Preferential expression of Th2-type chemokine and its receptor in atopic dermatitis

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Abstract

Lesional skin of patients with atopic dermatitis (AD) is histologically characterized by hypertrophy of the skin, and the infiltration of a large number of eosinophils and T cells into the dermis. Recent studies have indicated that Th2 cells play a crucial role in the pathogenesis of AD skin. Chemokines and their receptors are implicated in the development of symptoms of various skin diseases such as AD and psoriasis vulgaris (psoriasis). We have examined the in situ expression of a typical Th2-type chemokine, thymus- and activation-regulated chemokine (TARC), and its receptor (CCR4) using immunohistochemical techniques. TARC was found to be highly expressed in the basal epidermis of the lesional skin of AD patients and only slightly in the non-lesional skin. On the other hand, no positive cells were seen in the lesional skin of psoriasis. Consistently, CCR4+ cells were present predominantly in the lesional skin of AD patients, but not in the non-lesional skin. In contrast, in the lesional skin of psoriasis patients, cells positive for CCR5, which is expressed on Th1 cells, were abundantly present. Interestingly, psoralen plus ultraviolet A therapy reduced the number of CCR4+ cells in the AD skin lesions. These results suggest that Th2-type cytokines such as TARC are involved in the pathogenesis of skin lesions in AD patients through the preferential recruitment of Th2 cells.

Introduction

Atopic dermatitis (AD) is a common pruritic disease that occurs primarily in infancy and childhood (1–3). AD is characterized by itching, with patients having an individual or family history of atopic diseases in their background. Barrier dysfunction (4–8), immunological dysfunctions (type I and IV allergy) (9), genetic disorders (10,11) and psychological factors (12) contribute to the pathogenesis of AD (13).

Among these factors, CD4+ T, cells are reported to play a particularly crucial role in the pathogenesis of AD (14). It is commonly believed that allergens, e.g. aeroallergens, activate a particular subclass of T, cells recognizable by its cytokine profile (15). This fact allows the subdivision of mouse and human CD4+ T cells into two major subsets: Th1 cells that secrete IL-2, lymphotoxin and IFN-γ, but not IL-4, IL-5, IL-10 or IL-13, and Th2 cells that secrete IL-4, IL-5, IL-10 or IL-13, for example (16,17). In the immunological pathogenesis of AD, IgE and Th2 cells producing IL-4 play a key role in the onset and maintenance of the disease (18,19). On the other hand, it is reported that a Th1-type response is also associated with the pathogenesis of AD (20). In animal models of AD, Th1 cells appear to play pro-inflammatory roles, while Th2 cells may play an anti-inflammatory role (19,21). Thus, it is still controversial how these T cells with either Th1 or Th2 features are recruited into AD skin and contribute to the pathogenesis. Recent observations indicate that activated T cells acquire different migrating capacities (22). Given their different effector functions, it is likely that Th1 and Th2 cells are differentially recruited to the local sites.

Chemokines also contribute to the pathogenesis of AD (23). Chemokines and their receptors play a critical role in the selective recruitment of various subsets of leukocytes (24,25). There are indications that some chemokine receptors are differentially expressed on Th1 and Th2 cells (24,25). However, available data concerning the presence of T cells with Th1 or

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The features, and the expression of chemokines and their receptors in the lesional skin of AD are still limited (26). In the present study, we investigate the in situ expression of a T_{h}2-type chemokine and its receptor in the AD lesional skin using immunohistochemical techniques.

Methods

Patients

The subjects used in this study were 17 patients (10 men and seven women; age range 15–40 years; mean age 26.6 years)
diagnosed with AD according to the criteria proposed by Hanifin and Rajka (1). Of the 17 patients, nine had no history of either bronchial asthma or rhinitis, two had bronchial asthma, five had rhinitis, and one had both allergic asthma and rhinitis. All the patients were given the same therapy. For 3 days, systemic steroids and topical psoralen plus ultraviolet A (PUVA) therapy (27) were administered concurrently (steroid PUVA therapy) (28), while anti-histamines and topical treatment were continued. Clinical activity of skin lesions was measured by using the AD severity score (29) and the patients’ mean score was 7.58 ± 0.6. Total serum IgE levels of the AD patients were measured by the radioimmunosorbent test (RIST; Pharmacia, Uppsala, Sweden). The patients’ values ranged from 470 to 40,005 IU/ml and the mean value was 9015 IU/ml. The values for specific IgEs for various antigens were assessed by the radio allergosorbent test: 94.1% of AD patients were positive for house dust mite, 64.7% for Japanese cedar and 74.5% for fungus. As a disease control, five psoriasis vulgaris (psoriasis) patients (four men and one woman; age range 28–7 years; mean age 41.2 years) were entered into this study. In addition to the patient group, five healthy, non-atopic, age-matched individuals (three men and two women) were used as control subjects. All participants in this study gave their informed consent.

**Biopsy specimens**

Punch biopsy specimens (3 mm) were taken from those three groups (17 AD patients, five psoriasis patients and five normal control). In AD patients, before treatment and 1 month after treatment, biopsy specimens were taken from lesional and clinically non-lesional skin. In addition, 12 AD patients consented to have sequential biopsy specimens taken from areas subjected to burns by PUVA therapy, lesional and clinically non-lesional skin starting at 1 week after PUVA therapy. The biopsy specimens were snap-frozen in liquid nitrogen and stored at −80°C before use. Sections (4 µm) were cut on a freezing microtome and mounted on slides coated with 3-aminopropyltriethoxy-silane (Muto Glass, Tokyo, Japan).

**Immunohistochemical staining**

The following antibodies were used for immunohistochemical staining: mouse anti-human CCR4 antibody (KM2160; kindly provided by Dr Kouji Matsumoto, Department of Molecular Preventive Medicine, School of Medicine, University of Tokyo, Japan), mouse anti-human CCR5 antibody (36461A; Dako, Carpinteria, CA), mouse anti-human CD4 antibody (M716; Dako), mouse anti-human CD8 antibody (M707; Dako), rabbit anti-human thymus- and activation-regulated chemokine (TARC) polyclonal antibody (500-p107; PeproTech, London, UK), and mouse anti-human FceRI antibody (CRA-1; Kyokuto, Tokyo, Japan).

**Staining with mAb.** Sections were fixed with cold acetone (4°C) (Sigma, St Louis, MO) for 10 min and air-dried for 30 min. Primary antibodies were diluted in Tris–HCl (0.05 mol/l pH 7.6) and incubated with the sections for 8 h, then for 30 min in H2O2 in order to block endogenous peroxidase.

Slides were washed 2 times for 5 min with Tris–HCl (0.05 mol/l pH 7.6) after which a second layer of biotinylated donkey anti-mouse antibody (LSAB2 kit/HRP provided; Dako K0609) was applied for 10 min. After washing with Tris–HCl (0.05 mol/l pH 7.6) (2 times for 5 min each time), the sections were incubated with streptavidin-conjugated horseradish peroxidase (HRP) (LSAB2 kit/HRP provided; Dako K0609) for 10 min and washed 2 times for 5 min in Tris–HCl (0.05 mol/l pH 7.6). HRP reactivity was demonstrated with DAB solution [2% 3,3′-diaminobenzidine, 1 ml of stock solution, 99 ml of 50 mM Tris–HCl (pH 7.6), conc. H2O2 20 µl, for a total volume of 100 ml]. Slides were thereafet lightly counterstained with Methyl green (15944; Merck, LOCATION???) and embedded in a mounting medium (Malinol; Muto Pure Chemical, Tokyo, Japan).

**Staining with polyclonal antibodies.** Sections were fixed with cold acetone (−20°C) (Sigma) for 10 min, air-dried, and preincubated for 60 min in 2% BSA and 5% normal goat serum (NGS) in TBS (0.05 mol/l pH 7.6) + 0.05% Tween 20 (P-1379; Sigma). Primary antibodies were diluted in 2% BSA and 5% NGS in Tris–HCl (0.05 mol/l, pH 7.6) + 0.05% Tween 20, and incubated with the sections at 4°C for 8 h. Slides were washed for 30 min with TBS (0.05 mol/l, pH 7.6) + 0.05% Tween 20 and then in 2% BSA in TBS (0.05 mol/l, pH 7.6) + 0.05% Tween 20 for 30 min. After washing with PBS/ Tween (3 times for 5 min), HRP reactivity was demonstrated as in the previous method.

Evaluation of double-positive cells was done by first counting and photographing CCR4 or CCR5 stained sections, and thereafter the serial CD4 stained cells.

**Quantification of stained cells in the section**

The infiltrating mononuclear cells were not evenly distributed but showed an organized distribution in cell clusters. Cluster size was highly variable in different biopsy specimens. Therefore all cells located in the area from the cornified layer to the base of the hair follicles were counted at ×400 magnification by two independent observers. The number of positive cells was calculated per square millimeter and the average of five counts was taken. Cells adjacent to the hair follicles were excluded. Sequential sections of multiple 4-mm sections were stained with antibodies to CD4, CCR4 and CCR5 respectively, and were counted in 10–20 high-power fields (HPF) at ×400 and expressed as cells per HPF, with the mean ± SD calculated. T1 cells were calculated as CCR5/CD4 and T2 cells were calculated as CCR4/CD4. Statistical analysis of the immunohistology counts was performed using the Wilcoxon signed-rank test.

**Results**

Chemokine receptor CCR4 and CCR5 expression in skin specimens

Skin from patients with AD showed a very different picture from that of normal control subjects. In lesional skin, infiltrates containing mainly T cells and CCR4+ cells were found. The distribution of CCR4+ cells showed a patchy pattern (Fig. 1A). CCR5+ cells were distributed in a diffuse pattern in the lesional
A significant increase in the percentage of CCR5+ cells was observed in lesional AD skin as compared with normal skin. In contrast, a decrease in immunoreactivity was detected in non-atopic skin (Fig. 1C). CCR5+ cells were also observed, but their number was less than that of CCR4+ cells. The spindle shape of CCR5+ cells was also observed. Skin from patients with psoriasis showed a different picture from that of AD patients. Compared to the lesional skin of AD patients, there were less CCR4+ cells seen and the distribution of CCR4+ cells showed no distinct pattern (Fig. 1B). In contrast to the lesional skin of AD patients, there were more CCR5+ cells and their distribution showed a diffuse pattern (Fig. 1D). In addition, there were very few CCR4+ and CCR5+ cells in the skin of normal control subjects. CCR4 and CCR5 immunoreactivity was found predominantly in the mononuclear cells in the skin. The mononuclear cells in the epidermis and dermis were determined to be T cells (data not shown).

Quantification of CCR4+ and CCR5+ cells on in skin specimens

Increased numbers of CCR4+ cells were found in the sections of lesional AD skin (51.3 ± 1.4 per field) as compared with those in psoriasis skin (38.1 ± 17.1 per field) and the normal skin of control subjects (21.8 ± 16.7 per field) (Fig. 2).

These results suggest a significant increase in the percentage of CCR4+ cells in the lesional skin of AD patients as compared with normal control subjects (P < 0.027) and the lesional skin of psoriasis patients (P < 0.04). As for CCR4+ cells, an increase in immunoreactivity was detected in the non-lesional AD skin as compared with normal skin. In contrast, a significant increase in the percentage of CCR5+ cells was found in the lesional psoriasis skin as compared with normal control subjects (P < 0.027) and the lesional AD skin (P < 0.01).

Effect of steroid PUVA treatment on CCR4 and CCR5 expression in AD skin specimens

One month after treatment with steroid PUVA therapy, the skin lesions regressed. The number of CD4+ cells in the skin decreased to below normal levels, while that of CD8+ cells returned to normal levels (data not shown). The number of CD4+ cells and FcεRI+ cells decreased in treated lesional skin as compared with untreated lesional skin, but did not return to normal levels even after 1 month of treatment (data not shown). The numbers of both CCR4+ and CCR5+ cells decreased in treated lesional skin as compared with untreated lesional skin (Table 1).

Chemokine expression in AD skin specimens

Since one of the ligands for CCR4 is TARC, we further attempted to determine which ligand was expressed in the skin. Immunohistochemical studies on TARC expression were then carried out. TARC was markedly expressed in the non-lesional skin (Fig. 3A), but increasingly in the lesional AD skin before treatment (Fig. 3B). Weak expression was observed in normal skin (Fig. 3C) and psoriasis skin (data not shown). On the other hand, a decrease in the expression of TARC was observed in lesional AD skin after treatment (Fig. 3D). The expression of TARC was localized mainly to the basal layer of the epidermis, the secretary and ductal segments of the eccrine sweat glands (Fig. 3C).

Discussion

In this study, we examined the in situ expression of T2-type CCR (CCR4) and its ligand, TARC, in AD skin lesions by immunohistochemical analysis. The expression of CCR4 was markedly increased in lesional AD skin as compared with the skin of psoriasis patients and normal control subjects (non-atopic). In lesional AD skin, the expression of TARC was recognized in the basal cell of epidermis, venules and eccrine gland, but less immunoreactivity was recognized in non-lesional skin of AD patients. In skin of normal control subjects (non-atopic) and psoriasis, very little expression was seen. In this study, immunohistochemical staining could not detect the expression of macrophage-derived chemokine, although mRNA was recognized by RT-PCR (data not shown).

There are many mechanisms involved in the pathogenesis of AD such as type I allergy (18), type IV allergy (9) and barrier dysfunctions (4–8). Moreover, in recent years, a considerable body of evidence has implicated T cells as having a major role in the pathogenesis of AD (14). Increased numbers of T2 cells with a T2-type cytokine profile are present, especially in the initial phase of skin inflammation (16), whereas both T2-type cytokines (IL-5) and T1-type cytokines (IFN-γ) are up-regulated in chronic lesions (19). In recent studies, AD has been described to be a T2-type disease, at least in the initial phase, as lymphocytes invading the skin mainly produce T2-type cytokines.
cytokines such as IL-4, IL-5 and IL-10 (16), although the acute phase of AD revealed a cytokine profile of a Th1-type disease like psoriasis and contact dermatitis (30,31).

Recent data indicate that human Th1 and Th2 cells express distinct chemokine receptors (generated under the influence of IL-12 and IL-4 respectively) and are differentially recruited in response to chemokines (23). Th2 cells were shown to express CCR3 preferentially and selectively migrate in response to eotaxin (32). CCR4 and CCR8 were also shown to be expressed on Th2 cells (33,34). In contrast, CXCR3 and

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**Fig. 3.** Expression of TARC in skin biopsies. (A) TARC staining of non-lesional skin of AD patients before treatment; original magnification: ×100. (B) TARC staining of lesional skin of AD patients before treatment; original magnification: ×400. (C) TARC staining of non-lesional skin of normal control subjects; original magnification: ×200. (D) TARC staining of non-lesional skin of AD patients after treatment; original magnification: ×200. The sections were stained as described in Methods.
CCR5 were also shown to be expressed on Th1 cells (35). Thus, expression of the CXCR3 ligands, IP-10 and Mig, would lead to the preferential recruitment of Th1 cells, whereas the expression of CCR4 ligands such as TARC and macrophage-derived chemokine (MDC) would lead to preferential recruitment of Th2 cells (36,37). Both IP-10 and Mig are induced by IFN-γ (38), while IP-10 is found at sites of Th1-type immune responses and delayed-type hypersensitivity (39). IFN-α and transforming growth factor-β also have a major influence on Th1/Th2 polarization, and affect the expression of chemokine receptors (40). However, the regulation of chemokine expression is still poorly understood.

As shown in this study, expression of TARC in the skin of AD patients was mainly observed in keratinocytes and eccrine gland cells. This finding suggests that TARC produced by keratinocytes and eccrine glands could act as an early inducer of the Th2 response by attracting CCR4+ Th2 cells and lymphocytes in the dermis of lesional skin (44), and lesions of NC/Nga mice, and the production of IL-4 in mast cells. On the other hand, psoriasis is a Th1 disease. By immunohistochemical analysis, the expression of TARC and CCR4 was up-regulated in AD patients and correlated to the disease severity (22). Importantly, the number of CCR4+ cells, especially in non-lesional AD skin, may trigger the development of eczematous lesions. On the other hand, the number of dermal Langerhans’ cells (FerR1+) was still high, as was the number of mast cells. In a previous study, mast cells and lymphocytes decreased after PUVA therapy (58), but there are no data relating to the effects of UVA for chemokines and their ligands. Therefore, we suspect that UVA has some sort of effect especially on keratinocytes, mast cells and Langerhans cells (59).

Keratinocytes are known to produce many cytokines, such as IL-1α, IL-6 and IL-8 (60), and chemokine (TARC) (61). These cells work with each other to constitute one of the factors in the pathogenesis of AD (62). The results we presented support the previous reports on the expression of chemokines in NC/Nga mice treated with topical steroids (22). Interestingly, the levels of TARC produced in the skin correlated with the severity of the skin lesions, whereas MDC was produced by dermal dendritic cells in both lesional and non-lesional skin, although the level of MDC production increased in lesional skin, as did the number of MDC producing cells.

In conclusion, our data suggest that AD is a Th2 disease; on the other hand, psoriasis is a Th1 disease. By immunohistochemical analysis, the expression of TARC and CCR4 is up-regulated in AD patients and correlated to the disease activities of AD. To establish whether TARC and MDC are (49), promoting tissue inflammation and inducing tissue damage through the release of various kinds of chemical mediators, cytokines and substances like tumor necrosis factor (TNF)-α, IL, prostaglandins, leukotriens, PAF, MIP-1α, MIP-1β, RANTES, IL-8 and lymphotactin, which play a role (49) in type I allergy (50), cell adhesion (51), chemotaxis (52) and fibrosis (53). The attraction of Th2 cells to the site of inflammation in AD is of particular relevance as Th2-type cytokines, such as IL-4, IL-5 and IL-13, were reported to promote eosinophil, basophil and mast cell activation (48). Similarly, mast cells produce many kinds of Th2-type cytokines (48). TNF-α has been reported to be up-regulated in mast cells in the skin of AD lesions (54). Of all the cells, mast cells are the most important source of TNF-α (48). TNF-α induces expression of adhesion molecules in the endothelium of lesional AD skin (55). TNF-α is also an inducer of cytokines, chemokines and adhesion molecules (56). When murine keratinocytic cell line cells were cultured in the presence of several inflammatory cytokines, TNF-α was found to be the most potent inducer of TARC (22).

From the clinical perspective, serum levels of IgE, LDH and plasma levels of eosinophil cationic protein correlate with the severity of AD (57). Interestingly, in NC/Nga mice, the levels of TARC produced in the skin correlated with the severity of the condition (22). In our study, enhanced TARC and CCR4 expression was also found to be correlated with increased numbers of mast cells and their cytokines in AD skin lesions. Steroid PUVA treatments (28) for 1 month had an ameliorative effect on the CCR4+ cells of lesional AD skin. After treatment, regression of acanthosis and hyperkeratosis were observed. Infiltration was markedly reduced, leaving only very few CD4+ cells and almost no CD8+ cells. This fact may be due to a direct effect of steroids and PUVA therapy (28). Importantly, the number of CCR4+ and CCR5+ cells significantly decreased (Table 1). This finding is of particular interest, as activation of CCR4+ cells, especially in non-lesional AD skin, may trigger the development of eczematous lesions. On the other hand, the number of dermal Langerhans’ cells (FerR1+) was still high, as was the number of mast cells. In a previous study, mast cells, Langerhans cells and lymphocytes decreased after PUVA therapy (58), but there are no data relating to the effects of UVA for chemokines and their ligands. Therefore, we suspect that UVA has some sort of effect especially on keratinocytes, mast cells and Langerhans cells (59). Keratinocytes are known to produce many cytokines, such as IL-1α, IL-6 and IL-8 (60), and chemokine (TARC) (61). These cells work with each other to constitute one of the factors in the pathogenesis of AD (62). The results we presented support the previous reports on the expression of chemokines in NC/Nga mice treated with topical steroids (22). Interestingly, the levels of TARC produced in the skin correlated with the severity of the skin lesions, whereas MDC was produced by dermal dendritic cells in both lesional and non-lesional skin, although the level of MDC production increased in lesional skin, as did the number of MDC producing cells.

In conclusion, our data suggest that AD is a Th2 disease; on the other hand, psoriasis is a Th1 disease. By immunohistochemical analysis, the expression of TARC and CCR4 is up-regulated in AD patients and correlated to the disease activities of AD. To establish whether TARC and MDC are
selectively involved in \( T_{h2} \)-mediated skin disease, further investigations in other types of eczematous lesion are required. Our data show that chemokine receptor expression on T cells is controlled by its activation and by cytokines present in its specific environment such as an allergic status (acute to chronic). TARC/MDC–CCR4 interaction is likely to represent a major mechanism for promoting tissue inflammation and recruitment of inflammatory cells. The expression of chemokine receptors serves not only as a useful marker of T cell differentiation, but also indicates that distinct functional subsets such as CCR4 and CCR3 antagonists are potential candidates for immunotherapy in AD patients.

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Abbreviations

AD: atopic dermatitis  
HFP: high-power field  
HRP: horseradish peroxidase  
MDC: macrophage-derived chemokine  
NGS: normal goat serum  
psoriasis: psoriasis vulgaris  
PUVA: psoralen plus ultraviolet A  
TARC: thymus- and activation-regulated chemokine  
TNF: tumor necrosis factor

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