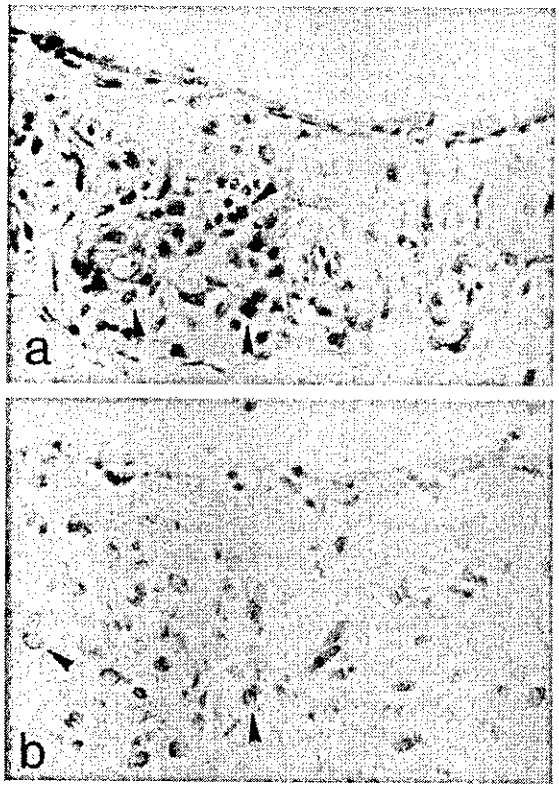

Figure 3. Immunohistochemical staining of CCR4 and CXCR3 in the lesional skin of patients with bullous pemphigoid. (a) CCR4 (arrowhead) was expressed on dermal infiltrating cells. (b) In addition, CXCR3 (arrowhead) was expressed on dermal infiltrating cells in a similar fashion.

Table 1. The average of CCR4 and CXCR3 positivity in CD4+ CD45RO+ cells of peripheral blood mononuclear cells in patients with bullous pemphigoid (BP) and healthy control subjects (HC)

	BP ($n = 3$)	HC ($n = 3$)
CCR4 positivity (%)	15·0 ± 2·7	5·2 ± 1·8[a]
CXCR3 positivity (%)	29·2 ± 2·1	24·5 ± 3·5[b]

[a]$P < 0.03$. [b]P = NS.

KC cell line, and human KCs produced TARC after stimulation with tumour necrosis factor-α and IFN-γ.[20,23] Consistent with these *in vitro* data, it was recently revealed that TARC is expressed in the lesional KCs of AD skin,[20,21] suggesting that KC is one of the main sources of TARC. In this study, we showed immunoreactive TARC in the lesional KCs of patients with BP and high fluid TARC levels in patients with BP. These results strongly suggest that the lesional KCs in patients with BP produce TARC, and that the secreted TARC from KCs results in high fluid TARC levels.

Very recently, we have clarified that serum TARC levels are high and correlate with the disease activity of AD.[21] In this paper we have shown that serum TARC levels in patients with BP were high and that these high serum TARC levels decreased after the treatment. Furthermore, we examined the correlation between serum TARC levels and eosinophil numbers in peripheral blood, which reflect the disease activity of BP,[27] and a significant correlation was obtained. From these results, it is suggested that the serum TARC levels in BP reflect disease activity.

Previous studies have revealed the simultaneous expression of TARC and CCR4, both of which were reported to indicate the presence of Th2 cells.[16–18,20,21] As TARC was expressed in the lesional KCs of BP skin, we speculated that CCR4, one of the receptors for TARC,[18] might also be expressed in lesional skin. Indeed, by an immunohistochemical procedure it was shown that CCR4+ cells exist beneath the bullae of patients with BP. These data strongly suggest that both TARC and CCR4 may be involved in the lesional skin of BP. Interestingly, CXCR3+ cells, reported to be preferentially expressed on Th1-type cells,[17] also exist beneath the bullae. The significance of this finding requires further clarification.

To elucidate the Th1/Th2 balance in patients with BP further, we also examined CCR4 or CXCR3 positivity in CD4+ CD45RO+ cells of PBMCs in three patients with BP and three healthy control subjects. CCR4 positivity in CD4+ CD45RO+ cells of PBMCs in patients with BP was significantly higher than that in healthy control subjects. In contrast, CXCR3 positivity in CD4+ CD45RO+ cells of PBMCs showed no significant difference between the two categories. This result is very similar to that observed in AD patients.[24] Taken together, these results suggest that TARC may be important for the pathogenesis of BP.

Acknowledgments

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Thymus and activation-regulated chemokine (TARC/CCL17) in mycosis fungoides: Serum TARC levels reflect the disease activity of mycosis fungoides

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Background: Mycosis fungoides (MF) belongs to cutaneous T-cell lymphoma and is clinically divided into 3 stages: patch, plaque, and tumor stage. Thymus and activation-regulated chemokine (TARC/CCL17) is a member of the CC chemokines and is a chemoattractant for CC chemokine receptor 4 (CCR4)- and CC chemokine receptor 8 (CCR8)-expressing cells.

Objective: In this study, we examined the involvement of TARC among patients with each stage of MF.

Methods: We investigated the expression of TARC, CCR4, and CXC chemokine receptor 3 in patients with each stage of MF by immunohistochemistry. We measured serum TARC levels in 20 patients with MF in varying degrees and compared them with 10 patients with psoriasis vulgaris and 10 healthy controls. In addition, we compared serum TARC levels in patients with MF with other laboratory data.

Results: Immunohistochemical staining revealed that TARC was expressed in the lesional keratinocytes in the patch, plaque, and tumor stages. CCR4 was expressed on the epidermotropic cells in both patch and plaque stages and on the large cell-transformed cells in the tumor stage, whereas CXC chemokine receptor 3 was constantly expressed on the small cells in the lesional dermis. Serum TARC levels in patients with MF were significantly higher than those in patients with psoriasis vulgaris or healthy controls. Moreover, serum TARC levels in patients with the tumor stage of MF ($n = 5$) were remarkably higher than those with patch stage ($n = 8$) or plaque stage ($n = 7$). Serum TARC levels significantly correlated with serum lactate dehydrogenase levels ($r = 0.62$), serum immunoglobulin E levels ($r = 0.60$), serum soluble interleukin 2 receptor levels ($r = 0.72$), and serum macrophage-derived chemokine levels ($r = 0.70$).

Conclusion: These data strongly indicate that serum TARC levels are useful for assessing the disease activity of patients with MF and that TARC and CCR4 may be involved in the pathogenesis of MF. (J Am Acad Dermatol 2003;48:23-30.)

Mycosis fungoides (MF) belongs to the subgroup of cutaneous malignant T-cell lymphoma.[1] It is clinically divided into 3 stages: patch, plaque, and tumor stage. Histopathologic findings of the lesional skin in the patch and plaque stages show the epidermotropism of atypical lymphoid cells, which is called Pautrier's microabscess, and the infiltration of these cells into the upper dermis. In the tumor stage, as the lymphoma progresses, tumor cells infiltrate into the deep der-

Abbreviations used:

AD:	atopic dermatitis
CCR4:	CC chemokine receptor 4
CXCR3:	CXC chemokine receptor 3
IgE:	immunoglobulin E
KC:	keratinocyte
LDH:	lactate dehydrogenase
MDC:	macrophage-derived chemokine
MF:	mycosis fungoides
sIL-2R:	soluble interleukin 2 receptor
TARC:	thymus and activation-regulated chemokine

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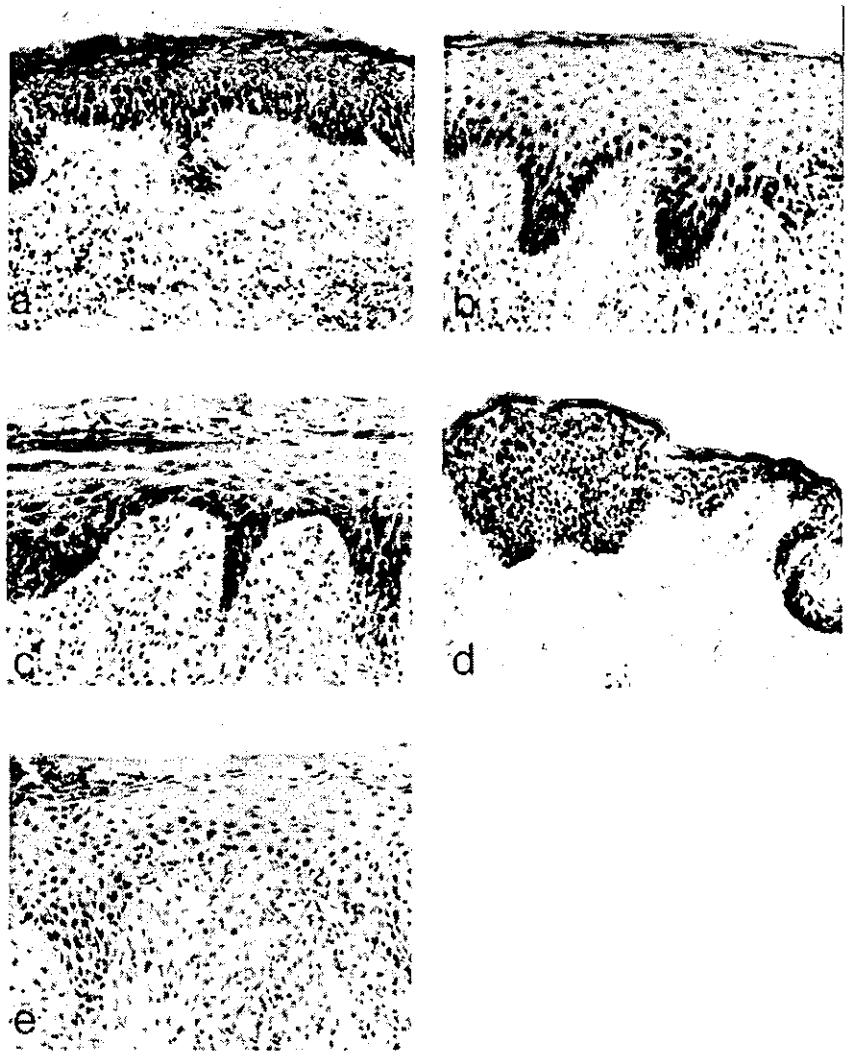

Fig 1. Immunohistochemical staining for TARC was investigated using the lesional skins of patients with each stage of MF. Immunoreactive TARC was shown in the lesional keratinocytes (KCs) in the patch **(a)**, plaque **(b)**, and tumor stages **(c)**. When an isotype-matched control (rabbit IgG) was used, no immunoreactivity was detected **(e)**. In normal skin, TARC expression was virtually negative in the epidermis **(d)**. (Original magnification ×200.)

mis and fat tissues and often develop into large cell transformation. To estimate the disease activity of MF, it was previously reported that the thickness of the cutaneous infiltrate or serum lactate dehydrogenase (LDH) level or soluble interleukin 2 receptor (sIL-2R) level were available.[2-4] A previous article indicated that type 2 helper T cell (T_H2) cytokine expression is predominant in the lesional skin of patients with MF,[5,6] whereas another article revealed that interferon gamma is the major cytokine secreted in the lesional skin of patients with MF.[7] Moreover, T-cell clones from early-stage MF show no T_H1/T_H2-polarized cytokine profile.[8] Thus, to date, it is unclear whether MF shows T_H1- or T_H2-type cytokine profiles in different stages.

Thymus and activation-regulated chemokine (TARC/CCL17) is a member of the CC chemokines.[9] It is a ligand for CC chemokine receptor 4 (CCR4)[10] and CC chemokine receptor 8 (CCR8)[11] and serves for the recruitment and migration of these receptor-expressing cells. We have previously shown that in NC/Nga mouse (regarded as a mouse model for human atopic dermatitis [AD]) and in human AD TARC is produced in the lesional keratinocytes

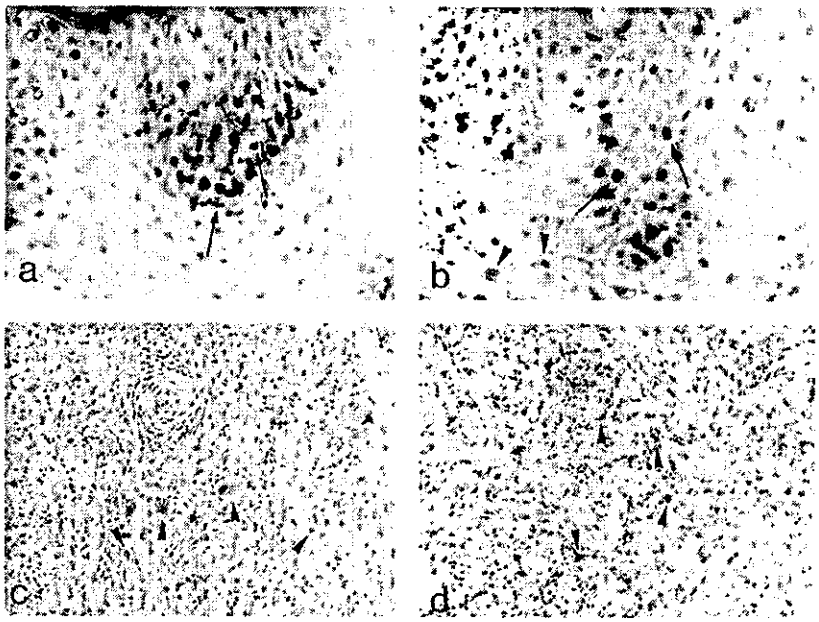

Fig 2. Immunohistochemical staining for CCR4 and CXCR3. CCR4 was mainly expressed on epidermotropic cells (*arrow*) **(a)**, whereas CXCR3 was expressed on both epidermotropic cells (*arrow*) and dermal infiltrating cells (*arrowhead*) **(b)** in plaque stage. In tumor stage, CCR4 was strongly expressed on large cell-transformed cells and small cells (*arrowhead*) in dermis **(c)**, and CXCR3 was expressed on small cells (*arrowhead*) but not on large cell-transformed cells **(d)**. (Original magnifications: **a** and **b**, ×400; **c** and **d**, ×200).

(KCs).[12,13] In addition, CCR4 is strongly expressed on the CLA^+ $CD4^+$ infiltrating cells in the lesional skin of patients with AD.[14] Moreover, we have shown that serum TARC levels and CCR4 expression on memory helper T cells in peripheral blood correlate with the disease activity of AD.[13,15] Thus, both TARC and CCR4 may play an important role in the pathogenesis of AD. However, there is no report of a correlation between serum TARC levels and the disease activity of other skin disorders.

To date, in MF, it has been reported that CCR4 is strongly expressed in the lesional skin, especially in the advanced stage, and that TARC is expressed in the endothelial cells.[16] However, the detailed significance of TARC and CCR4 in patients with each stage of MF is unknown.

In this study, to elucidate the participation of TARC in each stage of MF, we measured serum TARC levels, as well as serum macrophage-derived chemokine (MDC) levels in patients with MF, and compared these levels among the 3 stages. Next, we examined the correlation between serum TARC levels and the other laboratory data, such as serum LDH levels, sIL-2R levels, immunoglobulin E (IgE) levels, MDC levels, and eosinophil number in peripheral blood. Furthermore, we performed immunohistochemical staining of TARC, CCR4, and CXC chemokine receptor 3 (CXCR3) in the lesional skin of patients with each stage of MF.

MATERIAL AND METHODS

Samples and reagents

Twenty patients with MF (10 men and 10 women: age ± SE, 61.8 years ± 3.4) were enrolled in this study (Table I). MF was diagnosed on the basis of clinical features and findings of skin biopsy examinations according to clinical and histologic criteria.[17] The patients were divided into 3 stages by clinicohistopathologic findings: 8 patients with patch stage, 7 patients with plaque stage, and 5 patients with tumor stage. Serum samples were collected from all 20 patients. As a control, 10 patients with psoriasis vulgaris (5 men and 5 women; age ± SE: 58.5 years ± 5.2) characterized as T_H1-dominant disease and 10 healthy controls (5 men and 5 women; age ± SE: 54.0 years ± 8.1) were used. Laboratory data such as serum sIL-2R, LDH, and IgE levels, and eosinophil number in peripheral blood, were also examined and compared with serum TARC or MDC levels.

To perform the immunohistochemical procedure, the following antibodies (Abs) and isotype-matched controls were used: rabbit anti-human

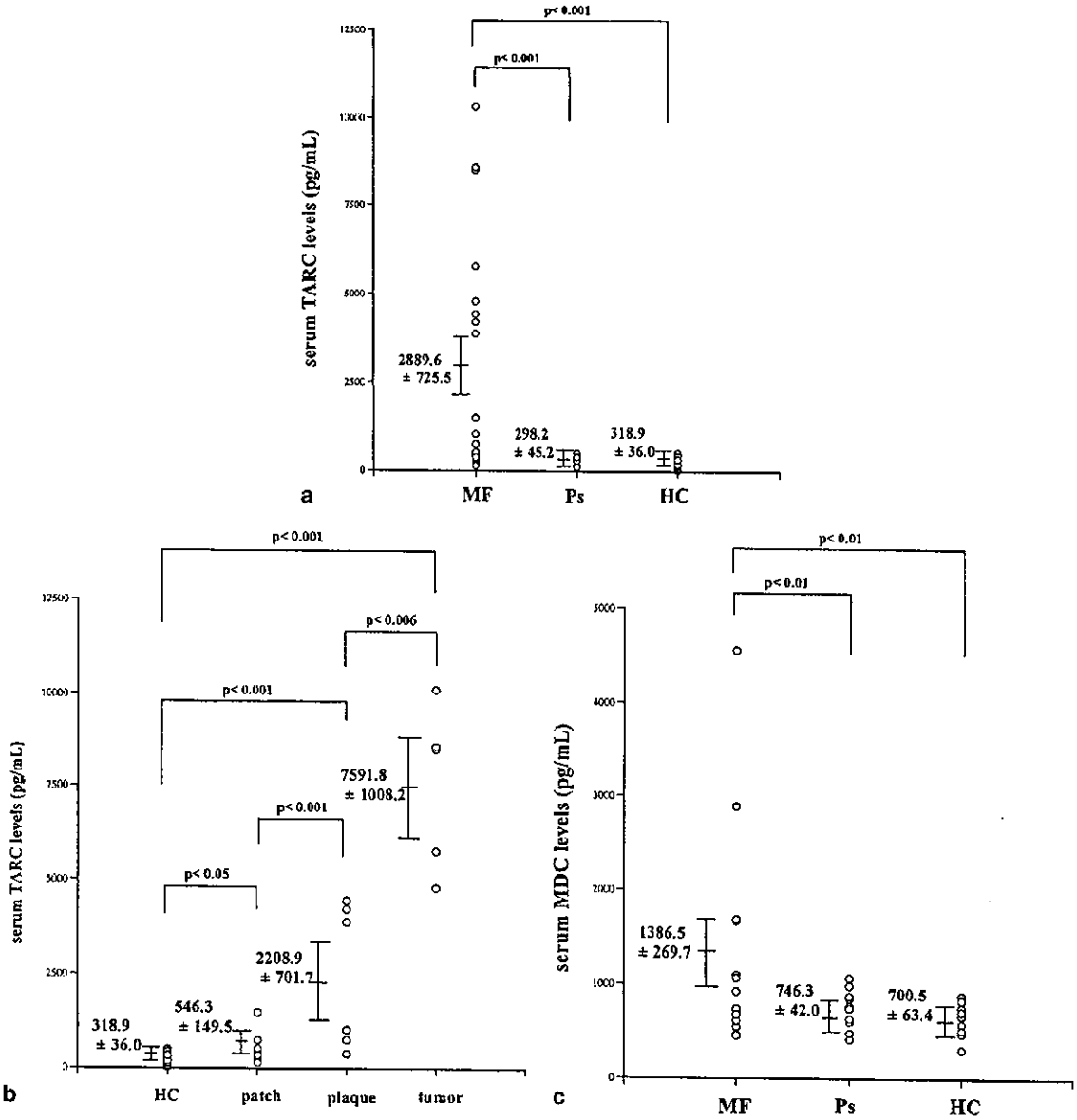

Fig 3. a, Serum TARC levels in patients with MF, psoriasis vulgaris patients (Ps), and healthy controls (HC). Serum TARC levels in patients with MF were much higher than those in patients with psoriasis vulgaris or healthy controls ($P < .001$, respectively). **b,** Serum TARC levels in patients with each stage of MF were examined. High serum TARC levels in tumor stage of MF were more remarkable than those in the patch or plaque stage of MF ($P < .001$, $P < .006$, respectively). **c,** Serum MDC levels in patients with MF, patients with psoriasis vulgaris, and healthy controls were also examined. Serum MDC levels in MF patients were higher than those in patients with psoriasis vulgaris or healthy controls ($P < .01$, respectively).

TARC Ab (Peprotech, Rocky Hill, NJ), mouse anti-human CXCR3 monoclonal (m) Ab (PharMingen, San Diego, Calif), mouse anti-human CD4 mAb (DAKO Co, Glostrup, Denmark), mouse anti-human CCR4 mAb (the characteristics of which are described elsewhere),[18] rabbit IgG (Jackson ImmunoResearch, West Grove, Pa), and mouse IgG (Jackson ImmunoResearch).

Table I. Summary of the patient's number, age, sex, stage, and laboratory data used in this study

No	Age (y)	Sex	Stage	Eosino*	LDH	sIL-2R	IgE	TARC	MDC	Duration (years)
1	57	M	Patch	424	174	474	N.T.	415	925	5
2	67	M	Patch	N.T.	N.T.	N.T.	N.T.	227	735	25
3	65	F	Patch	138	196	323	500	347	559	12
4	64	F	Patch	141	228	308	44	469	476	3
5	86	F	Patch	45	330	949	3	528	1098	17
6	57	M	Patch	188	207	577	1400	748	2888	6
7	50	F	Patch	87	225	231	N.T.	148	459	4
8	62	F	Patch	25	242	355	6	1488	1672	22
9	65	M	Plaque	123	199	558	10	1030	745	15
10	69	F	Plaque	70	292	648	18	4437	1066	6
11	70	F	Plaque	283	259	508	490	753	621	7
12	28	M	Plaque	92	209	340	50	760	439	10
13	75	M	Plaque	103	337	337	14	391	450	8
14	60	M	Plaque	60	168	1478	220	3878	1580	3
15	62	F	Plaque	N.T.	559	800	N.T.	4213	1524	10
16	74	F	Tumor	168	429	7490	26000	10310	4317	11
17	69	M	Tumor	0	362	191	810	8583	1236	6
18	73	M	Tumor	147	406	7837	340	8498	4564	15
19	57	M	Tumor	13	291	842	6200	5776	1686	20
20	27	F	Tumor	400	316	3972	2200	4792	686	13

IgE, Immunoglobulin E; *LDH*, lactate dehydrogenase; *MDC*, macrophage-derived chemoleine; *sIL-2R*, soluble interleukin 2 receptor; *TARC*, thymus and activation-regulated chemokine.
Each unit of the laboratory data is shown as follows: Eosino* (eosinophil number in peripheral blood): μL; LDH: U/mL, sIL-2R: pg/mL; IgE: IU/L, TARC: pg/mL, MDC: pg/mL.

Table II. Correlation coefficient between serum thymus and activation-regulated chemokine levels and other laboratory data in patients with mycosis fungoides

	TARC	
	r	*P*
Eosino	−0.14	.58
IgE	0.60	<.02
LDH	0.62	<.005
sIL-2R	0.72	<.0005
MDC	0.70	<.0006

IgE, Immunoglobulin E; *LDH*, lactate dehydrogenase; *MDC*, macrophage-derived chemokine; *sIL-2R*, soluble interleukin 2 receptor; *TARC*, thymus and activation-regulated chemokine.

Immunohistochemical staining of TARC, CCR4, and CXCR3 in the lesional skin of patients with MF

Biopsy samples were taken from the lesional skin of patients with MF (patch stage of 3 cases, plaque stage of 3 cases, and tumor stage of 2 cases) and the normal skin of 2 healthy control subjects. As a positive control for TARC, CCR4, and CXCR3, we used the acute lesional skin of patients with AD. The obtained skin samples were embedded in Tissue-Tek OCT compound (Miles Inc, Elkhart, Idaho) for at least 24 hours at −80. These frozen samples were cut with a cryostat set at 6 μm of thickness and fixed in 4% paraformaldehyde. After quenching endogenous peroxidase activity, they were incubated overnight with rabbit anti-human TARC Ab, mouse anti-human CCR4 mAb, mouse anti-human CXCR3 mAb, mouse anti-human CD4 mAb, rabbit IgG, or mouse IgG at 4°C. Next, they were incubated with biotin-conjugated anti-rabbit IgG (DAKO Co) or biotin-conjugated anti-mouse IgG (DAKO Co.) for 30 minutes. The samples were then washed with phosphate-buffered saline, incubated with avidin-biotin complex followed by diaminobenzidine solution until brown staining was visible, and counterstained with Mayer's hematoxylin, according to the manufacturer's instructions.

Measurement of serum TARC and MDC levels by enzyme-linked immunoabsorbent assay

We used a 96-well polystyrene microplate coated with a murine mAb against human TARC (TECHNE Corp, Minneapolis, Minn) and murine mAb against human MDC (R&D System Corp, Minneapolis, Minn). The serum TARC and MDC levels were measured in patients with MF, patients with psoriasis vulgaris, and healthy controls, according to the quantitative sandwich enzyme immunoassay technique. Optical densities were measured at 450 nm with a Bio-Rad Model 550 microplate reader (Bio-

Rad Laboratories, Inc, Hercules, Calif). The concentrations were calculated from the standard curve generated by a curve-fitting program. The minimum detectable doses of TARC and MDC were 7 and 10 pg/mL, respectively.

Statistical analysis

The data were analyzed using the Mann-Whitney *U* test to compare the significance of serum TARC or MDC levels between each category. A P value of $<.05$ was considered to be statistically significant.

Correlation coefficients (r) between serum TARC or MDC levels and the other laboratory data in patients with MF were determined using the Spearman rank correlation test. Statistical analysis was also performed, and a P value of $<.05$ was considered to be statistically significant.

RESULTS

TARC strongly expressed in the lesional KCs in the patch, plaque, and tumor stages

In the patch, plaque, and tumor stages of patients with MF, the lesional KCs had a strong immunoreactivity for TARC (Fig 1, *a–c*). Epidermotropic atypical cells and large cell-transformed cells stained negative for TARC (data not shown). In the lesional skin of patients with AD, TARC was also strongly immunoreactive in the epidermal KCs (data not shown). In the skin of healthy controls, the expression of TARC was virtually negative in the epidermis (Fig 1, *d*). When isotype-matched controls were used, no immunoreactivity of TARC was shown (Fig 1, *e*).

Both CCR4 and CXCR3 expressed in the lesional skin of patients in all 3 stages of MF

In the lesional epidermis of patch and plaque stages, CCR4 was expressed on the epidermotropic cells and CXCR3 expression was found to be distributed in a similar fashion (Fig 2, *a, b*). In the lesional dermis of patch and plaque stages, both CCR4 and CXCR3 were also observed on the dermal $CD4^+$ cells; however, the number of CCR4-expressing cells was smaller than that of $CXCR3^+$ cells. In the tumor stage, CCR4 was expressed on the large cell-transformed cells and a fraction of the small-sized cells (Fig 2, *c*), whereas CXCR3 was expressed on a large number of the small cells and entirely negative on the large cells (Fig 2, *d*). As a positive control for the immunohistochemistry of CCR4 and CXCR3, we used the serial sections of the acute phase of AD skin previously reported[14]; the immunoreactivity was described in a similar fashion with the previous report (data not shown).

Serum TARC levels and serum MDC levels in patients with MF higher than those in patients with psoriasis vulgaris and healthy controls

The serum TARC levels in the 20 patients with MF were 2889.6 ± 725.5 pg/mL, whereas those in patients with psoriasis vulgaris and healthy controls were 298.2 ± 45.2 pg/mL and 318.9 ± 36.0 pg/mL, respectively. Serum TARC levels in patients with MF were higher than in patients with psoriasis vulgaris ($n = 10$) or healthy controls ($n = 10$) ($P < .001$, respectively) (Fig 3, *a*). Next, we compared serum TARC levels in each stage of MF: patch stage ($n = 8$), plaque stage ($n = 7$), and tumor stage ($n = 5$). Serum TARC levels in the patch stage, plaque stage, and tumor stage were 546.3 ± 149.5 pg/mL, 2208.9 ± 701.7 pg/mL, and 7591.8 ± 1008.2 pg/mL, respectively. Those in the 3 groups were significantly higher than in healthy controls (patch stage vs healthy controls, $P < .05$; plaque stage vs healthy controls, $P < .001$; and tumor stage vs healthy controls, $P < .001$; respectively) or patients with psoriasis vulgaris (patch stage vs psoriasis vulgaris, $P < .05$; plaque stage vs psoriasis vulgaris, $P < .001$; and tumor stage vs psoriasis vulgaris, $P < .001$; respectively). In addition, serum TARC levels in the tumor stage were significantly higher than those in the patch or plaque stage (Fig 3, *b*). Serum MDC levels in 20 patients with MF were 1386.5 ± 269.7 pg/mL, whereas those in patients with psoriasis vulgaris and healthy controls were 746.3 ± 42.0 pg/mL and 700.5 ± 63.4 pg/mL, respectively. Serum MDC levels in patients with MF were significantly higher than those in patients with psoriasis vulgaris or healthy controls ($P < .01$, respectively), which was similar to the result for serum TARC levels (Fig 3, *c*). However, serum MDC levels in patients with MF showed no significant difference among the 3 stages: patch stage (1101.5 ± 292.2 pg/mL), plaque stage (917.9 ± 182.2 pg/mL), and tumor stage (2497.8 ± 809.7 pg/mL) (data not shown).

Serum TARC levels in patients with MF significantly correlated with serum sIL-2R, LDH, IgE, and MDC levels

Serum TARC levels strongly correlated with serum LDH ($r = 0.62$, $P < .005$), IgE ($r = 0.60$, $P < .02$), and sIL-2R levels ($r = 0.72$, $P < .0005$). However, serum TARC levels had no correlation with eosinophil number in peripheral blood (Table II). In addition, serum MDC levels also significantly correlated with sIL-2R ($r = 0.81$, $P < .0001$) (data not shown). Serum TARC and MDC levels significantly correlated with each other ($r = 0.70$, $P < .0006$). These correlation coefficients between serum TARC levels and other laboratory data are summarized in Table II.

DISCUSSION

In this study, we have shown the following results: (1) Immunohistochemical staining revealed that TARC was strongly immunoreactive in the lesional KCs in the patch, plaque, and tumor stages; (2) CCR4 was expressed on the epidermotropic cells in patch and plaque stages and on the large cell-transformed tumor cells in the tumor stage, whereas CXCR3 was constantly expressed on the small cells; (3) serum TARC and MDC levels in patients with MF were significantly higher than those in healthy controls or patients with psoriasis vulgaris; (4) serum TARC levels in patients with the tumor stage of MF were excessively higher than those in the patch or plaque stage, and serum TARC levels seem to have a tendency to increase in accordance with the development of the disease stage; and (5) serum TARC levels significantly correlated with serum LDH, IgE, sIL-2R, and MDC levels, and serum MDC levels also significantly correlated with serum sIL-2R levels.

TARC is a CC chemokine that has a chemoattractant for $CCR4^+$ or $CCR8^+$ cells.[10,11] A recent article has reported the involvement of TARC in various diseases such as Hodgkin's lymphoma,[19] fulminant hepatic failure,[20] and bronchial asthma.[21] Very recently, we have clarified that serum TARC levels are high in patients with AD and correlate with the disease activity of AD.[13] We have also observed that serum MDC levels correlate with disease activity of AD and that both serum TARC and MDC levels correlate with each other.[22] From these data, both TARC and MDC may be important for the pathogenesis of AD; however, little is known about the participation of TARC and MDC in cutaneous T-cell lymphoma such as MF. In this study we showed that serum TARC levels in patients with MF were higher than those in patients with psoriasis vulgaris or healthy controls, and serum TARC levels in the tumor stage showed a tendency to be higher than those in the patch or plaque stage. In addition, serum MDC levels in patients with MF were also higher than those in patients with psoriasis vulgaris or healthy controls, which is consistent with the previous report.[23] We also examined the correlation between serum TARC levels and other laboratory data in patients with MF. Serum TARC levels strongly correlated with serum LDH and sIL-2R levels, which are reported to reflect the disease activity of MF,[2-4] and serum MDC levels, respectively. In addition, serum MDC levels also correlated with serum sIL-2R. Our results strongly indicate that serum TARC and MDC levels correlate with the disease activity of patients with MF. As far as we know, this is the first report describing the relationship between these chemokines and the disease activity of MF.

A recent immunohistochemical study showed both TARC and CCR4 expression in the lesional skin of patients with tumor-stage MF (nodal stage).[16] Inasmuch as we showed that the serum TARC levels increased in accordance with the development of the stage, we also investigated the immunoreactivity for TARC, CCR4, and CXCR3 in the lesional skin of patients with the different stages of MF. The lesional KCs were strongly immunoreactive for TARC in the patch, plaque, and tumor stages. From these results, it is indicated that TARC may be mainly produced by the epidermal KCs in the lesional skin of patients with MF. CCR4, one of the receptors for TARC, was expressed on the epidermotropic cells in the patch and plaque stages and on both large cell-transformed cells and small cells in the lesional dermis in the tumor stage. In contrast, CXCR3, which is preferentially expressed on T_H1-type cells,[24,25] was constantly expressed on the small cells in the patch, plaque, and tumor stages, but not on the large cell-transformed cells in the tumor stage. From this immunohistochemistry, we clarified that CCR4 expression on large cell-transformed cells is more important for the staging of MF than CXCR3. Previous data support that TARC and CCR4 are important for the T_H2-type cytokine profiles.[18,24] In terms of TARC and CCR4, our results strongly indicate that MF may be polarized to T_H2-type cytokine profiles in the advanced stage. However, another recent article has revealed that $CCR4^+$ cells also produce T_H1-type cytokines, such as interferon gamma.[26] Thus, more accumulated evidence will be needed concerning whether CCR4 expression is biased to T_H1- or T_H2-type cells.

In conclusion, we showed that serum TARC levels correlated with disease activity of patients with MF and that coexpression of TARC and CCR4 may be important for the pathogenesis of MF, especially in the advanced stage. In this study, the sample size is small and it requires validation with larger sample sizes. Further investigation will be required to determine whether MF shows T_H1/T_H2-type polarization in the lesional skin.

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Demonstration of TARC and CCR4 mRNA Expression and Distribution Using *In situ* RT-PCR in the Lesional Skin of Atopic Dermatitis

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Abstract

Thymus- and activation-regulated chemokine (TARC/CCL17) and its receptor, CC chemokine receptor 4 (CCR4), have been proven to be involved in a number of allergic diseases, especially atopic dermatitis (AD). The purpose of this study was to examine the expression and distribution of TARC and CCR4 mRNAs in samples of AD (n=15, acute lesions 8, chronic lesions 7) and normal skin (n=6). The expression and distribution of TARC and CCR4 mRNAs were detected with the *in situ* reverse transcription (RT) -polymerase chain reaction (PCR) technique. TARC mRNA was expressed in epidermal keratinocytes, dermal endothelial cells and infiltrating cells. CCR4 mRNA was expressed in dermal endothelial cells and infiltrating cells. In acute AD lesional skin, there were more positive cells, and the staining intensity was stronger than in chronic lesions ($p<0.05$). The distribution of positive cells was as follows: In the epidermis, keratinocytes in the basal layer showed the strongest staining, and keratinocytes in the spinous layer showed moderate staining; the superficial area showed faint staining. In the dermis, infiltrating cells located in the superficial area of the dermis showed the strongest staining, positive staining intensity became weaker and the percentage of positive cells became less as the location became deeper. There were no positive cells in normal skin. These data further substantiate the role of TARC/CCR4 in the pathogenesis of AD.

Abbreviations: CCR4: CC chemokine receptor 4; DIG: digoxigenin; TARC/CCL17: thymus and activation-regulated chemokine

Key words: AD: atopic dermatitis; CCR4: CC chemokine receptor 4; TARC: thymus- and activation-regulated chemokine

Introduction

Chemokines (chemoattractant cytokine) belong to the super-family of cytokines (1–3). By virtue of the highly conserved cysteine residues in their sequences, they can be grouped into CC, CXC, C, and CX3C chemokines (2). The classification of their receptors is based on the structures of their ligands; CCR1-9, CXC1-5, XCR1, and CX-CR1 have been identified (3). Chemokines play important roles in recruiting selected subsets of leukocytes, are involved in a wide range of inflammatory processes, and have immuno-regulatory and hematopoietic functions (1–3). Thymus- and activation-regulated chemokine (TARC/CCL17), a CC chemokine, and its receptor, CC chemokine

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receptor 4 (CCR4), have been proven to be involved in a number of allergic diseases such as atopic dermatitis (AD) (4, 5).

AD, which is characterized by pruritic and eczematous lesions, is a common skin disorder. It has been reported that chemokines, especially TARC and CCR4, are involved in its pathogenesis (4, 6). It is generally believed that AD is a Th2 type disease, even though Th1 cytokines are also involved in its later phase. TARC was the first chemokine shown to be highly selective for Th2 cells. It is strongly induced in monocytes by cytokines, it is known to be produced by Th2 cells, and it induces chemotaxis of Th2 CD4+ T cells (7, 8). TARC levels are elevated in serum from AD patients, and this is closely related with the disease activity of AD (6). In addition, it has been reported that CCR4+ memory CD4+ T cells in blood are increased in AD patients and that CCR4+ lymphocytes infiltrate AD lesions (9). We have found that the expression of CCR4 protein on peripheral blood CD4+ T cells reflected AD disease activity (10). Although studies on protein expression have shown that TARC and CCR4 are involved in the development of AD, the data are controversial. Thus, the expression of TARC and CCR4 mRNAs in AD lesional skin should be clarified because the *in situ* reverse transcription (RT) polymerase chain reaction (PCR) technique can be used to construct the comprehensive mRNA expression profiles and semi-quantify mRNA expression levels. In this study, applying *in situ* RT-PCR techniques, we investigated the expressions of TARC and CCR4 mRNAs in AD lesional skin.

Materials and Methods

Study Participants

This study was approved by the Ethics Committees at Fukushima Medical University and Tokyo University. The diagnosis was established according to the criteria of Hanifin and Rajka and the Japanese Dermatological Association Criteria for atopic dermatitis (11). Fifteen patients (eight with acute lesions and seven with chronic ones) were included in this study. Disease activity was determined by the modified SCORAD (Scoring Atopic Dermatitis) system. We divided the AD patients into two groups, acute and chronic, according to the Japanese Dermatological Associatioin Criteria for atopic dermatitis (acute lesions: erythema, exudation, papules, vesiculopapules, scales, crusts; chronic lesions: infiltrated erythma, lichenification, prurigo, scales, crusts). Six healthy control subjects who did not have any history of allergy or skin disease were also included.

Sample Preparation

RNase-free conditions were used throughout. AD biopsy specimens were obtained during surgical procedures done for diagnostic pruposes. Specimens were fixed in 10% formalin and dehydrated prior to embedding in paraffin. Acid-cleaned slides coated with silane (3-aminopropyltriethoxy-silane, APES) or ready-coated slides were used. After routine dewaxing and rehydration, the sections were dried at 37°C and subjected to reverse transcription (RT) -polymerase chain reaction (PCR).

In situ RT reaction-cDNA synthesis (12, 13)

After incubation in 0.1 M HCl, sections were immersed in proteinase K buffer [50 mM Tris-HCl (pH 7.6)], and then were digested with proteinase K (10 μM/ml in proteinase K buffer). Preliminary experiments showed that a 30-minute digestion was optimal. After washing, the sections were treated with RNase-free DNase. Following stringent washing and inactivation of DNase, the RT reaction was carried out in a 25-μl chamber (TaKaRa, Japan) using a First cDNA kit (Invitrogen Co., CA, USA).

In situ PCR (12, 13)

In situ PCR amplification was carried out in a 25-μl chamber (TaKaRa, Japan). Oligonucleotide primers were synthesized for TARC and CCR4. The TARC primer sequences were: sense, 5′ CAC GCA GCT CGA GGG ACC AAT GTG 3′, antisense, 5′ TCA AGA CCT CTC AAG GCT TTG CAG G 3′. The CCR4 primer sequences were: sense, 5′ TAC TAT GCA GCA GAC CAG TGG GTT T3′, antisense, 5′ GGT TGC GCT CAG TAT AAC AAG TGC T 3′. PCR amplifica-

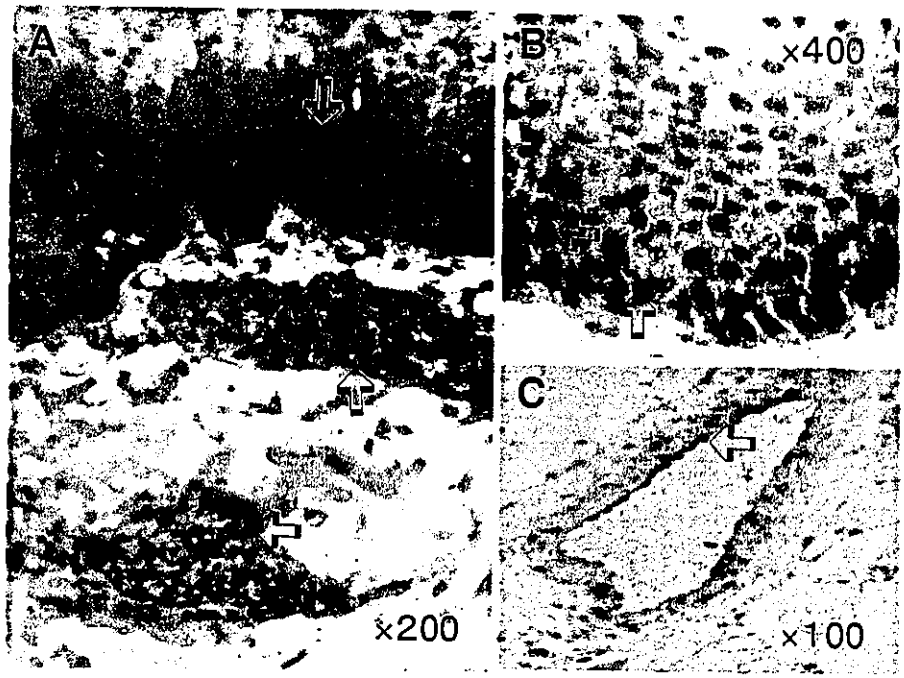

Fig. 1. Expression of TARC mRNA in the lesional skin of AD. A (×200) shows that TARC mRNA is expressed by epidermal keratinocytes and dermal infiltrating cells. B (×400) shows that TARC mRNA is expressed by epidermal keratinocytes. C (×100) shows that TARC mRNA is expressed by dermal endothelial cells. Arrows point to the positive cells.

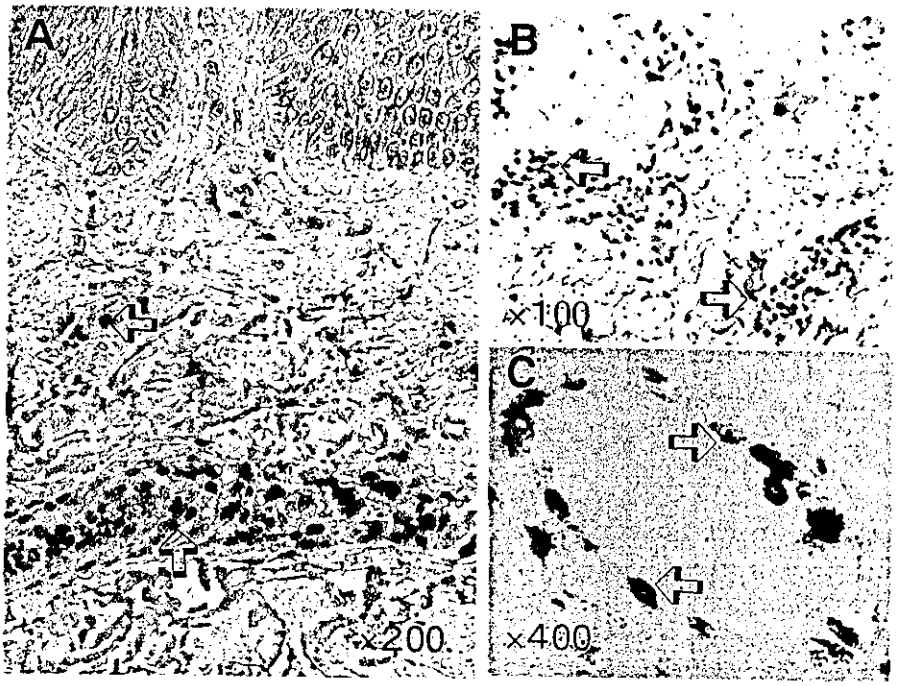

Fig. 2. Expression of CCR4 mRNA in the lesional skin of AD. A (×200) and B (×100) shows that CCR4 mRNA is strongly expressed by dermal infiltrating cells. C shows that CCR4 mRNA is expressed by dermal endothelial cells. Arrows point to the positive cells.

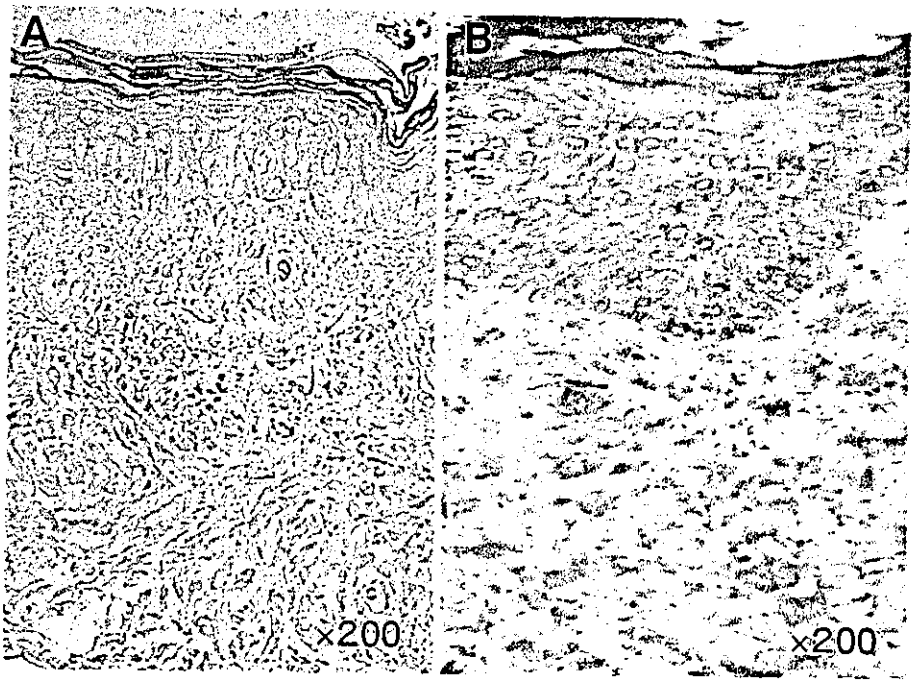

Fig. 3. Controls in this study. A. Normal skin control (×200). B. Control for the specificity of *in situ* RT-PCR (×200). RT-reaction was omitted. There were no positive cell controls.

tions were carried out in solutions containing cDNA, each primer (25 pmol), $MgCl_2$, dNTP-mix, Digoxgenin (DIG)-dUTP, and Taq polymerase (TaKaRa, Tokyo, Japan). The amplification conditions were 1 min of denaturation at 95°C, followed by 2 min of annealing at 60°C for TARC or 1 min of annealing at 57°C for CCR4, and 1 min of extension at 72°C. After amplification, the sections were washed stringently (first wash at 50°C). Color development was carried out using a DIG nucleic acid detection kit (Roche, Germany).

Negative Controls

Normal human skin samples (n=6) were used as negative controls. To confirm the specificity of the methods, the RT reaction, Taq polymerase, or anti-DIG antibody was omitted.

Analysis of Results and Statistical Analysis

The staining intensity of the mRNA expression was estimated semi-quantitatively (−, negative; +, moderate; ++, relatively strong; +++, strong; ++++, maximum). A semi-quantitative analysis of the number of positive cells was performed by counting a minimum of five microscopic high-power fields (400×). The average number of positive cells per field at 400× was scored as follows: −, no positive cells; +, <50; ++, 50− <150; +++, 150–300; ++++, >300. The analysis of results was based on a combination of the semi-quantitative grades of percentage of the numbers of positive cells and the scores for cell staining intensity. The specimens were examined and scored by two evaluators under blinded conditions. χ^2 test and Ridit test were used to see whether differences existed.

Results

Negative Controls for In situ TR-PCR

The absence of signal when the RT reaction was omitted showed that the *in situ* synthesized-cDNA was detected rather than genomic DNA. The absence of detectable signal after the omission of Taq polymerase indicated the necessity of PCR amplification. The absence of detectable signal after the omission of anti-DIG antibody confirmed the specificity of staining. Finally, the lack of positive signals on keratinocytes and endothelial cells in normal skin under the same conditions further confirmed the

Table 1. The results of semi-quantification of the number of positive cells for TARC mRNA

Group	Semi-quantification of the number of positive cells				
	−	+	++	+++	++++
Acute	0	0	1	4	3
Chronic	0	2	4	1	0
Control	6	0	0	0	0

There are significant differences among the three groups ($p<0.05$).

Table 2. The results of semi-quantification of the staining intensity of positive cells for TARC mRNA

Group	Semi-quantification of the staining intensity of positive cells				
	−	+	++	+++	++++
Acute	0	0	1	2	5
Chronic	0	2	3	2	0
Control	6	0	0	0	0

There are significant differences among the three groups ($p<0.05$).

Table 3. The results of semi-quantification of the number of positive cells for CCR4 mRNA

Group	Semi-quantification of the number of positive cells				
	−	+	++	+++	++++
Acute	0	0	1	5	2
Chronic	0	4	2	1	0
Control	6	0	0	0	0

There are significant differences among the three groups ($p<0.05$).

Table 4. The results of semi-quantification of the staining intensity of positive cells for CCR4 mRNA

Group	Semi-quantification of the staining intensity of positive cells				
	−	+	++	+++	++++
Acute	0	0	1	2	5
Chronic	0	2	3	1	1
Control	6	0	0	0	0

There are significant differences among the three groups ($p<0.05$).

specificity of our results.

TARC and CCR4 mRNA Expression and Distribution in AD Lesional Skin

TARC mRNA was expressed in the AD lesional skin in epidermal keratinocytes, dermal infiltrating cells, and endothelial cells (Fig. 1). CCR4 mRNA was expressed in infiltrating cells and endothelial cells (Fig. 2). The cells showed variable signal intensities, possibly indicating mRNA levels. In acute AD lesional skin, the positive cell numbers were larger and the staining intensities stronger than in chronic lesions ($p<0.05$). There were no positive signals on ker-

atinocytes or endothelial cells in normal skin (Fig. 3). The results are summarized in Tables 1–4. The distribution pattern of TARC mRNA positive cells was as follows: In the epidermis, keratinocytes in the basal layer showed the strongest staining and keratinocytes in the spinous layer showed moderate staining, and the superficial area showed faint staining. In the dermis, infiltrating cell located in the superficial area of the dermis showed the strongest staining and positive staining became weaker and the percentage of positive cells became less as the location became deeper. The distribution of CCR4 mRNA positive cells was as follows: CCR4 positive cells appeared only in the dermis. Cells located in the superficial area of the dermis showed the strongest staining. CCR4 mRNA positive staining intensity became weaker and the percentage of positive cells became less as the location became deeper. Analysis with both the χ^2 test and Ridit test showed significant differences between the acute group of AD and the chronic group of AD ($p<0.05$), and between the AD and normal skin group ($p<0.01$).

Descussion

In situ RT-PCR can detect low abundance mRNA and localize it in single cells. Consequently, it can be used to determine the frequency of gene expression. Furthermore, *in situ* PCR amplification in the presence of DIG-11-dUTP and subsequent binding with an anti-DIG antibody allows direct visualization of detected mRNA. Here, we used *in situ* RT-PCR to examine the expression and distribution of TARC and CCR4 mRNAs. We conducted a series of control experiments to ensure the specificity of the *in situ* RT-PCR, and confirmed the specificity of the products by direct sequencing. The sequencing results (data not shown) confirmed that the cDNAs derived from mRNA, rather than genomic DNA, were amplified.

It has been reported that TARC protein was positive in keratinocytes and in vascular endothelial cells, infiltrating T cells, and dentritic cells in the dermis of AD samples (4, 9, 14). In our study, we found that the TARC mRNA was expressed in keratinocytes, endothelial cells and infiltrating cells, in agreement with the previous findings. It has been shown that the number of CCR4-expressing cells was increased in AD patients, and CCR4 has been identified as being expressed preferentially on cutaneous-lymphocyte-antigen (CLA) positive skin homing memory T cells (6, 9). The proportion of CLA+ CCR4+ lymphocytes is increased in peripheral blood in AD and CCR4+ lymphocytes were infiltrating AD lesions (10). These reports indicate that CCR4 protein expression is involved in the pathogenesis of AD. In this report, using *in situ* RT-PCR, we found that CCR4 mRNA was strongly expressed by infiltrating cells and endothelial cells, in accordance with previous findings. Moreover, in active AD lesional skin, there were more cells that were positive and the staining intensity was stronger than in chronic lesions. Our results confirm previous findings with respect to mRNA levels and reinforce the argument that TARC and CCR4 are associated with the pathogenesis of AD. Recent data using inhibitory antibodies and chemokine antagonists suggest that interfering with chemokines and their receptors represents a new approach to allergy immuno-therapy (15). Based on the finding that TARC and CCR4 mRNAs were strongly expressed in AD lesions, we proposed that modulation at the pre-transcription level should be considered in exploring new approaches for allergy immuno-therapy.

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IFN-γ-inducible expression of thymus and activation-regulated chemokine/CCL17 and macrophage-derived chemokine/CCL22 in epidermal keratinocytes and their roles in atopic dermatitis

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Keywords: atopy, chemokine, chemokine receptor, IFN-γ, keratinocyte

Abstract

Thymus and activation-regulated chemokine (TARC)/CCL17 and macrophage-derived chemokine (MDC)/CCL22 are a pair of CC chemokines known to selectively attract T_h2 type memory T cells via CCR4. Here we examined circulating levels of TARC and MDC in patients with atopic dermatitis (AD) and control subjects by using plasma samples, which reflect blood contents of chemokines more accurately than serum samples. The plasma levels of TARC and MDC were significantly elevated in AD patients. These values also strongly correlated with disease severity and serum lactate dehydrogenase levels, and weakly correlated with serum total IgE levels and blood eosinophilia. Previous studies demonstrated TARC immunoreactivity in the epidermal layer of AD lesional skin and production of TARC by a human keratinocytic cell line HaCaT upon stimulation with IFN-γ. Here we demonstrated MDC immunoreactivity in the epidermal layer of AD skin at levels stronger than that of TARC. Furthermore, primary epidermal keratinocytes expressed both TARC and MDC mRNA upon stimulation with IFN-γ, but efficiently secreted only MDC. These results suggest a post-transcriptional regulation in TARC production. IFN-γ also induced TARC and MDC mRNA in mouse skin. Collectively, both TARC and MDC play important roles in the local accumulation of T_h2 cells in AD lesional skin. Production of T_h2-attracting chemokines by epidermal keratinocytes upon treatment with IFN-γ, which is also the potent inducer of T_h1-attracting chemokines, may underline the pivotal role of IFN-γ in the chronic phase of AD where both T_h1 and T_h2 responses are mixed.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory disease of the skin based on still unknown genetic predispositions, and is commonly characterized with dry skin, severe pruritis, high serum IgE levels and eosinophilia (1). The histological features of AD include epidermal hyperplasia, thickening of the papillary dermis and prominent perivascular infiltrates consisting predominantly of T cells (2). It is now considered that disregulated T_h2-dominant immune responses to environmental allergens in the skin are the central features of AD (1,2). In this context, it is likely that a group of chemotactic cytokines collectively called

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chemokines play important roles in AD pathogenesis by attracting various types of leukocytes into the lesional skin (3).

Previously, we have shown that LARC/CCL20, which is known to attract immature dendritic cells and effector/memory T cells via CCR6, is immunologically stained in epidermal layers of AD skin lesion, and can be induced in primary human epidermal keratinocytes upon stimulation with proinflammatory cytokines such as IL-1α and tumor necrosis factor (TNF)-α (4). Thus, LARC is likely to play an important role in AD pathogenesis through coupling innate and acquired immune responses in the skin (3). We have also shown that thymus and activation-regulated chemokine (TARC)/CCL17 and macrophage-derived chemokine (MDC)/CCL22, a pair of chemokines commonly acting on CCR4, selectively attract a subset of CD4+ memory T cells, which are mostly polarized to T_h2 (5). *In vitro* polarization of naive CD4+ T cells into T_h2 cells also selectively induced CCR4 expression (5). Thus, TARC and MDC are likely to play important roles in the T_h2-type immune responses by selectively recruiting T_h2-polarized memory/effector T cells into inflamed tissues (3). This notion has been amply supported by recent studies on murine models of AD and asthma (6–8). Furthermore, elevated 'serum' levels of MDC and TARC in AD patients as well as selective infiltration of CCR4-expressing T cells in AD skin lesions have been reported (9–12). TARC was also shown to be induced in a human keratinocytic cell line HaCaT upon stimulation with IFN-γ and TNF-α, and was immunologically stained in epidermal keratinocytes of AD lesional skin (13). Collectively, it is likely that TARC and MDC are produced in large quantities in lesional skin of AD patients.

The use of 'serum' samples in previous studies for evaluation of blood levels of TARC and MDC (9,10), however, had potential problems due to possibilities such as release of stored chemokines from platelets (14,15), release of chemokines from DARC, the chemokine scavenger receptor on erythrocytes (16,17) and/or adsorption of chemokines to newly formed blood clots (18). In fact, we have recently observed a substantial release of TARC from platelets during clotting (Fujisawa *et al.*, submitted). In the present study, therefore, we re-evaluated circulating levels of TARC and MDC in AD patients and control subjects by using 'plasma' samples. We have found that plasma levels of TARC and MDC are significantly elevated in AD patients, and correlate well with disease severity and serum LDH levels. We have also shown that both TARC and MDC are immunologically stained in the epidermal keratinocytes of AD skin lesions. Furthermore, we have demonstrated for the first time that primary human epidermal keratinocytes are induced to express not only TARC, but also MDC upon stimulation with IFN-γ. Unexpectedly, MDC, but not TARC, was efficiently secreted by IFN-γ-stimulated epidermal keratinocytes *in vitro*, suggesting a post-transcriptional regulation in the production/secretion of the latter.

Methods

Subjects

AD was diagnosed according to the criteria of Haniffin and Rajka (19). Fifty-two patients with AD aged from 14 to 56 years old (25 males and 27 females), all seen in Kobe University Hospital, were enrolled in this study. Levels of total IgE and lactate dehydrogenase (LDH) in patients' sera, and the numbers of eosinophils in peripheral blood were determined by routine laboratory tests. Skin symptoms of the patients were recorded and assessed using the eczema area and severity index (EASI) score (20). This scoring system evaluates area of involvement and erythema, infiltration/papulation, excoriation and lichenification in each area of head/neck, trunk, upper limbs and lower limbs. Based on the EASI score, AD patients were divided into three groups: mild (score <10), moderate (score 10–20) and severe (score >20). Healthy control subjects ($N = 8$) had no history of allergic diseases. Serum levels of IgE in control subjects were <160 IU/ml and no specific IgE antibodies to common inhaled allergens were detected using Phadiatope (Pharmacia Upjohn, Uppsala, Sweden). Peripheral blood samples were collected from AD patients and control subjects using sodium EDTA-containing tubes. After centrifugation, the plasma samples were store at –80°C until assay. Skin biopsies were taken from several donors as described previously (4). Informed consents were obtained from all subjects. This study was approved by the Ethical Committee of Kobe University Graduate School of Medicine.

Cells and reagents

Neonatal human epidermal keratinocytes were purchased from Clonetics (Walkersville, MD). The immortalized human keratinocytic cell line HaCaT was generously provided by Professor N. Fusenig (German Cancer Research Center, Heidelberg, Germany). Recombinant human and murine IL-1α, TNF-α, IFN-γ and IL-4 were all purchased from PeproTech (Rocky Hill, NJ). Lipopolysaccharide from *Escherichia coli* was purchased from Sigma (St Louis, MO). Recombinant human TARC/CCL17 and MDC/CCL22 were purchased from R & D Systems (Minneapolis, MN).

Mice

Female BALB/c mice, 6 weeks old, were purchased from Nippon Kurea (Osaka, Japan) and kept in specific pathogen-free conditions for 1 week before experiments. Mice were treated with depilatory cream (Kanebo, Tokyo, Japan) on the belly to remove hair. After 24 h, mice were anaesthetized by diethyl ether and injected intradermally with 100 μl of PBS alone or containing 10 ng of murine IFN-γ. After various time points, mice were sacrificed by cervical dislocation and skin tissues of 6-mm diameter were punched out at injected sites.

RT-PCR

Total RNA was prepared from skin biopsies and cultured keratinocytes using Trizol reagent (Invitrogen Corp., Carlsbad, CA). RNA samples were further purified using RNeasy (Qiagen, Hilden, Germany). Reverse transcription of total RNA (1 μg) was carried out using oligo$(dT)_{18}$ primer and SuperScript II reverse transcriptase (Invitrogen Corp.). First-strand DNA (20 ng total RNA equivalent) and original total RNA (20 ng) were amplified in a final volume of 20 μl containing 10 pmol of each primer and 1 U of Ex-Taq polymerase (Takara, Kyoto, Japan). The primers used were: +5′-ACTGC-TCCAGGGATGCCATCGTTTTT-3′ and –5′-ACAAGGGGAT-

GGGATCTCCCTCACTG-3′ for TARC; +5′-AGGACAGAG-CATGGCTCGCCTACAGA-3′ and –5′-TAATGGCAGGGAGG-TAGGGCTCCTGA-3′ for MDC; +5′-AAGAAGAACAAGGC-GGTGAAGATG-3′ and –5′-AGGCCCCTGCAGGTTTTGAAG-3 for CCR4; +5′-TGAGGTCACTTCAGATGCTGC-3′ and –5′-ACCAATCTGATGGCCTTCTTC-3′ for mouse TARC; +5′-TCTGATGCAGGTCCCTATGGT-3′ and –5′-TTATGGAGTAG-CTTCTTCAC-3′ for mouse MDC; +5′-GCCAAGGTCATCCAT-GACAACTTTGG-3′ and –5′-GCCTGCTTCACCACCTTCTT-GATGTC-3′ for G3PDH. Amplification conditions were denaturation at 94°C for 30 s (5 min for the first cycle), annealing at 60°C for 30 s and extension at 72°C for 30 s (5 min for the last cycle) for 32 cycles for TARC and MDC, 37 cycles for CCR4, 35 cycles for mouse TARC and mouse MDC, and 27 cycles for human and mouse G3PDH. Amplification products (10 μl each) were subjected to electrophoresis on 2% agarose and stained with ethidium bromide.

Immunohistochemistry

Skin biopsy specimens were snap frozen using liquid nitrogen. Cryostat sections were reacted with a mouse anti-TARC mAb 5F12 (Morita *et al.*, in preparation) or a mouse anti-MDC mAb 252Y (9). After washing, tissue sections were incubated with biotin-conjugated anti-mouse IgG (Dako, Kyoto, Japan). After washing, tissue sections were incubated with ultra-avidin–horseradish peroxidase (Sigma). After washing, sections were treated with diaminobenzidine and counter-stained with methylgreen or hematoxylin.

ELISA

A sandwich-type ELISA specific for MDC with a detection limit of 15 pg/ml was described previously (9). A sandwich-type ELISA specific for TARC with a detection limit of 0.6 pg/ml was developed by using two newly generated mouse anti-TARC mAb, 4A3 and 5F12, and will be described elsewhere (Morita *et al.*, in preparation). A sandwich-type ELISA for human IFN-γ with a detection limit of 8 pg/ml was purchased from American Research Products (Belmont, MA).

Statistical analysis

Data on plasma contents of TARC and MDC were expressed as geometric means since logarithmically transformed values of the data followed normal distribution. Differences were analyzed with unpaired *t*-test. Pearson's correlation coefficient was calculated between two parameters.

Results

Elevated plasma TARC and MDC levels in patients with AD

Using plasma samples obtained from eight normal subjects and 52 AD patients, we examined circulating contents of TARC and MDC in the blood. The patients were divided into three groups according to their EASI scores (20): mild <10, moderate 10–20 and severe >20. As shown in Fig. 1, plasma TARC and MDC levels were elevated in most AD patients and significantly correlated with the disease severity. The average values of TARC in normal subjects and AD patients with mild, moderate and severe disease groups were 37 ± 12, 101 ± 67, 369 ± 230 and 3769 ± 4712 pg/ml respectively. Similarly,

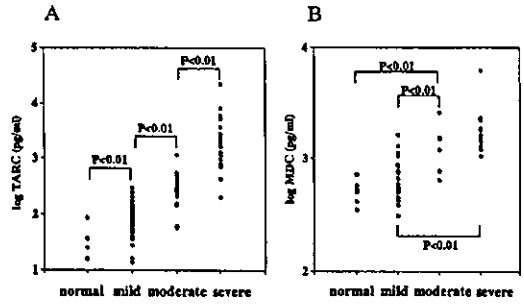

Fig. 1. Elevated plasma levels of TARC and MDC in patients with AD. Plasma samples were obtained from eight control and 52 AD subjects. Plasma contents of TARC and MDC were measured by ELISA. All measurements were done in duplicate and mean values were obtained. The AD subjects were grouped into three groups according to their EASI scores: mild (<10), moderate (10–20) and severe (>20). (A) TARC; (B) MDC.

those of MDC in normal subjects and AD patients with mild, moderate and severe disease groups were 671 ± 159, 692 ± 331, 1531 ± 756 and 1969 ± 1316 pg/ml respectively.

We next analyzed correlation of plasma TARC and MDC levels with other clinical parameters known to be elevated in AD patients. As shown in Fig. 2, the logarithmic values of plasma TARC levels strongly correlated with those of MDC levels ($r = 0.775$, $P < 0.001$) and the clinical scores ($r = 0.791$, $P < 0.001$). Plasma TARC levels also correlated strongly with serum LDH levels ($r = 0.717$, $P < 0.001$), and weakly with serum IgE levels ($r = 0.393$, $P < 0.01$) and blood eosinophilia ($r = 0.398$, $P < 0.01$). Similarly, the logarithmic values of plasma MDC levels correlated with serum LDH levels ($r = 0.735$, $P < 0.001$), serum IgE levels ($r = 0.632$, $P < 0.001$) and blood eosinophil counts ($r = 0.567$, $P < 0.001$) (not shown). Thus, plasma MDC levels appeared to correlate with serum IgE levels and eosinophil counts slightly better than plasma TARC levels.

Elevated expression of TARC and MDC in AD skin lesions

Elevated levels of circulating TARC and MDC in AD patients are likely to be due in part to elevated production of these chemokines in AD skin lesions. To test this possibility, we first carried out RT-PCR analysis on expression of TARC and MDC in skin tissues from normal donors ($n = 3$) and AD patients ($n = 3$). As shown in Fig. 3, TARC and MDC were clearly detected in skin tissues from all AD patients, but not in those from normal donors. Furthermore, CCR4 was also detected in lesional skin of two AD patients who had strong signals for TARC and MDC, suggesting infiltration of CCR4-expressing T cells by locally produced TARC and MDC in these patients. To determine the cells expressing TARC and MDC in AD lesional skin, we next carried out immunohistochemical staining of these chemokines in skin tissues obtained from normal donors ($n = 3$), AD patients ($n = 3$) and psoriatic patients ($n = 3$), using specific mAb. Typical results are shown in Fig. 4. We observed TARC immunoreactivity in epidermal keratinocytes, mainly those in the basal layers (Fig. 4A and B), and also in dermal vascular endothelial cells (Fig. 4C) in AD lesional skin ($n = 3$).