Roles of TGF-β/Smad signaling pathway in pathogenesis and development of gluteal muscle contracture

Xintao Zhang1*, Yukun Ma2*, Tian You1, Xiaopeng Tian3, Honglei Zhang1, Qi Zhu4, and Wentao Zhang1

1Department of Sports Medicine and Rehabilitation, Peking University Shen Zhen Hospital, ShenZhen, China, 2Department of Pediatric Surgery, Linyi People’s Hospital, Shandong Province, China, 3State Key Laboratory of Oncology in South China; Collaborative Innovation Center of Cancer Medicine, Sun Yat-sen University, Guangzhou, China, and 4Orthopaedics Hong Kong University, Shen Zhen Hospital, ShenZhen, China

Abstract

Purpose of the study: Gluteal muscle contracture (GMC) is a chronic fibrotic disease of gluteal muscles which is characterized by excessive deposition of collagen in the extracellular matrix. Transforming growth factor (TGF)-βs have been shown to play an important role in the progression of GMC. However, the underlying mechanisms are not entirely clear. We sought to explore the expression of TGF-β/Smad pathway proteins and their downstream targets in gluteal muscle contracture disease.

Materials and methods: The expression levels of collagens type I/III, TGF-β1, Smad2/3/4/7 and PAI-1 (plasminogen activator inhibitor type 1) in gluteal muscle contraction (GMC) patients were measured using immunohistochemistry, reverse transcription and polymerase chain reaction (RT-PCR) and western blot assays.

Results: The expressions of collagens type I/III and TGF-β1 were significantly increased in the contraction band compared with unaffected muscle. In addition, R-Smad phosphorylation and Smad4 protein expression in the contraction band were also elevated, while the expression of Smad7 was significantly decreased in the fibrotic muscle of the GMC patients compared to the unaffected adjacent muscle. The protein and mRNA levels of PAI-1 were also remarkably increased in the contraction band compared with adjacent muscle. Immunohistochemical analysis also demonstrated that the expression levels of TGF-β1 and PAI-1 were higher in contraction band than those in the adjacent muscle.

Conclusion: Our data confirm the stimulating effects of the TGF-β/Smad pathway in gluteal muscle contracture disease and reveal the internal changes of TGF-β/Smad pathway proteins and their corresponding targets in gluteal muscle contracture patients.

Keywords

Gluteal muscle contracture, PAI-1, Smad2/3, Smad7, TGF-β/Smad pathway

Introduction

Gluteal muscle contracture (GMC) is now recognized as a common clinical syndrome with multiple etiologies, characterized by fibrosis and contracture of the gluteal muscles and their tendons (1). The primary symptom of GMC is the difficulty in adducting the hip or squatting, which ultimately progresses to serious functional disorder of the hip (2). It is thought that repeated intramuscular injection into the gluteal region during childhood is a major cause for GMC (3). GMC is more widely reported in China than in the US and Europe, which is likely attributable to frequent use of benzyl alcohol as a diluent for intramuscular injections of penicillin in some regions of China (4). Previous studies have demonstrated that the extracellular matrix (ECM) deposition, which consists mainly of type I collagen, is increased during the development of fibrosis in GMC patients (5). Other studies have also confirmed that collagen type I and III are major components of the contraction band and are markedly elevated compared to the unaffected adjacent muscle (6).

It is known that GMC is a complicated pathological process involving various cytokines and multiple cell signaling pathways. Transforming growth factor (TGF)-βs are members of a superfamily of polypeptides that play an important role in the pathogenesis of many fibrotic diseases (7,8). One subtype, TGF-β1, is critical for tissue fibrosis due to its role as a potent fibrogenic cytokine involved in every step of the process by stimulating fibroblasts (9). TGF-β1 promotes excess ECM generation through the TGF-β/Smad signaling pathway, which leads to increases in the synthesis of matrix proteins and decreases in the production of matrix-degrading proteases (10). It is reported that the increased expression of TGF-β1 is associated with deposition of

*These authors contributed equally to this work

Correspondence: Wentao Zhang, Department of Sports Medicine and Rehabilitation, Peking University Shenzhen Hospital, No. 1120, Lianhua, 518036 ShenZhen, China. Tel: 075583923333-6135(6137). E-mail: zhangwtshenzhen@163.com
collagen types I and III in the fibrotic muscle of GMC patients. In addition, other forms of (TGF)-βs, namely TGF-β2 and β3, are known to contribute to the process of fibrotic diseases (6).

TGF-β proteins transmit their signal from the activated transmembrane receptor to the nucleus mainly through the Smad family proteins (11,12). It was found that Smad2 and Smad3 were highly phosphorylated in some tissue fibrosis, and inhibition of Smad2 and Smad3 by RNA interference resulted in a significant decrease in collagen expression (13,14). In contrast, the expression of Smad6 and Smad7 was reduced in diseased tissue compared with normal tissue (15). Consistent with these results, the fibrogenic roles of each of the three isoforms of TGF-β have been documented during GMC procession, although the detailed underlying mechanism remains unknown. This study sought to detect the changes of TGF-β1/Smad downstream signaling molecules which elaborate the effects of TGF-β1Smad pathway in the GMC pathological process.

Methods

Tissue specimens and antibodies

In this study, 28 patients (12 males and 16 females between the ages of 14 and 35 years) who had GMC release with radiofrequency energy under arthroscopic guidance were selected between May 2010 and January 2011 from the Department of Sports Medicine and Rehabilitation, Peking University Shenzhen Hospital. All of these selected patients had failed to improve with stretching exercises. In contrast to the traditional operation method, we applied radiofrequency coblation to cut the high tensor fascia and the gluteus medius (Figure 1). Tissues obtained from these 28 GMC patients (28 contraction bands and 28 adjacent muscles) during surgical release with coblation to cut the high tensor fascia and the gluteus medius muscle contracture patients. (A) The greater trochanter was blocked by the abnormal muscle (adjacent muscle). The red arrows indicate the thickening gluteal fascia (contraction band); the black arrows indicate the related atrophic muscle. (B) Abnormal thickening of the gluteal fascia and the related atrophic muscle. The red arrows indicate the thickening gluteal fascia (contraction band); the black arrows indicate the related atrophic muscle (adjacent muscle).

Figure 1. The morphology changes in gluteal muscle contracture patients. (A) The greater trochanter was blocked by the abnormal muscle contracture atrophic muscle (adjacent muscle). (B) Abnormal thickening of the gluteal fascia and the related atrophic muscle. The red arrows indicate the thickening gluteal fascia (contraction band); the black arrows indicate the related atrophic muscle (adjacent muscle).

Methods

Collagen assay

Tissue samples were accurately weighed and washed by PBS for three times. The tissues were cut into 1 mm cubes and incubated in 10 mL acetic acid (0.5 mol/L) overnight. Then the swelled tissue was homogenized on ice with normal saline for 5 min using the homogenizer (Allegre 64R, Beckman Coulter). The samples were stirred and digested by pepsin at 4°C for 6 h [enzyme: tissue (v/v) = 1:100]. The pH was adjusted to 7.4 after enzymolysis and the extraction was centrifuged at 12,400 × g (RCF = 1.118 × 10⁻⁵ × N² × R; N: rpm, R: 7.5 cm) for 45 min. The supernatant was then collected and salted out with 0.7 mol/L NaCl at 4°C overnight, then centrifuged at 6000 × g for 45 min at 4°C. Then the powder was weighed after lyophilization for 2 h. The samples were then dissolved in normal saline for other experiments.

Western blot analysis

Tissue samples were homogenized using a modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 150 mM NaCl and 1 mM EDTA) supplemented with protease and phosphatase inhibitors (1 mM phenylmethyl sulfonyl fluoride, 0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone, 1 mg/ml aprotinin, 1 mg/ml pepstatin, 0.5 mg/ml leupeptin, 1 mM NaF, 1 mM Na₄P₂O₄ and 2 mM Na₃VO₄). The extract was centrifuged at 16,800 × g (RCF = 1.118 × 10⁻⁵ × N² × R; N: rpm, R: 7.5 cm) for 15 min at 4°C to remove cell debris. The supernatant was harvested and the protein levels were quantified using the BCA protein assay (Rockford, MA), followed by boiling for 5 min with sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 12% β-mercaptoethanol, 20% glycerol and 0.01%
bromophenol blue) at the equivalent protein level. The samples were subjected to SDS-polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 10% fat-free skim milk in Tris Buffer Saline containing 0.1% Tween 20, then incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 2 h at room temperature after a series of TBST washes. The immunoreactivity proteins were visualized by ECL (Amersham Pharmacia Biotech, USA) and autoradiography. Densitometry analysis was carried out with Quantity One software (Bio-Rad, Hercules, CA).

Reverse transcription and polymerase chain reaction (RT-PCR) and real-time reverse transcription-polymerase chain reaction

The expression of various genes from GMC patient tissues was analyzed by RT-PCR and real-time PCR. Total mRNA of samples was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s protocol, and then converted to cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). cDNA was subjected to PCR with primers for collagen type I (forward, 5'-GTGTCGGCGACGACGAAAGCAG-3' and reverse, 5'-CAGATACGTTGTCAGCAAC-3'), collagen type III (forward, 5'-TGGCTCCAGAAGGATGTCACG-3' and reverse, 5'-GGGGTGGTCTGTTACCATTA-3'), TGF-β1 (forward, 5'-GCCAGAATCCTGTTCAAGC-3' and reverse, 5'-GTTG TTTCCACCATTAGCAC-3'), PAI-1 (forward, 5'-CGGAGC ACGGTCAGCAAGTG-3' and reverse, 5'-GTTGAGGCGCA GAGGAGGCCG-3'), and β-actin (forward, 5'-CTCCAT CTTGCGGCTCTGCTG-3' and reverse, 5'-GCTGTCACC TTACCGTTCC-3'). All target sequences were separately amplified for 30–31 cycles of the following protocol: 30 s at 94°C, 30 s at 55°C and 60 s at 72°C. The reaction products were separated by agarose gel electrophoresis, visualized by ethidium bromide staining, and photographed with 290 nm ultraviolet illumination. The density of each band was measured by Quantity One software (Bio-Rad, Hercules, CA).

Real-time PCR was then performed on each sample using SYBR Green PCR master mix (Applied Biosystems) in a total volume of 20 μl fast on the 7900HT Real-time PCR system (Applied Biosystems) as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 60 s. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. β-actin was used as the reference gene. The relative levels of gene expression were represented as ΔCt = Ctgene - Ctreference, and the fold change of gene expression was calculated by the 2−ΔΔCt Method. Experiments were repeated in triplicate.

Immunohistochemistry

Tissue samples were excised and embedded in paraffin blocks, then sectioned on slides. Slides were dried overnight at 37°C, dewaxed in xylenes, rehydrated through graded alcohol, and soaked in 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. For antigen retrieval, slides were boiled in tris (hydroxymethyl) aminomethane-EDTA buffer (pH 8.0) in a pressure cooker for 10 min.

The sections were incubated with 10% goat serum at room temperature for 20 min to block non-specific protein binding. Subsequently, the blocked sections were incubated with antibody against TGF-β1 (1:500) or antibody against PAI-1 (1:400) for 60 min at 37°C in a moist chamber. After rinsing five times with 0.01 mol/L phosphate-buffered saline (PBS, pH = 7.4) for 10 min, the slides were incubated with secondary antibody at a concentration of 1:100 for 30 min at 37°C. Slides were then stained with DAB (3,3-diaminobenzidine) after washing in PBS again to visualize immunoreactive protein. The staining intensities of TGF-β1 and PAI-1 in the contraction band and unaffected adjacent muscle were graded semi-quantitatively using the following scale: (−) = no staining; (+) = weak staining, less than 30% of the highest protein expression; (+++) = moderate staining, between 30% and 60% of the highest protein expression; and (++++) = strong staining, more than 60% of the highest protein expression. The degree of staining intensities was expressed as the mean of ten different fields in each slide. The results were scored by two independent pathologists who were blinded to the identities of the slides.

Statistical analyses

Statistical analysis was performed using the SPSS Software, Version 17.0 (SPSS Inc., Chicago, IL). All values were expressed as mean ± SD. Statistics between groups were compared using the Student’s t-test and the Mann–Whitney rank sum test, which was used for the degree of staining intensities. p < 0.05 were considered as statistically significant difference.

Results

Expression of collagen types I and III is upregulated in the contraction band

To determine if fibrosis within GMC contraction bands is associated with increased collagen production, we first aimed to examine the expression of collagen types I and III in these tissues. Expression of these proteins was upregulated significantly in contraction bands compared to unaffected adjacent muscle at both mRNA and protein levels as examined by Western blotting and RT-PCR, respectively (Figure 2A). Semi-quantitative densitometry analysis indicated that the protein expression of collagen types I and III was increased 4.2- and 5.6-fold, respectively, and the mRNA levels of collagen types I and III were increased 3.8- and 4.5-fold, respectively, in the contraction band tissue compared with those in unaffected muscle of the GMC patients (Figure 2B, mean of 28 cases, p < 0.01). Our real-time PCR results also confirmed that collagen types I and III mRNA expression was up-regulated in contraction band compared with that in unaffected adjacent muscle (Figure 2C). These results confirm that GMC tissues have increased collagen synthesis, which is characteristic of pathological fibrosis.

Expression of TGF-β1 is especially upregulated in contraction bands

Considering the key role of TGF-β1 in fibrosis, we next wanted to determine if this protein was also induced in GMC
contraction bands. As shown in Figure 3(A), protein expression of TGF-β1 measured by western blot and mRNA expression of TGF-β1 measured by RT-PCR assay in gluteal muscle contracture patients. (A) Top: Mouse anti-human monoclonal antibodies against collagen types I and III were used to detect the collagen protein in the contraction band and adjacent muscle in five GMC patients by SDS-PAGE gel electrophoresis. Bottom: mRNA levels of collagen types I and III in the contraction band and adjacent muscle in five GMC patients. (B) Densitometric analysis of protein expression and mRNA levels of collagen types I and III in the contraction band and adjacent muscle. The results are shown as the relative expression in the contraction band compared with that in adjacent muscle of 28 GMC patients. *p < 0.05 compared with the adjacent muscle. (C) Up-regulated expression of collagen types I and III mRNA was examined by real-time PCR in five GMC patients, when compared with adjacent muscle tissues. Expression levels were normalized to β-actin (n = 3).

Figure 2. Protein expression of collagen types I and III examined by western blotting assay and mRNA level of collagen types I and III by RT-PCR assay in gluteal muscle contracture patients. (A) Top: Mouse anti-human monoclonal antibodies against collagen types I and III were used to detect the collagen protein in the contraction band and adjacent muscle in five GMC patients by SDS-PAGE gel electrophoresis. Bottom: mRNA levels of collagen types I and III in the contraction band and adjacent muscle in five GMC patients. (B) Densitometric analysis of protein expression and mRNA levels of collagen types I and III in the contraction band and adjacent muscle. The results are shown as the relative expression in the contraction band compared with that in adjacent muscle of 28 GMC patients. *p < 0.05 compared with the adjacent muscle. (C) Up-regulated expression of collagen types I and III mRNA was examined by real-time PCR in five GMC patients, when compared with adjacent muscle tissues. Expression levels were normalized to β-actin (n = 3).

contraction bands. As shown in Figure 3(A), protein expression of TGF-β1 measured by western blot and mRNA expression of TGF-β1 measured by RT-PCR were both significantly upregulated in the contraction bands compared with those in the unaffected adjacent muscle (five representative cases). Semi-quantitative densitometry analysis demonstrated that the protein expression and mRNA level of TGF-β1 were increased 6.8- and 8.3-fold, respectively, in GMC contraction bands compared with the unaffected adjacent muscle (Figure 3B, mean of 28 cases, p < 0.01). The real-time PCR results also demonstrated that mRNA level of TGF-β1 was elevated in GMC contraction bands compared with the unaffected adjacent muscle (Figure 3D). Immunohistochemical analysis was also carried out to measure the expression of TGF-β1 in the contraction band and adjacent muscle (Figure 3C). Quantification of the staining confirms that TGF-β1 overexpression was largely restricted to the contraction band, but not unaffected adjacent muscle (Table 1).

R-Smad phosphorylation induced by TGF-β1 is upregulated in GMC contraction bands

Phosphorylation of R-Smads is a key event in the signaling pathway induced by TGF-β1. Western blotting results indicate that more phosphorylated Smad2 and Smad3 were present in GMC contraction bands compared to adjacent unaffected muscle, as shown in Figure 4(A). Semi-quantitative densitometry analysis of the blots confirmed that Smad2
and Smad3 phosphorylation in the contraction band was increased 5.3- and 9.4-fold, respectively, compared with that in adjacent muscle in the same patients (Figure 4B, mean of 28 cases, p < 0.01).

Expression of Smad4 is increased, while Smad7 is decreased, in GMC contraction bands

Following phosphorylation, R-Smads typically bind to the common mediator Smad and Smad4. Consistent with increased R-Smad phosphorylation, expression of Smad4 was also markedly increased in the contraction band compared with adjacent muscle of GMC patients analyzed by western blot. The expression of the inhibitor Smad and Smad7, was significantly decreased. Semi-quantitative densitometry analysis confirmed that Smad4 protein expression in the contraction band was increased 6.8-fold while Smad7 expression was decreased 9.2-fold, compared to that in adjacent muscle in the same patients (Figure 4D, mean of 28 cases, p < 0.01).

Expression of plasminogen activator inhibitor type 1 (PAI-1) in contraction band induced by TGF-β1

As shown in the Figure 5(A), the protein and mRNA levels of PAI-1 in the contraction band were both dramatically increased in GMC contraction bands compared to unaffected adjacent muscle (five representative cases are shown). Semi-quantitative densitometry analysis showed that the protein expression and mRNA level of PAI-1 were increased 4.4- and 9.6-fold, respectively (Figure 5B, mean of 28 cases, p < 0.01), in the contraction bands compared with adjacent muscle in the same patients (Figure 4D, mean of 28 cases, p < 0.01).
GMC patients. The real-time PCR results also certified that mRNA level of PAI-1 was enhanced in the contraction bands compared with the unaffected adjacent muscle (Figure 5D). Immunohistochemistry (Figure 5C) indicated that PAI-1 was weakly present in the adjacent muscle compared to the contraction band. Quantification confirmed that high expression of PAI-1 (++++) was mainly detected in the contraction band of GMC patients (Table 2).

Discussion

GMC is a clinical fibrotic disease of the gluteal muscle (16). In recent years, extensive work has focused on the clinical treatment and rehabilitation of affected patients, such as the precise diagnosis criteria, better operation methods and effective means of rehabilitation (17–20). Despite clinical progress, the mechanism of GMC still remains unclear. Researchers speculated that the trigger factors for GMC, and the resulting muscle stiffness and joint dysfunction, include the repeated injections of benzyl alcohol into the buttocks (21). Some retrospective studies have found that post-operative patients have a tendency to develop fibrosis in other organs, such as hypertrophy scars in the skin or keloid in the incision site (22,23). These secondary fibrotic lesions have a strong impact on the treatment and prognosis of GMC patients. Therefore, understanding the mechanism of GMC is essential to improve clinical treatment and post-operative rehabilitation. In this study, for the first time, we demonstrated that the expression of collagen types I and III was significantly increased in contraction bands compared to that in adjacent muscle, suggesting that an imbalance of extracellular matrix (ECM) proteins might be the driving force in the development of GMC disease (6).

TGF-β1 is well known as the crucial fibrogenic cytokine promoting ECM production and tissue fibrosis, which is well-established in GMC progression (17). Chenguang Zhao et al. recently discovered that Sphingosine-1-phosphate (S1P) significantly contributes to the process of gluteal muscle scarring in GMC patients, because it stimulates the expression of TGF-β1 and collagens in the contraction band (24). Our study has also shown that the protein and mRNA levels of TGF-β1 are remarkably increased in the contraction band compared with adjacent muscle. Immunohistochemistry also confirmed that high expression of TGF-β1 (++++) is mainly detected in the contraction band of GMC patients. It is implied that TGF-β1 production is higher than required under long-term fibrotic stimulation, especially in some scar diathesis. This is supported by evidence showing increased TGF-β3 in GMC patients, which could explain why TGF-β3 repression is not decreased in GMC patients by a negative feedback loop under long term stimulation of fibrotic factors (25,26). The negative feedback loop also could be seen in other tissue fibrosis such as keloid fibroblasts (27).
Smad family proteins are located downstream of TGF-β in the TGF-β/Smad signaling pathway and are responsible for transmitting the signal from cell surface receptors to the nucleus, leading to activation or inhibition of their target genes (28–32). R-Smads are receptor-regulated Smads. R-Smads are directly phosphorylated through their intracellular kinase domain on their C-terminus, leading to R-Smad activation (33). Many reports have shown that TGF-β receptors (TbR) I and II have serine/threonine protein kinase activity that can promote R-Smad phosphorylation (34). Phosphorylated R-Smad (Smad2 and Smad3) combine with Smad4 to form a complex in the nucleus, resulting in enhanced expression of intracellular and extracellular fibrogenic proteins and inhibition of ECM degrading-proteins (35). Numerous studies have demonstrated that Smad2 and Smad3 are key factors in tissue fibrosis, suggesting that down-regulation of the phosphorylation of Smad2/3 could inhibit the pathological process (36). We have shown that the protein expression of R-Smad is significantly elevated in the contraction band identified with other tissue fibrosis process. R-SMAD proteins bind to the SMAD4 protein and form a protein complex, which then moves to the cell nucleus. In the nucleus, the SMAD protein complex binds to specific areas of

Table 2. Immunohistochemical analysis of PAI-1 in the contraction band and adjacent muscle.

|       | PAI-1 |       |       |       |       |
|-------|-------|-------|-------|-------|-------|
|       | (−)   | (+)   | (+++) | (+++) | p     |
| Adjacent muscle | 13     | 8     | 6     | 1     |       |
| Contraction band | 3      | 4     | 8     | 13    | 0.05* |

Semi-quantitative scale: (−) = no staining; (+) = weak staining; (+++) = moderate staining; and (++++) = strong staining.

*p < 0.05 compare with the adjacent muscle.

Smad family proteins are located downstream of TGF-β in the TGF-β/Smad signaling pathway and are responsible for transmitting the signal from cell surface receptors to the nucleus, leading to activation or inhibition of their target genes (28–32). R-Smads are receptor-regulated Smads. R-Smads are directly phosphorylated through their intracellular kinase domain on their C-terminus, leading to R-Smad activation (33). Many reports have shown that TGF-β receptors (TbR) I and II have serine/threonine protein kinase activity that can promote R-Smad phosphorylation (34). Phosphorylated R-Smad (Smad2 and Smad3) combine with Smad4 to form a complex in the nucleus, resulting in enhanced expression of intracellular and extracellular fibrogenic proteins and inhibition of ECM degrading-proteins (35). Numerous studies have demonstrated that Smad2 and Smad3 are key factors in tissue fibrosis, suggesting that down-regulation of the phosphorylation of Smad2/3 could inhibit the pathological process (36). We have shown that the protein expression of R-Smad is significantly elevated in the contraction band identified with other tissue fibrosis process. R-SMAD proteins bind to the SMAD4 protein and form a protein complex, which then moves to the cell nucleus. In the nucleus, the SMAD protein complex binds to specific areas of
DNA, where it controls the activity of particular genes and regulates cell growth and division (proliferation). Other studies discovered that some molecules like MicroRNA or Small heat shock protein resist fibrosis through modulating Smad4 expression (37,38). Smad7 can restrain R-Smad phosphorylation by competitively inhibiting the TβRI receptor due to its similarity in structure with R-Smads (39,40). It has been established that a decrease in the expression of Smad7 is a feature of systemic sclerosis in skin fibroblasts (41). In our study, we found that R-Smad phosphorylation and protein expression of Smad4 were significantly increased in the contraction band compared with adjacent muscle of GMC patients, which corresponded with significantly suppression of Smad7 in contraction bands. These data suggest that the increased R-Smad phosphorylation and Smad4 expression could generate more complexes in the nucleus, resulting in superfluous fibrotic protein expression. In addition, decreased expression of Smad7 could exacerbate progression of fibrosis. Taking into account other findings on GMC, we propose that the formation of GMC is due, at least in part, to an increase in TGF-β1/Smad signaling.

Plasminogen activator inhibitor type 1 (PAI-1) is one of the furthest downstream target genes of TGF-β1/Smad signaling (42). It is the primary physiological inhibitor of the urokinase-plasminogen activator (uPA) and tissue-plasminogen activator (tPA) systems (43). The fibrinolytic system, which includes uPA and tPA systems, is the key to regulating the activity of matrix metalloproteinases (MMPs) and ECM degradation factors (42,44). Previous studies have demonstrated that overexpression of PAI-1 can induce fibrosis in many organs, especially in liver, where it can lead to hepatocellular carcinoma (45). Suppressing the expression of PAI-1 could postpone the progress of liver fibrosis (46). Our study has shown that the protein and mRNA levels of PAI-1 are both significantly increased in the contraction band, compared with the adjacent muscle of GMC patients. Immunohistochemistry confirmed that the expression of PAI-1 is higher in the contraction band than in adjacent muscle. These results suggest that the abnormally high expression of PAI-1 might play an important role in GMC disease development.

In this study, we have demonstrated that the expression of collagen types I and III, TGF-β1, and Smad4, as well as R-Smad phosphorylation, were significantly increased in contraction bands of GMC patients compared to adjacent unaffected muscle, whereas the expression of Smad7 was significantly decreased. In addition, the protein and mRNA levels of plasminogen activator inhibitor type 1 (PAI-1) in the contraction band were elevated in contraction bands compared to adjacent muscle. These results provide a clue for further research into the mechanism and treatment of GMC disease.

Conclusion

In summary, this study not only confirmed that GMC is a fibrotic disease of gluteal muscle characterized by accumulation of collagen types I and III, but also verified the importance of the TGF-β/Smad pathway and its downstream effectors in GMC. Our findings are beneficial to comprehending the development and progression of GMC and could provide a new avenue for treatment of this debilitating disease.

Acknowledgments

We thank Mr XiaoPeng Tian for technical assistance and Mrs XiaoMin Yin from the Department of Pathology of Peking University Shen Zhen Hospital for providing and processing samples. XTZ and WTW are the lead investigators and developed the design of the study, carried out data-acquisition, analysis, interpretations, and prepared the manuscript as primary authors. TY, HL and QZ assisted in carrying out data acquisition and were involved in preparing the study design. All authors read, edited, and approved the final manuscript.

Declaration of interest

The authors declare that they have no competing interests.

References

1. Liu GH, Cao FQ, Yang SH, Zhu JF. Factors influencing the treatment of severe gluteal muscle contracture in children. J Pediatr Orthop B 2011;20:67–9.
2. Cai JH, Gan LF, Zheng HL, Li H. Iliac hyperdense line: a new radiographic sign of gluteal muscle contracture. Pediatr Radiol 2005;35:995–7.
3. Liu G, Yang S, Du J, Zheng Q, Shao Z, Yang C. Treatment of severe gluteal muscle contracture in children. J Huazhong Univ Sci Technol Med Sci 2008;28:171–3.
4. Chen X, Tang X, Jiang X, Wang D, Peng M, Liu L. Diagnosis and treatment of unilateral gluteal muscle contracture. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 2011;25:530–2.
5. Zhang XT, Xiao DM, Shi JJ, Zhang WT. Familial osteopoikilosis in the pelvic region combined with bilateral gluteal muscle contracture: a case report. Zhongguo Gu Shang 2013;26:246–7.
6. Zhao CG, He XJ, Lu B, Li HP, Kang AJ. Increased expression of collagens, transforming growth factor-beta1, and -beta3 in gluteal muscle contracture. BMC Musculoskelet Disord 2010;11:15.
7. Cabello-Verrugio C, Santander C, Cofre C, Acuna MJ, Melo F, Brandon E. The internal region leucine-rich repeat 6 of decorin interacts with low density lipoprotein receptor-related protein-1, modulates transforming growth factor (TGF)-beta-dependent signaling, and inhibits TGF-beta-dependent fibrotic response in skeletal muscles. J Biol Chem 2012;287:6773–87.
8. Wang S, Sun A, Li L, Zhao G, Jia J, Wang K, Ge J, Zou Y. Up-regulation of BMP-2 antagonizes TGF-beta1/ROCK-enhanced cardiac fibrotic signalling through activation of Smurf1/Smad6 complex. J Cell Mol Med 2012;16:2301–10.
9. Choe JY, Jung HJ, Park KY, Kum YS, Song GG, Hyun DS, Park SH, Kim SK. Anti-fibrotic effect of thalidomide through inhibiting TGF-beta-induced ERK1/2 pathways in bleomycin-induced lung fibrosis in mice. Inflamm Res 2010;59:177–88.
10. Yang J, Zheng J, Wu L, Shi M, Zhang H, Wang X, Xia N, Wang D, Liu X, Yao L, Li L, Dou K. NDRG2 ameliorates hepatic fibrosis by inhibiting the TGF-beta1/Smad pathway and altering the MOMP2/TIMP2 ratio in rats. PLoS One 2011;6:e27710.
11. Shi Y. Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 2003;113:685–700.
12. Sun ZJ, Kim KS, Wager G, Reiner EL. Mechanisms contributing to T cell receptor signaling and assembly revealed by the solution structure of an ectodomain fragment of the CD3 epsilon gamma heterodimer. Cell 2001;105:913–23.
13. Phan TT, Lim UJ, Aalami O, Lorget F, Khoor A, Tan EK, Mukhopadhyay A, Longaker MT. Smad3 signalling plays an important role in keloid pathogenesis via epithelial-mesenchymal interactions. J Pathol 2005;207:232–42.
14. Gao Z, Wang Z, Shi Y, Lin Z, Jiang H, Hou T, Wang Q, Yuan X, Zhao Y, Wu H, Jin Y. Modulation of collagen synthesis in keloid...
fibroblasts by silencing Smad2 with siRNA. Plast Reconstr Surg 2006;118:1328–37.

15. Yu H, Bock O, Bayat A, Ferguson MW, Mrowietz U. Decreased expression of inhibitory SMAD6 and SMAD7 in keloid scarring. J Plast Reconstr Aesthet Surg 2006;59:221–9.

16. Tan AB, Kress S, Castro L, Sheppard A, Raghunath M. Cellular re- and de-programming by microenvironmental memory: why short TGF-beta1 pulses can have long effects. Fibrogenesis Tissue Repair 2013;6:12.

17. Chen L, Ge Q, Black JI, Deng L, Burgess JK, Oliver BG. Differential regulation of extracellular matrix and soluble fibulin-1 levels by TGF-beta1 in airway smooth muscle cells. PLoS One 2013;8:e65544.

18. Wang P, Nie X, Wang Y, Li Y, Ge C, Zhang L, Wang L, Bai R, Chen Z, Zhao Y, Chen C. Multiwall carbon nanotubes mediate macrophase activation and promote pulmonary fibrosis through TGF-beta1/Smad signaling pathway. Small 2013;9:3799–811.

19. Xu L, Zheng N, He Q, Li R, Zhang K, Liang T. Puerarin, isolated from Pueraria lobata (Wild.), protects against hepatotoxicity via specific inhibition of the TGF-beta1/Smad signaling pathway, thereby leading to anti-fibrotic effect. Phytotherapy 2013;20:1172–9.

20. Gao X, Cao Y, Yang W, Duan C, Aronson JF, Rastellini C, Chao C, Hellmich MR, Ko TC. BMP2 inhibits TGF-beta-induced pancreatic stellate cell activation and extracellular matrix formation. Am J Physiol Gastrointest Liver Physiol 2013;304:G804–13.

21. Ye B, Zhou P, Xia Y, Chen Y, Yu X, Xu S. New minimally invasive option for the treatment of gluteal muscle contracture. Orthopedics 2012;35:e1692–9.

22. Li Q, Lingyan Z, Yan L, Yulan P. The role of ultrasonography in the diagnosis of gluteal muscle contracture. Skeletal Radiol 2011;40:215–21.

23. Samarakoon R, Dobberfuhl AD, Cooley C, Overstreet JM, Patel S, Goldschmeding R, Meldrum KK, Higgins PJ. Induction of renal fibrotic genes by TGF-beta1 requires ERK activation, p53 and reactive oxygen species. Cell Signal 2013;25:2198–209.

24. Zhao C, Qin J, He X, Guan Y, Jia Y, Lei W. Sphingosine-1-phosphate is a possible fibrogenic factor in gluteal muscle fibrosis. Physiol Res 2013;62:691–9.

25. Kim DJ, Lee DC, Yang SJ, Potter JD, Newcomb PA, Sargent MD, Jenkins MA, Hopper JL, Gallinger S, Le Marchand L, Martinez ME, Ahnen DJ, Baron JA, Lindor NM, Haile RW, Gago-Dominguez M. Genetic variations in SMAD7 are associated with colorectal cancer risk in the colon cancer family registry. PLoS One 2013;8:e60464.

26. Wilkins-Port CE, Ye Q, Mazurkiewicz JE, Higgins PL. TGF-beta1 + EGFR-initiated invasive potential in transformed human keratinocytes is coupled to a psmma/MPM-10/MPM-1-dependent collagen remodeling axis: role for PAI-1. Cancer Res 2009;69:4081–91.

27. Bohn L, Serafin A, Akudugu J, Fernandez P, van der Merwe A, Aziz NA, uPA/PAI-1 ratios distinguish benign prostatic hyperplasia and prostate cancer. J Cancer Res Clin Oncol 2013;139:1221–8.

28. Solberg A, Holmdahl L, Falk P, Woltering M, Palmgren I, Ivarsson ML. Local and systemic expressions of MMP-9, TIMP-1 and PAI-1 in patients undergoing surgery for clinically suspected appendicitis. Eur Surg Res 2012;48:99–105.

29. Armendariz-Borunda J, Rincon AR, Munoz-Valle JF, Bueno-Topete V, Va´zquez-Del Mercado M, Troyo-SanRoman R, Arellano-Olivera F, Causse S, Marchal-Somme J, Fabre A, Crestani B, Kolb M, Gauldie J, Campos P, Garrido C, Bonniaud P. The small heat shock protein alphaB-crystallin is essential for the nuclear localisation of Smad4: impact on pulmonary fibrosis. J Pathol 2014;232:458–72.

30. Liu Y, Zheng WK, Gao WS, Shen Y, Ding WY. Function of TGF-beta and p38 MAPK signaling pathway in osteoblast differentiation from rat adipose-derived stem cells. Eur Rev Med Pharmacol Sci 2013;17:1611–19.

31. Gao Y, Lai WY. Transforming growth factor-beta1 (TGF-beta1) regulates cell junction restructuring via Smad-mediated repression and Clathrin-mediated endocytosis of nectin-like molecule 2 (Nect-2). PLoS One 2013;8:e64316.

32. Zhao L, Yee M, O’Reilly MA. Transdifferentiation of alveolar epithelial type II to type I cells is controlled by opposing TGF-beta and BMP signaling. Am J Physiol Lung Cell Mol Physiol 2013;305:409–18.

33. Wharton K, Derynck R. TGFbeta family signaling: novel insights in development and disease. Development 2009;136:3691–7.

34. Yumoto K, Thomas PS, Lane J, Matsuzaki K, Inagaki M, Ninomiya-Tsuji J, Scott GJ, Ray MK, Ishii M, Maxson R, Mishina Y, Kaartinen V. TGF-beta-activated kinase 1 (Tak1) mediates agonist-induced Smad activation and linker region phosphorylation in embryonic craniofacial neural crest-derived cells. J Biol Chem 2013;288:13467–80.

35. Thatcher JD. The TGF-beta signal transduction pathway. Sci Signal 2010;3:tr4.

36. He S, Yang Y, Liu X, Huang W, Zhang X, Yang S, Zhang X. Compound Astragalus and Salvia miltiorrhiza extract inhibits cell proliferation, invasion and collagen synthesis in keloid fibroblasts by mediating transforming growth factor-beta/Smad pathway. Br J Dermatol 2012;166:564–74.

37. Bellaye PS, Wettstein G, Burgy O, Bensard V, Joannes A, Colas J, Causse S, Marchal-Somme J, Fabre A, Crestani B, Kolb M, Gauldie J, Camus P, Garrido C, Bonniaud P. The small heat shock protein alphaB-crystallin is essential for the nuclear localisation of Smad4: impact on pulmonary fibrosis. J Pathol 2014;232:458–72.

38. He Y, Huang C, Sun X, Long XR, Lv XW, Li J. MicroRNA-146a modulates TGF-beta1-induced hepatic stellate cell proliferation by targeting SMAD4. Cell Signal 2012;24:1923–30.

39. Wang J, Zhao J, Chu E, Mok M, Go MY, Man K, Heuchel R, Lan HY, Chang Z, Sung JJ, Yu J. Inhibitory role of Smad7 in hepatocarcinogenesis in mice and in vitro. J Pathol 2013;230:441–52.

40. Jiang X, Castelao JE, Vandenberg D, Carracedo A, Redondo CM, Conti DV, Paredes CJ, Potter JD, Newcomb PA, Passarelli MN, Jenkins MA, Hopper JL, Gallinger S, Le Marchand L, Martinez ME, Ahnen DJ, Baron JA, Lindor NM, Haile RW, Gago-Dominguez M. Genetic variations in SMAD7 are associated with colorectal cancer family registry. PLoS One 2013;8:e60464.

41. Dong C, Zhu S, Wang T, Yoon W, Li Z, Alvarez RJ, Ten DP, White B, Wigley FM, Goldschmidt-Clermont PJ. Deficient Smad7 expression: a putative molecular defect in scleroderma. Proc Natl Acad Sci USA 2002;99:3908–13.

42. Wilkins-Port CE, Ye Q, Mazurkiewicz JE, Higgins PL. TGF-beta1 + EGFR-initiated invasive potential in transformed human keratinocytes is coupled to a plasmin/MPM-10/MPM-1-dependent collagen remodeling axis: role for PAI-1. Cancer Res 2009;69:4081–91.

43. Bohm L, Serafin A, Akudugu J, Fernandez P, van der Merwe A, Aziz NA. uPA/PAI-1 ratios distinguish benign prostatic hyperplasia and prostate cancer. J Cancer Res Clin Oncol 2013;139:1221–8.

44. Solberg A, Holmdahl L, Falk P, Woltering M, Palmgren I, Ivarsson ML. Local and systemic expressions of MMP-9, TIMP-1 and PAI-1 in patients undergoing surgery for clinically suspected appendicitis. Eur Surg Res 2012;48:99–105.

45. Armendariz-Borunda J, Rincon AR, Munoz-Valle JF, Bueno-Topete M, Oregon-Romero E, Islas-Carbajal MC, Medina-Preciado D, Gonzalez-Garcia I, Bautista CA, Garcia-Rocha S, Godoy J, Vázquez-Del Mercado M, Troyo-SanRoman R, Arellano-Olivera I, Lucano S, Alvarez-Rodriguez A, Salazar A. Fibrogenic polymorphisms (TGF-beta, PAI-1, AT) in Mexican patients with established liver fibrosis. Potential correlation with pirenfenone treatment. J Investig Med 2008;56:944–53.

46. Wang H, Zhang Y, Heuckeroth RO. PAI-1 deficiency reduces liver fibrosis after bile duct ligation in mice through activation of tPA. FEBS Lett 2007;581:3098–104.