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Elevation of Serum Soluble Tumour Necrosis Factor Receptors in Patients with Polymyositis and Dermatomyositis

T. Shimizu, Y. Tomita, K. Son, S. Nishinarita, S. Sawada and T. Horie

First Department of Internal Medicine, Nihon University School of Medicine, Tokyo, Japan

Abstract: The aim of the study was, to examine the relationship between serum levels of soluble tumour necrosis factor receptors (sTNF-R) and the gene expression of two types of receptor for TNF (TNF-R), a 55 kDa receptor (TNF-R1) and a 75 kDa receptor (TNF-R2), in peripheral blood mononuclear cells (PBMC) from patients with polymyositis and dermatomyositis (PM/DM). Soluble tumour necrosis factor receptor 1 (sTNF-R1) and soluble tumour necrosis factor receptor 2 (sTNF-R2) levels in sera from patients were measured by enzyme-linked immunosorbent assay. Expression of TNF-R1 and TNF-R2 mRNAs in PBMC was analysed by Northern blotting. Serum sTNF-R1 and sTNF-R2 levels were elevated significantly in 25 patients with active-stage PM/DM, compared to those in 18 patients with inactive-stage PM/DM and 32 normal controls. Serum concentrations of sTNF-R1 and sTNF-R2 were correlated with PM/DM disease activity. TNF-R1 gene expression was enhanced in freshly isolated PBMC from patients with active-stage PM/DM. In contrast, TNF-R2 mRNA was expressed constitutively in patients with active-stage PM/DM and in normal controls. The expression of TNF-R1 and TNF-R2 mRNAs in PBMC and elevation of their soluble forms in the sera of patients with active-stage PM/DM suggest increased proteolytic cleavage of cell surface TNF-R from PBMC in patients with active-stage PM/DM, and that sTNF-R may regulate TNF-α-mediated muscle fibre damage in PM/DM.

Correspondence and offprint requests to: Dr Y. Tomita, 30-1 Oyaguchikamimachi, Itabashi-ku, Tokyo 173-8610, Japan. Tel: 03-3972-8111; Fax: 03-3972-2893.

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Introduction

Polymyositis (PM) and dermatomyositis (DM) are inflammatory muscle diseases characterised clinically by systemic proximal muscle weakness, cutaneous legions (in DM), and systemic manifestations in other organs. Although little is known about the aetiologies of these diseases, evidence suggests that both cellular and humoral autoimmune mechanisms are involved in their pathogenesis and progression [1–3].

Proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1 and IL-6 play an important role in the inflammatory processes of many autoimmune diseases, including rheumatoid arthritis (RA) [4], systemic lupus erythematosus (SLE) [5] and multiple sclerosis (MS) [6]. However, the role of these cytokines in PM/DM is poorly understood. Immunohistochemical studies of muscle biopsy specimens of PM/DM patients have revealed that TNF-α-positive macrophages and lymphocytes are expressed in endomysium and around blood vessels in muscle from patients with PM [7,8]. Consequently, it has been suggested that TNF-α may play a role in the pathogenesis of PM/DM.

Two types of receptor for TNF-α (TNF-R), a 55 kDa receptor (TNF-R1) and a 75 kDa receptor (TNF-R2), have been identified [9–12] and shown to be expressed by a variety of cells [13–16]. The soluble forms, which originate from the extramembranous portions of TNF-R1 (soluble TNF-R1; sTNF-R1) and TNF-R2 (soluble TNF-

R2; sTNF-R2), can be found in supernatants of lymphocyte, monocyte and neutrophil cultures [14,17–19] and in biological fluids [20,21]. Soluble TNF-R (sTNF-R) levels in serum/plasma and biological fluids are elevated in several autoimmune diseases [22–26], and specific inhibitory effects of sTNF-R on TNF-α activities have been demonstrated [27].

In the present study we measured sTNF-R1 and sTNF-R2 concentrations in sera from patients with active-stage PM/DM, and analysed TNF-R1 and TNF-R2 mRNA expression in freshly isolated peripheral blood mononuclear cells (PBMC) from these patients. The results show that in patients with active-stage PM/DM increased serum levels of sTNF-R1 and sTNF-R2 are due, at least in part, to increased shedding of cell surface TNF-R from PBMC, and that sTNF-R may regulate TNF-α-mediated muscle fibre damage in PM/DM.

Patients and Methods

The study group comprised 43 patients with PM/DM (30 women and 13 men; mean age 49.0 years, range 15–78 years) classified according to the diagnostic criteria of Bohan and Peter [28], and 32 normal controls (14 women and 18 men). Disease activity was determined by (1) proximal muscle weakness, (2) elevated serum levels of creatine kinase (CK) and aldolase, (3) myogenic change on electromyogram (EMG), (4) muscle biopsy findings of inflammatory myopathy, and (5) skin lesions such as heliotrope rash or Gottron's sign. Patients who presented with at least three of these five features were described as having active disease. Of the 43 with PM/DM, 25 (14 PM and 11 DM) were noted to have active-stage disease; none had bacterial infections at the time of sampling. Age, treatment, clinical features and muscle enzyme levels for the active-stage PM/DM patients at the time of sampling are shown in Table 1. The serum samples from patients and controls were collected when blood was drawn and were stored at –20 °C until used.

Cell Preparation

PBMC were obtained by centrifugation of heparinised blood drawn from patients with PM/DM and from controls over Ficoll-Hypaque (Litton Bionetics, Kensington, MD) for 30 minutes at 400 g.

Measurement of Serum sTNF-R1/R2 and TNF-α Levels

Serum sTNF-R1/R2 and TNF-α levels were measured using commercially available enzyme-linked

Table 1. Clinical features and muscle enzyme levels in 25 patients with active PM/DM

PT	Age/ sex	Diagnosis	Disease duration (months)	CK* (IU/l)	Aldolase† (IU/l)	Muscle weakness	Muscle biopsy findings		Other clinical features§	Treatment daily dose¶ (mg)
							Inflammation	Myofiber atrophy		
1	53/F	PM	40	632	9.7	+	+	+	–	PSL45
2	22/F	PM	44	567	2.3	+	+	+	MCTD	PSL60
3	55/M	PM	43	7180	169.5	+	+	+	–	PSL30
4	58/M	PM	21	6138	49.5	+	+	+	–	PSL60
5	22/F	PM	23	5727	77.8	–	+	+	–	PSL50
6	32/M	PM	55	1701	20.4	–	–	+	–	–
7	15/F	PM	46	223	16.2	–	+	–	–	PSL40
8	22/F	PM	24	569	21.6	+	+	+	–	PSL50
9	78/F	PM	21	4578	71.8	+	–	+	PF	PSL50
10	59/M	PM	87	222	15.5	+	–	+	PF	PSL60
11	39/F	PM	18	5355	46.1	+	+	+	–	PSL40
12	55/M	PM	18	6790	118.8	+	+	+	AIN	PSL60
13	50/F	PM	7	1754	26.6	+	+	+	–	PSL60
14	63/F	PM	7	2239	26.4	+	–	+	PF	PSL60
15	16/M	DM	51	7315	51.4	+	+	+	–	PSL100
16	53/M	DM	48	5889	66.6	+	+	+	–	PSL60
17	31/F	DM	35	4116	55.5	+	+	+	–	PSL60
18	78/F	DM	24	2359	6.4	+	+	+	–	PSL50
19	40/M	DM	56	121	9.5	+	+	–	AIN/RA	mPSL1000/CY
20	47/F	DM	56	90	10.7	–	–	+	PF	PSL40
21	46/F	DM	15	2056	17.5	+	+	+	breast cancer	PSL70
22	48/F	DM	9	4326	21.6	+	ND	ND	–	PSL45
23	67/F	DM	3	2060	22.0	+	–	–	–	PSL55
24	73/M	DM	4	4059	125.5	+	–	+	–	PSL45
25	76/M	DM	4	632	12.0	+	–	+	PF	PSL30

*CK, creatine kinase (IU/l); †ND, not done; §PF, pulmonary fibrosis; AIN, acute interstitial pneumonia; ¶PSL, prednisolone; mPSL, methylprednisolone; CY, cyclophosphamide.

immunosorbent assay (ELISA) kits (Amersham International plc, Buckinghamshire, UK), according to the manufacturer's instructions. The sensitivities of the sTNF-R1, sTNF-R2, and TNF-α ELISAs were 2.5 pg/ml, 0.5 pg/ml and 4.4 pg/ml, respectively. All samples were assayed in duplicate.

Preparation of cDNA Probes

The cDNA probes specific for human TNF-R1 [10] and -R2 [11] were kindly provided by Dr W. Lesslauer (F. Hoffmann-LaRoche Ltd, Basel, Switzerland). The 0.77 kb NcoI-TaqI fragment of chicken actin cDNA was purchased from Oncor (Gaithersburg, MD). One hundred nanograms of purified cDNA were radiolabelled by random primer extension in the presence of ^{32}P-dCTP.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was prepared from freshly isolated PBMC using an RNA extraction kit (RNAzol B. TM, Tel-Test Inc, Friedswoods, TX) and the acid guanidine thiocyanate-phenol-chloroform extraction method. Fifteen micrograms of denatured RNA per lane were size-fractionated by electrophoresis on 1% agarose-formaldehyde gels and then transferred to a nylon membrane (Hybond N$^+$, Amersham, UK) by capillary transfer. The blots were prehybridised for 15 min at 65 °C and then hybridised for 3 h at 65 °C in Rapid Hybridisation Buffer (Amersham) with a ^{32}P-labelled cDNA probe. After hybridisation the blots were washed with 0.1 × SSC/0.5% SDS for 20 min at 65 °C and exposed to Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) with an intensifying screen for 16 h at –70 °C. For rehybridisation the blots were completely stripped of the TNF-R cDNA probe by washing with 'stripping buffer' (2.5 mM Tris, 0.1 mM EDTA, 0.025% sodium pyrophosphate, 0.05 × Denhardt's solution) for 40 min at 65 °C and then rehybridised with the ^{32}P-labelled actin cDNA probe for 3 h at 65 °C (29).

Statistical Analysis

Statistical significance was analysed using Fisher's exact test and Spearman's rank test was used for correlation. $P < 0.05$ was considered significant.

Results

Serum sTNF-R1/R2 and TNF-α Levels in PM/DM

Serum levels of sTNF-R1 and sTNF-R2 in the 43 PM/DM patients and 32 normal controls are shown in Fig. 1. Twenty-five of 43 PM/DM patients had active-stage disease and 18 were in the inactive stage. Both sTNF-R1

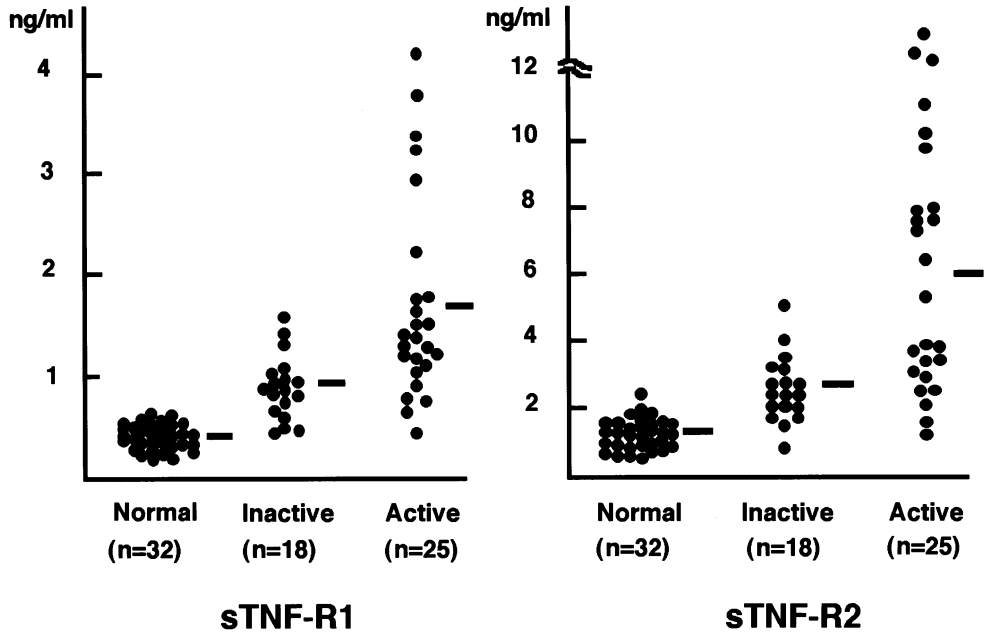

Fig. 1. Serum sTNF-R1/R2 levels in patients with PM/DM. Serum sTNF-R1 (left panel) and sTNF-R2 (right panel) levels of 25 patients with active-stage PM/DM, 18 patients with inactive-stage PM/DM and 32 normal controls were measured by ELISA. Horizontal short lines represent the mean of each group.

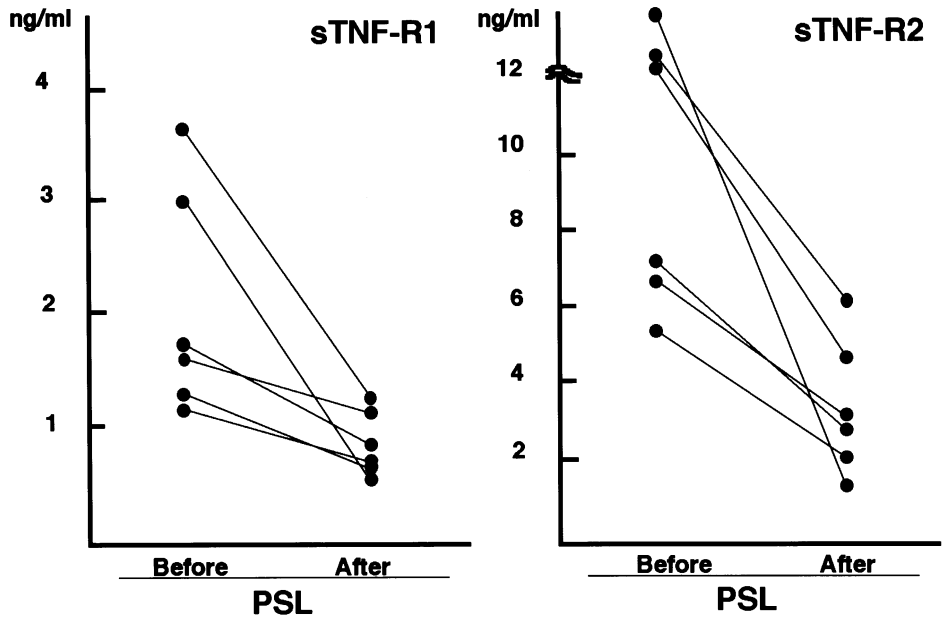

Fig. 2. Changes in serum sTNF-R1 and -R2 levels before and after steroid therapy in patients with PM/DM. Serum sTNF-R1 (left panel) and sTNF-R2 (right panel) levels in PM/DM patients before and after steroid therapy were measured by ELISA. Patients received 30–100 mg/day of prednisolone at the start of therapy, and the dose was gradually decreased. Samples were collected prior to steroid therapy and after reduction of the prednisolone dose to less than 20 mg/day. The disease activity of all patients decreased following prednisolone therapy.

and sTNF-R2 concentrations in serum were significantly higher in active-stage PM/DM patients (range 0.5–4.1 ng/ml; mean ± SE 1.8 ± 0.2 ng/ml for sTNF-R1 and range 1.7–14.7 ng/ml; mean ± SE 6.0 ± 0.8 ng/ml for sTNF-R2) than in inactive-stage patients (mean ±SE 0.9 ± 0.1 ng/ml for sTNF-R1, $P < 0.01$; and mean ±SE 2.6 ± 0.2 ng/ml for sTNF-R2; $P < 0.01$) and in normal controls (mean ±SE 0.5 ± 0.02 ng/ml for TNF-R1, $P < 0.01$; mean ±SE 1.3 ± 0.08 ng/ml for TNF-R2, $P < 0.01$). However, the difference in serum sTNF-R1 and sTNF-R2 levels between active-stage PM patients and active-stage DM patients (1.8± 0.2 ng/ml for PM vs. 1.6 ± 0.3 ng/ml for DM in sTNF-R1, and 6.2 ± 1.2 ng/ml for PM vs. 5.7 ± 0.9 ng/ml for DM in sTNF-R2) was not significant (NS). Of 25 active-stage patients, sTNF-R1 and sTNF-R2 levels were higher in those with severe muscle weakness than in those with moderate or mild muscle weakness. The relationships between sTNF-R1 and sTNF-R2 and steroid therapy in PM/DM patients are shown in Fig. 2. Six active-stage patients (three PM and three DM) received 30–100 mg/day of prednisolone. The disease severity in all patients decreased following therapy. Serum levels of sTNF-R1 and sTNF-R2 decreased after steroid therapy in all patients tested, suggesting that both sTNF-R1 and sTNF-R2 might be useful markers for assessing the level of disease activity in PM/DM. In contrast, serum levels of TNF-α were below detection limits for all active-stage PM/DM patients (data not shown).

Longitudinal Evaluation of Serum sTNF-R1/R2 Levels in a Patient with Chronic DM

Longitudinal evaluation of serum sTNF-R1 and sTNF-R2 levels was carried out to examine the relationship between serum sTNF-R1/R2 levels and the clinical course of a patient with chronic DM (PT 15). Prior to steroid therapy this patient had CK concentrations as high as 7315 IU/l and severe proximal muscle weakness. Muscle biopsy showed heavy lymphocyte infiltration around perivascular areas and necrosis of perifascicular muscle fibres. A skin biopsy showed lymphocytic infiltration around the vessels of the dermis. After therapy with high doses of prednisolone (100 mg/day initial dose), serum CK levels declined rapidly to 44 IU/l. When the prednisolone dose was reduced, the levels of CK increased to approximately 300 IU/l and did not change over more than 2 years. Serum aldolase levels were persistently elevated, and proximal muscle strength gradually improved but was still abnormal 2 years later. As shown in Fig. 3, serum levels of sTNF-R1 and sTNF-R2 were elevated initially, at 3.8 ng/ml and 12.6 ng/ml, respectively, and declined rapidly during high-dose prednisolone therapy. There was a significant relationship between sTNF-R1/R2 levels and CK levels during this initial response. When the prednisolone dose was reduced, both serum sTNF-R1 and sTNF-R2 levels increased with increased CK levels.

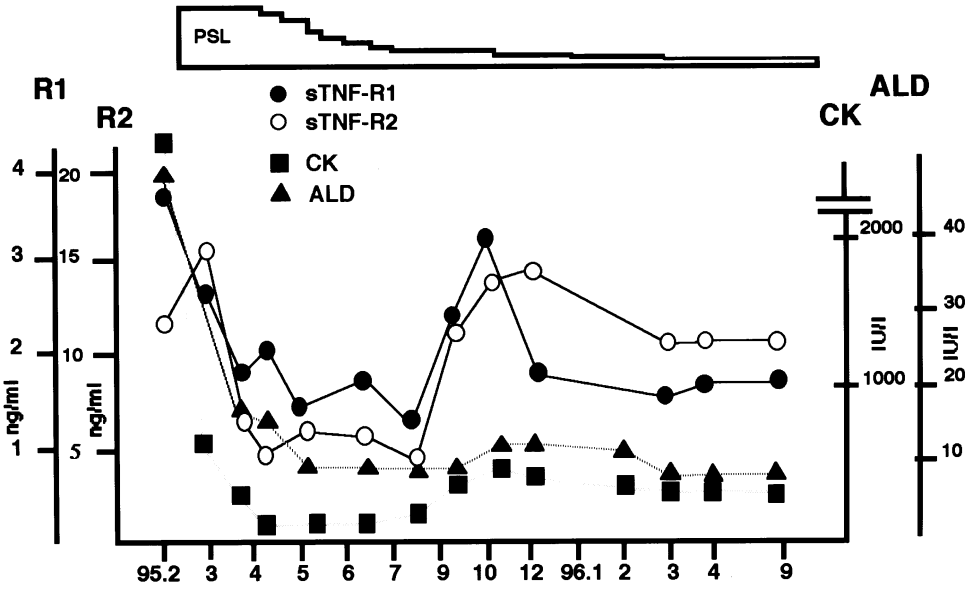

Fig. 3. Longitudinal evaluation of serum sTNF-R1/R2 levels in a patient with chronic DM. Changes in sTNF-R1 (●), sTNF-R2 (○), CK (■) and aldolase (▲) levels were evaluated during the course of this study. Data are from a 16-year-old male (PT 15) with elevated levels of CK (7315 IU/l) and aldolase (51.4 IU/l) and severe proximal muscle weakness at the time of diagnosis. Both TNF-R levels were declined rapidly during therapy with high doses of prednisolone (100 mg/day initial dose), and then increased concomitant with increased disease activity.

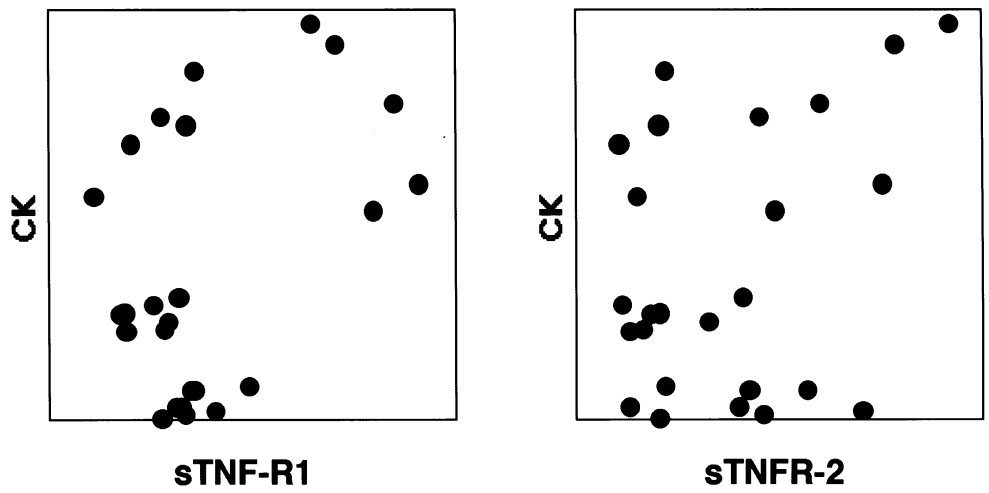

Fig. 4. Relationship between serum sTNF-R1/R2 levels and serum CK levels. The correlation between serum sTNF-R1 (left panel)/sTNF-R2 (right panel) levels and serum CK levels in 25 patients with active PM/DM were evaluated at the time of diagnosis. Neither serum sTNF-R1 nor sTNF-R2 levels were correlated with serum CK levels ($r = 0.420$, NS in R1; and $r = 0.331$, NS in R2).

Relationship between Serum CK or Aldolase Levels and Serum sTNF-R1/R2 Levels

The relationship between serum sTNF-R1/R2 levels and muscle enzyme levels was determined in 25 patients with active PM/DM. As shown in Figs 4 and 5, neither serum sTNF-R1 nor sTNF-R2 levels were correlated with serum CK levels ($r = 0.420$, NS in R1, and $r = 0.331$, NS in R2) (Fig. 4). In contrast, both sTNF-R1 and sTNF-R2 significantly correlated with serum aldolase levels ($r = 0.504$, $P < 0.05$ in R1, and $r = 0.442$, $P < 0.05$ in R2) (Fig. 5). There were significant correlations between serum sTNF-R1 and sTNF-R2 levels ($r = 0.739$, $P < 0.0001$) (Fig. 6) and between serum CK and aldolase levels ($r = 0.772$, $P < 0.0001$) (data not shown) in PM/DM patients.

Expressions of TNF-R1 and -R2 mRNAs in Freshly Isolated PBMC from PM/DM Patients

To investigate whether sTNF-R1 and sTNF-R2 are produced in the blood of PM/DM patients, the expression of sTNF-R1 and -R2 mRNAs was examined

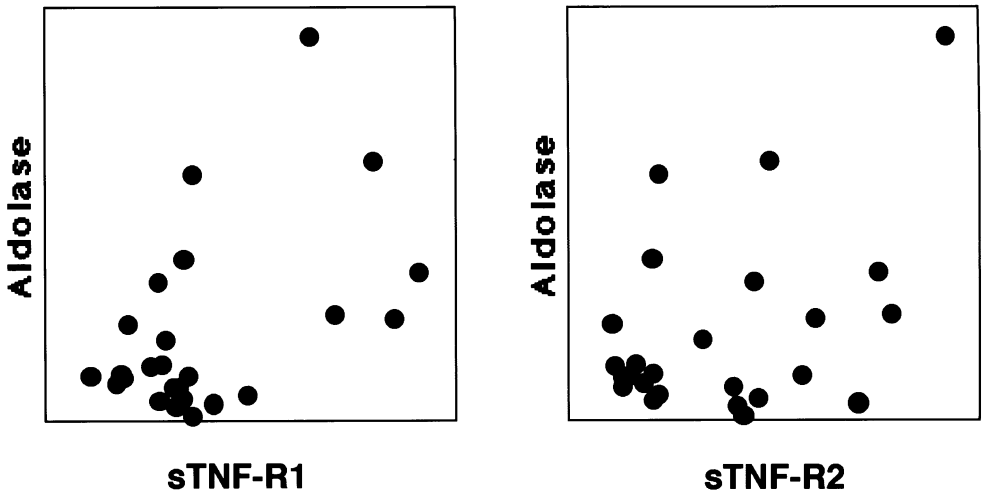

Fig. 5. Relationship between serum sTNF-R1/R2 levels and aldolase levels. The correlation between serum sTNF-R1 (left panel)/sTNF-R2 (right panel) levels and serum aldolase levels in 25 patients with active PM/DM were evaluated at the time of diagnosis. Both sTNF-R1 and sTNF-R2 significantly correlated with serum aldolase levels ($r = 0.504$, $P < 0.05$ in R1; $r = 0.442$, $P < 0.05$ in R2).

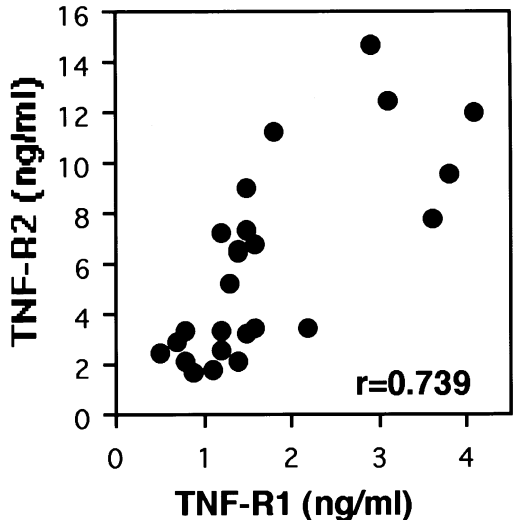

Fig. 6. Relationship between serum sTNF-R1 and serum R2 levels. The correlation between serum sTNF-R1 and sTNF-R2 levels in 25 patients with active PM/DM were evaluated at the time of diagnosis. There were significant correlations between serum sTNF-R1 and sTNF-R2 levels ($r = 0.739$, $P < 0.0001$).

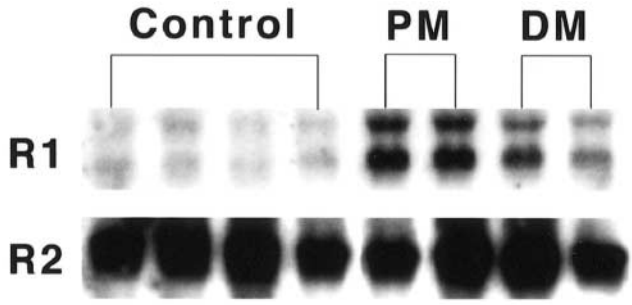

Fig. 7. Expression of TNF-R1 and R2 mRNAs in freshly isolated PBMC from PM/DM patients. Total cellular RNA was extracted from freshly isolated PBMC from four active-stage patients (two PM and two DM) and four normal controls, and Northern blot hybridisation was performed with TNF-R1 and -R2 cDNA probes.

by Northern blots of freshly isolated PBMC from patients with PM/DM. Total RNA was prepared from freshly isolated PBMC from active-stage PM/DM patients and normal controls and then hybridised with the TNF-R1/R2 cDNA probes. Figure 7 shows representative results from four active-stage patients (two PM and two DM) and four normal controls. None of the patients was receiving prednisolone at the time of sampling. Both TNF-R1 and TNF-R2 mRNAs were detectable in both patients and normal controls. Expression of TNF-R1 was weak in freshly isolated PBMC from normal controls, and was significantly enhanced in patients with active-stage PM/DM compared to that of normal controls. In contrast, TNF-R2 expression was strong but at similar levels in both patients and normal controls. A β-actin cDNA probe was used to determine the quantity of RNA in each lane on the Northern blots. The levels of β-actin mRNA were similar in all samples (data not shown).

Discussion

In the present study serum sTNF-R1/R2 levels were measured serially in patients with active-stage PM/DM. Our results showed that serum sTNF-R1/R2 levels are significantly higher in patients with active-stage PM/DM than in those with inactive-stage PM/DM or in normal controls. Longitudinal evaluation of sTNF-R1/R2 levels showed that changes are related closely to the clinical improvement of myositis. Moreover, TNF-R1 mRNA levels in freshly isolated PBMC from active PM/DM patients were higher than in normal controls. In contrast

to sTNF-R1, TNF-R2 mRNA was expressed strongly in both PM/DM patients and normal controls. These data suggest that monocytes and/or lymphocytes in the circulating blood may be a major source of sTNF-R1 in PM/DM patients.

Recent studies suggested that TNF-α plays a pathogenic role in several autoimmune diseases, including RA [4], SLE [5], and MS [6]. However, little information is currently available concerning the endogenous production of TNF-α in PM/DM patients. Immunohistochemical studies of muscle biopsy specimens revealed that TNF-α-positive macrophages and lymphocytes were expressed in endomysium and around blood vessels in muscle from patients with PM [7,8]. Although the TNF-α expression profiles in these studies were varied and inconsistent between patients with PM/DM, similar results were obtained from reverse-transcription polymerase chain reaction (RT-PCR) studies that examined TNF-α mRNA expression in muscle specimens [30,31]. These observations suggest that the TNF-α-mediated immune response plays an important role in the pathogenesis and progression of PM/DM.

The function of sTNF-R has been investigated. Soluble TNF-R can compete with TNF-R on the surface of cells and thus interfere with TNF activity [27]. Elevated sTNF-R levels in plasma/serum and biological fluids have been observed in several autoimmune diseases [22–26]. Moreover, Girardin et al. [32] showed that the ratio of TNF-α to sTNF-R in sera from patients with severe meningococcaemia was correlated with clinical outcomes. In addition, the protective effects of sTNF-R in sepsis [33,34] and the inhibitory effects of sTNF-R on pulmonary fibrosis in an animal model [35] have been demonstrated. These findings indicate that sTNF-R may contribute to the regulation of TNF-α-mediated diseases.

There have been few reports of sTNF-R in PM/DM. Gabay et al. [36] found that serum sTNF-R1/R2 levels were significantly higher in 15 PM/DM patients than in 12 normal controls, and Samsonov et al. [37] reported similar serum levels of sTNF-R1 from patients with PM/DM. These findings were similar to findings in the 25 patients with active-stage PM/DM. Taken together, these data suggest that sTNF-R might be a useful marker for monitoring the clinical course of myositis and classifying the levels of PM/DM activity. However, there was no significant correlation between CK levels and sTNF-R1/R2 levels in our study. Although the reason for this discrepancy is not clear, it may be related to observations that serum CK levels are not always elevated in patients with active-stage PM/DM, and especially in DM patients [38–41]. Thus, elevated serum CK levels are not sufficient for disease activity in PM/DM.

A previous study indicated that sTNF-R may contribute to regulation of TNF-mediated diseases [42]. Because TNF-α has been implicated in the pathogenesis of myositis, it could be that sTNF-R in PM/DM contributes to the regulation of inflammatory events in diseased muscle. The source of sTNF-R in PM/DM has not yet been established; however, determining its origin may provide useful information about the pathogenesis of myositis. Therefore, we analysed TNF-R1/R2 mRNA expression in freshly isolated PBMC from patients with PM/DM.

Our results clearly show that circulating monocytes and/or lymphocytes might be a major source of sTNF-R1 in the sera of PM/DM patients. TNF-R1 on PBMC might be shed in order to form soluble receptors. However, in regard to sTNF-R2, monocytes/lymphocytes and other types of cells may contribute to the high concentrations in sera. There are several possible mechanisms for elevation of TNF-R levels in PM/DM. First, activated monocytes and macrophages are stimulated to produce both TNF-α and sTNF-R via an autocrine mechanism in the cytokine network. This reflects induction of the inflammatory state and homoeostatic regulation during myositis. Because TNF-α levels were below detectable limits in PM/DM patients, it is possible that the concentrations of sTNF-R in patients with active PM/DM may be high enough to block TNF-α activity. Second, sTNF-R is produced by activated circulating monocytes as a result of increased TNF-α at the site of inflammation. Several studies using immunohistochemistry [7,8] and RT-PCR techniques [30,31] have indicated that TNF-α is produced by mononuclear inflammatory cells, atrophic muscle fibres in diseased muscle, and endothelial cells in the vascular wall. This cytokine may penetrate the tissue and enter the circulation. Although sTNF-R levels are thought to increase in response to endogenous TNF-α [14], sTNF-R levels may not be sufficient to block the effect of TNF-α in myositis. Third, elevation of serum sTNF-R, especially sTNF-R2, levels may be derived not only from peripheral monocytes/lymphocytes but also from other cell types, including inflammatory cells in muscle tissue. We are now investigating these possibilities using muscle biopsy specimens from normal controls and PM/DM patients.

Conclusion

Our study demonstrates elevation of serum levels of sTNF-R and increased gene expression of TNF-R1 in PBMC from patients with active-stage PM/DM. These results suggest that higher levels of serum sTNF-R may reflect increased TNF-R shedding in myositis, and that sTNF-R may regulate TNF-α-mediated muscle fibre damage in PM/DM.

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