Presence of Circulating CCR10+ T cells and Elevated Serum CTACK/CCL27 in the Early Stage of Mycosis Fungoides

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Abstract

Purpose: Mycosis fungoides (MF), a common type of cutaneous T cell lymphoma with an indolent clinical course, has the characteristic that malignant T cell clones are recruited into the skin from the early disease stages. The mechanisms of recruitment have been suggested from our knowledge of various chemokine-chemokine receptor interactions. Recently, CCR10 and CTACK/CCL27 were proposed to play a role in the recruitment of other types of cutaneous T cell lymphoma. We examined the expression of CCR10 in peripheral blood and serum CTACK/CCL27 levels in patients with MF.

Experimental Design: Eighteen patients with MF, six patients with atopic dermatitis, and nine healthy volunteers were enrolled in our investigation. We investigated the differences in CCR10+ CD4+ expression in peripheral blood mononuclear cells by flow cytometry. Serum CTACK/CCL27 levels were determined using a CTACK/CCL27 ELISA assay kit.

Results: The number of circulating CCR10+ CD4+ cells was significantly higher in MF peripheral blood than in controls, even during the early stages. In lesional MF skin, infiltrating tumor cells also showed extensive expression of CCR10. The serum level of CTACK/CCL27 was higher in patients with MF than normal controls, but no statistical difference was found compared with atopic dermatitis patients.

Conclusions: CCR10-CTACK/CCL27 interactions between circulating T cells and keratinocytes would seem to play an important role in the pathophysiology of MF from the early disease stages.

Mycosis fungoides (MF) is the most common cutaneous T cell lymphoma (CTCL), with an estimated incidence of 0.5 cases per 100,000 population per year in the western world (1). MF has a classically slow clinical course that progresses over years through the patch, plaque, and tumor stages, followed by lymph nodes and visceral involvement (2). In MF, although malignant T cells persist mainly in skin and only few cells circulate in peripheral blood, recent studies have revealed an aberrant T cell immunophenotype and circulating clonal cutaneous lymphocyte antigen (CLA)-positive T cells in patients’ blood (3–5). It was also shown that CLA+ CD4+ T cells express the CC chemokine receptor 4 (CCR4), which is suspected of playing a role in skin-homing. CCR4 was originally discovered in memory T cells (6, 7). Furthermore, Sokolowska-Wojdylo et al. reported that another CC chemokine receptor associated with skin-homing, CCR10, is expressed in circulating clonal CLA+ CD4+ cells in Sézary syndrome (8).

Another study showed that malignant T cells expressing CCR10 were infiltrated in skin tissues in Sézary syndrome, MF, and unspecified CTCL (9).

Cutaneous T cell attracting chemokine (CTACK/CCL27) is a skin-associated chemokine that attracts skin-homing memory T cells (10). CTACK/CCL27 is known to be the ligand for CCR10, and is mainly produced by activated keratinocytes in various diseases such as atopic dermatitis, psoriasis, drug reactions, and other inflammatory conditions (11, 12). CTACK/CCL27 is also expressed by dermal components and by the microvasculature, playing an important role in recruiting T cells into skin (13).

We hypothesized that CCR10-CTACK/CCL27 interactions play an early role in the pathophysiology of MF from the patch stage. In this report, we have examined the expression of CCR10 in CD4+ cells circulating in patients without apparent MF peripheral blood involvement. In addition, we have determined the concentration of CTACK/CCL27 protein contained in MF patients’ serum.

Materials and Methods

Materials. The following monoclonal antibody was used in this study: CD4 was from BD Bioscience (San Jose, CA), CCR10 from Immuno Detect, Inc. (Fayetteville, NY), CTACK/CCL27 from R&D systems (Minneapolis, MN). DMEM was purchased from Invitrogen (Groningen, Netherlands), collagenase from Wako Pure Chemical (Osaka, Japan), Ficoll from Amersham Biosciences Corp. (Piscataway, NJ), human CTACK/CCL27 ELISA assay kit was from R&D Systems. All other chemicals were of reagent grade or higher.
Patients and controls. Eighteen patients with MF (eight men and 10 women; mean age, 61.3 years old) from the Department of Dermatology at Hokkaido University Graduate School of Medicine were enrolled in this study (Table 1; Fig. 1). MF was diagnosed on the basis of clinical images, histopathologic findings from skin biopsies (all cases), and gene rearrangement analysis from skin tissue. Clinical stages were also evaluated according to the modified staging classification for CTCL proposed by Kashani-Sabet et al. (14). The clinical stages were also evaluated according to the modified staging classification for CTCL proposed by Kashani-Sabet et al. (14). The evaluations of clinical images, including the clinical stages, were done by a single qualified dermatologist. Blood and serum samples were collected under proper, informed consent. In two patients at the tumor stage, tumor samples were analyzed for flow cytometry analysis. For control purposes, nine healthy volunteers and six atopic dermatitis patients who had widespread skin lesions with moderate activity were also investigated.

Flow cytometry. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation using Ficoll. Two-color flow cytometry was done by incubation of cells (2 x 10^6) at 4°C. Staining for CCR10 done with monoclonal antibody against CCR10 followed by PE-labeled goat anti-mouse IgG (Rockland Immunochemical, Gilbertsville, PA) and FITC-conjugated antihuman CD4 monoclonal antibody (BD PharMingen, San Diego, CA). Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA), and CellQuest software (Becton Dickinson). Tumor cells were obtained from two patients' biopsy materials. The biopsy material was incubated with 200 units/mL collagenase in DMEM with 5% FCS at 37°C for 30 minutes. Suspensions were washed twice with PBS, and 50-μL suspensions were prepared for flow cytometry analysis. For control purposes, nine healthy volunteers and six atopic dermatitis patients who had widespread skin lesions with moderate activity were also investigated.

Flow cytometry. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation using Ficoll. Two-color flow cytometry was done by incubation of cells (2 x 10^6) at 4°C. Staining for CCR10 done with monoclonal antibody against CCR10 followed by PE-labeled goat anti-mouse IgG (Rockland Immunochemical, Gilbertsville, PA) and FITC-conjugated antihuman CD4 monoclonal antibody (BD PharMingen, San Diego, CA). Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA), and CellQuest software (Becton Dickinson). Tumor cells were obtained from two patients' biopsy materials. The biopsy material was incubated with 200 units/mL collagenase in DMEM with 5% FCS at 37°C for 30 minutes. Suspensions were washed twice with PBS, and 50-μm filters were used to remove larger tissue fragments. Cells (1 x 10^6) were prepared for two-color analysis by resuspending in PBS containing 1% FCS and 1% bovine serum albumin, and flow cytometry was done as described above.

ELISA. Serum CTACK/CCL27 levels were determined using a CTACK/CCL27 ELISA kit assay in patients with MF, patients with atopic dermatitis, and healthy volunteers. We used a 96-well polystyrene microplate coated with a murine monoclonal antibody against human CTACK/CCL27. Optical densities were measured at 450 nm with a Bio-Rad Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA). The protein levels were calculated from a standard curve generated by a curve-fitting program.

Immunohistochemistry. Paraffin-embedded skin tissues from patients with MF were cut into 4-μm-thick sections. To prepare the sections for immunohistochemical analysis, they were pretreated with 3% hydrogen peroxide for 10 minutes at 4°C. They were stained with avidin-biotin peroxidase complex procedure using a Vector ABC Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. In brief, samples were treated with 10% normal goat serum for 30 minutes at room temperature followed by overnight incubation with the anti-CTACK/CCL27 monoclonal antibody or anti-CCR10 monoclonal antibody at 4°C. Positive staining was visualized with dianinobenzidine as a chromogen using a streptavidin-biotin peroxidase system.

Table 1. Clinical characteristics of patients with MF

<table>
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<tr>
<th>Patient</th>
<th>Age, sex</th>
<th>Duration (mo)</th>
<th>Clinical stage</th>
<th>Tumor-node-metastasis-blood classification, stage</th>
<th>sIL-2R (units/mL)</th>
<th>LDH (IU/L)</th>
<th>CCR10 in PBMC (%)</th>
<th>Serum CTACK/CCL27 (pg/mL)</th>
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<td>3</td>
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<td>153</td>
<td>3.02</td>
<td>760</td>
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<td>82</td>
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<td>T3N0M0B0 stage IIIA</td>
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<tr>
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<td>T3N0M0B0 stage IIIA</td>
<td>1,200</td>
<td>188</td>
<td>0.64</td>
<td>850</td>
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</table>

Fig. 1. Clinical images. Patient 3, pink-red macules disseminated on the legs (patch stage). Patient 6, infiltrated erythematous plaques on the buttocks (plaque stage). Patient 16, poikilodermatous erythema, plaques and ulcerated nodules (tumor stage).
Statistics. Differences between various treatments were statistically tested using Mann-Whitney U tests. $P < 0.05$ was considered statistically significant. Data in the figures are shown as the mean ± SE of multiple samples.

Results

Flow cytometric analysis of CCR10 expression in circulating CD4+ cells. Representative flow cytometry analyses of the surface expressions of CD4 and CCR10 in a healthy volunteer, an atopic dermatitis patient, and MF patient 16 are shown in Fig. 2. Summary of percentages of CCR10+ CD4+ cells in the CD4+ T cell population is shown in Fig. 3. The average percentages of CCR10+ cells were 0.91 ± 0.13%, 0.55 ± 0.15%, and 2.19 ± 0.39% in healthy volunteers ($n = 9$), patients with atopic dermatitis ($n = 6$) and patients with MF ($n = 18$), respectively. Patients with MF express significantly more CCR10+ CD4+ cells ($P < 0.05$). When classified into clinical stages of MF, the average percentages were 2.68 ± 0.82%, 1.68 ± 0.41%, and 3.11 ± 1.35% in patch stage ($n = 5$), plaque stage ($n = 10$), and tumor stage ($n = 3$), respectively. Also, the average percentages were 2.31 ± 0.76% (stage I, $n = 6$), 1.86 ± 0.49% (stage II, $n = 8$), and 2.71 ± 1.04% (stages III and IV, $n = 4$). There was no statistical correlation between CCR10 expression and the MF patient clinical stage. Immunohistochemical analysis for CCR10 in MF skin tissue also showed strong CCR10 expression in tumor cells (Fig. 4, top). Flow cytometric analysis for CCR10 in skin tissue was also investigated in skin tumor cells. CCR10 was markedly expressed in 34.03% of mononuclear cells (patient 16, tumor stage; Fig. 4, bottom). These results are consistent with a recent study (9).

Serum CTACK/CCL27 levels by ELISA. The serum CTACK/CCL27 concentrations in patients with MF were 993.6 ± 84.3 pg/mL. Conversely, those in healthy volunteers ($n = 9$) and atopic dermatitis patients ($n = 6$) were 430.6 ± 26.0 and 887.0 ± 56.9 pg/mL, respectively (Fig. 5, top). Serum CTACK/CCL27 levels were statistically elevated in patients with MF compared with normal controls ($P < 0.05$), but no statistical difference was found between patients with atopic dermatitis, in which the elevation of serum CTACK/CCL27 had been previously reported (15). Compared with each MF clinical stage, serum CTACK/CCL27 levels were 652.5 ± 28.3, 1,126.6 ± 111.7, and 1,118.7 ± 196.4 pg/mL during the patch ($n = 5$), plaque ($n = 10$), and tumor stages ($n = 3$), respectively. Statistically significant increases were found between the patch stage and the other two stages (Fig. 5, bottom). According to the tumor-node-metastasis classification, the average levels were 656.5 ± 23.5 (stage I, $n = 6$), 1,158.8 ± 125.0 (stage II, $n = 8$), and 1,169.0 ± 147.7 pg/mL (stages III and IV, $n = 4$). Patients with stage II or more advanced stages also showed a statistically higher level of serum CTACK/CCL27 than those with MF disease stage I ($P < 0.05$).

Fig. 2. PBMCCCR10 expression. PBMCs were stained with the indicated monoclonal antibodies and analyzed by flow cytometry (left, healthy volunteer; middle, atopic dermatitis patient; right, MF patient). The MF patient expressed more CCR10+ in circulating T cells.

Fig. 3. CCR10 analysis using flow cytometry in PBMCs. Top, the percentage of CCR10+ T cells in PBMCs of MF patients were 0.55% to 5.3% (2.19 ± 0.39; average ± SE), which statistically exceeded those of healthy volunteers and patients with atopic dermatitis. Bottom, the expression of CCR10 was not correlated with MF clinical or disease stage.
Immunohistochemistry for CTACK/CCL27 in skin tissues. CTACK/CCL27 was strongly expressed in keratinocytes around both basal and suprabasal epidermal layers. CTACK/CCL27 diffusely stained the cytoplasm of keratinocytes (Fig. 6). In addition, endothelial cells of the superficial dermal plexus were stained. There was no correlation between the MF stages and extent of CTACK/CCL27 staining, or between serum CTACK/CCL27 levels and tissue expression (data not shown). Conversely, the CTACK/CCL27 expression in normal control skin was restricted to the basal cell layer (data not shown), as described previously (11).

Discussion

It has been shown that small numbers of clonal, aberrant T cells circulate in patients’ peripheral blood even in early stages of MF, which is associated with the skipped expansion and micrometastasis of the MF lesions (3–5). In addition, circulating CLA+ CD4+ T cells express CCR10, a CC chemokine receptor for skin-homing function, in patients with CTCL (especially Sézary syndrome; ref. 8). Another study showed that malignant T cells in the MF lesional skin express CCR10 (9). Therefore, we speculate that CCR10 and its ligand CTACK/CCL27 play an important role in progression and skin-homing in early MF. In the present study, we have confirmed the previous findings in patients with MF, in addition to the following new observations: (a) we have identified significantly increased numbers of CCR10+ CD4+ cells in MF patients’ PBMCs without any other peripheral blood involvement as shown by routine laboratory tests, (b) CCR10 expression levels of individual circulating T cells do not vary according to MF disease stages, and (c) serum and skin CTACK/CCL27 are also elevated in patients with MF.

There is still the possibility that the CCR10 expression in our patients with MF is a reflection of inflammatory changes, as seen in atopic skin and in PBMCs of patients with severe drug reactions (11, 12). In fact, CCR10 is expressed by only 10% of CD4+ T cells in two models of inflammatory skin diseases (16), whereas it was expressed by 34% of MF lesion cells in our study. As shown in Fig. 3, there was no increase in CCR10 expression in atopic dermatitis cases. From these findings, we hypothesize that CCR10 is an important receptor involved in the pathophysiology of MF skin-homing and epidermotropism processes. In addition, the increase of CCR10+ CD4+ cells in the PBMCs of patients with MF is likely further proof that malignant, clonal T cells may already be circulating from early MF disease stages.

During skin-homing of peripheral T cells, chemokine interactions between CCR4, the thymus, and the activation-regulated...
chemokine (TARC/CCL17) have also been shown. Soler et al. reported that CCR10 is expressed only in a minor population of "effector-memory" skin-homing T cells, which respond to recently seen antigens (16). They also concluded that CCR10-CTACK interactions are not directly necessary for skin-homing, using anti-CTACK antibodies in the murine allergy model. Furthermore, CCR4 was expressed in other systemic cells than skin-homing T cells; hence, CCR10 might be more specific to the skin-homing population (11). Our study revealed that CCR10+ T cells were statistically elevated in patients with early MF than those from normal healthy controls or patients with atopic dermatitis, a common allergic skin reaction. Further studies are needed, but our study already suggests a role for CCR10-CTACK/CCL27 interactions during epidermotropism and skin-homing, not only in Sézary syndrome, but also in MF, by a subtly different pathway from other allergic skin reactions.

CTACK/CCL27, a functional ligand for CCR10, is predominantly expressed in basal keratinocytes of the epidermis (11). The concentration of CTACK/CCL27 around basal keratinocytes is rather high compared with other chemokines, playing a role in steady T cell trafficking into the skin (17). In our study, the serum CTACK/CCL27 concentration in patients with MF was significantly increased, which suggests a dynamic interaction between basal keratinocytes and malignant T cells.

Conversely, CCR4, another chemokine expressed by malignant T cells, also plays a central role in T helper 2–mediated cutaneous inflammation (18). A recent report revealed that TARC/CCL17, a ligand for CCR4, is significantly elevated in MF patients’ serum, and that this is correlated with clinical and disease stages (19). It is also reported that TARC/CCL17 augments CTACK/CCL27 expression through tumor necrosis factor-α in atopic skin (20). These findings account for our observations that serum CTACK/CCL27 levels are elevated in patients with MF in accordance with the advance of the clinical stage. We believe that increases in the number of CCR10+ peripheral blood cells and serum CTACK/CCL27 levels may reflect the extent of increasing infiltration/inflammation during the course of the MF disease progress, as well as the importance of CCR4-TARC/CCL17 interactions during different disease stages.

A recent study has shown that single or multiple UV irradiation events can significantly down-regulate CTACK/CCL27 mRNA expression in mouse skin (21). Although no studies have been done to ascertain the relationship between CCR4 expression and UV therapy, one CTCL case has been reported in which the numbers of peripheral CCR4+ malignant T cells significantly decreased after retinoid plus psoralen UV A and IFN-γ therapy (22). These findings may explain one mechanism by which PUVA might be effective in treating CTCL skin lesions, by decreasing the affinity of skin-homing T cells and their rates of infiltration.

In summary, we have found that the CCR10+ CD4+ lymphocytes are significantly increased in the circulating PBMCs of patients with MF, regardless of clinical disease progression. An elevated concentration of serum CTACK/CCL27 was also noted in patients with MF, which together suggests an increase in lymphocyte skin-homing in the early stages of MF.

Acknowledgments

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