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The effects of FK506 on refractory inflammatory myopathies

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Abstract

We performed an observational clinical study, the effects of tacrolimus (FK506) on the thymic output in patients with refractory inflammatory myopathies. Sixteen patients with polymyositis (PM) and 15 with dermatomyositis (DM) were treated orally with tacrolimus. Serum CK levels significantly decreased 2 to 4 months after tacrolimus therapy ($p < 0.01$), and MRC (Medical Research Council) scores were significantly improved 2 months after tacrolimus therapy ($p < 0.01$). T-cell receptor excision circle (TREC) content, a proxy for thymic export was not significantly different from that in age-matched controls, except for an increase in the TREC content within CD8+ single positive cells in patients with DM. TREC contents within double-positive cells and $CD4^+$ single-positive cells were significantly decreased 4 M after tacrolimus therapy ($p < 0.05$) in PM/DM patients. Tacrolimus treatment significantly attenuated TREC content within cultured $CD4^+CD8^-$ cells from PM/DM patients ($p < 0.05$), but total cell counts were not significantly changed. These results indicate that tacrolimus therapy suppresses not only activated T-lymphocytes, but also some naïve T-cell subsets in both PM and DM.

Key words: Polymyositis; Dermatomyositis; T cell receptor excision circle; TREC; lymphocytes; cell culture.

Introduction

Idiopathic inflammatory myopathies, comprising polymyositis (PM), dermatomyositis (DM), and inclusion-body myositis, are characterized by inflammatory cell infiltrates in skeletal muscle tissue, muscle weakness, and muscle fatigue. Autoimmune responses are believed to be involved in both PM and DM, but the target antigens have not been identified. In PM and DM, the cellular infiltrates in muscle tissue are mainly composed of T-lymphocytes, macrophages, and B-lymphocytes. In particular, T-cell products are involved in the pathogenic process, including interleukin (IL)-1, IL-6, interferon-γ, and tumor necrosis factor α (TNF-α) (1).

It is well known that PM and DM are fundamentally different disorders in terms of pathogenesis, though some molecular pathways are shared between the subsets of inflammatory myopathies. DM is a humorally mediated autoimmune disorder; the humoral immune process is directed against the vascular endothelium. PM is caused by a cell-mediated immune phenomenon; autoinvasive CD8+ T-cells invade nonnecrotic muscle fibers expressing class 1 major histocompatibility complex antigen (MHC-1) (2). However, there is some evidence to indicate the common immune mechanism in PM and DM. The established T-cell lines from muscle tissue of patients with PM or DM showed a variable proportion of CD4+ and CD8+ T-lymphocytes that did not correlate with the diagnosis (3). Therapeutic agents that affect T-lymphocyte populations and that have been shown to be effective in polymyositis and dermatomyositis are methotrexate, cyclosporin A, tacrolimus, and antithymocyte globulin (4). In this paper, we examined the effects of tacrolimus on clinical symptoms and thymic T-cell export in patients with refractory PM/DM.

Patients and Methods

Clinical assessment

The subjects of this study were 17 patients with PM and 15 patients with DM. All were selected from Japanese patients who had been treated at Tokushima University Hospital from April 2003 to October 2008 and who agreed to participate in this study. The study was compliant with the Declaration of Helsinki and was approved by the institutional ethics committee and patients gave written informed

consent. None of the patients had inclusion body myositis (IBM), cancer, or other connective tissue disorders. The criteria for diagnosis of PM and DM were based on studies by Bohan *et al*. (5, 6) and Mastaglia *et al*. (1): symmetric muscle weakness, increased serum muscle enzyme, myopathic changes on electromyography, and typical histological findings on muscle biopsy and/or characteristic dermatological manifestations (heliotrope rash, periungual erythema, Gotron papules, and poikiloderma) for DM. All patients had a muscle biopsy. As for IBM, rimmed vacuoles and neurogenic changes were not observed in all cases and none of them fulfilled the criteria for IBM (7). The diagnosis of PM or DM was considered definite in all cases. The mean age of patients with PM was 59.5 ± 2.5 (mean ± SE); of those with DM it was 61.6 ± 4.0. All patients received an oral administration of prednisolone (PM patients, 7.1 ± 1.4 mg/day; DM patients 8.0 ± 0.9 mg/day). But corticosteroid therapy had not completely controlled the clinical manifestations and serum creatine kinase levels in all patients. Disease duration was from 2 y to 10 y, and mean duration was 4.1 (± 3.5) y. Tacrolimus was orally administered to all patients (3 mg/day). Corticosteroid therapy remained unchanged during the tacrolimus therapy. The patients were evaluated two and four months after treatment by a measurement of grip power, the Medical Research Council (MRC) score corresponding to a muscle strength of 18 proximal muscle groups (maximal score was 90) (8, 9) and serum creatine kinase (CK) concentration. For a measurement of MRC, the muscle strength of all patients was assessed by a neurologist, who was blind to the clinical information of the patients. Control samples were obtained from age-matched volunteers (n = 12; age 60.5 ± 5.2 y). Laboratory examinations were also performed before and after treatments, including peripheral blood cell count, CK, transaminases, cholesterol, and blood sugar.

Separation of CD4+ and CD8+ cell populations

A separation of CD4+ and CD8+ cell populations was carried out according to the previous report (10). In brief, mononuclear cells were separated from peripheral blood using Lymphoprep (Nycomed, UK). CD4+/CD4- and CD8+/CD8- cells were separated from mononuclear cells using a Magnetic cell-sorting system (MACS, Axis-Shield PoC, UK). The cells were counted using a hemocytometer. Some were used for primary culture, and the remaining cells were stored at -80°C until PCR analysis.

Quantification of T-cell receptor excision circle (TREC) DNA

Genomic DNA was extracted using a QIAmp Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For quantification TRECs, we used a Light Cycler PCR and detection system (Roche Diagnostics, Mannheim, Germany). Primers for δRec-ψJα signal joint TREC, probes, and PCR conditions were reported by Loeffler *et al*. (11).

Mononuclear cell culture

Single-positive cells ($CD4^+CD8^-$ and $CD4^-CD8^+$ cells) were resuspended in RPMI 1640 containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, according to the previous report of Horigome *et al*. (12). The cells were incubated for 72 h in 5% CO_2/air at 37°C in a humidified chamber in the presence (10 and 1000 ng/ml) or absence of FK506.

Statistical analysis

We used StatView for Windows (version 5.0) for statistical analysis. Paired data were analyzed by the nonparametric Wilcoxon's signed-rank test.

Results

In this study, we examined the effects of tacrolimus on clinical manifestations and on thymic output in patients with refractory PM/DM. Although the handgrips did not significantly change, MRC scores decreased 2 M after tacrolimus therapy in PM (0 M, 69.4 ± 2.1; 2 M, 81.9 ± 2.3 [mean ± SE]) and in DM (0 M, 49.23 ± 4.1; 2 M, 52.8 ± 4.2 [mean ± SE]), as shown in Fig. 1. Serum CK levels significantly decreased 2 M and 4 M after tacrolimus therapy in PM (-2 M, 311.2 ± 56.9; 0 M, 263.4 ± 41.7; 2 M, 217.6 ± 33.0; 4 M, 191.8 ± 30.6 [mean ± SE]) ($p < 0.01$) and DM (-2 M, 500.23 ± 40.0; 0 M, 416.3 ± 44.1; 2 M, 345.2 ± 58.3; 4 M, 297.6 ± 67.7) ($p < 0.01$).

We accessed TREC contents within single- and double-positive cells ($CD4^+CD8^+$), positive cells ($CD4^+CD8^-$, and $CD4^-CD8^+$ cells), and double-negative cells ($CD4^-CD8^-$). The TREC contents in PM patients (Fig. 2 and Table 1) were not significantly different from those of controls. On the other hand, the TREC contents within $CD4^-CD8^+$ cells was significantly decreased in DM patients, compared to controls ($p < 0.05$). In patients with PM, the levels in double-positive cells and $CD4^+CD8^-$ cells

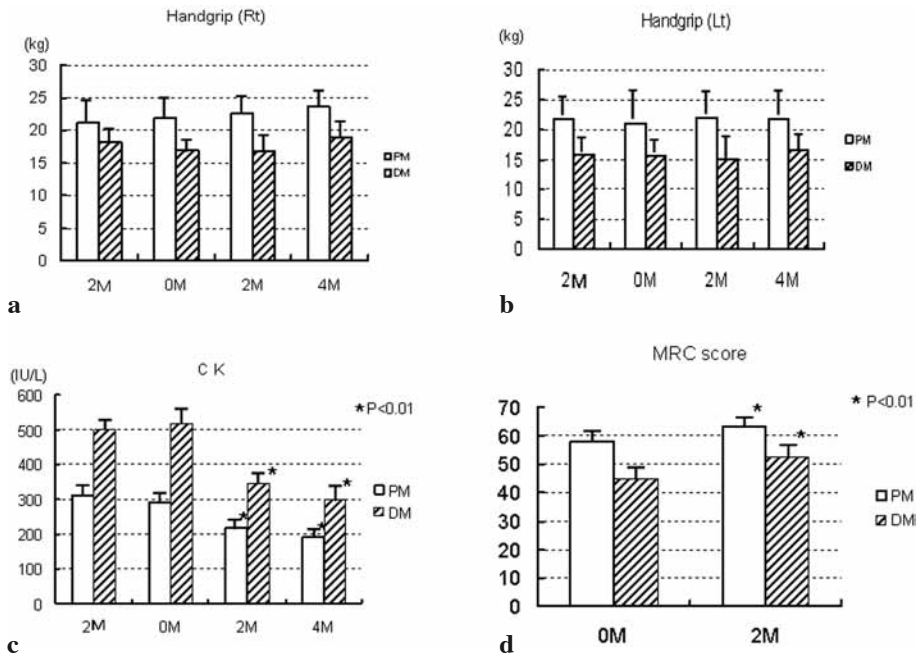

FIG. 1. — Handgrip power (**a** and **b**) and serum CK levels (**c**) in patients with polymyositis (PM) and dermatomyositis (DM) before and after oral administration with tacrolimus (3 mg/day). Serum CK levels significantly decreased 2 to 4 months after tacrolimus therapy. MRC scores were significantly improved 2 months after tacrolimus therapy (**d**).

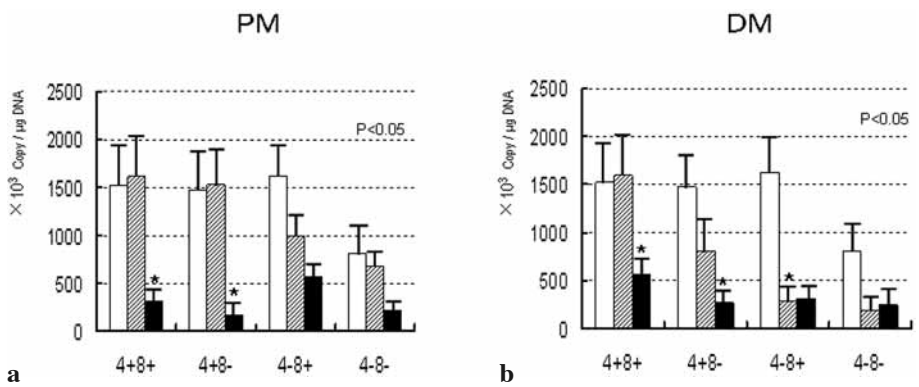

FIG. 2. — T-cell receptor excision circle (TREC) levels in single- and double-positive cells ($CD4^+CD8^+$), positive cells ($CD4^+CD8^-$ and $CD4^-CD8^+$ cells), and double-negative cells ($CD4^-CD8^-$) in patients with polymyositis (PM), dermatomyositis (DM), and age-matched controls. **a**, TREC levels in PM. In patients with PM (columns with slant lines) TREC levels were not significantly different from those of age-matched controls (open columns), but the levels in CD4+CD8+ cells and CD4+CD8- cells were significantly decreased 4 M after tacrolimus therapy (closed columns), respectively (stars, $p < 0.05$). **b**. TREC levels in DM. TREC levels in patients were not significantly different from those in age-matched controls, except for an increase in TREC level in CD8+ single-positive cells in patients with DM. The levels in double-positive and $CD4^+CD8^-$ positive cells were significantly decreased 4 M after tacrolimus therapy ($p < 0.05$).

were significantly decreased 4 M after tacrolimus therapy, respectively ($p < 0.05$). In patients with DM, the levels in double-positive and $CD4^+CD8^-$ positive cells were significantly decreased 4 M after tacrolimus therapy.

Next we examined the direct effects of tacrolimus on cultured T-lymphocytes from patients with PM (Fig. 3) and DM (Fig. 4). Single-positive cells were used in this study because double-negative/positive cells were not enough to examine. Tacrolimus treatment did not significantly change total cell counts of single-positive cells in PM patients (Fig. 3) or DM patients (Fig. 4). In PM patients, tacrolimus treatment fot 72 h significantly attenuated TREC contents within $CD4^+CD8^-$ cells (0 ng/μl, 2377 ± 431 copy/μg DNA; 10 ng/μl, 1377 ± 589 copy/μg DNA;

Table 1

T-cell receptor excision circle (TREC) contents within peripheral lymphoctes (copy/μg DNA, mean ± SE). Star, $p < 0.05$

	Control	Polymyositis		Dermatomyositis	
		FK506(-)	FK506(+)	FK506(-)	FK506(+)
CD4 (+)CD8 (+)	1515 ± 481	1613 ± 486	313 ± 78*	1593 ± 453	566 ± 175*
CD4 (+)CD8 (–)	1475 ± 511	1526 ± 425	170 ± 64*	808 ± 375	269 ± 112*
CD4 (–)CD8 (+)	1622 ± 476	997 ± 279	467 ± 145	290 ± 92	311 ± 111*
CD4 (–)CD8 (–)	813 ± 284	681 ± 170	217 ± 100	191 ± 170	244 ± 104

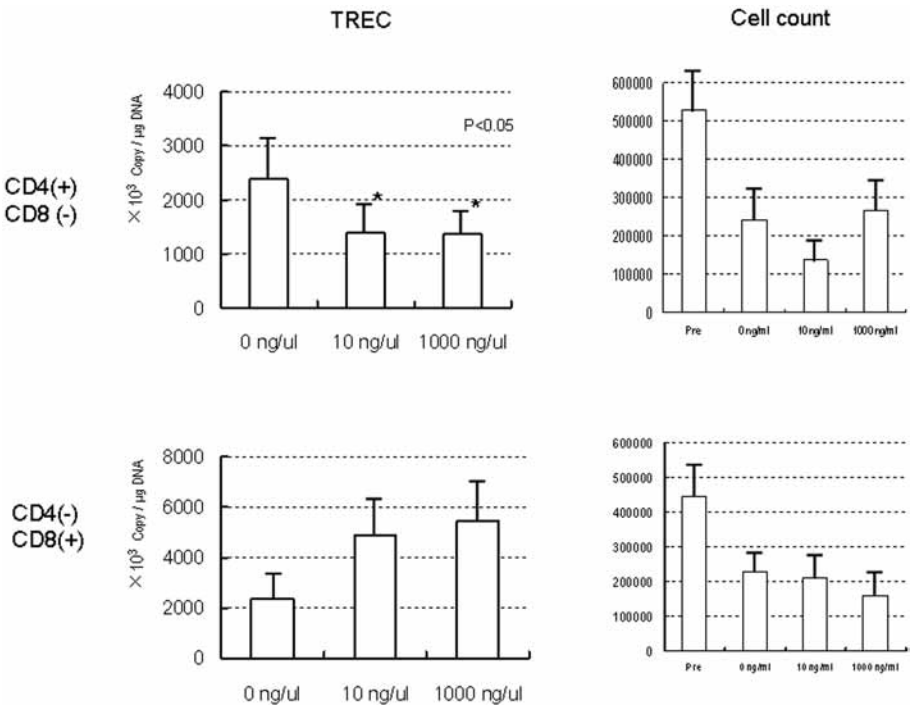

FIG. 3. — The effects of tacrolimus on cultured $CD4^+CD8^-$ cells and on $CD4^-CD8^+$ cells in patients with polymyositis. Tacrolimus treatment (10 and 1000 ng/μl, 72 h) significantly decreased T-cell receptor excision circle (TREC) levels in $CD4^+CD8^-$ cells, but not in $CD4^-CD8^+$ cells (stars, $p < 0.05$). Cell counts were not significantly changed by tacrolimus treatment.

1000 ng/μl, 1364 ± 639 copy/μg DNA; $p < 0.05$), but it did not those of $CD4^-CD8^+$ cells (0 ng/μl, 2345 ± 825 copy/μg DNA; 10 ng/μl, 4885 ± 1867 copy/μg DNA; 1000 ng/μl, 5427 ± 1869 copy/μg DNA) (Fig. 3). The TREC TREC contents within $CD4^+CD8^-$ cells in DM patients were significantly decreased under 1000 ng/μl tacrolimus treatment (72 h) (0 ng/μl, 410 ± 101 copy/μg DNA; 10 ng/μl, 335 ± 89 copy/μg DNA; 1000 ng/μl, 180 ± 80 copy/μg DNA; $p < 0.05$), but TREC contents within $CD4^-CD8^+$ cells were not (0 ng/μl, 286 ± 96 copy/μg DNA; 10 ng/μl, 210 ± 86 copy/μg DNA; 1000 ng/μl, 223 ± 109 copy/μg DNA) (Fig. 4). Therefore the direct effects of tacrolimus therapy on TREC seems prominent in CD4+CD8- lymphocytes from PM patients compared with those in CD4+CD8- lymphocytes from DM patients.

Discussion

PM and DM are diseases characterized by muscle weakness and muscle inflammatory infiltrates. A central role for endomysial autoaggressive CD8+ T-cells is suspected in PM and for perivascular B cells in DM (4). The pathogeneses of PM and DM are different; PM, but not DM, is an autoimmune CD8+ T-cell-mediated disease. In fact, the lymphocyte composition is different in peripheral blood levels. Dramatic perturbations of the T-cell repertoire were observed in the blood of PM patients, but not in DM

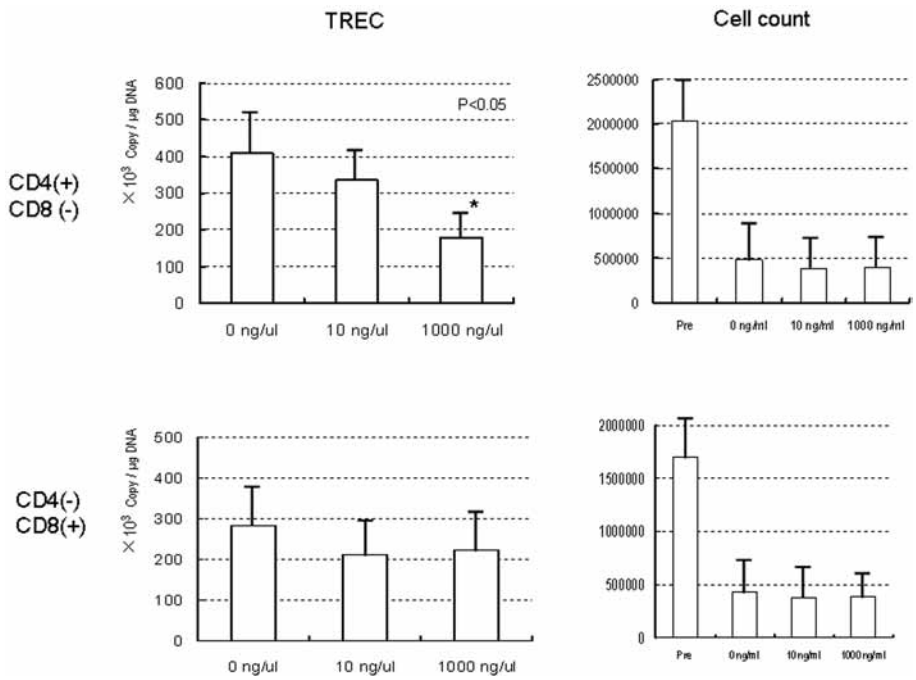

FIG. 4. — The effects of tacrolimus on cultured $CD4^+CD8^-$ cells and $CD4^-CD8^+$ cells in patients with dermatomyositis. Tacrolimus treatment (1000 ng/μl, 72 h) significantly decreased T-cell receptor excision circle (TREC) levels in $CD4^+CD8^-$ cells, but not in $CD4^-CD8^+$ cells (stars, $p < 0.05$). Cell counts were not significantly changed by tacrolimus treatment.

Table 2

T-cell receptor excision circle (TREC) contents within cultured lymphoctes with tacrolimus treatment (copy/μg DNA, mean ± SE). Star, $p < 0.05$

	Polymyositis			Dermatomyositis		
	0 ng/μl	10 ng/μl	1000 ng/μl	0 ng/μl	10 ng/μl	1000 ng/μl
CD4 (+)CD8 (–)	2377 ± 431	1377 ± 589*	1364 ± 639*	410 ± 101	335 ± 89	180 ± 80*
CD4 (–)CD8 (+)	2345 ± 825	4885 ± 1867	5427 ± 1869	286 ± 96	210 ± 86	223 ± 109

patients, the latter being undistinguishable from controls (4). These perturbations were due to oligoclonal expansions of CD8+ T-cells, and most blood clonal expansions were also found in muscle (13). In spite of the pathogenetic difference, 70% to 80% of patients with PM or DM respond satisfactorily to corticosteroids (1). In residual cases, the response is slow or incomplete, and it is then necessary to induce a second-line agent including various immunosuppressants. Recently, tacrolimus (FK506) has been used to treat several autoimmune disorders (14-18). It is an immunosuppressive agent similar to cyclosporin A, which inhibits the action of calcineurin, a serine/threonine phosphatase, thereby suppressing the interleukin-2 production (19, 20) and T-cell proliferation (21). We previously reported successful treatment with tacrolimus in a case of refractory dermatomyositis (9).

Phenotypic and T-cell receptor excision circle (TREC) analysis confirmed thymic origin of the expanded naïve T-cell subset. An analysis of the T-cell receptor repertoire showed the reconstitution of an overall broader clonal diversity, and TREC was used as a marker of thymic output. The altered thymic T-cell export has been reported in some autoimmune diseases (22-24). TREC production seems to play important roles in the development of autoimmune diseases, but the precise mechanism is unknown and may be complicated; TREC levels are reportedly increased in patients with autoimmune thyroid disease (23) and decreased in patients with multiple sclerosis (25) and juvenile arthritis (26). In thymomatous MG, naïve T-cells as well as TRECs were remarkably increased, and TREC levels decreased following thymectomy (10). We previously reported that tacrolimus has a beneficial effect

particularly in thymomatous MG (14). These findings encouraged us to perform an observational clinical study, the effects of tacrolimus on the clinical symptoms and TREC levels in refractory PM/DM.

It is well known that TREC concentrations depend on aging; they remain high for the first 20 years of life and gradually decrease thereafter as the thymus atrophies (11). In the present study, we compared TREC concentrations between patients and age-matched controls. TREC concentrations in patients were not significantly different from those in age-matched controls except for an increase in TREC content within CD8+ single-positive cells in patients with DM. The contents within double-positive cells and CD4+ single-positive cells were significantly decreased 4 M after tacrolimus therapy in patients with PM/DM. Taken together, serum CK levels and MRC scores were significantly decreased. These results indicate that tacrolimus therapy has some beneficial effects on refractory PM/DM and that TREC concentrations may become a marker of the curative effects of tacrolimus. Because TREC concentrations might not depend on the other immunosuppressive therapy using predonisone and azathioprine (16), we hypothesized that tacrolimus potentially acts directly on T-lymphocytes. We then examined the effects of it on cultured T-lymphocytes. It showed no toxic effects on them, but it significantly decreased the TREC content within $CD4^{+}CD8^{-}$ cells in PM/DM patients. The effects of tacrolimus therapy on TREC seem prominent in PM patients compared with DM patients. This may be related to the difference in lymphocyte repertoire between PM and DM. It is well known that tacrolimus inhibits the activation of T-lymphocytes via the suppression of calcineurin, and its action was considered to be primarily through activated T cells (21). The present results suggest that tacrolimus suppresses not only activated T-lymphocytes, but also naïve T-cells.

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