Expression levels of TGF-β1 and CTGF are associated with the severity of Duchenne muscular dystrophy

YANMIN SONG[1,2], SHUAI YAO[2,3], YUNHAI LIU[2], LILI LONG[2], HUAN YANG[2], QIUXIANG LI[2], JINGHUI LIANG[2], XINXIN LI[2], YULING LU[2], HAORAN ZHU[2] and NING ZHANG[2]

Departments of [1]Emergency and [2]Neurology, Xiangya Hospital, Central South University, Changsha, Hunan 410008; [3]Department of Rehabilitation Medicine, Mental Health Centre of Wuxi, Wuxi, Jiangsu 214151, P.R. China

Abstract. The present study aimed to analyze the association of transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) expression levels in skeletal muscle with the clinical manifestation of Duchenne muscular dystrophy (DMD). A total of 18 cases of DMD, which were confirmed by routine pathological diagnosis were recruited into the present study, along with 8 subjects who suffered from acute trauma but did not present any neuromuscular diseases and were enrolled as the healthy controls. Immunohistochemical staining was used to detect the expression levels of CTGF and TGF-β1 in muscle biopsy specimens. Furthermore, Spearman rank correlation analysis was conducted among the expression levels of CTGF and TGF-β1, age, clinical severity and pathological severity in DMD patients. The immunohistochemical staining results revealed that the expression levels of CTGF and TGF-β1 were significantly increased in the DMD group compared with those in the control group (P<0.05). These levels were not found to be significantly correlated with the onset age (P>0.05), but there was a significant correlation with the degree of pathology and clinical severity (P<0.05). In conclusion, an upregulated expression of CTGF and TGF-β1 was revealed in the skeletal muscle of DMD patients, which were in positive correlation with the degree of pathology and clinical severity. These two factors may be involved in the pathophysiology of fibrosis in DMD.

Introduction

Duchenne muscular dystrophy (DMD), an X-linked recessive disorder, is the most common muscular dystrophy that affects approximately 1 in 3,500 newborn boys (1). Mutations of the dystrophin gene cause an expression deficiency of dystrophin protein, and thus result in muscle degeneration, necrosis and atrophy (2,3). Furthermore, other mechanisms have been shown to serve key roles in the process of muscle atrophy. Recently, an increasing number of studies have focused on another prominent pathological feature of DMD, the fibrosis of connective tissue, which was considered to be compensatory for muscle cell loss (4-6).

Transforming growth factor-β (TGF-β) is a multifunctional polypeptide factor that promotes tissue fibrosis, cell growth and transformation. It is widespread in normal tissues, particularly in the skeleton and platelets (7-9). In humans, there are three subtypes of TGF-β, with TGF-β1 being the most abundant subtype (10). Connective tissue growth factor (CTGF) is also widespread in endothelial, smooth muscle, fibroblast, cartilage and specific tumor cells (11-13). It was revealed that TGF-β1 can significantly increase the expression level of CTGF in human foreskin fibroblasts, whereas CTGF can be important in the proliferation of fibroblasts, chemotaxis, extracellular matrix (ECM) production, vascular regeneration or other biological activities (14-16). Previous studies have observed overexpression of CTGF and TGF-β1 in patients with DMD (17-19). Furthermore, the levels of TGF-β and CTGF were revealed to correlate with fibrosis development in the skeletal muscle of DMD patients or of X chromosome-linked muscular dystrophy (mdx) mouse models of DMD (20,21). Inhibition of TGF-β1 or reduction of CTGF expression levels can also reduce the fibrotic phenotype in the mdx mouse model (22,23). Therefore, the present study aimed to examine the association of the expression of CTGF and TGF-β1 with the clinical severity of DMD in Chinese patients.

Subjects and methods

Subjects. Consecutive DMD patients admitted to the clinic of the Department of Neurology at Xiangya Hospital (Central South University, Changsha, China) between January 2013 and March 2014 were enrolled into the present study. A total of 35 suspected cases were enrolled, of which 18 cases were confirmed to be DMD through muscle biopsy and immunohistochemical methods. For pathological diagnosis, immunohistochemical staining with monoclonal

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Correspondence to: Dr Ning Zhang, Department of Neurology, Xiangya Hospital, Central South University, 87 Xiangya Road, Changsha, Hunan 410008, P.R. China
E-mail: zhangning_xy@163.com

anti-dystrophin antibody (1:10; Novocastra Laboratories Ltd., Newcastle, UK) was performed to establish whether dystrophin protein expression in the muscle fiber membrane was severely or completely absent (24).

In addition, 8 children who suffered from acute trauma but did not present any neuromuscular diseases were recruited from the Department of Orthopaedic Surgery as controls. All controls were identified as healthy subsequent to routine enzyme histochemical staining, as well as dystrophin protein immunohistochemical examination. The present study was approved by the Ethics Committee of Xiangya Hospital, and written informed consent was obtained from the patients. All the muscle samples were obtained from muscle biopsies and embedded in paraffin until further use.

Clinical data. The medical history and physical examination details of the DMD patients were recorded by two neurologists. The following details were obtained: Onset age, symptoms, course of disease, degree of muscle weakness and atrophy, pseudohypertrophy signs, tendon reflexes, Gowers' sign, gait and family history.

ATPase and immunohistochemical staining. ATPase (25,26) and immunohistochemical (27-29) staining were conducted according to previously described methods, using 8-μm sections from each sample. The primary antibodies used were monoclonal anti-dystrophin (Novocastra; Leica Biosystems, Wetzlar, Germany), polyclonal anti-CTGF and polyclonal anti-TGF-β1 (both from Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China). The secondary antibody used was the horseradish peroxidase-labeled goat anti-rabbit IgG antibody (P0448; Dako North America, Inc., Carpinteria, CA, USA). An immunohistochemical kit (Beijing Biosynthesis Biotechnology Co., Ltd.) and Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) were used to quantitatively measure the integrated optical density (IOD) value. The expression levels of CTGF and TGF-β1 in skeletal muscle samples were quantitatively determined by an streptavidin-peroxidase immunohistochemical method. Briefly, three different visual fields were randomly selected under x100 magnification light microscopy (30).

Assessment of the severity of DMD was conducted with ATPase staining to observe muscle fiber pathology, according to previously reported criteria (31,32). Briefly, in each pathological section of the ATPase staining, a semi-quantitative method was used to calculate the percentage of opaque muscle fibers and degeneration (32) The samples were classified according to the percentage of muscle fibers presenting necrosis, which indicates the pathological severity of DMD, as follows: <10% necrosis was classified as level 0, 10-40% as level 1, 40-70% as level 2 and >70% as level 3 (32)

The degree of dystrophin protein expression loss was determined compared with that in the control samples, using immunohistochemical staining. The results were divided into the following levels (32): Grade 0, no expression; grade 1, low protein expression (<10%); grade 2, small number of dystrophin-positive fibers (10-30%); grade 3, diffuse expression (30-70%); grade 4, positive muscle fibers with partial deletion, or mosaic distribution with dystrophin-positive or
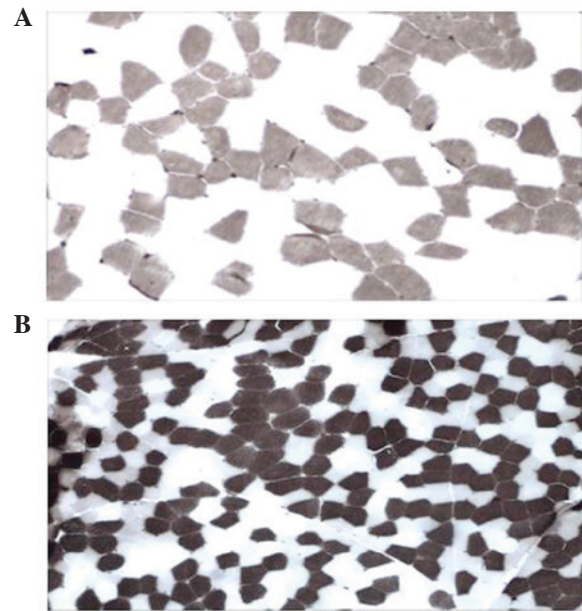

Figure 1. ATPase staining (magnification, x100; pH 4.2). (A) Duchenne muscular dystrophy and (B) control groups.

-negative muscle fibers (70-99%); grade 5, normal expression (100%); and grade 5+, enhanced expression (>100%).

Statistical analysis. SPSS version 19.0 software (IBM SPSS, Armonk, NY, USA) was used for data analysis. IOD values between the groups were compared with the Wilcoxon test. P<0.05 was used to indicate a statistically significant difference. The association among CTGF expression, TGF-β1 expression, pathological grading of severity and grading of clinical severity was analysed by the Spearman rank correlation analysis.

Results

Clinical data of DMD patients. All 18 cases of DMD patients were male, with an age distribution between 3 and 13 years and a mean age of 6.88±2.33 years. There was an onset age distribution between 0 months and 6 years and a mean onset age of 2.24±1.58 years. Furthermore, the course of disease distribution was between 2 and 7 years, and the average course was 4.63±1.65 years. Amongst all the patients, there were 3 children with a DMD family history (16.7%), 15 sporadic cases (83.3%) and parents of 2 cases with a consanguineous marriage history (11.1%). DMD patients demonstrated motor delay (50.0%), floppy infant symptoms (11.1%), difficulty in standing up (5.6%), frequent falling down (16.7%) and an abnormal gait (16.7%; Table I).

ATPase staining. The results of ATPase staining displayed two types of muscle fibers, type I and II, which were visible with a mosaic distribution in the DMD group (Fig. 1A) and the control (Fig. 1B) groups. The fiber type grouping phenomenon in muscular fiber type I or II was not identified. According to the aforementioned classification, there were 2 cases with level 0, 5 cases with level 1, 6 cases with level 2 and 5 cases with level 3 in the 18 DMD patients.

Table I. Clinical data of DMD patients.

Patient	Age (years)	Onset (years)	Course (years)	Family history	First symptom	Muscular force	Amyotrophy	Waddling gait	EMG
1	3	0.7	2.3	Y	Floppy infant	4/4	N	Y	-
2	7.7	1.3	6.4	N	Motor delay	4/4-	Y	Y	M
3	5.6	1.4	4.2	N	Difficulty in standing up	4/4-	Y	Y	M
4	6	1	5	N	Frequent falling down	4/4	Y	Y	M
5	7	3	4	N	Motor delay	4/3	Y	Y	M
6	7.6	4	3.6	N	Abnormal gait	4/3	Y	Y	M
7	8	3	5	N	Frequent falling down	4-/4-	Y	Y	M
8	5	2	3	N	Motor delay	5/4	Y	Y	M
9	9	2	7	N	Frequent falling down	4/4	Y	Y	M
10	6	3	3	N	Abnormal gait	4/4-	Y	Y	M
11	10	4	6	N	Motor delay	4/4	Y	Y	M
12	6	2	4	Y	Motor delay	4/4-	Y	Y	M
13	3	0	3	Y	Floppy infant	5/5	N	Y	-
14	7	2	5	N	Motor delay	4/4	Y	Y	M
15	7	1	6	N	Abnormal gait	4/4-	Y	Y	M
16	13	6	7	N	Motor delay	4/4	Y	Y	M
17	6	4	2	N	Motor delay	4/4-	Y	Y	M
18	7	0	7	N	Motor delay	3/3+	Y	Y	M

DMD, Duchenne muscular dystrophy; Y, yes; N, no; EMG, electromyogram; M, myogenic damage.
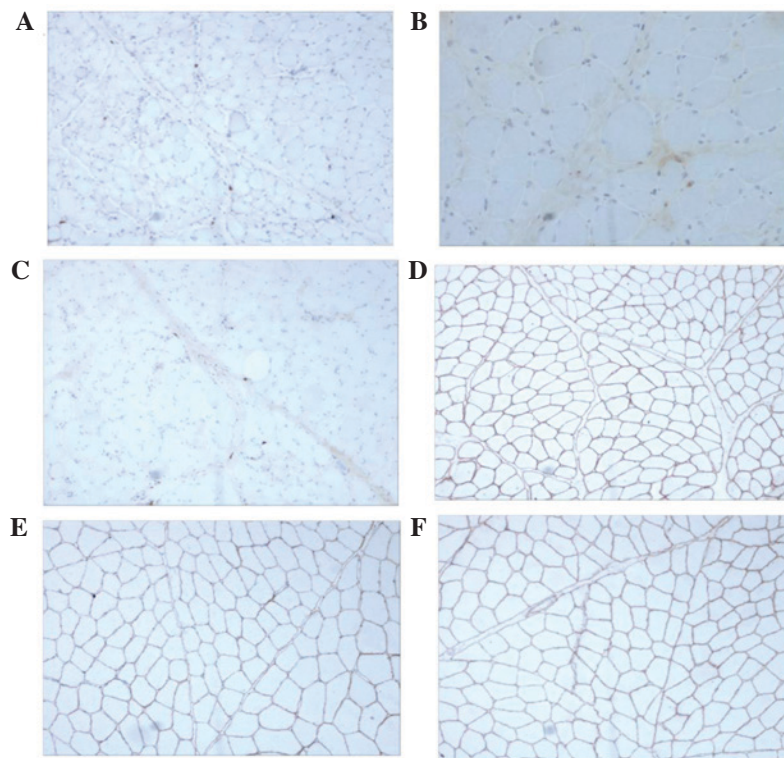

Figure 2. Immunohistochemical staining of dystrophin (magnification, x100). (A) Anti-dystrophin-N, (B) anti-dystrophin-C and (C) anti-dystrophin-R of the Duchenne muscular dystrophy group; (D) anti-dystrophin-N, (E) anti-dystrophin-C and (F) anti-dystrophin-R of the control group.

Dystrophin immunohistochemical staining. For all 18 patients with DMD, the expression of three antibodies against the Rod domain (DYS-1), C terminus (DYS-2), and N terminus (DYS-3) of dystrophin (Fig. 2A-C, respectively) in the muscle fiber membrane was severely lost or almost no expression was observed (grades 0-2, including 13 cases with grade 0

Table II. Expression levels of CTGF and TGF-β1 between groups.

Parameter	DMD (n=18)	Control (n=8)	P-value
CTGF	35,596.80±21,653.86	312.71±243.72	0.0001[a]
TGF-β1	40,110.80±22,410.68	319.98±289.40	<0.0001[a]

[a]P<0.05. CTGF, connective tissue growth factor; TGF-β1, transforming growth factor-β1; DMD, Duchenne muscular dystrophy.

Table III. Spearman rank correlation analysis.

	CTGF		TGF-β1	
Characteristic	Spearman correlation coefficient	P-value	Spearman correlation coefficient	P-value
Age	-0.089	0.589	-0.114	0.378
Degree of pathological severity	0.767	0.001[a]	0.465	0.018[a]
Degree of clinical severity	0.622	0.004[a]	0.487	0.022[a]

[a]P<0.05. CTGF, connective tissue growth factor; TGF-β1, transforming growth factor-β1.

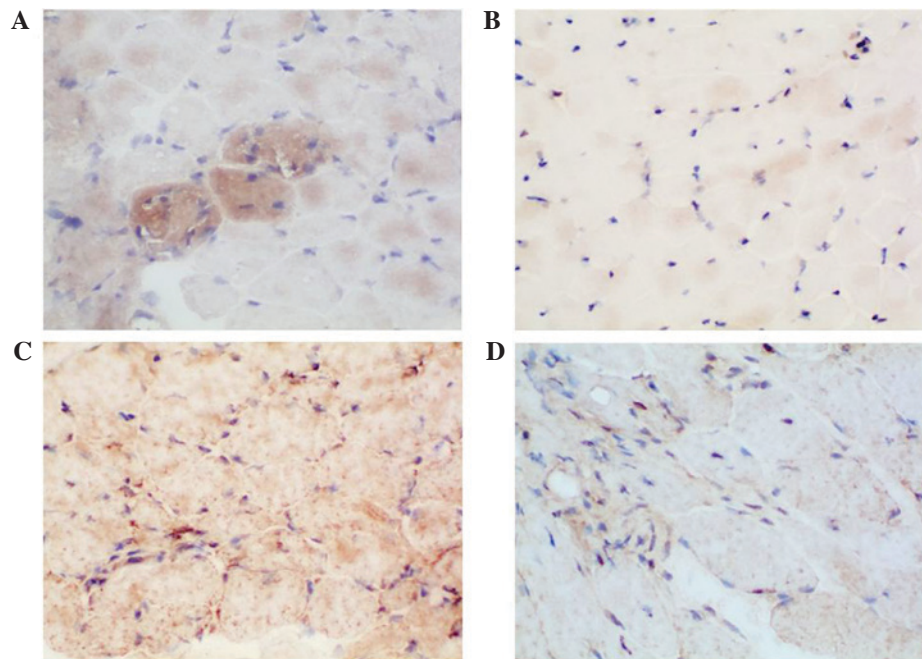

Figure 3. Immunohistochemical staining of CTGF and TGF-β1 (magnification, x100). (A) CTGF of the DMD group; (B) CTGF of the control group; (C) TGF-β1 of the DMD group and (D) TGF-β1 of the control group. CTGF, connective tissue growth factor; DMD, Duchenne muscular dystrophy; TGF-β1, transforming growth factor-β1.

and 5 cases with grade 1-2). By contrast, the control group presented a normal expression (all 8 cases were classified as grade 5), with a consistent and uniformly brown muscle fiber membrane (Fig. 2D-F).

CTGF and TGF-β1 immunohistochemical staining. As shown in Fig. 3, the results of CTGF and TGF-β1 immunohistochemical staining in the 18 DMD patients indicated upregulated expression of CTGF and TGF-β1 in the muscle cell plasma

and myenteric interstitium, with yellowish brown staining of the cells observed, when compared with the expression in the normal control group (Fig. 3B and D). The IOD values of those two proteins demonstrated a statistically significant difference between the two groups (P<0.05; Table II).

Correlation analysis of CTGF and TGF-β1 expression levels with clinical manifestation. Spearman rank correlation analysis was conducted to investigate the association of CTGF

and TGF-β1 expression levels with the age, clinical severity and pathological severity in DMD patients. The CTGF and TGF-β1 expression levels were significantly correlated with the degree of clinical and pathological severity (P<0.05); however, no significant correlation of the CTGF and TGF-β1 expression levels with the age of the patients was observed (P>0.05; Table III).

Discussion

The results of the present study demonstrated that an upregulated expression of TGF-β1 and CTGF was observed in the cytosolic and myenteric interstitia of the DMD skeletal muscle. There were significant differences in the expression of those two proteins between the DMD and control groups. The expression levels of CTGF and TGF-β1 were positively correlated with the pathological and clinical severity, which can provide important information for the evaluation of DMD severity. Furthermore, CTGF and TGF-β1 were involved in the fibrosis process, which is known to be one of key pathogenesis factors in DMD.

Nevertheless, further research is required on the involvement of TGF-β1 and CTGF in skeletal muscle fibrosis. *In vitro* experiments confirmed that TGF-β1 can downregulate myogenic protein to induce fibrosis-associated proteins (33). In addition, it has been shown that TGF-β1 can promote a skeletal muscle fiber cascade and induce differentiation from myogenic to fiber cells *in vivo* (33). In the fibrosis process of the skin, liver or kidney, the upregulated expression of CTGF can directly promote cell matrix adhesion, ECM deposition, and synthesis of collagen I and III, integrin-β1 and fibronectin (34-36). Furthermore, the inflammatory response is capable of activating macrophages or fibroblasts in order to increase TGF-β1 secretion, which then activates CTGF (37). TGF-β1 and CTGF are considered to participate in the promotion of ECM synthesis and fibroblast chemotaxis through the following signaling pathways: Smad, cyclic adenosine monophosphate-protein kinase A, mitogen-activated protein kinases and c-Jun N-terminal kinase-dependent signaling (38-40). In the mdx animal models, overexpressed CTGF can directly result in a muscular dystrophy phenotype, whereas anti-CTGF may reverse the muscular dystrophy phenotype and improve the effect of cell therapy (23). It has been hypothesized that myenteric interstitial fibrosis causes shortage of blood supply by surrounding muscle cells, which then inhibits the regeneration of muscle satellite cells (20).

CTGF and TGF-β1 are important in the fibrosis process of DMD, and thus may assist in the development of novel treatments. The results of the present study indicated that, as common pathogenic factors, CTGF and TGF-β1 are important in the fibrosis processes of DMD. TGF-β1 receptors are distributed widely. In animal models, blocking of TGF-β1 can inhibit CTGF release, as well as inhibit the carcinogenic and pro-inflammatory response and other side effects (41). In addition, CTGF is mainly confined to the connective tissue to take function, with low expression observed in normal physiological conditions; thus, blocking CTGF expression in order to antagonize fibrosis may be safe (6). Therefore, the TGF-β1/CTGF signaling pathway may also be promising in the search for therapeutic agents against DMD.

In conclusion, in the present study, the upregulated expression of CTGF and TGF-β1 was identified in the skeletal muscle of DMD patients, which were in positive correlation with the degree of pathology and clinical severity. Finally, these two factors may be involved in the pathophysiology of fibrosis in DMD patients.

Acknowledgements

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