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〈Original Article〉

## Frequent Establishment of Long-term-cultured Myofibroblast Cell Lines Derived from Dupuytren's Nodules, Which Are Implantable into Nude Mice

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Key Words : Dupuytren's contracture; Myofibroblasts; Established cell lines;  
 $\alpha$  smooth muscle actin; Transforming growth factor  $\beta$ 1

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### ABSTRACT

The purpose of this study is to establish myofibroblast cell lines derived from Dupuytren's nodules. Cells were obtained from 6 patients with Dupuytren's contracture, and those from 4 out of the 6 patients were serially passaged. The morphologic characteristics were observed by light and electron microscopies. Each karyotype was analyzed by a trypsin-Giemsa banding technique. The production of  $\alpha$  smooth muscle actin ( $\alpha$ SMA) was detected by immunological methods and reverse transcription polymerase chain reaction. Transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) was measured by enzyme immunoassay. Xenografting was performed by inoculating the cultured cells into 5-week-old nude mice (BALB/c nu). The cell lines could be subcultured for more than 1 year, therefore, we considered them to be established. Spindle-shaped cells were arranged irregularly, and showed myofibroblast-specific ultrastructural characteristics. The karyotype analysis did not reveal any deletion of the chromosomes where  $\alpha$ SMA and TGF $\beta$ 1 genes were localized. The cell lines showed electron-dense bundles and maintained the production of  $\alpha$ SMA, its messenger RNA, and TGF $\beta$ 1. The implantability into nude mice was 33-100%. Our results indicated that the established cell lines were myofibroblastic cell lines. We could implant the cell lines into nude mice and they may have beneficial applications in animal models of contractile diseases.

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## INTRODUCTION

Dupuytren's contracture is characterized by a progressive digital flexion deformity caused by the shortening of the palmar fascia. The disease consists histologically of a fibrous nodule in the palm, called Dupuytren's nodule containing a dense population of myofibroblasts.<sup>1)</sup> The myofibroblasts are spindle-shaped and  $\alpha$  smooth muscle actin ( $\alpha$ SMA)-rich fibroblasts, which occurs in granulation tissue during the wound-healing process<sup>2)</sup> and in some benign proliferative disorders, such as desmoid tumor. They appear also in nodules of Dupuytren's contracture.<sup>3)</sup> Sanders et al.<sup>4)</sup> reported that the myofibroblasts have a contractile force, leading to contracture in Dupuytren's contracture.

A serious problem in the therapy of the Dupuytren's contracture is recurrent contracture after surgery at varying rates of 34 to 78%.<sup>5), 6)</sup> Based on a finding that the production of  $\alpha$ SMA is inhibited by interferon- $\gamma$  (IFN $\gamma$ ),<sup>7)</sup> clinical open pilot studies on the treatment of Dupuytren's contracture were performed using IFN $\gamma$ .<sup>8)</sup> Recently, a possible method for preventing recurrent contracture after surgery has been examined *in vitro* using 5-fluorouracil.<sup>9)</sup> Many studies revealed that myofibroblasts were transformed from fibroblasts by stimulation with cytokines, such as transforming growth factor  $\alpha$  (TGF $\alpha$ ), TGF $\beta$ , tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), epidermal growth factor (EGF), basic fibroblast growth factor ( $\beta$ FGF), platelet derived growth factor (PDGF), and interleukin 1 $\alpha$  (IL-1 $\alpha$ , IL-4, IL-6 and so on).<sup>10), 11)</sup> Cells from Dupuytren's nodule in culture were characterized as showing an abnormal behavior,<sup>12)</sup> an increase in absolute content of  $\alpha$ SMA,<sup>13)</sup> and a contractile force of  $\alpha$ SMA.<sup>14)</sup> Recently, Bisson et al.<sup>1)</sup> have examined the proportions of myofibroblasts relative to fibroblasts in cultured cells derived from Dupuytren's nodule, cord and normal flexor retinaculum.

In all previous studies cited above, cells from Dupuytren's contracture were cultured in a short term, that is, within ten passages. We could not find any report on the establishment of long-term-cultured cell lines derived from Dupuytren's nodules. Long-term-cultured cell lines are preferable for the studies on the pathophysiology of Dupuytren's contracture and chemotherapy against recurrence. Since long-term-cultured myofibroblast cell lines have not been established, Tanaka et al.<sup>15), 16)</sup> examined the influences of IFN $\gamma$

on  $\alpha$ SMA production using a TGF $\beta$ 1-stimulated human fibroblast cell line in lieu of a Dupuytren's myofibroblast cell line. The inhibition of  $\alpha$ SMA production of the model was similar to that of Dupuytren's myofibroblasts. In this study, we succeeded in culturing cells from Dupuytren's nodules for more than one year as the established long-term-cultured cell lines. We characterized them and attempted to establish an animal model of the contractile disease.

## MATERIALS and METHODS

### Cell culture

Before undergoing surgery, a written permission for the use of the lesion in the study was given from each patient. All the specimens were dislinked by randomized anonymousness. Surgical specimens of Dupuytren's nodules were separated from the cord and other surrounding tissue. The nodules were minced and cultured in 25 cm<sup>2</sup> plastic flasks (BD Falcon, SA, France). The cells were maintained in Eagle's minimum essential medium (E-MEM) (Nissui, Tokyo, Japan) containing 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO<sub>2</sub>. After the proliferation of cells were confirmed once to have grown up confluent, they were continuously subcultured for more than 2 years. The established cell lines were named Dup-1, -2, -3, and-4. A fibroblast cell line, WI-38 (Riken BRC, Ibaragi, Japan) was used as a control culture. The cells were analyzed using a WST-8 kit (Dojin, Kumamoto, Japan), which is a modification of water-soluble formazan assay.

### Morphological analysis

The morphology of the cells was examined under an inverted-type phase-contrasted light microscope (IMT-2 Olympus, Tokyo, Japan). For immunohistochemistry, the cells were cultured in 4-well chamber slide glass with E-MEM containing 10% FBS, and fixed with 1% acetone for 3 min at room temperature. The fixed cells were stained by an immunohistochemical technique. Briefly, after blocking with 5% bovine serum albumin, the cells were reacted with an anti- $\alpha$ -smooth muscle actin ( $\alpha$ SMA) monoclonal antibody (Sigma, St. Louis, USA) at 37 °C for 30 min and rinsed in a gentle stream of phosphate-buffered saline (PBS) 3 times for 30 min at room temperature. The cells were reacted then with a fluorescein-labeled anti-mouse IgG goat antibody (KPL Inc., Maryland, USA) at 37 °C for 30 min. After the rinse in PBS 3

times at room temperature, the cells were observed under a fluorescence microscope (BX-51, Olympus, Tokyo, Japan). For electron microscopy, cultured cells were fixed with 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 5 min in culture flasks, and harvested using a rubber scraper. The cells were collected in a conical tube and centrifuged at 900 rpm for 5 min to obtain cell pellets. The pellets were fixed with the same fixative at 4 °C for 60 min, and washed 5 times with 0.05 M cacodylate buffer (pH 7.2), followed by the postfixation with 1% osmium tetroxide in the same buffer at 4 °C for 60 min. The specimens were washed with the same buffer, dehydrated in ethanol, and embedded in Epoxy resin. Ultrathin sections were cut using an MT6000 ultramicrotome (Sorvall Instruments, DuPont, CT, USA), doubly stained with uranyl acetate and lead citrate, and observed under an H-7100 electron microscope (Hitachi, Tokyo, Japan). The difference in the ultrastructure between the Dup-1, -2, -3 and -4 cell lines and a fibroblast cell line, WI-38, was examined.

### **Karyotype**

Chromosome preparations were made from cell lines by trypsin-Giemsa banding techniques.<sup>17)</sup>

### **Western blot (WB) analysis**

After washing confluent cells in a flask 3 times in PBS, the cells were trypsinized, counted with a hemacytometer and lysed in sodium dodecylsulfate (SDS) lysis buffer containing 0.1% SDS, 125 mM Tris (pH6.8), and proteinase inhibitor complete cocktail (Sigma, St. Louis, USA). Lysates were boiled and sonicated. Equal amounts of proteins were loaded onto a 10-20% gradient SDS-polyacrylamide gel and separated by electrophoresis for 1 hour in a mini-gel system (Daiichikagaku, Tokyo, Japan). Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer system (Biorad, California, USA). The membrane was rinsed in PBS containing 0.1% Tween20 (PBS-T) and blocked by incubation in 5% w/v skim milk dissolved in 0.1% PBS-T. The membrane was washed in 0.1% PBS-T, incubated with mouse anti- $\alpha$ SMA (Sigma, St. Louis, USA), and anti- $\beta$ ,  $\gamma$ SMA (Neomarkers, California, USA) monoclonal antibody solutions (1:500 diluted) at 4 °C overnight. Following the PBS-T washes, the membrane was incubated with peroxidase-labeled sheep anti-mouse IgG (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 1 hour. The

membrane was then washed in 0.1% PBS-T and subjected to immunoperoxidase color development using diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories, California, USA). The  $\beta$  and  $\gamma$ SMA was used as an internal marker for cell protein loading.

### **Reverse transcription polymerase chain reaction (RT-PCR)**

The primer pair used in RT-PCR for  $\alpha$ SMA was purchased from Stratagene (CA, USA). The expected size of the PCR product was 590 bp. Prior to gene amplification with Gene Amp PCR system 9700 (PE Applied Biosystems, CA, USA), mRNAs were isolated using a Micro-Fast Track 2.0 kit (Invitrogen, CA, USA) from  $1 \times 10^5$  cells. PCR was performed in a 50  $\mu$ l volume with temperature profiles as follows: one cycle of denaturation for 60 s at 94 °C and annealing and reverse transcription for 30 min at 60 °C, followed by 35 cycles of denaturation for 30 s at 94 °C and annealing for 60 s at 65 °C, and a final extension for 10 min at 65 °C. The PCR products obtained were mixed with loading buffer and subjected to electrophoresis. The gel was stained with a SYBR Green I nucleic acid gel staining kit (FMC Bioproducts, ME, USA) and observed through a transilluminator at a wavelength of 312 nm.

### **Measurement of TGF $\beta$ 1**

TGF $\beta$ 1 in culture supernatant was measured using an enzyme-linked immunosorbent assay kit (Duo Set, R & D systems, MN, USA). FBS, 10% of which is added to culture medium, may contain substance(s) cross-reacting with TGF $\beta$ 1. To confirm that the levels of TGF $\beta$ 1 in culture supernatant were not augmented by TGF $\beta$ 1 in FBS, the culture supernatant was diluted tenfold and the final value of TGF $\beta$ 1 was compared with that of a fresh medium in which no cells were cultured. Statistical significance was assessed using Student's *t* test.

### **Implantation of cells into nude mice**

The established cultured cells were inoculated to 5-week-old nude mice (BALB/c nu) and observed for 3 weeks. Each cell line was suspended in PBS, and  $2 \times 10^7$  cells (per 0.2 ml) were inoculated to 3 mice, respectively. The mice were examined by hand every week after the inoculation. When a palpable tumor was detected, the lesion was harvested for histopathological examinations.

## RESULTS

### ***Establishment of cell lines and their microscopic morphology***

Five cell lines from 6 different samples were successfully subcultured for 1 to 3 years (70 to 150 generations). One of the cell lines was maintained for more than 1 year but accidentally lost, and another one from the 6 samples was shortly subcultured but failed to establish. The remaining 4 cell lines were named Dup-1, -2, -3 and -4, and analyzed in this study. Inverted light microscopy showed that spindle-shaped cells were arranged irregularly, occasionally with a cramped pattern, i.e., whorls or pinwheels of cells radiating out from a center (Fig. 1-a, -b, -c and -d) and the TGF- $\beta$ 1-treated human fibroblast cell line (Fig. 1-e). In contrast, untreated fibroblasts were aligned parallel, and the cells grew in 2 to 3 layers under confluent conditions (Fig. 1-f).

### ***Karyotype***

The karyotype analysis of the cell lines derived from Dupuytren's nodules did not reveal specific chromosomal abnormalities. Dup-1 was 45 X, -Y, 47, XY+7 and 46, XY, and Dup-2, -3 and -4 were, 46 XY, 46 XY, and 46 XY, respectively. A trisomy 8<sup>18)</sup> was not observed in any cell lines.

### ***Ultrastructure of established cell lines***

To determine whether the established cell lines possess myofibroblast-specific structures, electron microscopic observation was performed. All the cell lines were rich in rough endoplasmic reticulum with somewhat dilated lumina containing flocculent material. Golgi apparatus and mitochondria were moderately developed. Bundles of microfilaments of 0.1 - 0.5  $\mu$ m width were found especially beneath the cell membrane, running parallel to the long axis of the cell (Fig. 2-a, -b, -c and -d). Dense patches were interspersed among the microfilaments. Pinocytotic vesicles

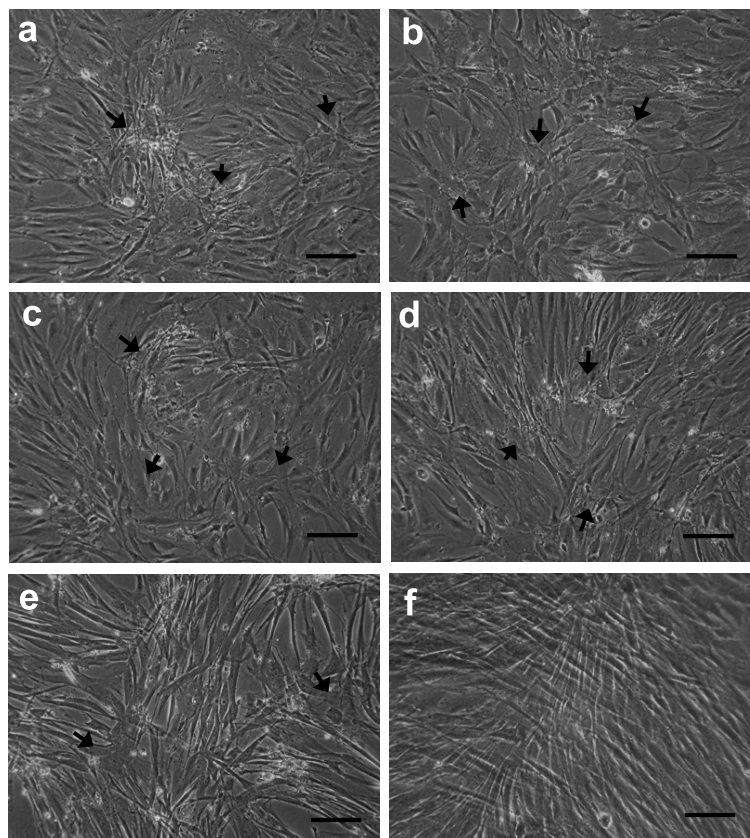


Fig. 1 Inverted light microscopy photographs of established cell lines in culture. Spindle-shaped cells of Dup-1 (a), Dup-2 (b), Dup-3 (c) and Dup-4 (d) cells, and TGF $\beta$ 1-treated fibroblasts (e) showed an irregular arrangement, occasionally with a cramping formation (arrows), while untreated fibroblasts (f) were aligned parallel. bars=100 $\mu$ m

were frequently found in these cells. Similar features were found in TGF $\beta$ 1-treated fibroblasts (Fig. 2-e), but not in untreated fibroblasts (Fig. 2-f).

#### RT-PCR

The production of  $\alpha$ SMA-specific mRNA was detected by RT-PCR in all cell lines (Fig. 3, lanes a-d) but not in untreated fibroblast cell line (Fig. 3, lane e).

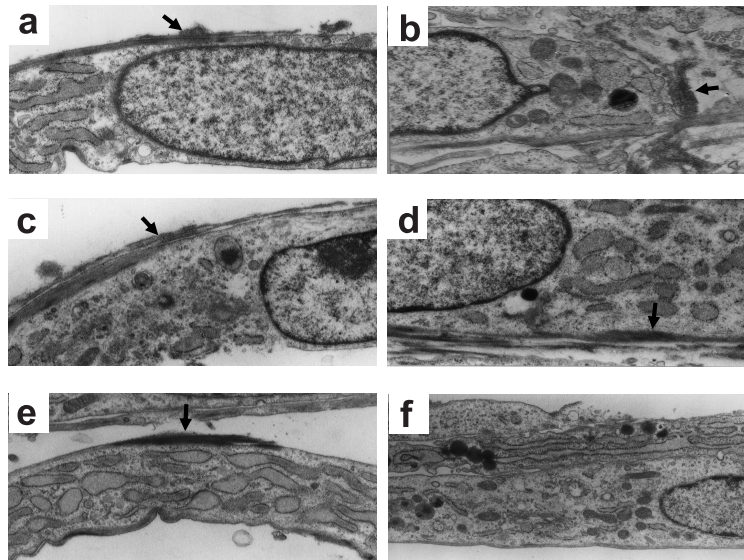


Fig. 2 Ultrastructure of established cell lines

Electron microscopy photographs of Dup-1 (a), Dup-2 (b), Dup-3 (c), and Dup-4 (d) cells, TGF $\beta$ 1-treated fibroblasts (e), and untreated fibroblasts (f). Arrows indicate the bundle structure in the cells (X16000).

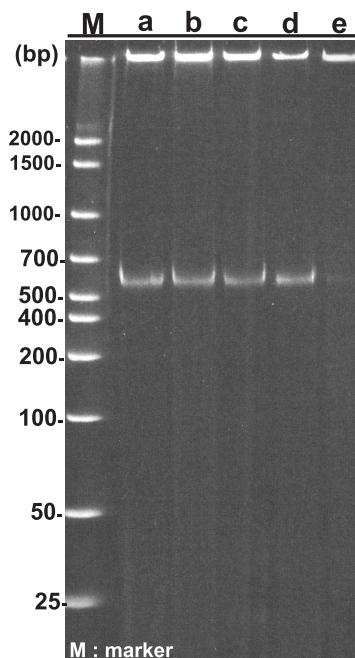


Fig. 3 Expression of  $\alpha$ SMA mRNA in established cell lines

Extracted mRNA samples from Dup-1 (lane a), Dup-2 (lane b), Dup-3 (lane c), and Dup-4 (lane d) cells and fibroblasts (lane e) were amplified for  $\alpha$ SMA-specific nucleotide sequence by RT-PCR. M: molecular size marker. The specific band of 590bp was visualized in all Dup cell lines but not in fibroblast.

### WB analysis for $\alpha$ SMA and localization of $\alpha$ SMA

WB analysis was performed to determine whether the  $\alpha$ SMA molecule is abnormal in the Dupuytren's cell lines. The assay revealed a single band of 43 kDa for each Dupuytren's cell line (left panel of Fig. 4A, lanes a-d) and TGF $\beta$ 1-treated fibroblast cell line (Fig. 4A, lane e). No significant difference in the molecular weight of  $\alpha$ SMA was seen among these cell lines. Expression of  $\beta$  and  $\gamma$ SMA protein, which is expressed in fibroblasts as well as smooth muscle cells, was detected in all the cell lines and TGF $\beta$ 1-treated fibroblast cell line (right panel of Fig. 4A, lanes a-d vs. lane e). To detect whether these cell lines possess  $\alpha$ SMA-rich filaments, immunofluorescence microscopy was performed. All the cell lines revealed strong fluorescent signals, either in bundle-like structures or in dots (Fig. 4B-a, -b, -c and -d). Similar signals were observed in fibroblasts cell line treated with TGF $\beta$ 1 (Fig. 4B-e), but not in untreated fibroblasts (Fig. 4B-f).

### Production of TGF $\beta$ 1

All cell lines produced abundantly  $\alpha$ SMA for more than one year. Since the production of  $\alpha$ SMA may be due to the TGF $\beta$ 1 autocrine system, culture supernatants were measured for TGF $\beta$ 1. The concentrations of TGF $\beta$ 1 in the culture supernatants of the Dup-1, -2, -3, -4 cell lines were significantly higher than that of the normal fibroblast cell line. (Fig. 5, a-d vs. e,  $p < 0.01$ , Student's *t* test,  $n = 16$ )

### Implantation of cell line in animals

To determine whether cell lines are implantable, cells were inoculated to 5-week-old nude mice (BALB/c nu), and observed for 3 weeks. Each cell line was suspended in PBS, and  $2 \times 10^7$  cells (per 0.2 ml) were inoculated to the 3 mice and 33-100% of animals revealed a nodular lesion at injected subcutaneous portion (Table 1). The size of removed nodules ranged between 3 to 5 mm in diameter (Fig. 6-a). Histopathological findings of the lesions were similar to those of

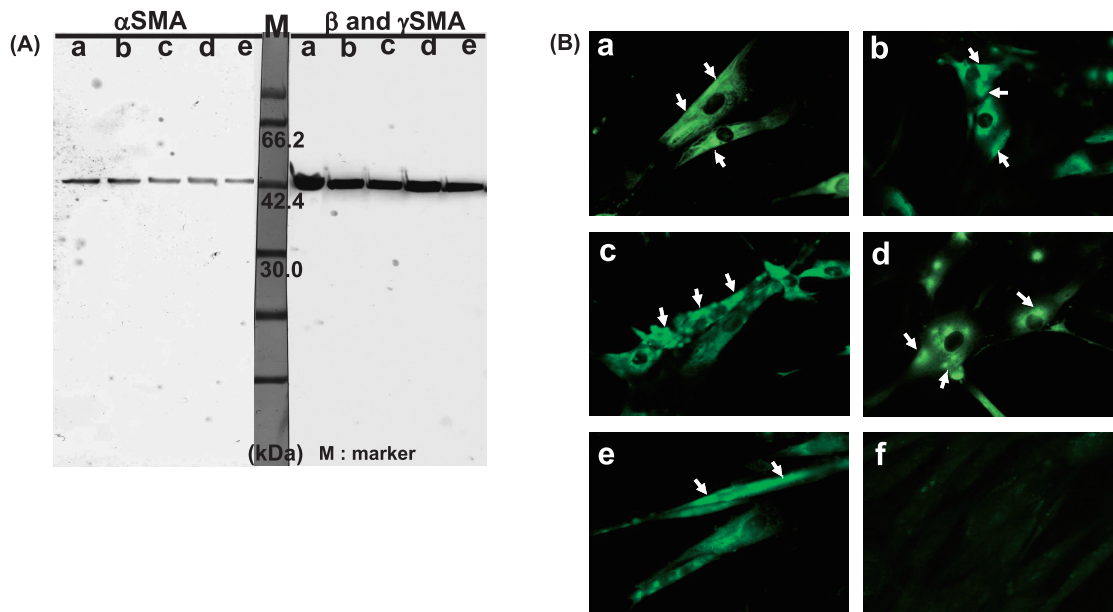


Fig. 4 (A) Production of  $\alpha$ SMA protein in established cell lines

The left membrane is a Western blot for  $\alpha$ SMA in lysates from Dup-1 (lane a), Dup-2 (lane b), Dup-3 (lane c) and Dup-4 (lane d) cells, and TGF $\beta$ 1-treated fibroblasts (lane e). The right membrane is a Western blot for an isoform of smooth muscle actin,  $\beta$  and  $\gamma$ SMA, which is universally expressed in fibroblasts. The alphabetical representation is the same as that in left membrane.

(B) Immunofluorescence microscopy photographs of  $\alpha$ SMA bundle in established cell lines

Microfilaments specific for  $\alpha$ SMA (arrows) either in bundles or dots occurred in Dup-1 (a), Dup-2 (b), Dup-3 (c) and Dup-4 (d) cells, and TGF $\beta$ 1-treated fibroblasts (e), but not in untreated fibroblasts (f).

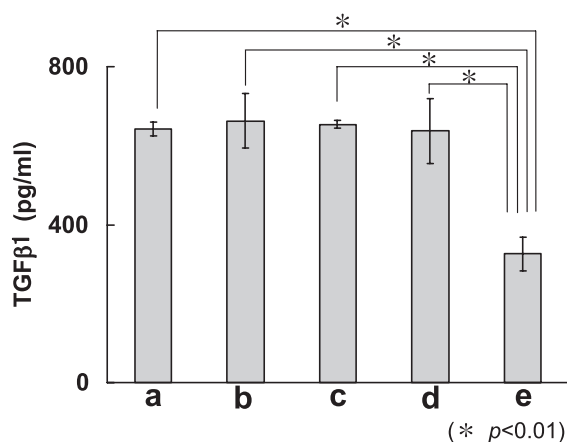


Fig. 5 Production of TGFβ1 in established cell lines  
Culture supernatants from Dup-1 (a), Dup-2 (b), Dup-3 (c) and Dup-4 (d) cells contained higher levels of TGFβ1, compared to normal fibroblasts (e).

Table 1 Implantability of Dupuytren's contracture cell lines into nude mice

Name of cell line	Numbers of mice	Numbers of mice with nodules		Implantability (%)
		positive	negative	
Dup 1	3	3	0	100
Dup 2	3	1	2	33
Dup 3	3	1	2	33
Dup 4	3	2	1	66

Each cell line was suspended in PBS, and  $2 \times 10^7$  cells in 0.2 ml were inoculated sc into the back of 5-week-old nude mice. Mice were sacrificed 3 weeks later.

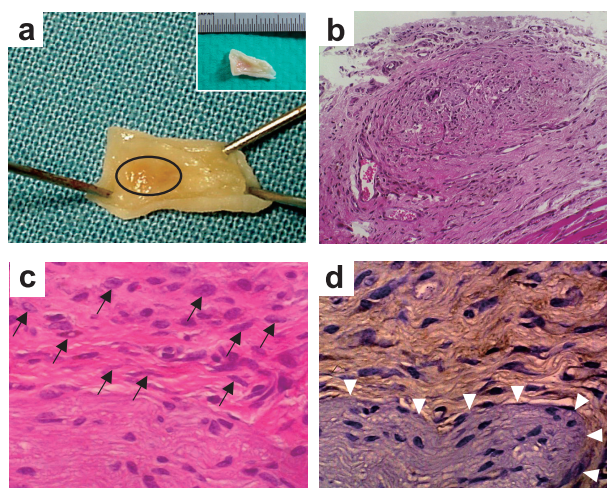


Fig. 6 Implantation of cell line in animals  
Millimeter-sized nodule is circled in a macroscopic photograph of a nodule from an implanted mouse (a). Nodular tissue is seen at subcutaneous region by HE stain at low magnification. (b) (X100) At high magnification, myofibroblasts are aligned disorderly in vascular-rich tissue by HE staining. (c, arrows) An immunohistochemical study showing αSMA-expressing cells is distinguishable from negative nervous tissue. (d, arrow heads) (X400)

Dupuytren's nodule at the early phase of involutinal stage. That is, myofibroblasts aligned disorderly in mature capillary-rich nodule. Myofibroblasts had spindle shaped nucleoli which contained fine and homogeneous chromatin, and mitotic activity was rarely observed (Fig. 6-b and -c). In immunohistochemical examination, myofibroblasts in the nodule reacted to anti-human  $\alpha$ SMA antibody (Fig. 6-d).

## DISCUSSION

Myofibroblasts are specially differentiated fibroblasts with a high expression level of  $\alpha$ SMA. We have established myofibroblastic cell lines from Dupuytren's nodules, which maintain a high level of  $\alpha$ SMA. The cell lines we established hold at least 10q and 19q chromosomes, in which  $\alpha$ SMA and TGFb genes are localized, and do not show a trisomy 8, one of the most common aberrations in Dupuytren's disease.<sup>18)</sup> The cell lines were confirmed to express  $\alpha$ SMA mRNA by RT-PCR method and the  $\alpha$ SMA protein by WB analysis. Immunofluorescence microscopy revealed that the cells were rich in filamentous structure specific for  $\alpha$ SMA. Electron microscopy showed the characteristics of myofibroblasts, i.e., bundles of microfilaments with interspersed dense patches, especially beneath the plasma membrane and well-developed rough endoplasmic reticulum. The cultured cells were arranged irregularly, running in random direction and occasionally with a cramped formation. The cell lines were transplantable to nude mice, in which a nodular lesion similar to the original Dupuytren's nodule was formed. All these findings indicate that the cell lines are myofibroblast cell lines.

The arrangement of cells was different between established cell lines and normal fibroblast cell line. The hypothesis on cell arrangement has recently been accepted, that is, the proliferation of transformed cell is regulated by the availability of hormone-like peptides or cytokines such as growth factors, not by cell contact and density-dependent regulation.<sup>19)</sup> We consider this hypothesis also applicable to these cell lines. Peter et al.<sup>20)</sup> reported the presence of TGF $\beta$ 1 in the serum-free conditioned medium of primary cultures of Dupuytren's cells and normal fibroblasts. The cytokine is responsible for the induction of  $\alpha$ SMA production in fibroblasts<sup>21)</sup> and for the increase in myofibroblast proliferation.<sup>22)</sup> We observed in the present study that the high levels of TGF $\beta$ 1 were secreted into the

supernatants of established cell lines, compared with normal fibroblast cell lines. The established cell lines produce TGF $\beta$ 1, which may stimulate themselves. Since the presence of TGF $\beta$  receptor and the production of other cytokines were not examined in the present cell lines, further studies are required.

Almost all *in vitro* studies on myofibroblasts from contractile lesions have been performed using primary culture, at most within 10 passages. The investigators of those previous studies would like to eliminate characteristic modification during repeated cell passages, such as transformation to the fibroblastic phenotype with reduction of  $\alpha$ SMA production. Vande Berg and Rudolph<sup>23)</sup> reported that myofibroblasts from both humans and laboratory animals could be successfully cultured *in vitro* through multiple passages, while, in later passages, dedifferentiation and cell degeneration occurred, leading to no growth by passage 15. In addition, Azzarone et al.<sup>12)</sup> reported that cells from Dupuytren's nodules could be cultured for six months, and that the cells revealed tumoral features. As they did not culture the cells longer, it is not clear whether the cell line was established as a long-time-cultured cell line. Indeed a primary culture is theoretically preferable for studying the characteristics of the cells from a contractile lesion, but our stable cell lines seem useful in the study of the characteristics of myofibroblasts because of maintained expression of  $\alpha$ SMA.

Bisson et al.<sup>1)</sup> mentioned that Dupuytren's nodule cells are different in the morphology as well as in the response to TGF $\beta$ 1 from cells of surrounding tissues such as the cord and flexor retinaculum. Whereas previous reports indicated that IFN $\gamma$  reduced  $\alpha$ SMA production in myofibroblasts *in vitro*<sup>7), 16), 17)</sup>, and *in vivo*,<sup>8)</sup> Hindman et al.<sup>24)</sup> demonstrated that IFN $\gamma$  did not reduce the production of  $\alpha$ SMA in cultures from surrounding tissue. The discrepancy may be due to the difference in the stage of the disease and/or sampling of lesions. To explain such discrepancy, a stable long-term-cultured cell line will be useful.

An animal model of Dupuytren's contracture was successfully reported as implants of Dupuytren's nodules into nude mouse.<sup>25)</sup> Since the implanted tissue in the mouse decreased in volume and production of tissue matrix, Kischer et al.<sup>25)</sup> stated that it is difficult to use the implant as a proposed model for the evaluation of therapy. The reason of such decrease may cause by a missing factor in tissue of Dupuytren's patient or

residual capacity for rejecting foreign tissue in host animals. Tissue implantation is related to many factors of graft. We may simplify an animal model by using implantation of culture cell. In this study, we implanted Dupuytren's cell lines and successfully observed a small nodule bearing human  $\alpha$ SMA antigen in nude mice. Whereas the implantability may indicate that the nodule is a possible model of Dupuytren's contracture, further studies are requiring on this nodule.

In conclusion, we established 4 myofibroblastic cell lines from Dupuytren's nodules. The cell lines continuously produced  $\alpha$ SMA and TGF $\beta$ , and were implantable into nude mice. Further studies on the beneficial use of the cell lines are required.

### ACKNOWLEDGEMENTS

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