

CTLA-4 expression in T cells of patients with atopic dermatitis

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Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4; CD152) is a surface molecule of activated T cells with sequence homologous to CD28, and may act as a negative regulator of T-cell activation. In murine animal models, cross-linkage of CTLA-4 molecules on the cell surface results in decreased T-cell proliferation, accompanied by increased interleukin (IL)-2 production and apoptosis. To clarify the activation of peripheral blood T cells, we studied the CTLA-4 expression in 32 patients with atopic dermatitis who visited our institution, and 19 normal children who visited for pre-operative laboratory examination were used as normal controls. Whole blood was obtained from all subjects and stained with anti-CD3, anti-CD4, anti-CD8 monoclonal antibodies (mAb). After erythrocyte lysis with lysing solution, the cells were stained with anti-CTLA-4 mAb, and stained cells were analysed by fluorescence-activated cell sorter (FACScan) flow cytometer. Intracellular expression of CTLA-4 was significantly upregulated in peripheral blood CD3⁺ T cells (36.8%), CD4⁺ T cells (21.7%) and CD8⁺ T cells (18.7%) of patients with atopic dermatitis, compared with normal control (18.3%, 9.7%, 9.8%; respectively). Furthermore, CTLA-4-positive CD3⁺ T cells in patients with severe atopic dermatitis were significantly higher compared with milder group (42.8% vs. 32.2%). However, no significant difference was obtained in CD4⁺ and CD8⁺ T cells. Mean percentage of T cells expressing CTLA-4 in patients with atopic dermatitis was higher than the control group. These observations suggest the possibility that the disease activity can be correlated with the CTLA-4 level.

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Atopic dermatitis (AD) is a chronically recurrent inflammatory skin disorder characterized by pruritus, specific distribution and family history, and is likely found in the individuals with immunoglobulin E (IgE)-borne diseases such as asthma or allergic rhinitis, or have a genetic factor capable of increasing IgE (1). Histological characteristics of AD are different depending on acuteness and disease period, therefore, the infiltration of inflammatory cells, whose majority are CD4⁺ T cells, is detected in the acute period, and the mononuclear cell macrophages are observed in the chronic period (2). CD4⁺ T cells are normally divided into two types, Th₁ and Th₂, according to cytokine types secreted and functions, and a critical pathogenic mechanism of AD has been suggested to be

an imbalance of Th₁/Th₂ cytokines to environmental allergens (3). Th₁ secretes interleukin (IL)-2 and interferon (IFN)- γ , and generally participates in cell-mediated immunity, rheumatic arthritis or chronic inflammatory diseases like multiple sclerosis. On the contrary, Th₂ secretes IL-4 and IL-5, and usually concerns in allergic diseases such as AD (4). The increases of IL-4 and IL-13 can be observed, when Th₂ cells are increased in the acute skin lesion and blood of the AD children, while accumulation of macrophages or eosinophiles and the increase of cytokines such as IL-12 and IFN- γ secreted from Th₁ cells are observed in the chronic inflammation (5). Most of the T cells are activated by two signals; by TCR/CD3 complex through the recognition of major histocompatibility complex

(MHC)/peptide structure, and by stimulant molecules expressed on the surface of antigen-presenting cell (APC) (6, 7). CD28, which is the representative stimulant molecules expressed in the T cells, promotes the proliferation of T cells to enhance the production of cytokines such as IL-2, tumour necrosis factor (TNF)- α , IFN- γ , lymphotaxin and granulocyte-macrophage colony-stimulating factor (GM-CSF). This CD28 holds two ligands, B7.1 and B7.2. The activated T cells express cytotoxic T lymphocyte associated antigen-4 (CTLA-4) that has 30% homology to CD28 and combines to B7 family with high affinity (8). Contrary to CD28, however, CTLA-4 is known to act as an inhibiting modifier, but not as a promoter for the immune response (9); the cross-linking of CTLA-4 reduces T-cell promotion and IL-2 production, and induces apoptosis in the already activated T cells (10). Tissue destruction, spleen hypertrophy, lymphoproliferation and elevation of blood immunoglobulin because of severe histological deposition of lymph nodes were observed in the CTLA-4 knock out mouse, and the animal finally succumbed within 3–4 wk (11). Recent studies indicate a correlation between CTLA-4 and autoimmune diseases, and correlations of CTLA-4 genetic polymorphism with Graves' disease (12), type 1 diabetes mellitus (13) and systemic lupus erythematosus (SLE) (14). Moreover, many attempts to treat immune diseases by using the immunological characteristics of CTLA-4 have been made to control the activated T cells through the synthesis of immunoglobulin combining fused protein (15, 16). In the allergic diseases like bronchial asthma, Th₂ cytokine was decreased in the atopic asthma patients through the relation of bronchial hypersensitivity and CTLA-4 genetic polymorphism and the treatment with CTLA-4 immunoglobulin fused protein (17).

However, there is no report on the correlation between CTLA-4 and AD. Therefore, this study was designed to examine the expression of CTLA-4 related to T-cell activation and possible correlation with AD. And furthermore, to understand interrelationship of the CTLA-4 expression levels with the severity of AD, we compared the values of CTLA-4 between AD child patients and normal control children. And we also measured serum levels of IL-16, a useful marker of disease activity in childhood AD (18).

Materials and methods

Study population

A total of 32 patients with AD, fulfilling the criteria of Hanifin and Rajka (19), were enrolled

in this study. They consisted of 20 boys and 12 girls (age range: 7 months to 13 yr; mean: 6.55 ± 4.1 yr). The clinical severity of AD was evaluated according to the SCORAD index (20). Patients were further divided into 18 mild (SCORAD: <15 ; mean: 7.69 ± 3.96) and 14 moderate-to-severe AD patients (SCORAD: ≥ 15 ; mean: 46.01 ± 17.89) according to objective (extent and intensity of the lesions) SCORAD (21). None of the patients had asthma or allergic rhinitis, and they had not received any topical and/or oral medication for at least 2 wk before entering the study. The mean IgE of the subjects was 316.6 ± 376.0 IU/ml (10–1440 IU/ml). Control serum samples were obtained from 19 children who had routine blood work before elective surgical procedures. They consisted of 12 boys and seven girls (age range: 4–18 yr; mean: 8.59 ± 4.5 yr) and had no personal or family history of allergic rhinitis, asthma, or AD. The mean IgE of the non-atopic children was 206.5 ± 121.0 IU/ml (22–370 IU/ml). Informed consent of the parents was obtained for the children included in this study.

Measurement of CTLA-4

Peridinin chlorophyll protein (PerCP)-conjugated anti-CD3 monoclonal antibodies (mAb; Becton-Dickinson, Mountain View, CA, USA), fluorescein isothiocyanate (FITC)-conjugated anti-CD4 mAb (Becton-Dickinson) and FITC-conjugated anti-CD8 mAb (Becton-Dickinson) were added to the whole blood (200 μ l) obtained from the subjects, and this mixture was incubated for 15 min at 4°C for the staining. Erythrocytes in the sample were treated with 2 ml of lysing solution (Becton-Dickinson) at room temperature for 10 min, and washed with washing buffer (phosphate-buffered saline containing 0.5% bovine serum albumin and 0.1% NaN₃). Subsequently, cytofix/cytoperm solution (Becton-Dickinson) was added for 20 min and washed with FACS permeabilizing solution. Then, phycoerythrin (PE)-conjugated anti-CD152 mAb (CTLA-4; Becton-Dickinson) was added to the sample to react in the dark for 1 h, and the sample was analysed by a FACScan flow cytometer.

Flow cytometer

The sample was resuspended in a buffer solution, and stained cells were analysed by a FACScan flow cytometer equipped with a 15-Mw argon ion laser and a filter set for FITC (530 nm), PE (585 nm) and PerCP (677 nm). Intracellular

expression of CTLA-4 was analysed by PE staining. PerCP-conjugated mouse IgG1, PE-conjugated mouse IgG2, FITC-conjugated mouse IgG1 served as an isotype control (Becton-Dickinson). The number of CTLA-4-positive cells was represented as the percentage of CD3⁺, CD4⁺, or CD8⁺ T cell.

IL-16 ELISA

Serum levels of IL-16 were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D System, Minneapolis, MN, USA), according to manufacturer's instructions. All sera were evaluated in duplicate. The detection limits of each assay were 8 pg/ml.

Statistics

Student's *t*-test was used to compare the CTLA-4-positive in the CD3⁺, CD4⁺ and CD8⁺ T cells between AD group and control group, and the AD disorder between mild group and

moderate-to-severe group of the AD patients at the level of significance ($p < 0.05$). Data were analysed statistically using the ANOVA test for comparison of the three groups; moderate-to-severe AD group, mild AD group and control group. Afterwards, correlation coefficients between the value of CTLA-4 and the levels of IL-16 were obtained through Pearson correlation.

Results

Fig. 1 shows typical flow cytometric results of peripheral blood lymphocytes from severe AD and control subjects. The children with AD had significantly higher levels of CTLA-4-positive CD3⁺ ($p = 0.001$), CD4⁺ ($p = 0.001$) and CD8⁺ T cells ($p = 0.002$) than the control group (Table 1). In the AD group, CD4⁺ T cell showed more CTLA-4 expression than in CD8⁺ T cell, however, with no statistical significance.

In order to compare the levels of CTLA-4 as the function of severity of the disease, the AD patients were divided into moderate-to-severe group and mild groups. As seen in Table 2,

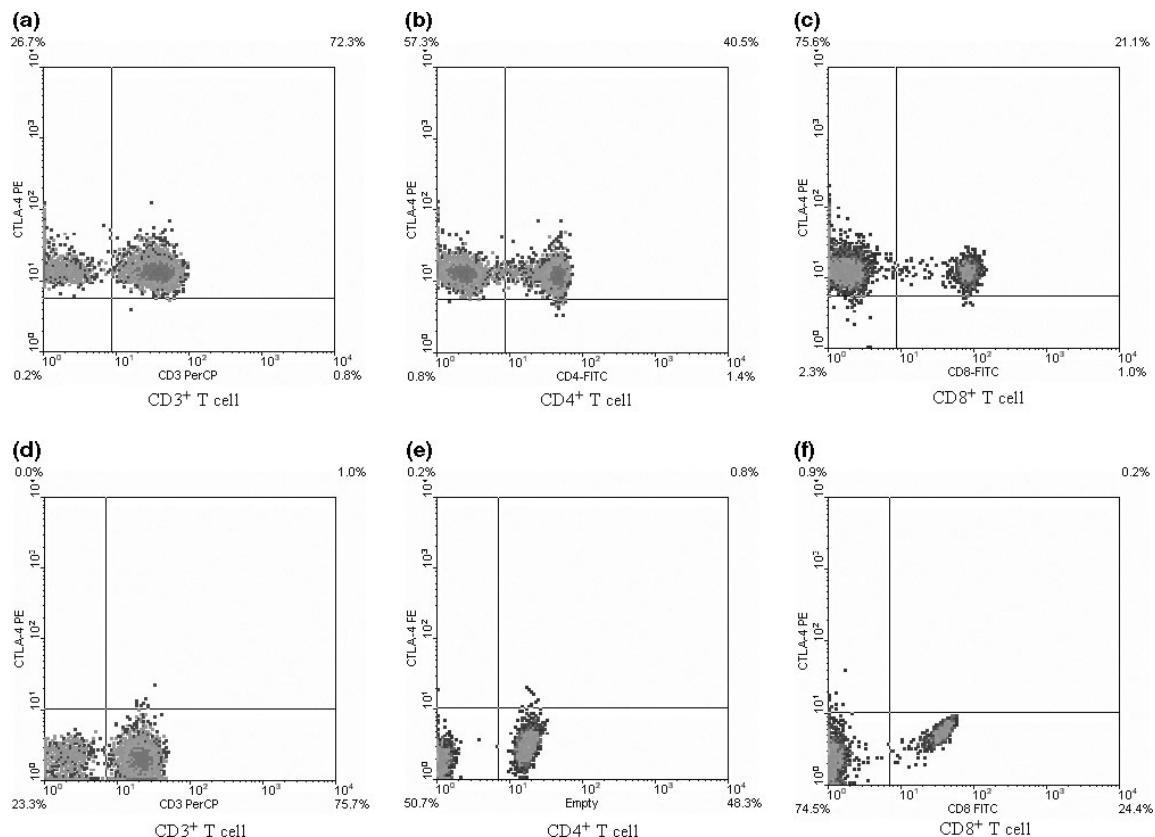


Fig. 1. Typical dot plot of intracellular expression in CD3⁺ T, CD4⁺ T and CD8⁺ T cells from severe atopic dermatitis (a-c) and control groups (d-f). Percentages represent cells expressing cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) among CD3⁺ T, CD4⁺ T, CD8⁺ T cells. Upper quadrant, CTLA-4-positive cells. CTLA-4 expression was increased in all CD3⁺ T, CD4⁺ T and CD8⁺ T cells in atopic dermatitis, compared with control.

Table 1. Frequency of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) expressing T cells in atopic dermatitis group and control group

	N	CD152 ⁺ CD3 ⁺ T cell	CD152 ⁺ CD4 ⁺ T cell	CD152 ⁺ CD8 ⁺ T cell
Atopic dermatitis	32	36.8 ± 15.1*	21.7 ± 10.4*	18.7 ± 6.7*
Normal	19	18.3 ± 13.7	9.7 ± 9.3	9.8 ± 9.7

Values are expressed as mean ± s.d. (%).

* $p < 0.05$, significant increase than control group.

Table 2. Differences of CTLA-4 expression of T cells as a function of severity of atopic dermatitis

	N	CD152 ⁺ CD3 ⁺ T cell	CD152 ⁺ CD4 ⁺ T cell	CD152 ⁺ CD8 ⁺ T cell
AD (moderate-to-severe)	14	42.8 ± 17.7*,**	23.8 ± 11.9*	18.9 ± 7.4*
AD (mild)	19	32.2 ± 11.1*	20.0 ± 9.2*	18.5 ± 6.4*
Normal	19	18.3 ± 13.7	9.7 ± 9.3	9.8 ± 9.7

Values are expressed as mean ± s.d. (%).

* $p < 0.01$, significant increase between the three groups in ANOVA test.

** $p < 0.05$, significant increase than the mild group in *t*-test.

CTLA-4, cytotoxic T lymphocyte-associated antigen 4; AD, atopic dermatitis.

significant difference between these groups were observed ($p < 0.01$): CTLA-4-positive CD3⁺ T cells ($p = 0.04$) in the moderate-to-severe group were significantly higher than in the mild groups. The CTLA-4 expression in CD4⁺ and CD8⁺ T cells of the moderate-to-severe patients were more increased than in the mild group, but not significantly ($p > 0.05$).

The IL-16 levels correlated well with the CTLA-4 values in AD patients. CTLA-4-positive CD3⁺ and CD4⁺ T cells showed correlation with the IL-16 levels ($p < 0.01$, Fig. 2), but no correlation was found between the CTLA-4-positive CD8⁺ T cells (data not shown).

Discussion

The exact mechanism of AD as a chronically recurrent inflammatory disease, has not yet been verified, however, the immune system disorders related to T cells can play an important role. There has practically been no study on the relation between CTLA-4, expressed only on the activated T cell, with AD and allergic diseases. The present study showed increased levels of CTLA-4 in all of CD3⁺, CD4⁺ and CD8⁺ T cells of the AD child patient group, in comparison with those of the normal control group. Moreover, although CD4⁺ or CD8⁺ T cells did not show any significant differences depending on the severity, significantly increased level of CTLA-4 were noted in CD3⁺ T cell of moderate-to-severe AD. In the CD3⁺, CD4⁺ T cells, the CTLA-4 value was significantly related

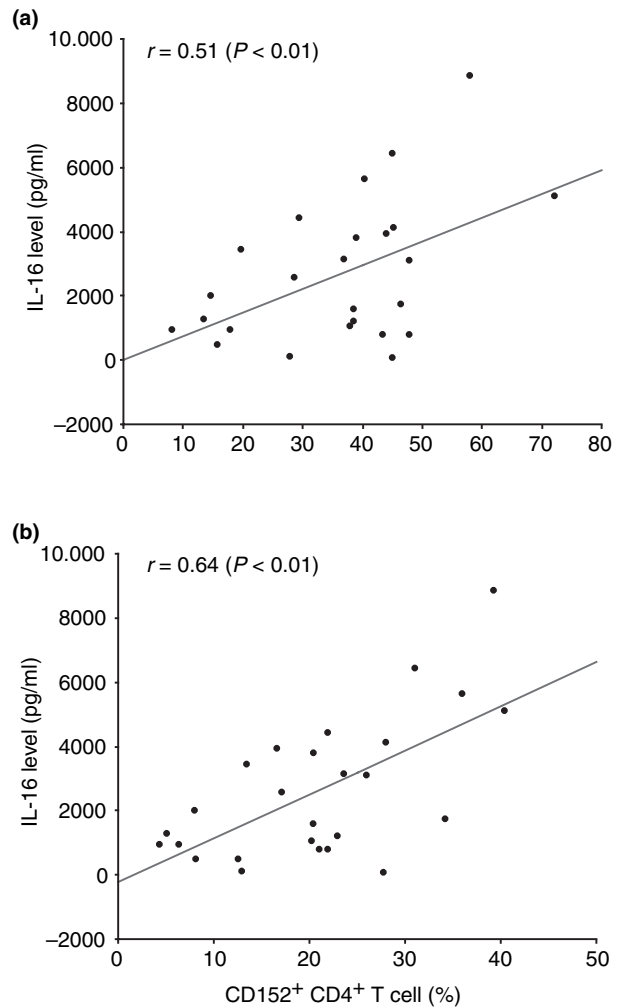


Fig. 2. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)-positive CD3⁺ T and CD4⁺ T cells were significantly correlated with circulating interleukin (IL)-16 levels in atopic dermatitis patients (a, b), as determined by the Pearson correlation coefficient (*r*).

to the presence of circulating IL-16, a serologically useful marker of disease activity in childhood AD (18, 22). The fact that CTLA-4 is increased in AD children can suggest the regulatory role of T cells in AD.

The genetic base sequence to express the proteins and the amino acid sequence of both CD28 and CTLA-4 are very similar with each other, and both have the same ligands such as B7-1 (CD80) or B7-2 (CD86). The chromosomes of both molecules are equally located at the long arm of chromosome 2 (2q33–34) in human and at the long arm of chromosome 1 (1c1–3) in rat (23). However, CD28 is expressed in naive T cells, but CTLA-4 is expressed only in activated T cells. Furthermore, the functions are also contrary to each other. In stimulation of CD28, initiation of IL-2 transcription and IL-2 production are

upregulated to ultimately promote T-cell proliferation. However, stimulation of CTLA-4 inhibits the proliferation of T-cell population or the cytokine production to play an important role in the immune response as a down-regulator (24, 25). Indirect inhibitory effects have been implicated to be involved in the induction of transforming growth factor (TGF)- β or IL-10 expression by CD152 (26). Accordingly, CD28 can balance the immune system by stopping the immune responses, which are involved by the CTLA-4 molecules after costimulation in the early immune responses (27).

The antigen (Ag) of this CTLA-4 has two significant characteristics. The first is the immunological characteristic to affect biological immune response as described above, and the second is the cellular biological characteristic that this Ag is expressed on the cell surface by only the stimulation (28). CTLA-4 has been shown to be temporarily expressed on the cell surface and remain expressed at low concentration through the cellular endocytosis. However, its expression level is quite low, being 1/20 of the CD28 expression level, although CD152 binds to CTLA-4 with 20- to 50-fold higher affinity than CD28 (8). Because of these characteristics and its low level, the CTLA-4 expression on the surface is not easily observed by the flow cytometric method. In the mouse model, the expression level on the cell surface after the Ag stimulation was low and the intracellular CTLA-4 level was increased (29). Only 2% of the activated T cells were found to express surface CD152, while > 80% of them express intracellular CD152 (30). On consideration of these characteristics, we measured in the present study intracellular level of the CTLA-4 of CD3⁺ T cell, CD4⁺ T cell and CD8⁺ T cell through intracellular staining of whole blood collected from the child patients of the acute stage on the first admission to this hospital.

The CTLA-4 knock out mouse model has recently been shown to have increased circulating cytokines, highly concentrated immunoglobulin and lymphoproliferative syndrome (11). Several studies have reported on the relationship between CTLA-4 and autoimmune diseases. SLE patients express more CTLA-4 than normal individuals under the resting state, but decrease the upregulated CTLA-4 level after the stimulation (31). Furthermore, several studies have investigated the polymorphism related to CTLA-4 genes in SLE and in other immune diseases such as type 1 diabetes mellitus and autoimmune thyroiditis (12–14). The roles of CTLA-4 have been indicated in the human immunodeficiency virus (HIV) infection which is highly associated with T cells;

expression levels were significantly increased selectively on CD4⁺ T cell during all stage of HIV infection, however, after stimulation with the mitogen phytohaemagglutinin (PHA), CTLA-4 levels were severely impaired in T cells from HIV patients. In other words, by reflecting the dysfunction of T cells, the activation-induced cell death can be much easier in T cells expressing CTLA-4 in the HIV patients (32). By measuring CTLA-4 level in the infective diseases like malaria, it was found that T cell was an activation marker, because CTLA-4 was increased at the admitting time but was decreased after the medication (33). CD4⁺ T cell generally expressed increasingly more CTLA-4 in the infective diseases such as malaria or HIV infection and autoimmune diseases like SLE.

In conclusion, the findings of the present study indicates the possibility that T cell participates in the mechanism of AD, and CTLA-4 molecule acts as a regulatory factor, providing the possibility that the disease activity can be correlated with the CTLA-4 level.

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