Increased Expression of the TGF-β Isoform and Changed Contents of Collagen in Tendon of Cerebral Palsy Patients

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**Purpose:** This study measured the expression level of the transforming growth factor-β (TGF-β) isoform expression and the collagen composition within the Achilles tendon from cerebral palsy (CP) patients. **Materials and Methods:** The Achilles tendons were obtained from 15 CP patients with spastic equinovarus. The presence of the TGF-β isoform and the composition of the collagen were examined histologically, performing by immunohistochemical staining (IHS) and determining the mRNA expression level using a reverse transcriptase polymerase chain reaction (RT-PCR). **Results:** IHS revealed the presence of TGF-β1 and TGF-β2 expression in 2/15 cases and 4/15 cases respectively, and weak TGF-β3 expression in 7/15 cases. The TGF-β1 and TGF-β2 expression levels were uniform in all 15 cases according to RT-PCR, while TGF-β3 expression was observed in 8 out of 15 cases. IHS and RT-PCR showed strong TGF-β3 expression in 6/7 non-ambulatory patients. An immature form of collagen, type III collagen, was observed more abundantly in the non-ambulatory patients. **Conclusion:** These results suggest that contracture in CP may induce changes in the type of collagen via growth factors such as TGF-β. **Key Words:** Cerebral palsy, Achilles tendon, Transforming growth factor-β (TGF-β), Collagen

Spasticity is the main feature of the many symptoms of cerebral palsy (CP). Continuous spasticity in CP causes injury to the muscles and tendons, leading to their subsequent regeneration and degeneration. Among the cytokines and growth factors participating in these regeneration and degeneration processes, the transforming growth factor-β (TGF-β) plays an important role in the formation of fibrosis while upregulating wound healing at the time of a muscle injury. TGF-β induces cellular proliferation and increases the rate of synthesis of both collagen and the cell matrix by stimulating various cells. This study examined the expression of the TGF-β isoforms and collagen composition within the Achilles tendon of CP patients with an equinovarus deformity.

**MATERIALS AND METHODS**

From March 2001 to March 2002, 15 CP patients underwent an Achilles tendon lengthening for spastic equinovarus. A 2 × 2 × 5 mm³ specimen of the Achilles tendon was obtained from the mid-substance. As a control, the same samples were obtained from healthy persons who had undergone surgery for a traumatic rupture of the tendon (Table 1). The expression level of the TGF-β isoform and the collagen composition were determined using histological studies, immunohistochemical staining (IHS) and the mRNA expression level was examined using a reverse transcriptase polymerase chain reaction (RT-PCR).

1. **Immunohistochemistry**

Immunohistochemical analyses of TGF-β1, TGF-β2, and TGF-β3 as well as, type I and III collagen were performed using the avidin-biotin complex (ABC) method. The specimens were frozen and stored at -70°C prior to immunostaining. Four-micrometer parallel sections were cut on a cryostat, mounted on a slide on plus slides, and air-dried. The
sections were incubated with the primary antibodies against TGF-β1, TGF-β2 and TGF-β3 for 2 hours, and type I and III collagen for 1 hour in a humidity chamber at room temperature (listed in Table 1). Biotin-labeled secondary antibodies (Zymed Laboratories, USA) were utilized for 7 minutes at 45°C. The streptavidin-horseradish peroxidase (Zymed Laboratories, USA) detection system was then applied to the sections. The sections were counterstained with hematoxylin and mounted on a Universal Mount (Re- search Genetics, USA).

2. RNA isolation and RT-PCR

The specimen was freshly frozen in liquid nitrogen. The total RNA from the tissue was extracted using the methodology for the TriZol reagent (Gibco BRL, Life Technologies). The RNA (1 μg) was reverse-transcribed using the Superscript First-Strand Synthesis System for RT-PCR (Gibco BRL, Life Technologies).

The primers for TGF-β1, -β2, -β3, type I, type III collagen, and β actin, which is a constitutively expressed housekeeping gene, were designed from a reference paper. Table 2 shows the primer sequences. The PCR reaction mixture contained 2 μL of each cDNA sample, 10 pM each of sense and antisense primers, and the other PCR reagents in a final volume of 20 μL. The PCR reagents, dNTP, Taq DNA polymerase, 10 × reaction buffer (40 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM MgCl₂, stabilizer and tracking dye) were obtained from Accupower® PCR PreMix (Bioneer, Korea). The PCR cycles were at 94°C for 5 minutes, and 35 cycles of denaturation at 94°C for 1 minute. The annealing temperature was set for 1 minute, and polymerization was performed at 72°C for 2 minutes followed by 72°C for 10 minutes.

The PCR products were electrophoresed on 1.0 percent agarose gel, visualized by ethidium bromide staining, and photographed under UV light. The TGF-β1 cDNA, -β2 cDNA, -β3 cDNA, the type I cDNA collagen, type III cDNA collagen, and β-actin cDNA were semi-quantified by IMAGERTM & 1D MAIN (Bioneer, Korea).

The mRNA integrity and amplification efficiency of the TGF-β isoforms, as well as the type I and type III collagen transscripts were evaluated by amplifying the β-actin sequence from the equivalent amounts of the total RNA from each sample.

### RESULTS

1. Clinical characteristics

The average age of the 15 patients (10 males and 5 females) was 10 years (6-17 years). Seven patients were spastic paraplegic and 8 were hemiplegic. All 7 paraplegics were non-ambulatory and the 8 hemiplegics were ambulatory.

2. Histological findings

The control specimens collected from a 38-year-old female
3 days after the trauma showed that the Achilles tendon was composed of fibroblasts and matrix including collagen fibers. The collagen fibers were parallel to the longitudinal axis of the tendon with fibroblasts located between the fibers. Compared with the control, no difference was observed in the ambulatory CP. However, tendinolipomatosis and decreased cellular distribution were observed in 2 cases of nonambulatory CP (Fig. 1).

3. Immunohistochemical staining (IHS)

1) TGF-β isoforms

Optical microscopy after IHS revealed the presence of TGF-β1 and TGF-β2 expression in 2/15 cases and 4/15 cases respectively, and weak TGF-β3 expression in 7/15 cases (Fig. 2). It was also noted that TGF expression was observed only in the tenocytes but not in the matrix. This study did not determine the matrix composition and the level of tenocyte proliferation according to the type of paralysis and the degree of TGF-β expression.

2) Collagen staining

Type I collagen showed relatively dark stain and type III collagen light stain in the ambulatory CP patients. How-
ever, type I and type III collagen were stained the same in the nonambulatory CP patients with a relative increase in the immature type III collagen level (Fig. 3). No correlation was found between the expression level of the TGF-β isoforms and the degree of collagen staining.

4. The level of mRNA according to RT-PCR
TGF-β1 and TGF-β2 expression were observed in all cases, whereas TGF-β3 expression was observed only in 8 out of 15 cases. The expression of the TGF-β isoform was strong in 6 out of the 7 non-ambulatory patients and weak in all 8 hemiplegic patients. The type III collagen level was upregulated in all 7 cases showing TGF-β3 upregulation. Regarding the collagen composition, immature type III collagen was observed in all 15 CP cases. The distribution of type III collagen was more prominent in the non-ambulatory CP cases than that of the type I collagen. However, the expression level of the TGF-β isoform increased evenly and the level of collagen synthesis was also increased in the trauma group. These results are similar to those of the spastic non-ambulatory CP cases, which might coincide with the phase of the active inflammatory repair process.

5. TGF-β isoform expression and collagen composition
According to the IHS, the expression level of the TGF-β isoform was not uniform. Therefore, no correlation was found between the degree of collagen staining and TGF expression. However, the mRNA level according to RT-PCR showed increased synthesis of the TGF-β isoform and collagen in the non-ambulatory CP group (Fig. 4).

**DISCUSSION**

Muscle spasticity in CP induces muscle and tendon injury. However, the complete regeneration of the injured tissues is hindered as a result of repetitive spasms, eventually leading to muscle contracture and a secondary joint deformity. A normal tendon is composed of fibroblasts and collagen fibers. Collagen fibers form a triple-stranded helix and are composed of type I collagen in 95% of cases. On the other hand, type III collagen is present in an immature form and is usually present during the healing stage. There are few reports on the growth factor or cytokines associated with muscle damage that occurs after CP, which is the main subject of this study.

Chang et al. reported, after experimenting with TGF-β1 antibodies in rabbits, that TGF-β1 participates in the formation of excessive scarring by improving the tendon excursion. Shin et al. reported that the expression of type I collagen mRNA and collagen formation, as well as cellular proliferation in the tendon cells are promoted with the introduction of TGF-β1 alone or in combination with other growth factors, indicating that TGF-β actively participates in the healing process of injured tendons inducing fibrosis. In our CP patients, it is believed that the continued spasms were probably the cause of the increase in the TGF-β level rather than the uncontrollable action of the TGF-β isoform according to the clinical findings.

The increase in the type III collagen level in the rupture site of the human Achilles tendon is probably due to con-
continued microtrauma and the subsequent healing process\(^3\). The quantitative measurement of the type I to type III collagen ratio was measured in the tendon using IHS\(^18\). This study was also able to determine a difference in collagen staining and the mRNA level. The injured tendons produce type III collagen during the inflammatory phase and the type I collagen during the reparative phase. In addition, collagen formation is reduced and the collagen is arranged longitudinally in the remodeling phase, which can last for 20 weeks\(^13\). This study using IHS and RT-PCR also confirmed the increase in the type III collagen level within the CP tendon. A continued spasm in CP patients would initiate a microtrauma and induce an injury, which may increase the collagen III level via the participation of TGF-\(\beta\). It is possible that the type III collagen level would decrease when further tissue damage is prevented by controlling the spasms in CP patients. The histological examination revealed hypoxic degenerative tendinopathy, mucoid degeneration, tendinolipomatosis or combination of these in the injured tendons\(^12\). In the fresh trauma which was used as the control, the TGF-\(\beta\) expression level was increased in the rupture site. However, the alignment of the collagen fibers was regular and the major composition was collagen I. This study also observed similar findings in the Achilles tendon of the severe CP cases. Alioto et al.\(^1\) experimented with the palma fascia of Dupuytren’s disease and predicted that the contracture could be prevented by inhibiting the TGF-\(\beta\) and collagen receptors. However, in CP, the tendon injury induced by a spasm would produce growth factors such as TGF-\(\beta\) to repair the injury, which in turn would increase the level of collagen synthesis. The initial pulse would repeat and continue with time, which is in contrast to traumatic rupture. Therefore, it is important to continue with physical therapy to decrease the spasm and prevent the trauma from spasticity. The early and active rehabilitation will help in the formation of a mature matrix.

The Type III collagen mRNA level was upregulated in the tendons of the non-ambulatory patients as result of the severe spasm at the time of the upregulated TGF-\(\beta\) mRNA level.

However, this study could not determine whether or not the presence of cellular proliferation is related to the upregulation of TGF-\(\beta\) mRNA.

A further study with a larger number of subjects and the standardization of objective measurements in the spasticity in CP and in vivo studies will clarify the direct relationship between TGF-\(\beta\) and collagen formation.

**CONCLUSION**

This study focused on a histological study of the Achilles tendon of spastic CP patients, the degree and pattern of TGF-\(\beta\) expression, and the changes in the collagen types. The results of this study can provide the basic data for understanding the functions of the growth factors not only in CP but also in those diseases related to tendon contracture. It can also be used to implement the logical treatments and to improve the clinical evaluations of the disease.

**REFERENCES**

뇌성마비 환자의 아킬레스건에서 TGF-β 발현 및 콜라겐 조성의 변화

정성택 ∙ 서형연 ∙ 이재준 ∙ 김명선 ∙ 김양경 ∙ 김계진
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목적: 뇌성마비 환자의 아킬레스건 내 TGF-β isoform의 발현 정도와 콜라겐 구성도 알기 위해 수행하였다.

대상 및 방법: 뇌성마비로 인한 경직성 첨족마비 환자 중 수술적 연장술을 시행하였던 15명 환자의 아킬레스건 중앙부를 약 2×2×5 mm3 크기로 채취한 후, 면역조직화학 염색과 RT-PCR을 이용하여 TGF-β와 콜라겐 구성도 조사하였다.

결과: 아킬레스건 중앙부의 구성은 섬유모세포와 콜라겐 섬유 등의 기질로 구성되어 있었다. 면역조직화학 염색상 TGF-β1과 TGF-β2는 15예 중 각각 2예, 4예에서만 발현되었으며, TGF-β3의 경우는 7예에서 약하게 발현되었다. 경직성이 심한 비보행성 마비를 보인 7예 중 6예에서 TGF-β3의 양성 소견을 보였다. 콜라겐 염색상 경직성이 심한 비보행성 마비 환자에서는 섬유성형태가 제3형의 콜라겐이 증가됨을 알 수 있었다. 면역조직화학 염색 및 RT-PCR상 TGF-β3가 증증이 늘어진 7예에서 제3형 콜라겐이 증가되어 있었다.

결론: 뇌성마비로 인한 경직성 근관측은 건내 콜라겐 성분의 변화를 초래하고 이 과정중 TGF-β가 관여할 것으로 사료 된다.

색인단어: 뇌성 마비, 아킬레스 건, TGF-β, 콜라겐