High glucose promotes collagen synthesis by cultured cells from rat cervical posterior longitudinal ligament via transforming growth factor- β 1

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Abstract Non-insulin-dependent diabetes is known as a risk factor of ossification of posterior longitudinal ligament, but the mechanism has not been well understood. We hypothesized that hyperglycemia, as a typical characteristic of diabetes, is closely associated with ligament hypertrophy in ossification of posterior longitudinal ligament. In this in vitro study, we investigated the effect of high glucose on collagen synthesis and transforming growth factor- β 1 (TGF- β 1) production using cells isolated from rat cervical posterior longitudinal ligament. The cells were subjected to high D-glucose concentration (25 mM) media for 4 days. Notable increases were observed in gene expression and protein synthesis of collagen types I, III in the cells. The increase was inhibited in the presence of anti-TGF- β 1 antibodies. Production of TGF- β 1 by the cells was also increased significantly by high glucose concentration. Exogenous application of TGF- β 1 was confirmed to increase collagen synthesis of the cells. These data suggested that high glucose could promote collagen synthesis in the posterior longitudinal ligament mainly via endogenous TGF- β 1, resulting in hypertrophy of the ligament.

Keywords High glucose · Collagens · Transforming growth factor- β 1 · Ossification of posterior longitudinal ligament

Introduction

Ossification of posterior longitudinal ligament (OPLL) is a pathological extracellular hyperplasia and ectopic ossification of the posterior longitudinal ligament at the cervical and thoracic spine, causing myeloradiculopathy as a result of chronic compression on the spinal cord and nerve roots. Both systemic and local factors play important roles in the onset and progression of OPLL [21]. Among them, non-insulin-dependent diabetes (NIDDM) was confirmed as an independent risk factor for the onset and progression of OPLL [4, 25]; however, the mechanism has not been well understood.

Hyperglycemia, as a typical characteristic of NIDDM, is closely associated with extracellular matrix hypertrophy and collagen production in renal and cardiac fibroblasts by autocrine secretion of transforming growth factor- β 1 (TGF- β 1). So far, no literature exists on what exact glucose levels the ligament cells do see in vivo, however, 25–100 mmol/l glucose were utilized in most in vitro studies mimicking hyperglycemia [19, 20, 31, 37]. On the other hand, hypertrophied ligament characterized by an increase in collagen content was also observed in OPLL [27]. Among different kinds of collagen, overproduction of type XI collagen was histochemically demonstrated in surgical specimens of OPLL [9]. Increased secretion of type I collagen was also observed in the ligament cells isolated from OPLL in vitro study [8, 14, 17]. In the ttw mice, a model mice of OPLL, it was found that type I collagen positive cells proliferated and type II collagen positive cells decreased in the posterior longitudinal ligament during the formation process of OPLL [9]. Moreover, polymorphisms in genes of collagen VI, XI are closely associated with OPLL [7, 23, 30], although their precise roles in pathogenesis of the OPLL remain unknown. TGF- β 1 has been

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thought to play a critical role in the regulation of extracellular matrix and confirmed as an important local factor in the pathology of OPLL [11]. Therefore, we hypothesized that high glucose may stimulate the proliferation of extracellular matrix characterized as an increase in collagen production of posterior longitudinal ligament cells by autocrine secretion of TGF- β 1 and, thus, contribute to the onset or progression of OPLL. In the present study, we investigated the effect of high glucose on the in vitro production of TGF- β 1 and collagen synthesis in cultured cells derived from the posterior longitudinal ligament of rat cervical spine.

Materials and methods

Cell isolation and cell culture

This study was approved prospectively by the Animal Care and Use Committee of authors' institution. Three-month-old male Sprague–Dawley rats were euthanized by intravenous administration of 150 mg/kg pentobarbital sodium (Abbott Laboratories). Posterior longitudinal ligament tissues of cervical spine (C2–T1) were then obtained aseptically, avoiding ligament-bone insertions. The ligaments were washed with phosphate-buffered saline several times, minced into about 1 mm² pieces, and then placed in 35 mm culture dishes (Corning) containing Dulbecco's modified Eagle's media (DMEM, Sigma) supplemented with 10% heat inactivated fetal bovine serum (FBS, Sigma) and 0.2 mM L-ascorbic acid 2-phosphate (Sigma). The explants were incubated in controlled 95% air/5% CO₂ atmosphere at 37°C. Cells migrating from the explants and becoming confluent were harvested with 0.25% trypsin with 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma), and replanted in 60 mm culture dishes (Corning) to form a monolayered culture. The second and fifth passage cells were used in the following studies. The subcultured ligament cells were observed under a phase-contrast microscope before they were provided for each experiment to confirm that they had retained the morphologic characteristics as the posterior longitudinal ligament specimen.

In vitro application of stimulation to cultured cells

For these experiments, 10⁵ cells/well were plated into 12-well cell culture plates (Corning) in DMEM and 10% FBS, and at confluence, they were made quiescent by incubation in DMEM with 0.5% FBS. After 12 h, cells were cultured for 4 days in 3 ml DMEM with 1% FBS, 0.2 mM L-ascorbic acid 2-phosphate, and one of the following additives (Table 1): 5.6 mM D-glucose (low

glucose), 25 mM D-glucose (high glucose), 5.6 mM D-glucose plus 19.4 mM mannose (osmotic control), low glucose media in the presence of 0.01, 0.1, 1, 10 ng/ml rh TGF- β 1 (R&D), low- or high-glucose media in the presence of 2 μ g/ml neutralizing rabbit anti-rat TGF- β 1 antibody or a similar concentration of normal isotypic rabbit IgG (R&D). The preliminary study showed that the cells could not survive under this condition over 6 days. Therefore, after 4 days, culture supernatants were harvested and measured for secreted proteins in enzyme-linked immunosorbent assay (ELISA), and cells were lysed for RNA isolation and semi-quantitative RT-PCR.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

The gene expressions of alpha-1 type I collagen (COL1a1), alpha-1 type II collagen (COL2a1), alpha-1 type III collagen (COL3a1), alpha-2 type VI collagen (COL6a2), alpha-1 type X collagen (COL10a1), alpha-1 type XI collagen (COL11a1) mRNA and TGF- β 1 were examined by semi-quantitative RT-PCR. Total RNA was extracted by the guanidinium thiocyanate–phenol–chloroform extraction method (TRIzol Reagent, Gibco). The quantity and purity of the isolated RNA were measured at two optical densities, OD₂₆₀ and OD₂₈₀, and analyzed on a 0.5× TBE (0.045 mol/l tris borate, 0.001 mol/l EDTA) 1% agarose gel to check the integrity of the RNA. First strand cDNA was synthesized from 1 μ g of total RNA using the cDNA synthesis kit (Takara). For PCR amplification, specific oligonucleotide primers to rat sequences were designed by program Oligo 4.0 (National Biosciences, Inc) on the basis of sequences in GenBank (Table 2). Reactions were carried out using the Taq Hot Start PCR Kits (Takara) performed in a Perkin-Elmer 9600 thermal cycler according to manuals. The samples were cycled for 35 times, each consisting of 30 s at 94°C, 30 s at primer-specific annealing temperature indicated in Table 2, 1 min at 72°C, and then 72°C for 5 min. PCR products were electrophoresed in a 1.5% agarose gel and then stained with ethidium bromide. Photos of the gels were taken on an ultraviolet (UV) light box (UV Transilluminator UVP, Inc.) and scanned by a computer scanning densitometer. The density of each band was referred to that of GAPDH on the same sample to standardize any variations in the amount of mRNA between the lanes. Each sample was evaluated in triplicate.

Enzyme linked immunosorbent assay (ELISA)

The amount of procollagen type I amino-terminal peptide (PINP), procollagen type III amino-terminal peptide (PIIINP) and TGF- β 1 secreted into the media during 4 days

Table 1 Cell culture and stimulation conditions

Experiment	Basic culture medium	Additives	Culture appliance
Tissue culture	DMEM, 10% FBS	0.2 mM L-ascorbic acid 2-phosphate	35 mm culture dish
Subculture	DMEM, 10% FBS	0.2 mM L-ascorbic acid 2-phosphate	60 mm culture dish
Cell quiescence	DMEM, 0.5% FBS	0.2 mM L-ascorbic acid 2-phosphate	12 well culture plate
Low	DMEM, 1% FBS	5.6 mM D-glucose	12 well culture plate
		0.2 mM L-ascorbic acid 2-phosphate	
High	DMEM, 1% FBS	25 mM D-glucose	12 well culture plate
		0.2 mM L-ascorbic acid 2-phosphate	
Osmotic	DMEM, 1% FBS	5.6 mM D-glucose	12 well culture plate
		19.4 mM mannose	
		0.2 mM L-ascorbic acid 2-phosphate	
TGF- β 1	DMEM, 1% FBS	0.01, 0.1, 1, 10 ng/ml TGF- β 1	12 well culture plate
		5.6 mM D-glucose	
		0.2 mM L-ascorbic acid 2-phosphate	
High + anti-TGF	DMEM, 1% FBS	2 μ g/ml anti-TGF- β 1 antibody	12 well culture plate
		25 mM D-glucose	
		0.2 mM L-ascorbic acid 2-phosphate	
High + IgG	DMEM, 1% FBS	2 μ g/ml IgG	12 well culture plate
		25 mM D-glucose	
		0.2 mM L-ascorbic acid 2-phosphate	

All of the cultures and stimulations were incubated in controlled 95% air/5% CO₂ atmosphere at 37°C. All of the stimulations last for 4 days. *Low* cells cultured in low glucose media, *High* cells stimulated by high glucose, *Osmotic* cells stimulated by osmotic control, *High + anti-TGF* cells subjected to high glucose medium with 2 μ g/ml of TGF- β 1 antibody, *High + IgG* cells cultured in high glucose medium with 2 μ g/ml of rabbit IgG

Table 2 Sequences of RT-PCR primers used in the present study

Gene	Primer sequences	Product size (bp)	Anneal temperature(°C)
COL1a1	5' CCAACAACCCAACTCAAT 3' 5' GTTAGGCTCCTTCAATAGTCC 3'	509	53.0
COL2a1	5' GAGCGGAGACTACTGGATTGA 3' 5' TCTGGACGTTAGCGGTGTT 3'	242	57.5
COL3a1	5' ATGGTGGCTTTCAGTTCAG 3' 5' CAATGTCATAGGGTGCGATA 3'	351	53.8
COL6a2	5' GGGTGTTTCGCAGTGGT 3' 5' CAGGCGATGGAGTAGAGG 3'	160	56.2
COL10a1	5' GGGGACTCACGTTTGGGTAG 3' 5' ATCGGGCGATGGAATGG 3'	291	55.8
COL11a1	5' TGAGTCTCCTCGGCGGGTAT 3' 5' TCTTCATTAGGGGAGGTCGT 3'	243	54.2
TGF- β 1	5' GCGGCTGCTCGCTTTGTA 3' 5' GCCACTCAGGCGTATCAG 3'	362	57.0
GAPDH	5' TCAACGGCACAGTCAAGG 3' 5' ACCAGTGGATGCAGGGAT 3'	470	57.7

incubation after the stimulation was assayed using ELISA kits (Takara) according to manuals. Measurements of total TGF- β 1 levels required prior activation of the samples with acid. The results were standardized by the total mRNA

content of the cells and expressed as nanogram (PINP, PIIINP or TGF- β 1) per microgram (RNA) or percentage to avoid the influence of the high glucose on cell proliferation. Each sample was evaluated in triplicate.

Statistical analysis

The data are presented as mean $\pm$ standard deviation of six samples of triplicate cultures. Differences between various culture conditions were evaluated by one-way ANOVA, followed by Student–Newman–Keul (SNK) for multiple comparisons using the SPSS 11.5 statistical software program. A value of $P < 0.05$ was considered to represent a significant difference.

Results

Phenotypic characteristic of the cells

The posterior longitudinal ligament cells (PLLs) displayed mainly typical fibroblastoid morphology with a fusiform spindle shape. The morphological characteristics did not differ from each specimen or each passage of cells.

Effect of high glucose on the expression of collagen mRNA and the production of PINP, PIIINP

The cells expressed alpha-1 type I collagen (COL1a1), alpha-1 type III collagen (COL3a1), alpha-2 type VI collagen (COL6a2), alpha-1 type X collagen (COL10a1), alpha-1 type XI collagen (COL11a1) mRNA (Fig. 1); COL2a1 mRNA was absent (data not shown). The mRNA expression of COL1a1 and COL3a1 in cells stimulated by high glucose for 4 days was increased by 93.7 and 126%, respectively, when compared with those in low glucose media ($P < 0.05$, Fig. 1a). The synthesis of PINP and PIIINP in supernatant of cells induced by high glucose were raised by 188 and 123%,

respectively, compared to low glucose ($P < 0.05$, Fig. 1b). This increase was not due to the high osmolarity to which the cells were exposed because there were no effects on the mRNA expression when cells were exposed to the osmotic control media (Fig. 1a). No significant alteration was observed in COL6a2, COL10a1 and COL11a1 mRNA expression of cells after stimulation (Fig. 1a).

Effect of high glucose on the expression of mRNA and the production of TGF- β 1

Figure 2 shows the mRNA expression of TGF- β 1 exhibited by the cells both in low and high glucose media. The mRNA expression of TGF- β 1 in cells cultured in high glucose media for 4 days was increased by approximately 30% compared with those in low media ($*P < 0.05$; Fig. 2a). The concentration of TGF- β 1 in the low, high glucose media and osmotic control media for 4 days was approximately 0.123, 0.254, and 0.122 ng/ μ g RNA, respectively ($*P < 0.05$; Fig. 2b). The concentration of TGF- β 1 in high glucose media is approximately 2.1 times greater than that in low glucose media. No significant change of gene expression or protein production of TGF- β 1 was shown in cells in the osmotic control media compared with those in low glucose media (Fig. 2b).

Effect of TGF- β 1 antibodies on collagen mRNA expression

To determine the neutralizing effect of anti-TGF- β 1 antibodies on endogenously produced TGF- β 1 by the PLL cells, the cells were subjected to high glucose for 4 days in the presence or absence of TGF- β 1 antibody at a

Fig. 1 Effect of various media on collagen synthesis in the PLL cells for 4 days. (low cells cultured in low glucose media, high cells stimulated by high glucose, osmotic cells stimulated by osmotic control). **a** PCR products of COL1a1, COL3a1, COL6a2, COL10a1, COL11a1 mRNA were electrophoresed in agarose gel stained with ethidium bromide. The density of each band was numerically quantitated using NIH Image software. Each bar represents the mean value of six experiments. The Y-axis represents the ratio of collagen

values to GAPDH values. Expression of COL1a1 and COL3a1 is significantly increased ($*P < 0.05$) when the cells are subjected to high glucose for 4 days ($n = 6$). No alteration was shown in expression of COL6a2, COL10a1 or COL11a1. **b** The content of PINP and PIIINP in cell supernatants. The Y-axis represents the content of PINP or PIIINP in nanograms per micrograms RNA. The contents of PINP and PIIINP in high glucose media were significantly higher than those in other media ($*P < 0.05$, $n = 6$)

Fig. 2 Effect of high glucose on TGF- β 1 synthesis in the PLL cells (*low* cells cultured in low glucose media, *high* cells stimulated by high glucose, *osmotic* cells stimulated by osmotic control). **a** Expression of TGF- β 1 mRNA is significantly increased ($*P < 0.05$)

in cells subjected to high glucose for 4 days. **b** The release of TGF- β 1 into the culture medium of cells subjected to high glucose for 4 days is significantly higher ($*P < 0.05$) than that of the controls

concentration of 2 μ g/ml. The increase in mRNA expression of COL1a1 and COL3a1 in response to high glucose was significantly inhibited by the addition of TGF- β 1 antibody (COL1a1 100%, COL3a1 62%, $P < 0.05$), but it was not inhibited by the addition of rabbit IgG (Fig. 3a). Notably, the mRNA expression of COL1a1 induced by high glucose was completely inhibited by TGF- β 1 antibody (Fig. 3a). The addition of TGF- β 1 antibody significantly inhibited the increment of PINP and PIIINP synthesis induced by high glucose (PINP 96%, PIIINP 91%, $P < 0.05$, Fig. 3b). An equal amount of rabbit IgG had no effect on PINP and PIIINP synthesis (Fig. 3b).

Effect of exogenous TGF- β 1 on the expression of collagen mRNA

The mRNA expression of COL1a1 and COL3a1 in cells increased in a dose-dependent manner when the cells were treated with recombinant human (rh) TGF- β 1 ($P < 0.05$, Fig. 4a). After standardization by GAPDH from the same sample, these are the approximate ratios of the increments of COL1a1 and COL3a1, respectively, at various concentrations of rh TGF- β 1: 133.3 and 157.9% at a concentration of 0.01 ng/ml rh TGF- β 1; 151.1 and 236.4% at a concentration of 0.1 ng/ml; 175.2 and 272% at a concentration of 1 ng/ml; and 193.1 and 364.8% at a concentration of 10 ng/ml ($P < 0.05$, Fig. 4a). The PINP and PIIINP synthesis also rose in a dose-dependent manner when the cells were treated with rh TGF- β 1 ($P < 0.05$, Fig. 4b). The content of PINP and PIIINP synthesis, respectively, in supernatant at different concentrations of rh TGF- β 1 was measured: 0.33 (109%) and 1.26 (208%) ng/ μ g RNA at a concentration of 0.01 ng/ml rh TGF- β 1; 0.69 (203%) and 3.88 (391%) ng/ μ g RNA at a concentration of 0.1 ng/ml; 1 (394%) and 6.19 (514%) at a concentration of 1 ng/ml; and 2.84 (762%) and 13.08 (937%) at a concentration of 10 ng/ml ($P < 0.05$, Fig. 4b).

Discussion

The rationale for conducting these studies relates to observation of the close association of OPLL with NIDDM [1, 4, 25]. NIDDM was confirmed as an independent risk factor in OPLL clinically [25]. The key factor in the mechanism still remains unknown, however, so it is feasible to assume that hyperglycemia, as a typical characteristic of NIDDM, may play important roles in the pathology of OPLL. The high glucose concentration used in this research was 25 mM. In prior tests, the cells could not tolerate higher glucose concentrations of 50 or 100 mM for less than 8 h, possibly due to the high osmolality, and they seem not to respond to concentrations below 25 mM (data not shown). To avoid the interference by growth factor in serum, we use low serum (1% FBS) medium during the stimulation of high glucose. However, in this medium, the cells could not survive for 6 days regardless of addition of low or high glucose. We thought that the long-term lack of nutrition mainly contribute to the death of the cells and did not measure apoptosis in the cells. Therefore, according to our prior tests and literatures [3, 16, 34], the term of culture in the present study was determined as four days. On the other hand, spinal ligaments, like the ligaments in other sites, contain few cells and abundant extracellular matrix. They are parallel-fibered collagenous tissues. The content and type of collagen directly influence the structure and property of ligaments. The potential role of collagen types in the pathogenesis of OPLL is supported by evidence from gene analysis and histopathological examination [7, 23, 32]. Therefore, the research on the alteration of collagen in ligament could provide a clue for the onset or progression of OPLL. Since the presence of collagen types I, II, III, VI, X and XI had been confirmed in the spinal ligament of OPLL [7, 30, 32], all of these collagen types were examined in the present study.

Fig. 3 Effect of TGF- β 1 antibodies on COL1a1 and COL3a1 mRNA expression and PINP and PIIINP synthesis induced by high glucose in the PLLs for 4 days. Each column represents mRNA expression or supernatant of the specimen. (*low* cells cultured in low glucose media, *high* cells stimulated by high glucose, *osmotic* cells stimulated by osmotic control, *low + anti-TGF* cells cultured in low glucose medium with 2 μ g/ml of TGF- β 1 antibody, *high + anti-TGF* cells subjected to high glucose medium with 2 μ g/ml of TGF- β 1 antibody, *high + IgG* cells cultured in high glucose medium with 2 μ g/ml of rabbit IgG, *low + IgG* cells cultured in low glucose medium with 2 μ g/ml of rabbit IgG.) **a** The increase in COL1a1 mRNA expression caused by high glucose is completely inhibited by the addition of TGF- β 1 antibody (* P < 0.05). The increase in COL3a1 mRNA expression caused by high glucose is notably inhibited by the addition of TGF- β 1 antibody (* P < 0.05). The equal amount of rabbit IgG had no effect on the COL1a1 and COL3a1 mRNA expression of cells in low or high glucose media. **b** The increment of PINP and PIIINP in supernatant of cells induced by high glucose was notably inhibited by the addition of TGF- β 1 antibody (* P < 0.05). The equal amount of rabbit IgG had no effect on PINP and PIIINP synthesis of cells in low or high glucose media

The main results of this study show that high glucose concentration in the culture media increases gene expression and protein synthesis of collagen types I and III, particularly, in cells of the posterior longitudinal ligament of rat cervical spine; this action mimics the effects of rh TGF- β 1 on ligament cell collagen synthesis. In fact, the stimulation of collagen synthesis by high glucose appears to be mediated largely, or at least in part, by bioactivities of endogenous TGF- β 1.

Fig. 4 Effect of various concentration of exogenous TGF- β 1 on COL1a1 and COL3a1 mRNA expression and PINP and PIIINP synthesis in the PLLs (0 0 ng/ml TGF- β 1, 0.01 0.01 ng/ml TGF- β 1, 0.1 0.1 ng/ml TGF- β 1, 1 1 ng/ml TGF- β 1, 10 10 ng/ml TGF- β 1). **a** Expression of COL1a1 and COL3a1 mRNA increases in a dose-dependent manner when the cells are treated with various concentrations of exogenous TGF- β 1 (* P < 0.05 vs. 0 ng/ml TGF- β 1). **b** PINP and PIIINP synthesis increases in a dose-dependent manner when the cells are treated with various concentrations of exogenous TGF- β 1 (* P < 0.05 vs. rest)

Similar results have been reported in collagen synthesis in smooth muscle cells [12], cardiac fibroblasts [31] and mesangial cells [37] stimulated by high glucose; however, different results were obtained from skin and ileum fibroblasts in which collagen synthesis was not affected by glucose concentration [5, 34]. This difference may be attributed to the various origins or types of fibroblasts, different glucose concentrations, or stimulation time. Collagen I and III are the main collagen protein content in the ligament and their increment is an important cause of ligament tissue hypertrophy [6]. The ligamentous hypertrophy may play significant roles in both onset and progression of OPLL. First, it is unclear how a normal ligament develops into an OPLL and whether there is some transition state between the two conditions. Hypertrophy of the posterior longitudinal ligament (HPLL) has been considered to be a precursor of OPLL, although the mechanism and whether or not HPLL even develops into OPLL remains unclear [18, 35]. Local factors, such as extruded disc material, were

thought to be the cause of thickening of the PLL [35]. It is also possible that other systemic factors, like hyperglycemia, initiate the process of ligamentous hypertrophy. The results showing that high glucose could induce collagen I and III production may provide some evidence for the systemic hypothesis. Second, if ectopic bone formation is limited to the original thickness of the posterior longitudinal ligament, ossification of the posterior longitudinal ligament never compresses the spinal cord. Specifically, symptoms caused by ossification of the posterior longitudinal ligament should be attributed mainly to the ligament's growth in thickness, including ligament tissue hypertrophy and the differentiation of hypertrophic ligament fibrous tissue into cartilage tissue, and subsequent ossification [27]. The results of the present study indicated that the hyperglycemia-induced elevation of collagen I and III may increase the thickness of hypertrophic posterior longitudinal ligament and, thus, aggravate the compression of hypertrophic ligaments on the spinal cord clinically.

Type II collagen is distributed mainly at the ligament insertion of the vertebra body or in chondrocytes differentiated from ligament cells in the stage of a cartilage template formation in OPLL. Subsequently, the proliferating chondrocytes undergo a process of hypertrophy and express the gene of collagen X. In the present study, collagen type II gene expression could not be detected regardless of stimulation. In this study, the specimens were harvested from the ligament insertion of the vertebra body of a normal rat. The less dominant chondroblastic cells may have been curtailed during cell passage, thus the cultured cells may have included only the predominant fibroblastic cells that did not have the potential to produce collagen type II. However, the implication of collagen X gene expression in these cells remains unknown although its expression was not affected by high glucose. The polymorphisms in COL6a1 gene, encoding type VI collagen, and collagen 11a2 (COL11a2) gene, encoding type XI collagen, were found to have a significantly higher prevalence in patients with OPLL than those in control subjects [23, 24, 32]. However, it is unknown what roles they may play in the pathology of OPLL. Both of them are minor components of extracellular matrix in ligament and whether they have relationship with the ligament hypertrophy is also unknown. Furthermore, OPLL is a multifactor disease, so it is impossible to use single factor to clarify the whole pathological mechanisms. Therefore, according to the result that the gene expression of these types of collagen was not affected by high glucose, we only think that the two types of collagen may be not involved in our hypothesis. Of course, in other hand, we also could not exclude the possibility that the 4-day- culture is not enough for the stimulation of process of hypertrophy and ossification, and that the change of these kinds of collagen may

not appear. Therefore, we also think that further experiments need to be devised to testify the possibility.

Several biochemical mechanisms explaining the effects of high glucose on cells have been proposed, including osmotic, activation of protein kinase C, and endogenous TGF- β 1, among others [31, 36]. No effect of osmosis on collagen synthesis was observed, therefore, we focused on TGF- β 1, which is well known as a regulatory polypeptide that enhances matrix synthesis and an important factor associated with the extracellular matrix of OPLL [10, 11, 13, 15, 28]. The biological activity of TGF- β 1 is governed by dissociation of mature TGF- β from an inactive, latent TGF- β complex in a process that is critical to its role in vivo [2]. Activation of the samples with acid was required before obtaining measurements of total TGF- β 1 levels released in the culture media. The effect of high glucose concentrations on the production of TGF- β 1 in the cells was confirmed. The gene expression and protein synthesis of TGF- β 1 in the cells after 4-day stimulation of high glucose increased by approximately 1.3 and 2.1 times, respectively, compared to those in the controls. Similar results have been reported by other investigators; high glucose increased the production of TGF- β 1 1.76 to 2-fold in mesangial cells [20, 37], and 11.1-fold in smooth muscle cells [22]. Thus, the increase in TGF- β 1 production induced by high glucose seems to vary among cell types. Next, the effect of TGF- β 1 on the production of collagen I and III was analyzed. Exogenous TGF- β 1 increased the mRNA expression and synthesis of collagen types I and III in a dose-dependent manner. The fact that high glucose-induced collagen production was neutralized significantly by TGF- β 1 antibody and mimicked by rh TGF- β 1 suggested that high glucose induces endogenously active TGF- β 1 production in ligament cells. It suggested, too, that the active TGF- β 1 is the main factor for the increment of collagen synthesis in ligament cells. Notably, the endogenous expression of TGF- β 1 was observed in normal cells and the collagen production in control cells was not inhibited by TGF- β 1 antibody (Fig. 3b, low versus low + anti-TGF), suggesting that the normal cells had the potential to produce collagens independently of TGF- β 1. Also of note in the present results is the finding that the maximal level of TGF- β 1 released from the cells was approximately 0.34 ng/ μ g RNA, bearing a concentration of approximately 0.14 ng/ml in the culture medium. This amount of endogenous TGF- β 1 stimulates production of collagen type I and III much less significantly than does almost an equal amount of exogenous TGF- β 1. There are two possible explanations for this difference. First, glucose-induced endogenous TGF- β 1 may interact with the extracellular matrix changed by high glucose, and the matrix-TGF- β 1 interaction may influence its bioactivity. These interactions, however, do not affect the results of this study,

which showed high glucose-induced promotion of collagen synthesis in the PLL cells by endogenous TGF- β 1. The proportion of active TGF- β 1 among the high glucose-induced endogenous TGF- β 1, though, had not been measured. Second, normal cells were the targets of exogenous TGF- β 1 while transformed cells affected by high glucose were the targets of endogenous TGF- β 1. Namely, other alterations, which may exist after high glucose stimulation, had an impact on TGF- β 1 function on cells, although the detail alterations require further investigations. In addition, a possibility remains that the cells could release not only TGF- β 1, but also other growth factors which could affect collagen production in response to high glucose, independently or in concert with each other, such as CTGF and IGF-1 [19]. The present study strongly suggested that the improvement of collagen synthesis is mainly caused by high glucose-induced active TGF- β 1.

In previous in vitro studies, cells were always derived from patients with OPLL or other degenerative spine diseases, such as cervical disc herniation, cervical spondylosis myelopathy, and cervical canal stenosis, and they had already acquired some characteristics differing from normal ligament cells [29]. Thus, it may influence research on initiation mechanisms in the pathogenesis of OPLL. Therefore, in the present study, we used the cells cultured in vitro from animals to avoid the aforementioned interference to investigate the correlation between diabetes and the onset of OPLL. Indeed, animal experiment always has some limitations which are difficult to avoid completely. Although thus, there can be found some studies from other species for OPLL [1, 9, 26, 33]. The cultured cells used in the present study were isolated from rat posterior longitudinal ligament of cervical spine. Although special attention was paid in harvesting and trimming the surgical specimens, it is possible that the cultures contained multiple cell populations. The phenotypic similarity of each cell culture was confirmed under a phase-contrast microscope before starting each study. Since no considerable difference was noted in phenotype or arrangement of the cells, it was believed that the polygonal cells were the predominant populations of posterior longitudinal ligament of cervical spine that produced collagen and TGF- β 1. It was not possible, by any means in the present study, to identify from which type of cells collagens and TGF- β 1 originated.

This is the first report known to investigate the correlation, if any, between the ligaments' hypertrophy of OPLL and hyperglycemia by in vitro test. It was concluded that high glucose has a significant promotion on some types of collagen production in posterior longitudinal ligament cells; it was also assumed that these alterations caused by high glucose may contribute to the ligament hypertrophic process in the pathogenesis or progression of OPLL.

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