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: T1 cells that secrete IL-2, lymphotoxin and IFN-γ, but not IL-4, IL-5, IL-10 or IL-13, and T2 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 T1-type response is also associated with the pathogenesis of AD (20). In animal models of AD, T1 cells appear to play pro-inflammatory roles, while T2 cells may play an anti-inflammatory role (19,21). Thus, it is still controversial how these T cells with either T1 or T2 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 T1 and T2 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 T1 and T2 cells (24,25). However, available data concerning the presence of T cells with T1 or
Th2 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 Th2-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, AD patients consented to have sequential biopsy specimens taken from areas subjected to burns by PUVA therapy, lesional and non-lesional skin starting at 1 week after PUVA therapy. The biopsy specimens were taken from the chronically lesional skin and clinically non-lesional skin of psoriasis patients and normal skin of control subjects. 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 Matsushima, Department of Molecular Preventive Medicine, School of Medicine, University of Tokyo, Japan), mouse anti-human CCR5 antibody (36461A; Dako, Carpenteria, 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 thereafter 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 clusters. Cluster size was highly variable in different biopsy specimens. Therefore all cells located in the area from the cornified layer 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 T1,2 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 skin of control subjects. 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).
In this study, we examined the in situ expression of Th2-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 Th2 cells with a Th2-type cytokine profile are present, especially in the initial phase of skin inflammation (16), whereas both Th1-type cytokines (IL-5) and Th1-type cytokines (IFN-γ) are up-regulated in chronic lesions (19). In recent studies, AD has been described to be a Th2-type disease, at least in the initial phase, as lymphocytes invading the skin mainly produce Th2-type cytokines (20).

**Table 1. Effect of PUVA on CCR4+ and CCR5+ cells**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR4</td>
<td>36.42 ± 13.93</td>
<td>22.08 ± 6.39</td>
<td>0.04</td>
</tr>
<tr>
<td>CCR5</td>
<td>6.25 ± 2.08</td>
<td>4.47 ± 1.27</td>
<td>0.14</td>
</tr>
<tr>
<td>CCR4/CD4</td>
<td>73 ± 7.96%</td>
<td>32.2 ± 27.4%</td>
<td>0.08</td>
</tr>
<tr>
<td>CCR5/CD4</td>
<td>17.8 ± 4.1%</td>
<td>10 ± 5.5%</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Cell numbers of CCR4+ and CCR5+ cells, and percentage of CCR4+ and CCR5+ cells among CD4+ cells in pre-treatment and post-treatment AD skin were calculated as described in Methods.
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

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 T1 cells, whereas the expression of CCR4 ligands such as TARC and macrophage-derived chemokine (MDC) would lead to preferential recruitment of T2 cells (36,37). Both IP-10 and Mig are induced by IFN-γ (38), while IP-10 is found at sites of T1-type immune responses and delayed-type hypersensitivity (39). IFN-α and transforming growth factor-β also have a major influence on T1/T2 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 activated keratinocytes and eccrine glands could act as an early inducer of the T2 response by attracting CCR4+ Th2 activated keratinocytes and eccrine glands could act as an important source of chemokine expression (41,42). The sharing of chemokine receptors by Th2 and the effector cells involved in allergic reactions is likely to represent a key mechanism in the generation of allergic responses (22). It is possible that TARC production is differentially regulated according to the degree of surrounding inflammation and that such inflammation is not present in the microenvironment of non-lesional skin of AD patients and normal control subjects. CCR3 and CCR4 ligands may selectively attract not only eosinophils and basophils, but also T2 cells, that, upon activation by an antigen, could provide a source of IL-4 and IL-5 required for activation and survival of the effector cells (42).

TARC/MDC and CCR4 have been shown to be involved in the T2-mediated disease process in murine models (22). We previously reported the production of IL-4 and IL-5 in skin lesions of NC/Nga mice, and the production of IL-4 in mast cells and lymphocytes in the dermis of lesional skin (44), and thus proposed NC/Nga mice as a model for human AD (43). These reports indicate that expression of both TARC and CCR4 was elevated in resident cells such as keratinocytes and endothelial cells as well as in mast cells and mononuclear cells recruited to the site of inflammation in NC/Nga mice. The up-regulation of TARC by basal keratinocytes correlates with the progression of lesions in the skin of NC/Nga mice. Thus, our present results in human AD are consistent with with those in NC/Nga mice. This also suggests that TARC may play a key pathogenic role (22).

The chemokine receptor predominantly expressed on T1-type human T cells is CCR5 (34), CCR7 (45) and the ligands for this receptor are RANTES and MIP1α/β. In this study, we demonstrated expression of CCR5+ cells in lesional skin of AD and psoriasis patients. CCR5+ cells are increased in both patients, but in psoriasis patients, predominantly expression of CCR5 rather than of CCR4. Recent study suggests that RANTES and MIP-1α have been shown to be involved in the skin of psoriasis, even in that of AD (40,46).

The pathologically of AD lesions shows numerous lymphocytes, degranulated eosinophils, Langerhans cells and mast cells (47). In AD, mast cells serve as important effector cells (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 lymphotacticin, which play a role (49) in type I allergy (50), cell adhesion (51), chemotaxis (52) and fibrosis (53). The attraction of T2 cells to the site of inflammation in AD is of particular relevance as T2-type cytokines, such as IL-4, IL-5, IL-10 and IL-13, were reported to promote eosinophil, basophil and mast cell activation (48). Similarly, mast cells produce many kinds of T2-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 (FoxR1+) 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 UVB for chemokines and their ligands. Therefore, we suspect that UVB 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 T2 disease; on the other hand, psoriasis is a T1 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 Th2-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
HPF = 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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