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Transforming Growth Factor-β1 in Polymyositis and Dermatomyositis Correlates with Fibrosis but not with Mononuclear Cell Infiltrate

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Abstract. The idiopathic inflammatory myopathies are diseases of unknown etiology characterized by T cell-mediated myocytotoxicity in polymyositis and complement-mediated angiopathy of muscle fibers in dermatomyositis. A variable degree of fibrosis is present in muscles in these conditions both perimysially and endomysially. We evaluated the expression of TGF-β1, a pleiotropic cytokine with fibrogenic and immunomodulating activity, by means of quantitative-polymerase chain reaction and immunocytochemistry in DM and PM muscle biopsies. TGF-β1 mRNA was significantly higher in DM compared with controls, whereas in PM the values were not significantly different when compared with controls and DM. TGF-β1 was localized in connective tissue but did not correspond with mononuclear cell infiltrates. These findings suggest a correlation between TGF-β1 and connective tissue proliferation in inflammatory myopathy, while its immunomodulatory role remains to be elucidated.

Key Words: Connective tissue; Dermatomyositis; Fibrosis; Inflammatory myopathies; Polymyositis; Transforming growth factor-β1.

INTRODUCTION

The idiopathic inflammatory myopathies (IM) are diseases of unknown etiology; the two main forms, polymyositis (PM) and dermatomyositis (DM), are now considered to have an autoimmune pathogenesis in view of evidence of cytotoxic attack on muscle fibers in PM, mediated by T lymphocytes, and complement-mediated angiopathy of muscle fibers in DM (1–3). Positive responses to immunosuppressive therapy are reported for both conditions (1, 4). In PM the infiltrate is mainly composed of $CD8^+$ cytotoxic T lymphocytes that surround and eventually invade the muscle cells, and myofiber toxicity has been shown to occur as a result of perforin release from these $CD8^+$ cells, leading to muscle fiber necrosis (5). In DM high concentrations of B cells and $CD4^+$ helper T cells are found at perimysial and perivascular sites; perifascicular muscle fibers present a characteristic atrophy related to complement-mediated vascular damage and local ischemia (1).

It has been shown recently that T cell-derived transcripts of interleukin (IL)-4, IL-2 receptor (IL-2R), tumor necrosis factor (TNF)-α, and interferon (IFN)-γ can be amplified by reverse transcriptase PCR in muscles of patients with inflammatory myopathies (6, 7). Additionally, Tews and Goebel have found enhanced expression of several pro-inflammatory and immunomodulatory cytokines (IL-1, IL-2, IL-4, TNF-α and -β, and IFN-γ) in IM muscles by immunocytochemistry (8).

Areas of inflammation are also observed in the muscle of Duchenne muscular dystrophy (DMD) patients; the infiltrating mononuclear cells are mostly macrophages (9), although T cells are also found that have molecular characteristics that are noticeably different from the T cells infiltrating PM muscle (9, 10). Analysis of the cytokine profile in DMD muscle (6–8) has revealed significantly increased expression, correlated with the degree of fibrosis and patients' age (11), of transforming growth factor-β1 (TGF-β1), a pleiotropic cytokine mediating a wide range of biologic processes (12), including an immunomodulatory and fibrotic role (13). Because PM/DM and DMD share some pathological similarities, including an increase of connective tissue proliferation (14), and because of the controversial effects of TGF-β1 on cells of the immune system (13), we decided to quantify its expression in muscle biopsies from PM and DM patients and to seek to relate that expression to pathological alterations such as myofiber necrosis, presence of mononuclear cell infiltrates, and connective tissue proliferation.

MATERIALS AND METHODS

Patients

The IM patients (11 DM and 8 PM) were diagnosed on the basis of clinical, electromyographic and histological criteria (2). Muscle samples, obtained by needle biopsy, were frozen and stored in liquid nitrogen pending assay. No patients received immunosuppressive drugs before muscle biopsy. Sixteen subjects who had undergone muscle biopsy for diagnosis, but whose clinical, electromyographic, and histological findings showed them to be free of muscle disease, were used as normal controls.

Quantitative-PCR (Q-PCR) Analysis

Quantitative analysis of the TGF-β1 transcript was performed on total RNA extracted from muscle biopsies according to a

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TABLE 1
Histopathological Features of Inflammatory Myopathy Patients

Disease	Patient	Age (years)	Disease duration* (months)	Degree of Inflammation**	Degree of Connective Tissue***	TGF-β** positivity
DM	P.V.	35	3	++	++	++
	C.M.	60	18	−	+++	+++
	P.M.	71	36	+	+++	+++
	P.C.	15	12	+	normal	+
	P.A.	6	15	+	+	+
PM	F.A.	50	8	+	++	++
	C.M.	64	12	++	+	++
	G.A.	35	3	+	normal	++
	M.L.	55	12	++	normal	+
	T.G.	57	2	++	normal	+
	C.A.	60	24	+++	+	+
	D.F.	51	8	++	normal	+
	G.C.	72	1	++	++	++

* Before muscle biopsy.
** (+) low; (++) medium; (+++) high.
*** Degree of connective tissue, expressed as percentage per unit area, was determined by image analysis according to the procedure described; normal = <10%; (+) = 10–20%; (++) = 20–30%; (+++) = >30%.

protocol described elsewhere (11), with the modification that the internal competitor concentrations, co-amplified with the cDNA in the PCR reaction, were: 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 fg. As control, the cDNAs were also amplified with β-actin-specific primers.

Quantitative Analysis of Connective Tissue

Quantitative evaluation of intramuscular connective tissue was performed on hematoxylin-eosin-stained muscle transverse sections, as described (9). The area corresponding to the image obtained on a video connected to a light microscope at ×6 magnification was adopted as unit area for the calculations. Within this area, connective tissue was outlined by a digitizer linked to a computer and its area measured as the percentage of the total area using Image Measure® software (Microscience Inc., USA). In patients and controls, a mean of 4 areas (range 3–6) of the muscle biopsy slide were analyzed and the total amount of fibrosis was expressed as the gross mean of all the values obtained for each biopsy. As a reference value the normal muscles exhibited a fibrosis percentage up to 10%. A fibrosis percentage of >10 up to 20 was defined as +, >20 up to 30 as ++, and >30 as +++ (see Table 1).

Immunocytochemical Analysis

For the diagnosis of IMs, cellular infiltrates were characterized by immunocytochemistry on acetone-fixed 4-μm-thick cryosections of muscle biopsies. The following mouse monoclonal antibodies (mAbs) were used: anti-CD3 (Becton Dickinson, Mountain view, CA), anti-CD4 (helper T cells), anti-macrophages (clone Ber-MAC3), anti-CD8, anti-CD22, anti-MHC class I and II (all from Dako, Copenaghen, Denmark), anti-α/β and anti-γ/δ TCR (T Cell Sciences, Inc., Cambridge, MA). The mAbs were applied to the sections for 2 hours (h) at room temperature in a humid chamber, followed by a 60 minutes (min) incubation with the secondary biotinylated horse anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA). Rhodamine-avidin D (Vector) was then applied for 60 min. Sections were mounted in a glycerol-based medium containing *p*-phenylenediamine and observed in a Zeiss microscope. In several cases, paired immunofluorescence was employed to localize $CD3^+$ infiltrating cells and delineate muscle fibers; for the latter a polyclonal anti-dystrophin D8 antibody against the rod-domain of dystrophin was used (15).

Immunohistochemical analysis for TGF-β1 was performed on muscle biopsies from 13 IM patients (8 PM and 5 DM) and 6 controls; 2 normal skin biopsies (kindly provided by Prof. E. Berti) were also included in the analysis. TGF-β1 was detected by immunofluorescence with a mouse mAb to human TGF-β1 (Serotec Ltd, Oxford, UK) used at a dilution of 1:100 on unfixed cryosections according to the protocol described elsewhere (11). Serial sections stained with anti-TGF-β1, hematoxylin-eosin, anti-CD3 and anti-macrophage antibodies were obtained to correlate TGF-β1 location with tissue histology and mononuclear cell infiltrates. The possible expression of TGF-β1 on muscle fiber sarcolemma was analyzed by means of a paired immunofluorescence using anti-dystrophin D8 antibody. As control the primary antibody was omitted or substituted with a nonimmune mouse isotype-specific $IgG_{1\kappa}$ (Sigma, St. Louis, MO) on adjacent sections.

Statistical Analysis

Data are presented as mean ± standard deviation (SD) and compared using the ungrouped two-tailed Student *t*-test.

RESULTS

Quantification of TGF-β1 Transcripts by Q-PCR

All samples stained positively and isointensely for β-actin transcripts. The results of the analyses are presented in Figure 1. In 10 DM patients, values ranged from

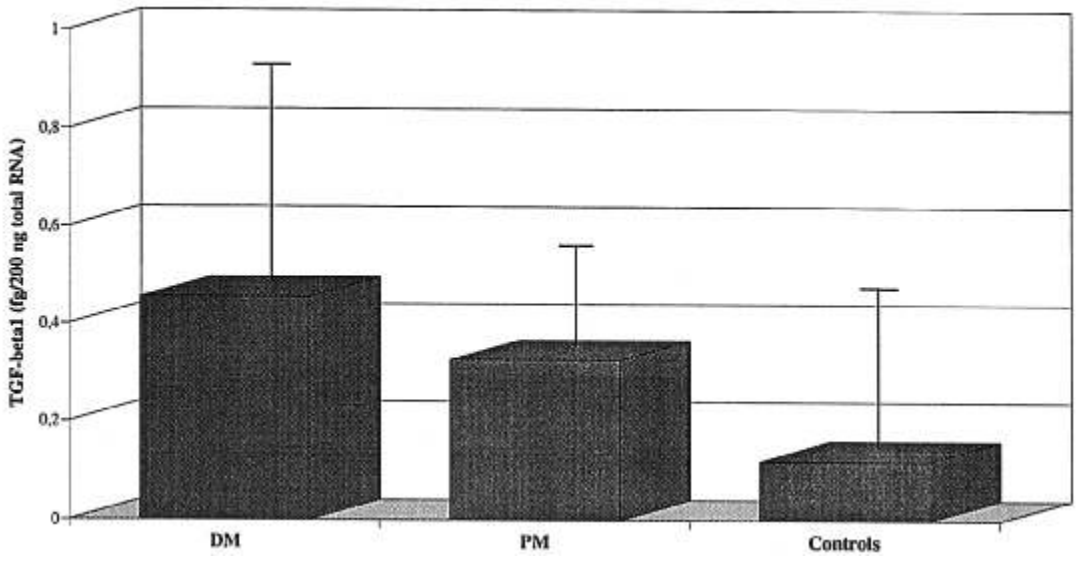

Fig. 1. Quantitation of TGF-β1 transcript in muscles of DM and PM patients. TGF-β1 was measured by the Q-PCR technique. Statistical analysis was by two-tailed Student *t*-test: DM TGF-β1 mRNA level vs controls P = 0.0363; PM TGF-β1 mRNA level vs controls P = 0.1022 and vs DM P = 0.4874.

0.091 to 1.516 fg/200 ng total RNA with a mean ± SD of 0.455 ± 0.449, which differed significantly (P = 0.0363) from controls (0.116 ± 0.331); in one DM patient no muscle was available for Q-PCR. In 8 PM muscles the level of TGF-β1 transcript ranged from 0.106 to 0.653 fg/200 ng RNA (0.33 ± 0.19), which did not differ significantly from control (P = 0.1022) and from DM (P = 0.4874).

Histopathological Features

In PM muscles, immunostaining for muscle-infiltrating mononuclear cells revealed their presence mainly in the endomysium, with T lymphocytes prevalent, and $CD8^+$ more frequently than $CD4^+$. Typically these cells surrounded and invaded muscle fibers and were sometimes grouped in large infiltrates. Macrophages were occasionally present in the extracellular matrix but were mainly found within muscle fibers, apparently engaged in phagocytosis. In DM variable quantities of T cells, B cells and macrophages were found.

In Table 1 the extent of mononuclear cell infiltration (inflammation) is reported; quantitative evaluation of connective tissue proliferation revealed that 3 out of 5 DM patients (60%) showed a percentage of fibrosis higher than 20% of the total area, while in PM only 2 out of 8 patients (25%) showed a similar increase, and no patients had a degree of fibrosis higher than 30%. Regarding the disease duration before muscle biopsy, DM patients had a longer disease duration than PM patients: 16.8 ± 12.1 months vs 8.75 ± 7.5 (Table 1); however, the statistical analysis did not show any significance (P = 0.1628).

Immunolocalization of TGF-β1

In all PM and DM patients, TGF-β1-positive immunostaining was observed in thickened and fibrotic connective tissue ramifications, and appeared to correspond to the degree of fibrosis (Table 1).

In DM patients TGF-β1 positivity was particularly marked at the perimysial level, corresponding with increased fibrous tissue (Fig. 2a, b) and often in relation to perifascicular atrophy of muscle fibers, when it appeared to infiltrate the muscle fascicles and surround atrophic fibers (Fig. 2c, d).

In PM patients, areas of TGF-β1-positive connective tissue proliferation were observed at the perimysial level but were more commonly observed in circumscribed areas of endomysial connective tissue (Fig. 2e, f). Normal control muscles were never positive for TGF-β1 (data not shown). Mononuclear infiltrating cells (lymphocytes and macrophages) did not show TGF-β1 positivity in any PM or DM patient. This was so when isolated lymphocytes were invading (Fig. 2e, f) the muscle fibers and when they were grouped in large infiltrates (Fig. 3). Muscle fibers, either of normal appearance, degenerating or necrotic, did not usually show TGF-β1 positivity either at the sarcolemmal or sarcoplasmic levels; occasionally faint sarcolemmal positivity was evident in fibers close to areas of connective tissue; however, paired TGF-β1 immunolocalization with dystrophin demonstrated that TGF-β1-positivity surrounds and does not involve muscle fiber sarcolemma (data not shown). A positive staining for TGF-β1 was also found in connective tissue of human dermis.

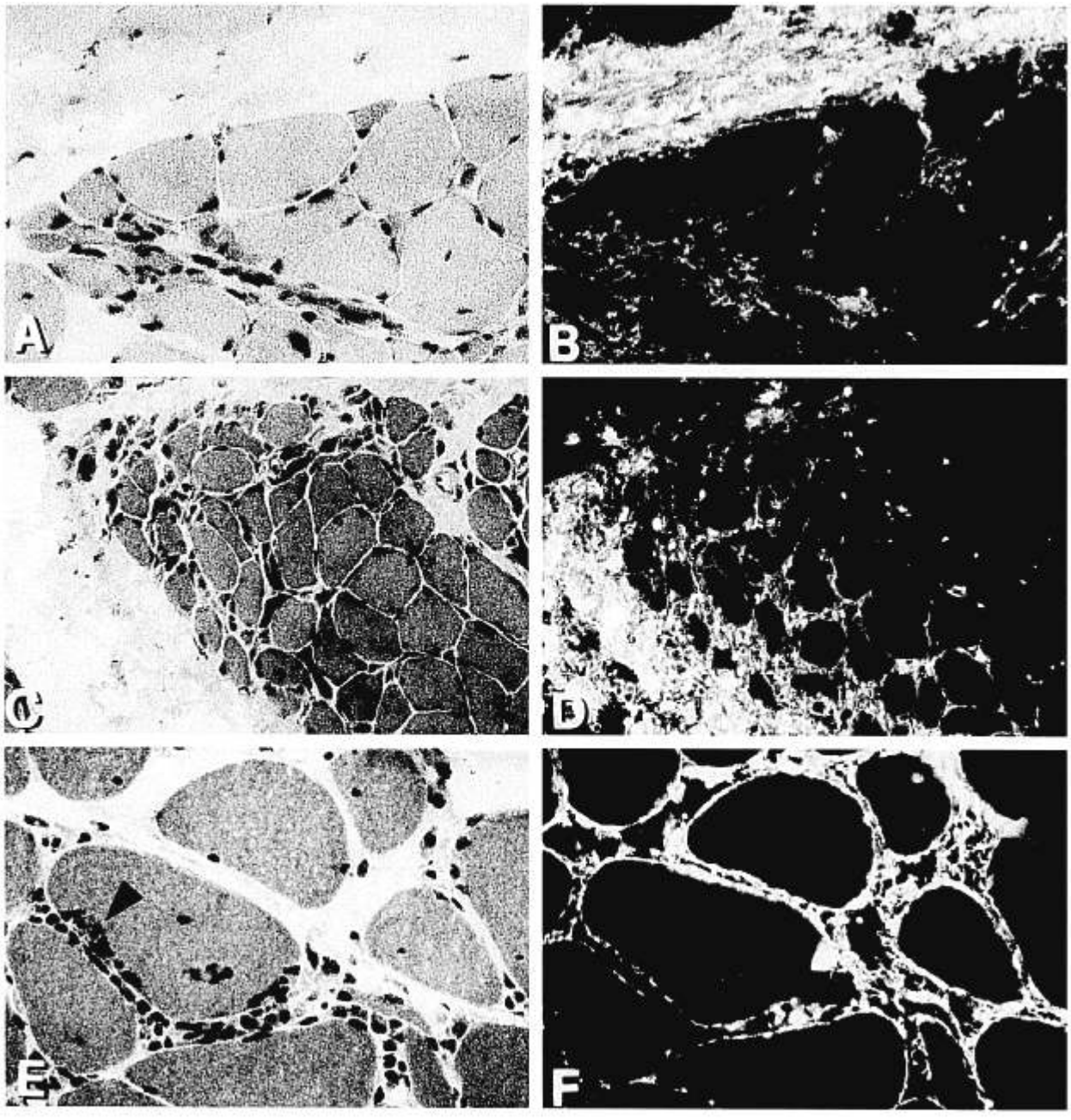

Fig. 2. Hematoxylin-eosin staining (A, C, E) and TGF-β1 (B, D, F) immunolocalization in muscles of 2 DM (A–D) and 1 PM (E, F) patients. TGF-β1 positivity is mainly located perifascicularly in correspondence to fibrous tissue, sometimes diffusing among atrophic fibers (B, D). In (F) TGF-β1 is located in proliferating endomysial connective tissue, and not in or with infiltrating mononuclear cells. Arrow indicates mononuclear cells infiltrating a non-necrotic muscle fiber. (A, B, E, F: ×400; C, D: ×250).

DISCUSSION

Our study on the presence of TGF-β1 in PM/DM muscles confirms the findings of Lundberg et al (7), who reported a strong expression of TGF-β1 mRNA in IM and dystrophic muscles. We have further expanded the analysis by a quantitative evaluation, identifying a significant difference in TGF-β1 levels only between DM and controls ($P = 0.0363$). This difference may be related either to the particular pathogenetic mechanism of DM or to the different degree of fibrosis, and, to a certain degree, to the longer disease duration before muscle biopsy of DM with respect to PM (see Table 1); however, it is relevant to note that the absolute TGF-β1 Q-PCR values of DM were lower than those detected in the highly fibrotic DMD muscles: 0.455 ± 0.449 vs 16.7 ± 25.4 fg/200 ng total RNA (11), respectively. Immunocytochemical analysis of TGF-β1 protein in both PM and DM muscles revealed a strong relation between this cytokine

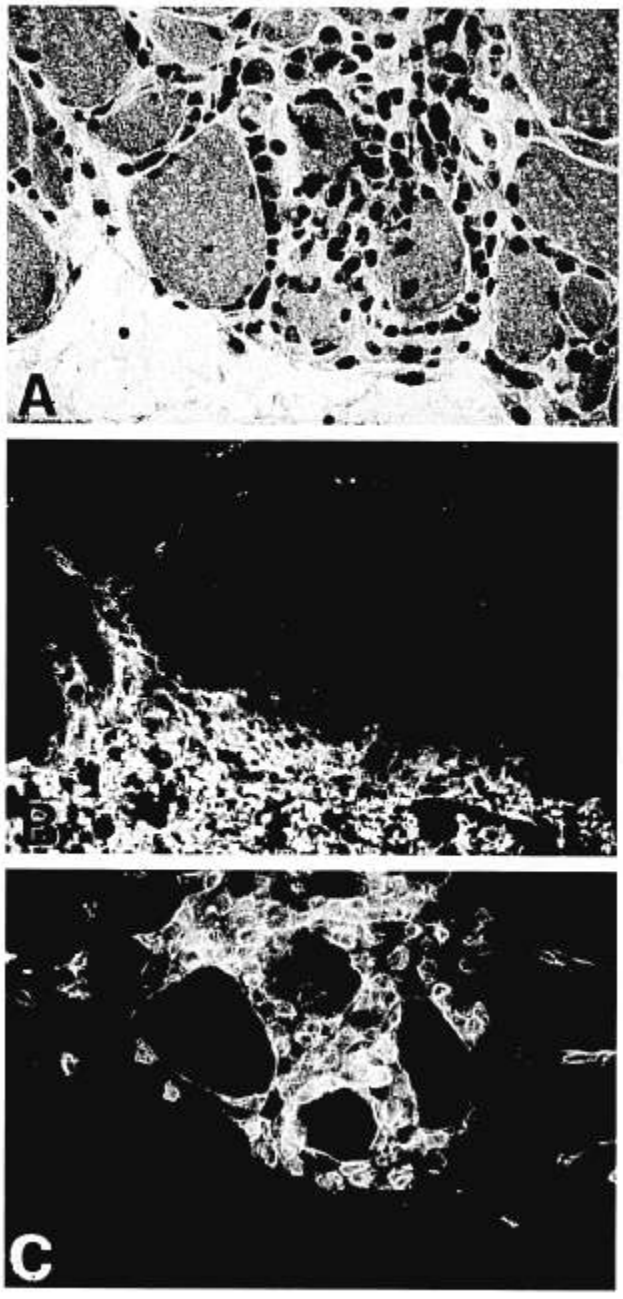

Fig. 3. Hematoxylin-eosin staining (A), TGF-β1 (B) immunolocalization and $CD3^+$ cells and dystrophin (C) paired immunolocalization in PM muscle. The distinct location of infiltrating cells and TGF-β1 positivity is clearly evident. ×400.

and the presence of connective tissue: in DM patients TGF-β1 was mostly found in the connective tissue surrounding the areas of perifascicular atrophy and also showed a tendency to infiltrate the endomysial space, while in PM patients TGF-β1 positivity was also localized in circumscribed areas of increased endomysial connective tissue. These data resemble those obtained analyzing the fibrotic process in DMD muscles: TGF-β1 increased progressively with the increase of connective tissue (11). The different distribution of TGF-β1 in DM and PM may reflect the different pathological features of their muscles and may resemble that described for the expression of IL-4 and IFN-γ in IM by Tews et al (8), who observed a disease-specific distribution of cytokine-secreting cells in the areas of inflammation in the muscle. The extracellular matrix may store high quantities of TGF-β1 bound to proteoglycans (decorin) complexed to collagen (16); collagen I and III were found increased in the fibrotic areas of DMD and PM/DM muscles (14). In IM the finding of TGF-β1 in the extracellular matrix probably reflects its activation at particular sites. An immuno-mediated angiopathy inducing an ischemic muscular damage is the main pathological alteration of DM; it has been reported that tissue hypoxia may promote fibroblast activity and collagen production and upregulate synthesis of TGF-β1 in brain, myocardium and kidney (17–19). Similarly, we like to speculate that the increase of TGF-β1 mRNA and the high TGF-β1 perifascicular positivity in our DM patients may be linked to the ischemic muscle damage.

Muscle fibers, as a rule, did not show any sarcoplasmic or sarcolemmal staining for TGF-β1; the occasional faint sarcolemmal positivity for TGF-β1 was not confirmed by double immunostaining with anti-TGF-β1 and anti-dystrophin antibodies and was found only in close proximity to TGF-β1-positive connective tissue; confocal analysis will help discriminate the possible overlapping signal.

The active form of TGF-β1 was not detected within or in the proximity of mononuclear cell infiltrates; this suggests that a transient release of cytokines may occur only at the beginning of the inflammatory process, as also hypothesized by others (20). A similar TGF-β1 distribution was observed in multiple sclerosis, a central nervous system demyelinating disease characterized by foci of inflammation: TGF-β1 was mainly associated with the extracellular matrix around blood vessels, and not in relation to cellular infiltrates (21). Furthermore, it should be emphasized that $TGF\text{-}\beta1^{-/-}$ knock-out mice develop an inflammatory cell infiltration in striated muscles that resembles to a variable degree that seen in PM (22).

Our findings point to a possible active role of TGF-β1 in the fibrotic process in the course of PM/DM and once more underline the need for further studies to elucidate its immunosuppressive or proinflammatory activity.

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REFERENCES

1. Engel AG, Hohlfeld R, Banker BQ. Polymyositis and dermatomyositis syndromes. In: Engel AG, Franzini-Armstrong C, eds. Myology. New York; McGraw-Hill, 1994:1335–83
2. Dalakas MC. Immunopathogenesis of inflammatory myopathies. Ann Neurol 1995;37(S1):S74–S86
3. Hohlfeld R, Engel AG. The immunobiology of muscle. Immunol Today 1994;15:269–74
4. Dalakas MC. Current treatment of the inflammatory myopathies. Curr Opin Rheumatol 1994;6:595–601
5. Orimo S, Koga R, Goto K, et al. Immunohistochemical analysis of perforin and granzyme A in inflammatory myopathies. Neuromusc Disord 1994;4:219–26
6. Andreetta F, Bernasconi P, Torchiana E, Baggi F, Cornelio F, Mantegazza R. T-cell infiltration in polymyositis is characterized by coexpression of cytotoxic and T-cell-activating cytokine transcripts. Ann N Y Acad Sci 1995;756:418–20
7. Lundberg I, Brengman JM, Engel AG. Analysis of cytokine expression in muscle in inflammatory myopathies, Duchenne dystrophy, and non-weak controls. J Neuroimmunol 1995;63:9–16
8. Tews DS, Goebel HH. Cytokine expression profile in idiopathic inflammatory myopathies. J Neuropathol Exp Neurol 1996;55:342–47
9. Engel AG. Quantitative morphological studies of muscle. In: Engel AG, Franzini-Armstrong C, eds. Myology. New York; McGraw-Hill, 1994:1018–45
10. Mantegazza R, Andreetta F, Bernasconi P, et al. Analysis of T cell receptor repertoire of muscle-infiltrating T lymphocytes in polymyositis. J Clin Invest 1993;91:2880–86
11. Bernasconi P, Torchiana E, Confalonieri P, et al. Expression of transforming growth factor-β1 in dystrophic patient muscles correlates with fibrosis. J Clin Invest 1995;96:1137–44
12. Wahl SM. Transforming growth factor β: The good, the bad, and the ugly. J Exp Med 1994;180:1587–90
13. Wahl SM. Transforming growth factor beta (TGF-β) in inflammation: A cause and a cure. J Clin Immunol 1992;12:61–74
14. Foidart M, Foidart JM, Engel WK. Collagen localization in normal and fibrotic human skeletal muscle. Arch Neurol 1981;38:152–57
15. Morandi L, Mora M, Bernasconi P, et al. Very small dystrophin molecule in a family with a mild form of Becker dystrophy. Neuromusc Disord 1993;3:65-70
16. Ruoslahti E, Yamaguchi Y. Proteoglycans as modulators of growth factors activities. Cell 1991;64:867–69
17. Lehrmann E, Kiefer R, Finsen B, Diemer NH, Zimmer J, Hartung HP. Cytokines in cerebral ischemia: Expression of transforming growth factor beta-1 (TGF-β1) mRNA in the postischemic adult rat hippocampus. Exp Neurol 1995;131:114–23
18. Herskowitz A, Choi S, Ansari AA, Wesselingh S. Cytokine mRNA expression in postischemic/reperfused myocardium. Am J Pathol 1995;146:419–28
19. Goes N, Urmson J, Ramassar V, Halloran PF. Ischemic acute tubular necrosis induces an extensive local cytokine response. Evidence for induction of interferon-gamma, transforming growth factor beta-1, granulocyte-macrophage colony-stimulating factor, interleukin-2, and interleukin-10. Transplantation 1995;59:565–72
20. Dalakas MC, Sivakumar K. The immunopathologic and inflammatory differences between dermatomyositis, polymyositis and sporadic inclusion body myositis. Curr Opin Neurol 1996;9:235–39
21. Cannella B, Raine CS. The adhesion molecule and cytokine profile of multiple sclerosis lesions. Ann Neurol 1995;37:424–35
22. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor β-1 gene results in multifocal inflammatory disease. Nature 1992;359:693–99

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