSGL-1 (clones 8 and 19) were preincubated or not with mouse IgG, PL1, or KPL1, then stained with phycoerythrin (*PE*)-labeled KPL-1 (*left panel*) and CCL27-Fc (*right panel*), and analyzed by flow cytometry. Mean fluorescence intensity of the cells preincubated in the absence of antibodies is expressed as 100%. *D*, effect of the cell surface expression of PSGL-1 on CCL27-induced chemotaxis. L1.2 cells expressing CCR10 and L1.2 clones expressing both CCR10 and human PSGL-1 (clones 53, 13, and 65) were assayed for CCL27-induced chemotaxis. The data are the means ± S.E. from triplicate wells. One of three similar experiments is shown.

were also shown to contribute to high affinity binding of CCR5 ligands (31). Whether *O*-glycans are attached to CCR10 and contribute to the binding of CCL27 has not yet been shown.

rPSGL-Ig is a recombinant, soluble, and chimeric form of PSGL-1 that was developed as an antagonist to P-selectin. rPSGL-Ig has been shown to exert anti-inflammatory effects in many models of inflammation; it reduces hepatic ischemia/reperfusion injury in rats (45), accelerates thrombolysis and prevents reocclusion in a porcine model (19), ameliorates acute traumatic shock in rats (16), and protects against myocardial ischemic reperfusion injury in cats (17). A recent report showed that rPSGL-Ig binds the murine chemokine KC, which may in part account for its anti-inflammatory effect (20). Our results also show that rPSGL-Ig binds to several chemokines, supporting the idea that its effect in various inflammatory conditions is not exclusively due to its inhibition of selectin function but also of chemokine function. The mechanisms by which rPSGL-Ig inhibits chemokine function may involve the inhibition of chemokine binding to its receptor or the inhibition of chemokine-induced signal transduction.

Although the *in vivo* relevance of the interaction of PSGL-1 with CCL27 is not yet clear, the modulation of CCL27-induced cell responses by PSGL-1 may confer an additional level of regulation on the trafficking of skin-homing T cells. CCR10 is expressed on a subset of skin-homing T cells, which also express PSGL-1. PSGL-1 on the surface of T cells that have migrated into the skin may bind CCL27 and regulate its function in the local microenvironment. Alternatively, soluble PSGL-1 in the vicinity of these cells may bind CCL27 and affect cell responses to CCL27. A previous study showed that the phorbol ester PMA induces the ectodomain shedding and secretion of PSGL-1 from human neutrophils (46). Several proteases such as BACE1 have been implicated in mediating PSGL-1 shedding (47). In support of this idea, soluble PSGL-1 is found in human bronchoalveolar lavage fluids and in serum (46, 48). A raised concentration of soluble PSGL-1 in serum is associated with a lower frequency and severity of pulmonary fibrosis in systemic sclerosis (48), suggesting that soluble PSGL-1 could function as a protective factor by binding to certain chemokines that promote the progression of the disease. Because CCL27 is critically involved in various T cell-mediated inflammatory diseases of the skin such as atopic dermatitis and contact dermatitis (23), the identification of molecules that regulate the function of CCL27 should lead to the development of treatments to control these human skin diseases.

Acknowledgments—We thank Mahiru Kamiya for preparing the hPSGL-Fc construct and Dr. Myoung-Ho Jang and Tomoharu Kikuchi for preparing the CCL27-Fc protein. We also thank Drs. Toshiyuki Tanaka, Toshiyuki Murai, and Haruko Hayasaka for valuable comments and Shinobu Yamashita and Miyuki Komine for secretarial assistance.

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Journal of Dermatological Science (2004) 36, 186–188

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LETTER TO THE EDITOR

Effects of MAPK inhibitors on CCR4-mediated chemotaxis against thymus and activation-regulated chemokine (TARC/CCL17)

To the Editor,

Tissue recruitment of immunocompetent cells is a central event in the inflammatory response. Most of peripheral blood T cells polarized to type 2 helper T (Th2) cells express CC chemokine receptor 4 (CCR4) and also include almost all skin-seeking memory T cells positive for cutaneous lymphocyte antigen [1,2]. Thymus and activation-regulated chemokine (TARC/CCL17) is one of the ligands for CCR4 and the ligation of TARC and CCR4 plays an important role in the migration of Th2 cells into inflamed tissues [1,3]. Moreover, serum levels of TARC were shown to correlate well with disease severity in Th2-prone skin disorders, such as atopic dermatitis [4]. Keratinocytes also express TARC in vivo and in vitro under an appropriate condition [4]. Therefore, TARC- and CCR4-mediated chemotaxis may play a crucial role in the initiation and perpetuation of the allergic inflammation.

Various cellular events such as proliferation, differentiation and migration responses are associated with phosphorylation of intracellular signaling molecules such as p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases1/2 (ERK1/2) and MAPK/Erk kinase 1/2 (MEK1/2). Recently, several researchers have reported on the differential involvement of MAPKs in chemotaxis [5,6]. However, the intracellular signaling pathways linking to TARC–CCR4-mediated chemotaxis are not well characterized. We have investigated the effects of various MAPK inhibitors on CCR-4-mediated chemotaxis to TARC using mouse T-cell lymphoma cell line EL4 constitutively expressing recombinant human CCR4 (CCR4/EL4 cells) with high level [7].

Recombinant human TARC was obtained from Sigma, St. Louis, MO. SB203580 (p38 MAPK inhibitor), SD-282 (p38 MAPK inhibitor), U0126 (MEK 1/2 inhibitor) and PD098059 (ERK 1/2 inhibitor) were obtained from Calbiochem (Dermstadt, Germany). CX-659S (MEK 1/2 inhibitor) was obtained from Kowa Co., Japan [8]. CCR4/EL4 cells were obtained from Kyowa Hakko Kogyo Co., Ltd., Japan. These cells expressed large amounts of cell surface CCR4 [7] and exhibited marked chemotactic activity to TARC (Fig. 1). All the inhibitors inhibited the TARC-induced chemotaxis in a dose-dependent manner (Fig. 1 and Table 1). However, the inhibition was partial in each agent. Both p38 MAPK inhibitors (SB203580 and SD-282) showed similar level of inhibition. Among MEK/ERK inhibitors, U0126 exhibited the most potent inhibitory capability. Neither agent suppressed the viability of the CCR4/EL4 cells under the experimental concentrations examined.

The inhibitory effects on chemotaxis by MAPK inhibitors are diverse, depending on the cell types and kinds of stimuli. In granulocytes, IL-8-mediated chemotaxis was inhibited by PD98059 (about 40% inihibition) but not by SB20358 [6], whereas the inhibition of MEK1/2 by PD98059 did not block the chemotaxis in response to classical nonchemokine chemoattracts such as fMLP [5]. In monocyte chemoattractant protein-1 (MCP-1)-induced chemotaxis, p38-MAPK, but not MEK1/2, was involved in chemotaxis of CCR2-expressing THP-1 monocytic cells [9].

Ligation of TARC to CCR4 is known to activate the pertussis toxin-sensitive G proteins not only in the aggregation of platelets [10]. However, little is known on the involvement of MAPKs on TARC–CCR4 chemotaxis. In the present study we investigated the inhibitory effects of five different MAPK inhibitors on CCR4-expressing EL4 cells. All the inhibitors partially inhibited the TARC-induced chemotaxis in a dose-dependent manner. These results suggest that p38-MAPK and MEK/ERK pathways are both involved in the CCR4-mediated chemotaxis to TARC. Although these results using CCR4/EL4 do not exactly correspond to human Th2 lymphocytes, those inhibitors might be candidates that suppress the migration of T cells expressing CCR4, and could be therapeutic reagents for allergic skin diseases.

0923-1811/$30.00
doi:10.1016/j.jdermsci.2004.08.013

Table 1 Effects of various MAPK inhibitors on TARC–CCR4-mediated chemotaxis

	6.25 μM	12.5 μM	25 μM	50 μM
SB203580 (p38 MAPK inhibitor) (%)	19.1 ± 0.68	24.4 ± 2	27.3 ± 0.6	47.2 ± 0.9
	5 μM	10 μM	20 μM	40 μM
SD-282 (p38 MAPK inhibitor) (%)	20.8 ± 0.6	31.5 ± 2	38.9 ± 0.8	38.4 ± 1.2
	5 μM	10 μM	20 μM	40 μM
U0126 (MEK 1/2 inhibitor) (%)	48.3 ± 0.6	52.6 ± 3	54.3 ± 0.4	62.8 ± 1.1
		10 μM		100 μM
CX-659S (MEK 1/2 inhibitor) (%)		0		16.6 ± 0.8
	5 μM	10 μM	20 μM	40 μM
PD098059 (ERK 1/2 inhibitor) (%)	7 ± 1.5	23.2 ± 0.9	30 ± 1.2	32.8 ± 2.5

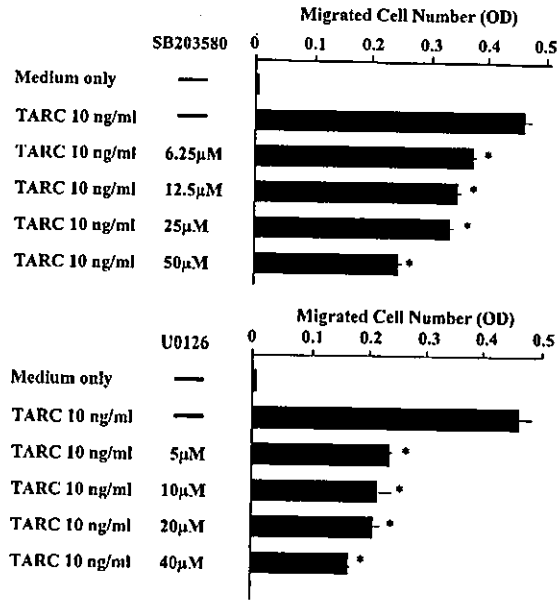

Fig. 1 Inhibitory effects of SB203580 and U0126 on the TARC-mediated chemotaxis of CCR4/EL4 cells. Number of migrated cells through 5-μm pore was determined using a cell counting kit (cell proliferation kit, Biovision Inc., Mountain View, CA). TARC induced a potent chemotaxis of CCR4/EL4 cells compared to negative control (medium only). Both agents significantly and dose-dependently inhibited the chemotaxis. $^{*}p < 0.01$.

Acknowledgements

We greatly appreciated Kyowa Hakko Kogyo Co. Ltd. for allowing us to use CCR4/EL4 cells. This work supported by Health Science Research Grants from the Ministry of Health, Labour and Welfare, and by Ministry of Education, Culture, Sports, Sciences and Technology, Japan.

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7 June 2004

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Cytokine 28 (2004) 186–189

Reciprocal regulation of permeability through a cultured keratinocyte sheet by IFN-γ and IL-4

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Received 24 November 2003; received in revised form 19 May 2004; accepted 2 August 2004

Abstract

The T cell cytokines profoundly modify the phenotypic and functional characteristics of keratinocytes. Until now, no study has focused on the effect of Th1 and Th2 cytokines on keratinocyte permeability. Using a two-layer well culturing system, permeability was assessed through cultured keratinocyte sheet in the presence or absence of various concentrations of IFN-γ and IL-4. Transepithelial electrical resistance (TER) and the flux of 40 kDa FITC-dextrans were measured across the cultured keratinocyte sheet. IFN-γ significantly increased the TER in a dose- and time-dependent manner, suggesting that IFN-γ profoundly inhibited the permeability of ions through the keratinocyte sheet. In contrast, IL-4 did not affect the TER. When compared to medium control, the flux of FITC-dextran of the IFN-γ group was significantly decreased in a dose-dependent fashion. In sharp contrast, the flux of FITC-dextran was significantly and dose-dependently increased in the presence of IL-4. A significant increase in TER and a significant decrease in the flux of dextran suggested that IFN-γ clearly reduced the permeability of both ions and high molecular weight material through the keratinocyte sheet. Although IL-4 did not affect the permeability of the ions, it significantly enhanced the permeability of high molecular weight material. A flow cytometric assay revealed that the expression of desmoglein-3 was suppressed by IL-4, but was enhanced by IFN-γ. The reciprocal regulation of permeability of the cultured keratinocyte sheet by IFN-γ and IL-4 may be partly related to the modification of intercellular adhesion molecules.

Keywords: Keratinocyte; Permeability; IFN-γ; IL-4

1. Introduction

Epidermis, a squamous epithelium of the skin, serves as the outermost barrier of the body, separating it from the surrounding environment and preventing the loss of body fluids and proteins. Very frequently, immunological and inflammatory processes result in skin damage in eczematous disorders such as contact dermatitis and atopic dermatitis [1,2]. Eczema is an orchestrated cellular response conducted by various immunocompetent cells, including Th1 and the Th2 cells, that produce large amounts of cytokines such as IFN-γ and IL-4, respectively [3,4]. One of the characteristic manifestations of eczema is serous papules with spongiosis and oozing, which may result from an increased permeability of body fluids into the epidermal compartment.

Previous studies have shown that T cell cytokines profoundly modify the phenotypic and functional characteristics of keratinocytes [5,6]. However, there have been no reports investigating the effect of Th1 and

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1043-4666/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cyto.2004.08.003

Th2 cytokines on the permeability of keratinocytes. We recently demonstrated that IL-4 down-regulated the cell surface expression of an adhesion molecule, E-cadherin, whereas IFN-γ up-regulated its expression [7]. This finding suggested that IL-4 and IFN-γ may modify the permeability of keratinocyte. In the present study, we examined the effects of IFN-γ and IL-4 on the trans-epithelial electrical resistance (TER) and the flux of 40 kDa FITC-dextrans across a cultured keratinocyte sheet.

2. Results

2.1. IFN-γ increased the TER of the keratinocyte sheet

We first examined the effects of IFN-γ and IL-4 on the TER of the keratinocyte sheet. The TER values represent the permeability of water-soluble ions. IFN-γ significantly increased the electrical resistance in a dose- and time-dependent manner (Fig. 1). In particular, the values of TER after treating the cultured sheet with 2 ng/ml of IFN-γ were approximately three times higher than those of the medium control, suggesting that IFN-γ profoundly inhibited the permeability of ions through the keratinocyte sheet. In contrast, IL-4 did not affect the TER (Fig. 1).

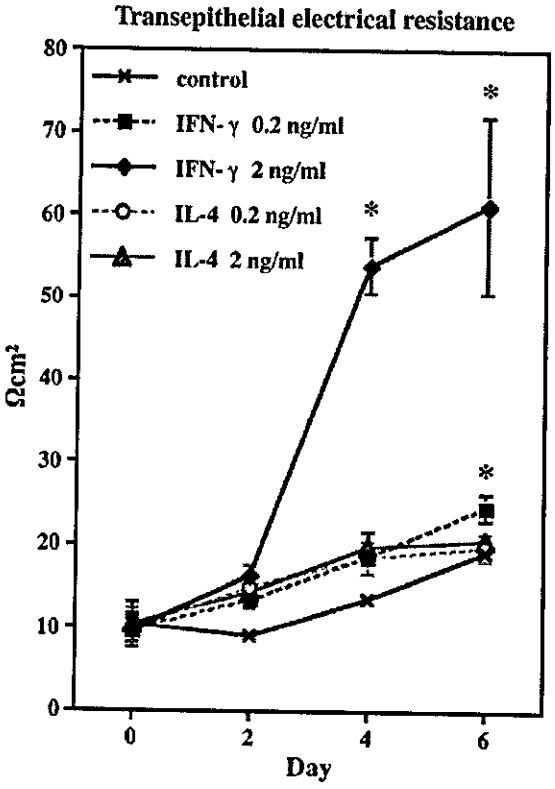

Fig. 1. The TER was measured across the confluent HaCaT keratinocyte sheet every second day using a Millicell-ERS epithelial voltohmmeter. IFN-γ significantly increased the TER in a dose- and time-dependent manner. In contrast, IL-4 did not affect the TER. The values indicated are means ± SD ($n = 4$). *$p < 0.01$ compared with the control.

2.2. Down-regulation of the flux of FITC-dextran through the keratinocyte sheet by IFN-γ and its up-regulation by IL-4

Next, we examined whether IFN-γ or IL-4 may modify the permeability of high molecular weight material through the keratinocyte sheet using 40 kDa FITC-dextran. As shown in Fig. 2, IFN-γ significantly decreased the flux of FITC-dextran in a dose-dependent fashion compared to the medium control (81.3% inhibition at 2 ng/ml of IFN-γ). In sharp contrast, IL-4 dose-dependently up-regulated the flux of FITC-dextran (92.3% up-regulation at 2 ng/ml of IL-4).

2.3. Desmoglein-3 expression was up-regulated by IFN-γ, but was down-regulated by IL-4

Desmoglein-3 is one of the major intercellular adhesion molecules between keratinocytes. Therefore, we investigated whether the expression of desmoglein-3 is modulated by IL-4 or IFN-γ. The expression of desmoglein-3 was dramatically up-regulated by IFN-γ and was down-regulated by IL-4 (Fig. 3).

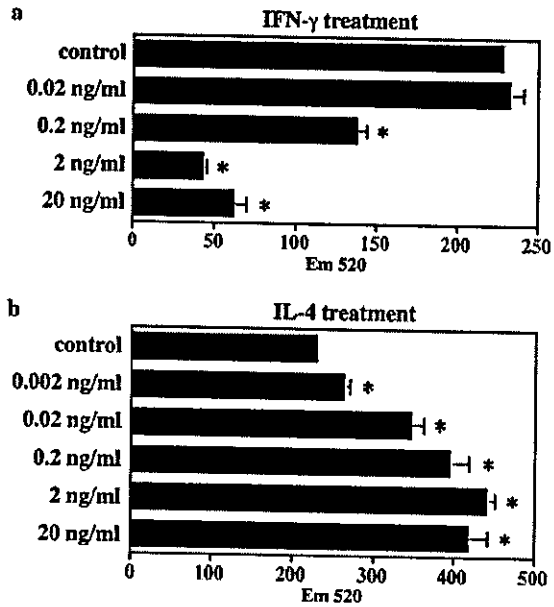

Fig. 2. The flux of 40 kDa FITC-dextrans was measured using a fluorometer (excitation, 492 nm; emission, 520 nm). IFN-γ dose-dependently inhibited the permeability of FITC-dextran, whereas IL-4 enhanced its permeability. The values indicated are means ± SD ($n = 4$). *$p < 0.01$ compared to the control.

3. Discussion

TER and the flux assay have been demonstrated to be a suitable in vitro models to determine the permeability of water-soluble ions and solutes containing various molecular weight materials, respectively [8,9]. FITC-labeled dextrans with a molecular weight of 40 kDa, which are similar in molecular weight to plasma albumin, were utilized in the flux assay as a tracer. In this study, we demonstrated a significant increase in TER and a significant decrease in the flux of dextran by IFN-γ, suggesting that IFN-γ may reduce the permeability of both ions and high molecular weight material through the keratinocyte sheet. In contrast, IL-4 significantly enhanced the permeability of dextran. We did not detect an effect of IL-4 on the TER of the keratinocyte sheet. Interestingly, Ahdieh et al. recently investigated the permeability of cultured lung epithelial cells and found that IL-4 treatment resulted in a 70–75% increase in permeability, as assessed by electrophysiological and mannitol flux measurements. In contrast, IFN-γ markedly reduced the permeability [10]. However, the means that IFN-γ and IL-4 modify the permeability are unknown.

Ye et al. have reported that the expression of IL-1 and its receptor plays a pivotal role in permeability homeostasis in the epidermis [2]. IFN-γ and IL-4 may alter the IL-1/IL-1 receptor signaling system. In fact, IFN-γ augments IL-1 production [11]. The expression of adhesion molecules may be related to the permeability of keratinocytes. Trautmann et al. demonstrated a reduced expression of E-cadherin in areas of spongiosis in acute eczematous dermatitis. They also showed that the induction of keratinocyte apoptosis is accompanied by a rapid cleavage of E-cadherin [12]. Recently, we found that IL-4 down-regulated the cell surface expression of E-cadherin, whereas IFN-γ up-regulated its expression [7]. In the present study, we showed that the expression of desmoglein-3 was also enhanced by IFN-γ but was inhibited by IL-4. The modification of these intercellular adhesion molecules occurred earlier than the permeability change and required higher concentrations of cytokines, however, it may be partly responsible for the permeability of keratinocytes.

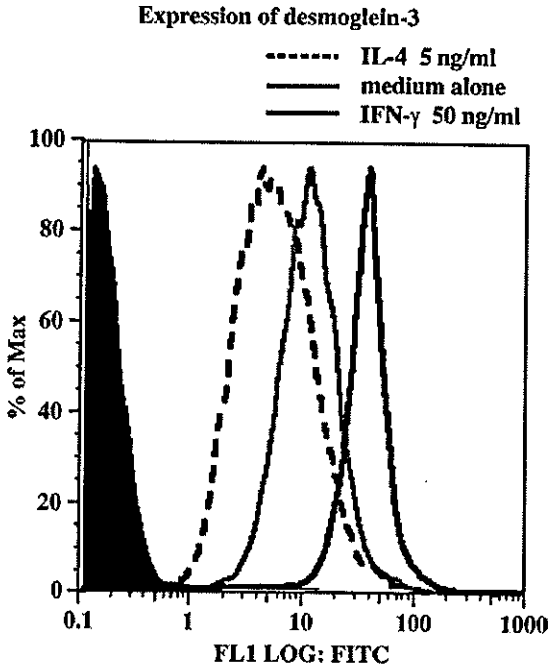

Fig. 3. Keratinocytes were cultured with IFN-γ (50 ng/ml), IL-4 (5 ng/ml) or medium alone for 48 h and the expression of desmoglein-3 was analyzed by flow cytometry. IFN-γ augmented the expression of desmoglein-3, while IL-4 decreased its expression.

4. Material and methods

4.1. Cell culture

HaCaT keratinocytes (a kind gift from Dr. N.E. Fusening in Heidelberg) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL, Rockville, MD/USA). Using a two-layer well culturing system, cells were plated at a confluent density, at about 3×10^5 cells/cm^2, on 12-mm membrane culture inserts (0.6 cm^2; Millipore Corp., Bedford, MA/USA) in a 24-well plate. After an overnight incubation for cell attachment, we added various concentrations of IFN-γ (0.02–20 ng /ml) or IL-4 (0.002–20 ng/ml) into the outer layer culture medium, and further cultured the cells for up to 6 days. Assays for the permeability of keratinocyte sheets were performed as described below.

4.2. Measurement of the TER

The TER was measured using a Millicell-ERS epithelial voltohmmeter (Millipore), as described previously [13]. The TER values were calculated by subtracting the contribution of the bare filter and medium and multiplying by the surface area of the filter. The TER represents the transepithelial permeability of the water-soluble ions. A higher value of TER means a lower permeability of ions. These data were analyzed using the Student's t test. A p value of less than 0.01 was considered to be statistically significant. The experiment was repeated at least three times in quadruplicate.

4.3. Measurement of flux of FITC-dextran

The cells were cultured at confluence with or without various concentrations of IFN-γ or IL-4 for 6 days on culture insert. The flux was measured as described previously [13]. In brief, the culture medium was replaced with P buffer (10 mM Hepes, pH 7.4, 1 mM sodium pyruvate, 10 mM glucose, 3 mM $CaCl_2$, 145 mM NaCl), and 1 mg/ml FITC-dextran (40 kDa: Sigma, St. Louis, MO/USA) was added to the outer wells. At 120 min after the addition of FITC-dextran into the outer chambers, the media from the inner wells were collected, and the flux of FITC-dextran was measured with a fluorometer RF-1500 (Shimadzu, Tokyo, Japan). The flux of FITC-dextran represents the permeability of the solutes with high molecular weight material beyond the keratinocyte sheet. These data were analyzed using the Student's t test. A p value of less than 0.01 was considered to be statistically significant. The experiment was repeated at least three times in quadruplicate.

4.4. Flow cytometry

The HaCaT keratinocytes were cultured in the presence or absence of IL-4 (5 ng/ml) or IFN-γ 50 ng/ml) for 48 h. Cells were detached from the plates using trypsine–EDTA and were incubated with mouse anti-desmoglein-3 antibody (Zymed, San Francisco, California, USA) or isotype-matched control antibody (Zymed) for 30 min on ice. After washing, the cells were incubated with FITC-conjugated goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR). The stained cells were analyzed by flow cytometry (EPIX-XL, Coulter, Fullerton, CA).

Acknowledgements

This work was supported in part by grants from Koga Hideya Funds and Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (Nos. 08770015, 09770010 and 11770008).

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Journal of Dermatological Science (2004) 35, 35–42

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Enhanced TARC production by dust-mite allergens and its modulation by immunosuppressive drugs in PBMCs from patients with atopic dermatitis

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Received 26 September 2003; received in revised form 2 February 2004; accepted 5 February 2004

KEYWORDS
Atopic dermatitis (AD);
Dust mite allergen;
Thymus and activation-regulated chemokine (TARC);
Disease activity;
CC-chemokine receptor 4 (CCR4);
Dexamethasone (Dex);
Cyclosporine (CsA);
FK506 (Tacrolimus)

Summary ***Background:*** Thymus and activation regulated chemokine (TARC) is a CC chemokine that attracts $CCR4^+$ T cells. We reported previously that TARC is an important chemokine that defines Th2 imbalance in the pathogenesis of atopic dermatitis (AD). ***Objectives:*** This study was undertaken to clarify TARC producing cells in peripheral blood mononuclear cells (PBMCs), the regulation of dust mite-allergen clude extract (DME) and different immunosuppressive drugs (Tacrolimus (FK506), cyclosporine (CsA), dexamethasone (Dex)) on TARC production by peripheral PBMCs from AD patients in vitro. ***Methods:*** Monocyte derived dendritic cells (MoDCs) were generated from and TARC mRNA levels were examined and comapared with those from T cells in PBMCs from AD patients. PBMCs were cultured with or without DME and/or immunosuppressive drugs (Tacrolimus, CsA, Dex) for 7 days and TARC levels were measured. ***Results:*** PBMCs from AD patients which were cultured with DME stimulation for 7 days showed significantly higher levels of TARC production than those from healthy controls. RT–PCR demonstrated that TARC mRNA was expressed in $CD4^+$ T cells, $CD8^+$ T cells and MoDCs. Tacrolimus, CsA and Dex individually suppressed TARC production by PBMCs from AD patients which were co-cultured with DME for 7 days. Gel shift analysis revealed differential inhibitory effects of these immunosuppressive drugs on NFκB activity in PBMCs from AD patients. ***Conclusion:*** Our data demonstrate that TARC producing cells are MoDCs, T cells as well as epidermal keratinocytes in AD. We suggest that MoDCs might regulate the immune responses by attracting T cells and $CD25^+$ T cells in the pathogenesis of AD. We also showed the important role of DME on TARC production and the inhibitory effect of the immunosuppressive drugs on TARC production by PBMCs from AD patients, that can regulate ongoing immune responses in the pathogenesis of AD.

Abbreviations: AD, atopic dermatitis; DME, dust mite allergen extract; TARC, thymus and activation-regulated chemokine; CCR4, CC chemokine receptor 4; PBMC, peripheral blood mononuclear cell; Dex, dexamethasone; CsA, cyclosporine

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0923-1811/$30.00
doi:10.1016/j.jdermsci.2004.02.008

1. Introduction

Chemokines are chemoattractants which bring different types of leukocytes to sites of inflammation [1]. They are produced locally in tissues and act on leukocytes through specific receptors [2]. Chemokines are divided into four subfamilies, CXC, CC, CX_3C and C, depending on the position of their first two N-terminal cysteine residues [1,2]. Thymus and activation-regulated chemokine (TARC/CCL17) is a member of the CC chemokine superfamily, and is produced by monocyte-derived dendritic cells (DCs), endothelial cells and keratinocytes [3–6].

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is associated with an elevated serum IgE level, IgE specific to environmental allergens such as mites, and tissue and the occurrence of peripheral blood eosinophilia. It is characterized by predominant infiltration of Th2-type cells, increased secretion of Th2-type cytokines in acute phase of lesional skin [7] and high responsiveness of peripheral blood mononuclear cells (PBMCs) to IL-4 [8]. The production of Th2 cytokines, such as IL-4, IL-10 and IL-13, are significantly increased when PBMCs of AD patients are stimulated with dust mite-allergen extracts (DME) compared without stimulation [9].

We showed previously that serum TARC levels, and the expression of CC chemokine receptor 4 (CCR4) are significantly increased in patients with AD [10]. These data strongly suggest that the interaction of TARC with CCR4 may be important for recruiting Th2-type T cells in the pathogenesis of AD. However, little is known about the effects of DME and various immunosuppressive drugs (Taclorimus (FK506), cyclosporine (CsA), dexamethasone (Dex)) on TARC production by PBMCs of AD patients. In the present study, we quantified the effect of DME and the inhibitory effects of immunosuppressive drugs on TARC production by DME-stimulated PBMCs of AD patients in order to clarify the action of immunosuppressive drugs on Th2 chemokine regulation.

2. Materials and methods

2.1. Samples and reagents

Eight AD patients (mean age ± S.D., 25.4 ± 7.9) and eight healthy controls (mean age ± S.D., 29.8 ± 5.8) were examined. The levels of IgE RIST, peripheral blood eosinophil numbers, SCORAD indexes were listed in Table 1. All patients with AD were diagnosed according to the criteria of Hanifin and Rajka [11] and were treated with topical corticosteroids in combination with oral anti-histamines.

2.2. Culture of PBMCs

PBMCs were isolated from peripheral blood samples by centrifugation on a Ficoll-metrizoate density gradient (Pharmacia Biotech, Uppsala, Sweden) at 2000 rpm for 20 min at room temperature. PBMCs were washed three times with PBS, and dispensed into 10^6 cells per tube in RPMI1640 medium. Isolated PBMCs were cultured at 37 °C for 7 days in RPMI 1640 medium with dust-mite clude extract (DME) (Greer Laboratories Inc., NC) (10 μg/ml) with or without various immunosuppressive drugs (Tacrolimus, CsA or Dex) (10^{-6} M to 10^{-10} M).

2.3. Cell viability

After culture, cell viability was evaluated with trypan blue dye exclusion. The viable cells and dead cells were counted in each experiment.

2.4. ELISA

Polystyrene microplates were coated with a murine mAb against human TARC (TECHNE Corp., Minneapolis, MN). TARC levels in the supernatants of cultured PBMCs were measured [6]. Briefly, the samples were added to each well and the plates were incubated for 2 h at room temperature (RT). After washing, anti-TARC Ab conjugated to

Table 1 The laboratory data of AD patients examined in this experiment are shown

Age	Sex	IgE (IU/ml)	Dust mite specific IgE	Eosinophils (mm^{-3})	SCORAD
22	Female	420	0.86	210	28.9
21	Female	6100	65.3	610	37.6
23	Male	2100	69.9	120	41.2
37	Female	9000	76.3	730	48.4
39	Female	83	0.39	260	29.2
21	female	16000	78.0	690	44.6
20	Male	36	5.69	60	28.8
20	Female	4200	68.7	360	40.3

horseradish peroxidase (HRP) was added to each well and the plates were incubated for 1 h at RT. After washing, a substrate solution (including tetramethylbenzidine) was added and the plates were incubated for 30 min. The optical density of each well was determined using an ELISA reader (Spectra and Rainbow Readers). The minimum amount of TARC detectable was less than 7 pg/ml. ELISAs were performed at least three times in each experiment.

2.5. Purification of $CD4^+$ T cells, $CD8^+$ T cells and monocytes, and separation of monocyte derived dendritic cells in PBMCs from AD patients

PBMCs were incubated with or without DME for 24 h, and $CD4^+$ T and $CD8^+$ T cells were purified from PBMCs using RosetteSep™ antibody cocktail (Atemcell Technologies Inc., Vancouver, Canada). These cells were used for RT–PCR analysis. Monocytes were isolated from un-stimulated PBMCs and monocyte derived dendritic cells (DCs) were generated, according to published methods [12]. Briefly, to generate immature monocytes derived DCs (MoDCs), monocytes purified from PBMCs using RosetteSep™ antibody cocktail were cultured for 7 days with recombinant human GM–CSF (R&D) (50 ng/ml) and recombinant human IL-4 (R&D)(50 ng/ml). These MoDCs were then cultured with or without DME for 24 h and used for RT–PCR analysis.

2.6. Preparation of mRNA and RT-PCR

$PolyA^+$ mRNAs were extracted from cells using a Micro-FastTrack™ 2.0 kit (Invitrogen Co., Carlsbad, CA) [13]. Briefly, cells were lysed and transferred to vials containing oligo (dT) cellulose. The oligo (dT) cellulose was washed and the $polyA^+$ mRNA was retrieved. Approximately 1 μg of $polyA^+$ mRNA was reverse-transcribed using a cDNA CYCLE™ kit (Invitrogen Co.). The reaction mixture contained 1 μg of $polyA^+$ mRNA, 1 μl of random primers, 1 μl of RNase inhibitor, 4 μl of 5 × RT buffer, 1 μl of 100 mM dNTPs, 1 μl of 80 mM sodium pyrophosphate and 0.5 μl of AMV reverse transcriptase. After incubation at 42 °C for 60 min and 95 °C for 3 min, the resulting cDNA was used for PCR. Sequences were amplified from cDNAs using specific primers for TARC and β-actin. The TARC primer sequences were: sense, 5′-CAC GCA GCT CGA ACC AAT GTG-3′, antisense, 5′-TCA AGA CCT CTC AAG GCT TTG CAG G-3′. The β-actin primer sequences were: sense, 5′-GAC TAT GAC TTA GTT GCG TTA-3′, antisense, 5′-GCC TTC ATA CAT CTC AAG TTG-3′. PCR amplifications were carried out in cDNA, each primer (25 pmol), $MgCl_2$, dNTPmix and Taq polymerase (TaKaRa, Tokyo, Japan). Each cycle consisted of denaturation at 94 °C for 1 min, annealing at 58 °C for TARC or at 55 °C for β-actin for 1 min, and extension at 72 °C for 2 min. For negative controls, cDNA was omitted from the PCR reaction. After amplification, the PCR products were separated by electrophoresis on a 1.5% agarose gel.

2.7. Gel shift assay

Nuclear extracts were prepared from PBMCs (3×10^6) incubated with or without DME and immunosuppressive drugs using a Cell Lytic™ Nu kit (Sigma Co., St Louis, Ms.). The cells were collected by centrifugation, washed with PBS, and resuspended in buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 mM dithiothreitol. The cells were incubated on ice for 30 min. The cells were then treated with 10% Igepal, and centrifuged at 1300 rpm for 3 min. The pellets were collected and treated with extraction buffer containing 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, and 25% glycerol. The suspension was centrifuged at 14,000 rpm for 30 min at 4 °C. The pellets were freeze-thawed in liquid N_2 three times and centrifuged to remove debris. Approximately 7 μg of protein was incubated with or without an excess of unlabeled competitor. The probe was annealed to form oligomers and labeled using T4 polynucleotide kinase (Promega), [γ-^{32}P]ATP (Amersham Pharmacia Biotech). P-labeled oligonucleotide probe (5000 cpm) was added and the incubation was continued for additional 10 min at room temperature. The free and the protein-bound DNAs were separated on 5% polyacrylamide gels (29:1–acrylamide: bisacrylamide). The gels were pre-run for 30 min in 1 times TBE buffer and then run for 2–2.5 h at 125 V. The gels were transferred onto filter paper, and exposed to x-ray film (X-Omat, Kodak Co.) at −70 °C for 24–48 h with screen ontensifiers. The band density was scanned and analyzed using NIH image scanning.

2.8. Statistical analysis

The data were analyzed using the Mann–Whitney's *U* test. A *P* value less than 0.05 was considered to be significant.

3. Results

3.1. Levels of TARC production by PBMCs from patients with atopic dermatitis (AD)

Peripheral blood mononuclear cells (PBMCs) from AD patients cultured for 7 days produced high levels of TARC (440.3 ± 72.8 pg/ml) which were significantly higher than those from healthy controls (24.4 ± 10.1 pg/ml) ($P < 0.001$) (Fig. 1a and b). PBMCs from AD patients which were cultured with DME for 7 days showed enhanced levels of TARC production up to 1652.3 ± 368.5 pg/ml, which was significantly higher than by those cultured without DME ($P < 0.001$). TARC levels of PBMCs from AD patients which were cultured with DME for 7 days were significantly higher than those of healthy controls (163.4 ± 23.3 pg/ml, $P < 0.001$).

3.2. TARC mRNA expression by CD4$^+$ T cells, CD8$^+$ T cells and monocyte-derived dendritic cells (MoDC) from AD patients analyzed by RT-PCR analysis

We examined TARC mRNA expression of purified CD4$^+$ T and CD8$^+$ T cells derived from non-stimulated or DME-stimulated PBMCs from AD

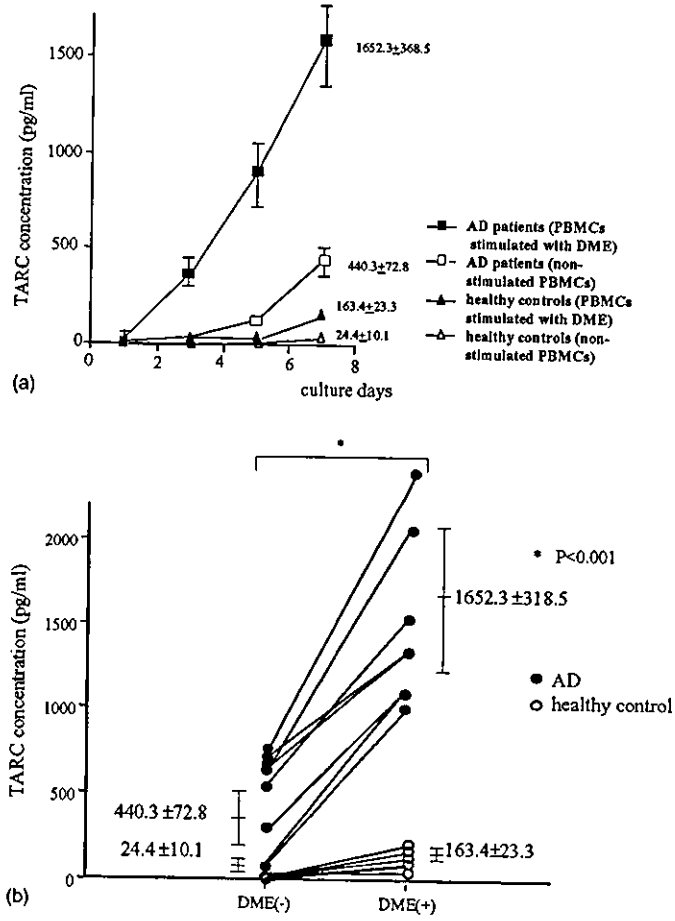

Fig. 1 TARC production from atopic dermatitis (AD) patients and healthy controls cultured with dust-mite allergen extract (DME). (a) Time course of TARC production by PBMCs is shown. PBMCs isolated from AD patients and healthy controls were cultured with DME (10 μg/ml) up to 7 days and TARC levels in the culture supernatants were measured at 3, 5 and 7 days. The levels of TARC production by PBMCs from AD patients were significantly higher than healthy controls ($P < 0.001$). The levels of TARC production by PBMCs from AD patients which were cultured with DME for 7 days were significantly higher than by those cultured without DME ($P < 0.001$). (b) TARC production by PBMCs cultured with DME (10 μg/ml) for 7 days is shown. The TARC levels by cultured PBMCs isolated from AD patients is significantly higher compared with those from healthy controls. Closed circles show TARC levels of AD patients and open circles show those of healthy controls.

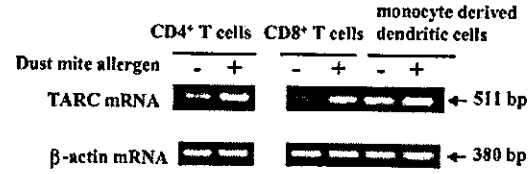

Fig. 2 TARC mRNA expression in $CD4^+$ T cells, $CD8^+$ T cells and monocyte derived dendritic cells (MoDCs) in AD patients. $CD4^+$ T cells and $CD8^+$ T cells were purified from non-stimulated or DME-stimulated PBMCs from AD patients for 24 h. Very little TARC mRNA was detected in non-stimulated T cells. After stimulation with DME for 24 h, TARC mRNA levels were enhanced in purified $CD4^+$ T cells and $CD8^+$ T cells. Monocytes were purified from non-stimulated PBMCs and monocyte derived DCs (MoDCs) were generated. MoDCs were stimulated with DME for 24 h and TARC mRNA expression was examined. Both non-stimulated and DME-stimulated MoDCs expressed high levels of TARC mRNA. Each experiment was performed at least three times and representative data are shown. Upper panel shows bands for TARC mRNA (511 bp) and lower panel shows bands for β-actin (380 bp).

patients. When the PBMCs were not stimulated, $CD4^+$ T and $CD8^+$ T cells expressed very little TARC mRNA, however TARC mRNA expression was dramatically enhanced in both $CD4^+$ T and $CD8^+$ T cells, when PBMCs from AD patients were stimulated with DME for 48 h (Fig. 2). We next examined TARC mRNA expression in MoDCs. Both DME-stimulated and non-stimulated MoDCs from AD patients strongly expressed TARC mRNA (Fig. 2).

3.3. Effects of immunosuppressive drugs on TARC production by DME-treated PBMCs from AD patients

We next investigated TARC production by PBMCs from AD patients which had been cultured with DME and immunosuppressive drugs (Tacrolimus, CsA or Dex) for 7 days and found that the drugs dose dependently inhibited TARC production by PBMCs as shown in Fig. 3. There were no significant differences of viability between non-treated and immunosuppressive drugs-treated groups (data not shown). ELISA showed that the inhibitory rates of TARC production by PBMCs cultured with Tacrolimus, CsA and Dex in AD patients were as follows; Tacrolimus: 87.4% (10^{-6} M), 79.4% (10^{-8} M), 47.1% (10^{-10} M); CsA: 79.5% (10^{-6} M), 29.2% (10^{-8} M), 13.8% (10^{-10} M); Dex: 74.2% (10^{-6} M), 29.1% (10^{-8} M), 2.1% (10^{-10} M), respectively.

3.4. Effects of immunosuppressive drugs on NFκB DNA binding by DME-treated PBMCs from AD patients

We investigated the effects of immunosuppressive drugs on NFκB activity in PBMCs from AD patients which were stimulated with DME. As shown in Fig. 4, NFκB DNA binding activity increased when

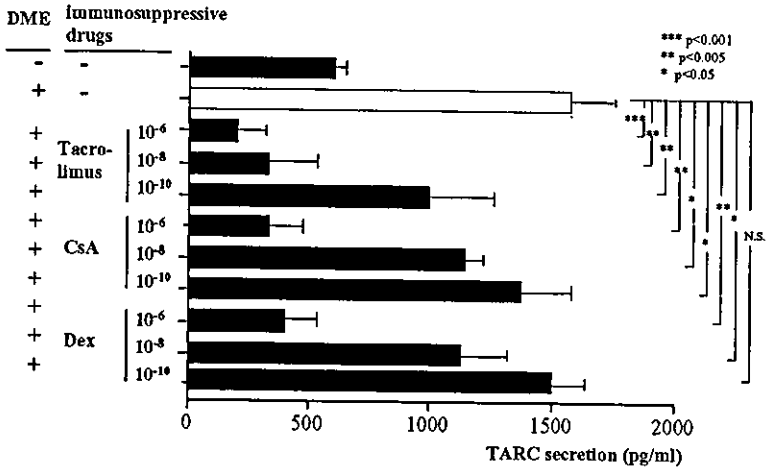

Fig. 3 Inhibitory effects of immunosuppressive drugs on TARC protein production by PBMCs from AD patients cultured with DME. PBMCs were cultured with DME (10 μg/ml); and various immunosuppressive drugs (10^{-8} M); for 7 days, and TARC levels in the supernatants were measured. The inhibitory effects of various immunosuppressive drugs on TARC protein production by PBMCs from AD patients cultured with DME for 7 days were as follows: Tacrolimus: 87.4% (10^{-6} M) 79.4% (10^{-8} M); 47.1% (10^{-10} M); CsA: 79.5% (10^{-6} M), 29.2% (10^{-8} M), 13.8% (10^{-10} M); Dex: 74.2% (10^{-6} M), 29.1% (10^{-8} M), 2.1% (10^{-10} M), respectively. The each experiment was performed at least three times and the average inhibitory rate was determined.

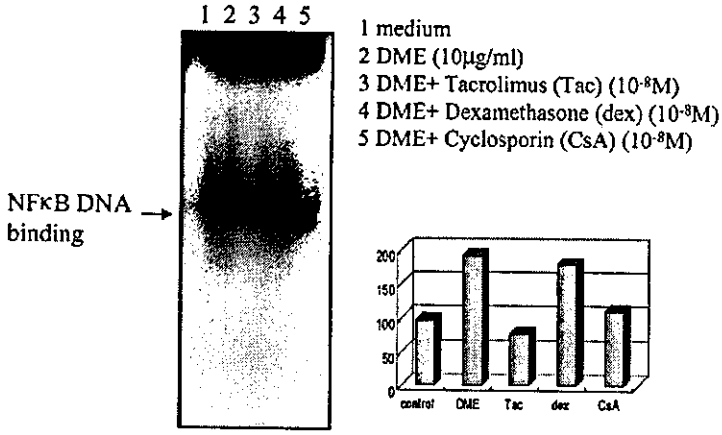

Fig. 4 Inhibitory effects of immunosuppressive drugs on NFκB activity in PBMCs from AD patients cultured with DME. The inhibitory effects of immunosuppressive drugs on NFκB activity by PBMCs cultured with DME were examined. NFκB activity was enhanced by DME- stimulation of PBMCs from AD patients. The inhibitory effects of Tacrolimus and CsA on NFκB activity were dramatic, while Dex inhibited NFκB activity only slightly. Each experiment was performed at least three times and representative data are shown. The band density was shown in the right panel.

PBMCs were cultured with DME (lane 2). Tacrolimus (10^{-8} M) and CsA (10^{-8} M) strongly suppressed NFκB binding while Dex (10^{-8} M) slightly suppressed NFκB DNA binding in nuclear protein obtained from stimulated PBMCs from AD patients.

4. Discussion

This study demonstrated the following: (1) PBMCs from AD patients which were cultured without stimulation for 7 days produced significantly higher levels of TARC protein (440.3 ± 72.8 pg/ml) compared to those from healthy controls (24.4±10.1 pg/ml, $P<0.01$). (2) When PBMCs from AD patients were stimulated with DME for 7 days, the levels of TARC production markedly increased up to 1652.3 ± 368.5 pg/ml, which was significantly higher than those without stimulation ($P < 0.001$). (3) PBMCs from AD patients which were stimulated with DME for 7 days produced significantly higher levels of TARC compared to those from healthy controls (163.4 ± 23.3 pg/ml, $P < 0.001$). (4) RT–PCR analysis demonstrated that in AD patients TARC mRNA was expressed in $CD4^+$ T cells, $CD8^+$ T cells and MoDCs. (5) ELISA showed that immunosuppressive drugs such as Tacrolimus, CsA and Dex suppressed TARC production by PBMCs from AD patients stimulated with DME for 7 days. (6) Gel shift assays revealed that immunosuppressive drugs differentially suppress NFκB activity in PBMCs from AD patients which had been stimulated with DME.

AD is an inflammatory skin disease characterized by infiltration of eosinophils, mast cells and Th2-type T cells in the lesional skin [7]. The immunological abnormality is characterized by overproduction of Th2 cytokines such as IL-4, IL5 and IL-13 and GM–CSF [7,8,14]. Among several factors which induce skin manifestations of AD, dust mite allergens is one of the major triggers that affects Th2 cytokine production in PBMCs of AD patients [9]. We showed previously that DME induces secretion of Th2 cytokines such as IL-4, IL-5 and IL-13 by PBMCs from AD patients [9]. Thus these data indicate that leukocytes in peripheral blood from AD patients produced predominantly Th2 cytokine patterns. The recent paper revealed that the lesional skin of AD showed Th2 cytokine mRNAs in acute lesional skin and the participation of Th1 cytokines such as IL-12 mRNA in chronic phase, and the mechanism of Th1/Th2 balance in the lesional skin of AD patients has not been completely elucidated [15].

TARC is a Th2 chemokine which can bind and attract $CCR4^+$ Th cells. We reported high levels of TARC in the sera of AD patients and demonstrated that TARC plays an important role in defining Th2 shift imbalance in the pathogenesis of AD [6]. In this paper we demonstrated that TARC production by PBMCs from AD patients is increased by the stimulation with DME. It has been reported that monocytes and DCs secrete significant amounts of TARC protein when stimulated, however un-stimulated T cells did not express TARC mRNA [16]. In this paper we clearly demonstrated that $CD4^+$ T and $CD8^+$ T cells from AD patients have the capacity to express TARC mRNA. Interestingly, when MoDCs were purified from PBMCs from AD patients, they strongly expressed TARC mRNA with or without the stimulation

by DME. These observation, strongly suggest that DCs, as well as $CD4^+$ T and $CD8^+$ T cells, produce TARC in PBMCs from AD patients, and we suggest that the TARC producing cells in AD are DCs, T cells in peripheral blood in addition to epidermal KCs as we previously demonstrated [6]. It has been reported that TARC/CCL17-deficient mice showed diminished contact hypersensitivity responses with decreased levels of antigen presenting capacity of TARC-deficient DCs [17]. Thus, we also suggest that TARC producing DCs attract Th2 cells as well as $CD25^+$ T cells and that these may participate in the regulation of immune responses in AD [18].

Immunosuppressive drugs are very effective and widely used for the treatment of AD. Tacrolimus and CsA bind calcineurin resulting in the inhibition of NF–AT nuclear translocation [6]. Glucocorticosteroids bind glucocorticoid receptors and inhibit IL-2 promoter activities by the interaction of glucocorticoid receptor with AP-1 to prevent it from binding to the IL-2 promoter [19]. In addition, glucocorticoids inhibit IL-5 production through RE-II region which contain NF–AT transcription region [20]. It has been reported that Tacrolimus inhibits IL-4 production through NF–AT activity in basophils [21]. We examined the effects of DME and the immunosuppressive drugs on NFκB activity in PBMCs from AD patients. Our data showed that DME strongly induced NFκB activity in PBMCs of AD patients. When PBMCs were stimulated with PMA, the enhancement of TARC production by PBMCs was detected in both AD patients and healthy controls, however, the enhanced levels was lower than those stimulated with DME stimulation (data not shown). Our data suggest that TARC production by PBMCs in AD patients may be enhanced by DME through the enhanced NFκB DNA binding activity.

In addition, we demonstrated that immunosuppressive drugs differentially inhibited NFκB activity of PBMCs. The inhibitory effect of tacrolimus and CsA on NFκB activity in PBMCs of AD patients was observed prominent. Because it is reported that the NFκB binding site may be located in TARC promoter lesion [22], we suggest that the inhibitory effect of immunosuppressive drugs such as Tacroimus and CsA on TARC production from PBMCs in AD may be partially explained by the inhibition of NFκB DNA binding activity in PBMCs. Further investigation will be required to clarify the mechanism of the regulation of TARC promoter activity in PBMCs of AD.

This paper demonstrated that overproduction of TARC levels was induced by DME in PBMCs of AD patients and that TARC mRNA was produced by $CD4^+$ T cells, $CD8^+$ T cells and MoDCs in AD patients. Immunosuppressive drugs inhibited TARC protein by PBMCs in AD patients. We conclude that Th2 chemokine, TARC, is produced by MoDCs and lymphocytes as well as epidermal KCs in AD, and that that these may be important in the regulation of T cells recruitment in peripheral blood in AD patients. Further study will be required to elucidate Th1/Th2 balance in the pathogenesis of AD.

Acknowledgements

This work was supported in part by Health Sciences Research Grants from the Ministry of Health, Labour and Welfare, and by Grants-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Journal of Dermatological Science (2004) 36, 165–172

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Effect of an antiallergic drug (Olopatadine hydrochloride) on TARC/CCL17 and MDC/CCL22 production by PBMCs from patients with atopic dermatitis

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Received 5 April 2004; received in revised form 31 August 2004; accepted 3 September 2004

KEYWORDS
Atopic dermatitis (AD);
Dust mite allergen;
Thymus and activation-regulated chemokine (TARC);
Monocyte-derived chemokine (MDC);
Olopatadine hydrochloride;
Disease activity

Summary

Background: Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by the predominant infiltration of Th2-type cells in lesional skin. Thymus and activation-regulated chemokine (TARC/CCL17) and monocyte-derived chemokine (MDC/CCL22) are Th2-type cytokines, and it has been reported that serum CCL17 and CCL22 levels are associated with AD disease activity. Olopatadine hydrochloride (Olopatadine) is an antiallergic drug with selective histamine H_1 receptor antagonist activity. The effect of Olopatadine on chemokine production by peripheral blood mononuclear cells (PBMCs) in AD patients has not been completely elucidated.
Objectives: This study was undertaken to clarify the effects of Olopatadine on CCL17 and CCL22 production by PBMCs from patients with AD during the treatment.
Methods: We measured plasma levels of CCL17, CCL22, IFNγ, IL-12 and IL-18 in 15 patients with AD before and after treatment with oral Olopatadine (10 mg/day) for 4 weeks. We also examined disease activity using SCORAD index, eosinophil numbers in peripheral blood and serum levels of LDH. PBMCs from the patients were taken before and after the treatment and cultured with or without dust mite allergen extract (DME) for 3 or 5 days. CCL17, CCL22, IFNγ, IL-12 and IL-18 levels in the supernatants of cultured PBMCs were measured.
Results: SCORAD index and eosinophil numbers in peripheral blood significantly decreased during treatment of AD patients with oral Olopatadine and topical corticosteroids for 4 weeks. The plasma levels of CCL17 and CCL22 significantly decreased

Abbreviations: AD, atopic dermatitis; DME, dust mite allergen extract; TARC, thymus and activation-regulated chemokine; PBMC, peripheral blood mononuclear cell; MDC, monocyte-derived chemokine

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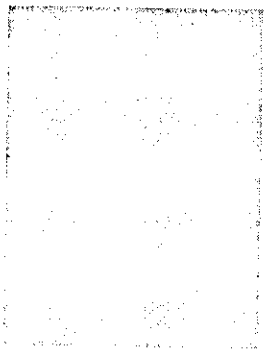
doi:10.1016/j.jdermsci.2004.09.001



after the treatment compared with before the treatment ($p < 0.05$) and were significantly correlated with SCORAD index. PBMCs from AD patients taken after the treatment and cultured with DME for 5 days, showed significantly lower levels of CCL17 production than those taken before the treatment ($p = 0.018$). PBMCs from AD patients taken after the treatment and cultured with DME for 5 days, also showed significantly lower levels of IFNγ production than those taken before the treatment ($p = 0.012$).

Conclusion: Our data demonstrate that Olopatadine inhibits CCL17 and CCL22 production by PBMCs from AD patients, which are important regulators of Th2 recruitment in the skin.

1. Introduction

Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disease that is associated with elevated serum IgE specific to environmental allergens such as mites and tissue and peripheral blood eosinophilia. It is characterized by predominant infiltration of Th2-type cells, increased secretion of Th2-type cytokines in acute phase lesional skin [1] and high responsiveness of peripheral blood mononuclear cells (PBMCs) to IL-4 [1,2].

Olopatadine has an antagonistic action against the histamine H_1 receptor [3]. It also inhibits the release of chemical mediators, such as platelet-activating factor (PAF), leukotrienes (LTs), and thromboxane $(TX)A_2$ from neutrophil-enriched human polymorphonuclear leukocytes [4]. Furthermore, it inhibits TNF-α- and histamine-induced expression of E-selectin and ICAM-1 on human umbilical vein endothelial cells [5], and LFA-1 and Mac-1 expression in eosinophils [6]. Olopatadine also shows inhibitory effects on neuropeptide, such as tachykinin release from sensory nerve endings [7]. The inhibition of cytokines such as IL-6 and IL-8 production from human conjunctival epithelial cells by Olopatadine has also been clarified [8].

Chemokines are chemoattractants which bring different types of leukocytes to sites of inflammation [9]. They are divided into four subfamilies, CXC, CC, CX_3C and C, depending on the position of their first two N-terminal cysteine residues [9,10]. Thymus and activation-regulated chemokine (TARC/CCL17) is a member of the CC chemokine superfamily, produced by monocyte-derived dendritic cells (DCs), endothelial cells and keratinocytes [11–14]. Macrophage-derived chemokine (MDC/CCL22) is also a CC chemokine produced by DCs [15]. CCL17 and CCL22 were identified as selective chemoattractants for cells expressing CC chemokine receptor 4 (CCR4), expressing such as Th2-type cells [16–18].

We showed previously that serum CCL17 and CCL22 levels, and the expression of CCR4 are significantly increased in patients with AD, and that the serum CCL17 and CCL22 levels are correlated significantly with the severity of AD [14,19]. This strongly suggests that the interaction of CCL17 and CCL22 with CCR4 may be important for recruiting Th2-type T cells in the pathogenesis of AD. However, little is known about the inhibitory effects of antiallergic drugs on CCL17 and CCL22 production by PBMCs of AD patients.

In the present study, we quantified the inhibitory effect of an antiallergic drug, Olopatadine hydrochloride (Olopatadine), on the levels of CCL17 and CCL22 in plasma of AD patients in order to clarify the action of antiallergic drug on Th2 chemokine production. We also compared CCL17 and CCL22 levels in the culture supernatants of dust mite allergen extract (DME)-stimulated PBMC taken from AD patients before and after the treatment.

2. Materials and methods

2.1. Samples and reagents

Fifteen AD patients were examined in this study. Their average age patients are 23.1 ± 3.3 years old ranging from 19 to 30. Five males and 10 females were in the study. All patients were diagnosed with AD according to the criteria of Hanifin and Rajka [20]. Specific IgE levels for dust mite allergens was 40.1 UA/ml in average (0.35–100 UA/ml). All patients were treated with oral Olopatadine at 10 mg/day for 4 weeks in combination with topical corticosteroids of the under the strong class. The patients were not treated with other antiallergic drugs, immunosuppressive drugs, or corticosteroids 1 week before treatment with Olopatadine. The class and topical amounts of topical corticosteroids per day were not altered between before and after the treatment with Olopatadine. Olopatadine hydrochloride (Olopatadine),(Z)-11-[(3-dimethylamino)polylidene]-6,11-dihydrodibenz [b,e] oxepin-

2-acetic acid monohydrochloride,is an orally active antiallergic drug with selective and potent histamine H_1 receptor antagonist activity [21,22].

Before and after the treatment, disease activities were determined by the SCORing Atopic Dermatitis (SCORAD) system. The mean SCORAD index was 37.3 ± 3.7. Serum LDH, IgE levels and eosinophil numbers in peripheral blood were examined and peripheral blood samples were taken before and after the treatment.

2.2. Culture of PBMCs

PBMCs were isolated from peripheral blood samples by centrifugation on a Ficoll-Metrizoate density gradient (Pharmacia Biotech, Uppsala, Sweden) at 2000 rpm for 20 min at room temperature. PBMCs were washed three times with PBS, and dispensed into culture dishes at 3×10^6 cells per well in RPMI1640 medium. They were cultured at 37 °C for 3 or 5 days in RPMI1640 with or without DME (Greer Laboratories Inc., NC) (10 μg/ml).

2.3. ELISA

We used 96-well polystyrene microplates coated with a murine mAb against human CCL17, CCL22, IFNγ, IL-12 (TECHNE Corp., Minneapolis, MN), or IL-18 (MBL Corp., Japan). The levels of these chemokines in plasma and in the culture supernatants of PBMCs taken from the patients before and after the treatment were measured [8]. The optical density of each well was determined using an ELISA reader (Spectra and Rainbow Readers).

2.4. Statistical analysis

The data were analyzed using the Wilcoxon test. A p value less than 0.05 was considered significant.

3. Results

3.1. Analysis of SCORAD index, laboratory data, and plasma levels of CCL17, CCL22, IFNγ, IL-12 and IL-18 in AD patients before and after treatment with oral Olopatadine and topical corticosteroids

The SCORAD index and eosinophil numbers decreased significantly after oral Olopatadine compared to before the treatment ($p < 0.001$) ($p < 0.05$) (Table 1). Serum LDH levels tended to decrease after treatment, but it was not significant (Table 1). Serum IgE levels did not significantly change from the treatment (Table 1). The level of plasma CCL17 was significantly lower after the treatment (669.6 ± 163.0 versus 1146.0 ± 249.9) ($p < 0.05$) (Table 1) and this was also true of the level of plasma CCL22 (1069.8 ± 129.6 versus 1589.9 ± 365.6) ($p < 0.05$) (Table 1). There were significant correlations between the SCORAD index and plasma CCL17 and CCL22 levels (CCL17: $r = 0.77$, $p < 0.001$; CCL22: $r = 0.49$, $p = 0.007$). There was also a significant correlation between the numbers of peripheral blood eosinophils and plasma levels of CCL17 ($r = 0.76$, $p < 0.001$) but this was not the case with CCL22 ($r = 0.39$, $p = 0.066$) (Fig. 1). There were no significant differences in the plasma levels of IFNγ, IL-12 and IL-18 before and after the treatment.

3.2. Levels of CCL17, CCL22, IFNγ, IL-12 and IL-18 in the supernatants of cultured PBMCs

We examined the levels of CCL17 and CCL22 in the supernatants of cultured PBMCs which were taken before and after the treatment. CCL17 levels in the supernatants of cells taken after Olopatadine

Table 1 SCORAD index, numbers of eosinophils in peripheral blood, serum levels of LDH, IgE, plasma levels of CCL17, CCL22, IFNγ, IL-12 and IL-18 before and after the treatment with oral Olopatadine

	Before	After	p
SCORAD	37.3 ± 3.7	22.3 ± 2.3	<0.001
Eosinophils (cells/μl)	490.5 ± 138.6	462.7 ± 119.8	<0.05
LDH (U/l)	235.3 ± 18.5	223.3 ± 12.8	0.124
IgE (U/l)	2251.4 ± 951.7	2102.8 ± 856.0	0.594
CCL17 (pg/ml)	1146.0 ± 249.9	669.6 ± 163.0	<0.05
CCL22 (pg/ml)	1598.9 ± 365.6	1069.8 ± 129.6	<0.05
IFNγ (pg/ml)	28.5 ± 6.4	27.5 ± 7.1	0.553
IL-12 (pg/ml)	19.0 ± 1.4	24.8 ± 5.0	0.272
IL-12 (pg/ml)	363.3 ± 34.6	373.4 ± 43.8	0.735

Fifteen AD patients were treated with oral Olopatadine 10 mg per day for 4 weeks. Before and after the treatment, disease activity was determined by SCORing Atopic Dermatitis (SCORAD) system.