Int J Clin Exp Med 2015;8(2):2233-2239
www.ijcem.com /ISSN:1940-5901/IJCEM0004358

*Original Article*

# TGF-β1 related inflammation in the posterior longitudinal ligament of cervical spondylotic myelopathy patients

Jia-Zeng Wang[1,2*], Xiu-Tong Fang[1*], E Lv[3], Fang Yu[1], Zhen-Wei Wang[1], Hong-Xing Song[1]

*[1]Department of Orthopaedics, Beijing Shijitan Hospital, Capital Medical University, Beijing 100038, China; [2]Shandong Medical College, Linyi 276000, Shandong, China; [3]Department of Neurology, Key Laboratory for Neurodegenerative Disorders of The Ministry of Education, Capital Medical University, Beijing 100069, China. [*]Equal contributors.*

Received December 2, 2014; Accepted February 29, 2015; Epub February 15, 2015; Published February 28, 2015

**Abstract:** Aim: This study aimed to elucidate the pathogenesis of posterior longitudinal ligament (PLL) hypertrophy. Methods: Cervical PLL specimens were collected from CSM patients during surgery (n = 30) and during routine autopsy (n = 14), and processed for histological examination (HE staining and Masson's Trichrome staining) and IHC (CD3, CD68, CD31, TGF-β1 and collagen II). In addition, the mRNA expression of collagen I was detected in cervical PLL specimens from 16 CSM patients (n = 16) and from routine autopsy (n = 16) by RT-PCR. Results: Obvious fibrosis, cartilage metaplasia and calcification were found in the cervical PLL of CSM patients. In the degenerated PLL, $CD68^+$ macrophages were frequently identified, $CD3^+$ T lymphocytes were occasionally found, and many newly generated small vessels were also present. In the degenerated PLL, of the number of TGF-β1 positive cells increased markedly when compared with control group. IHC indicated TGF-β1 was secreted by macrophages. RT-PCR showed a significantly lower mRNA expression of collagen I in the PLL of CSM patients as compared to control group. Conclusions: Macrophages are the major type of inflammatory cells involved in the cervical PLL degeneration, and TGF-β1 is related to the cervical PLL degeneration. TGF-β1 is mainly secreted by macrophages. Anti-inflammation may serve as an alternative non-surgical treatment and prophylactic strategy for PLL degeneration.

**Keywords:** Cervical spondylotic myelopathy, posterior longitudinal ligament, degeneration, inflammation, transforming growth factor beta 1

## Introduction

Cervical spondylotic myelopathy (CSM) is a common disease in middle-aged and elderly people, and the degeneration of posterior longitudinal ligament (PLL) is one of major causes of CSM [1-4]. Surgery is a main treatment of choice for CSM. However, clinical findings show that simple excision of the herniated intervertebral disc or osteophyte fail to achieve favorable outcome in some patients, but symptoms may be relieved significantly after removal of the degenerated PLL simultaneously. This may be ascribed to more effective spine decompression after resection of the degenerated PLL [5]. The roles of PLL degeneration in the pathogenesis and treatment of cervical spondylosis have attracted increasing attentions. Although numerous studies have been conducted to investigate the ossification of PLL, few studies focus on the PLL degeneration before its ossification, and the specific mechanism of PLL degeneration is still not clear [6-8].

Previous pathological studies showed the fracture and disordered arrangement of collagenous and elastic fibers and an increase in scar tissues following PLL degeneration, causing ligament hypertrophy and fibrosis, and compromised elasticity [9, 10]. Our previous studies indicated cyclooxygenase-2 expression increased in the PLL of patients with CSM, suggesting that PLL degeneration is closely related inflammation [11]. Nevertheless, the inflammatory mechanism of PLL degeneration is still poorly understood. In the present study, histology, immunohistochemistry (IHC) and RT-PCR were employed to investigate: 1) the pathologi-

**Table 1.** Primers and their sequence

| Gene | Primer Sequence (5' 3') | Product Size (bp) |
|---|---|---|
| CollA1 | Forward: AAGAGGAAGGCCAAGTCGAG | 156 bp |
| | Reverse: AGATCACGTCATCGCACAAC | |
| CybB | Forward: CCGCATCGTTGGGGACTGGA | 109 bp |
| | Reverse: CAAAGGGCCCATCAACCGCTATCT | |

cal characteristics of degenerated cervical PLL; 2) collagen I mRNA expression in the PLL of CSM patients and healthy controls; 3) the cellular compositions (macrophages, vascular endothelium and T lymphocytes) of PLL of CSM patients; 4) the expression of transforming growth factor beta1 (TGF-β1) in the degenerated PLL. Our findings may provide evidence for the inflammatory mechanism of PLL degeneration and offer a new approach for the prevention and non-surgical treatment of PLL degeneration.

## Materials and methods

### *Sample collection*

Cervical PLL specimens were collected during the anterior decompression surgery from patients with CSM (38-82 years old, mean age 53.7, n = 30) and from subjects without CSM during routine autopsy (43-80 years old, mean age 61.4, n = 14). All the specimens were collected at C4-6 of PLL. The protocol was approved by the Ethics Committee at Medical University Graz (EK-number: 21-059 ex 09/10, LKH, Graz) and written informed consent obtained before study.

### *Histological examination*

PLL specimens were fixed in 10% neutral formaldehyde for 48 h, embedded in paraffin and cut into sections (3 μm). Sections were processed for HE staining and Masson trichrome staining, followed by histological examination.

### *Immunohistochemistry for CD3, CD68, CD31 and TGF-β1*

Rabbit anti-human CD3 monoclonal antibody, mouse anti-human CD31 monoclonal antibody and mouse anti-human CD68 antibody were purchased from Fuzhou Maxim Biotech Development Co., Ltd. Rabbit anti-human TGF-β1 polyclonal antibody was from Wuhan Boster Bioengineering Co., Ltd. The corresponding second antibody and IHC kits were from the Germany Vector Company. Immunohistochemistry was performed using Streptavidin-Peroxidase (SP method). The details of IHC for collagen II have been reported in our previous study [11].

### *Biological detection*

Additional PLL specimens were collected during anterior surgery for CSM from 16 patients (32-82 years old, mean age 49.2), and 18 samples were collected during autopsy from subjects without CSM (47-80 years old, mean age 56.3). Total RNA was isolated and processed for real-time reverse transcription-polymerase chain reaction (RT-PCR) according to previously reported [11]. Primers were designed according to the published sequences of humans (**Table 1**). CybB gene was used as an internal reference.

### *Statistical analysis*

Data are expressed as mean ± standard deviation (SD) and comparisons were done with an unpaired Student's t-test. A value of $P < 0.05$ was considered statistically significant.

## Results

### *Pathological characteristics of degenerated cervical PLL*

PLL is mainly composed of fibrous tissues with regular arrangements. The fibroblasts with fusoid nuclei in these fibrous tissues were distributed in the same direction (**Figure 1A**, **1B**). The regular structure is destroyed in the presence of PLL degeneration. Fracture and disordered arrangement of collagenous and elastic fibers were present following PLL degeneration, and compensatory fibrosis appeared in the degenerated PLL. Small vessels were also found in the PPL with severe degeneration, and even patchy calcification was present in some ligaments (**Figure 1C**, **1D**). A progressive transition area was not observed between degenerated PLLs and control PLLs. A wide variety of cells infiltrated the degenerated PLL (**Figure 1E**). Collagen II expression was found around the chondrometaplastic cells and in the matrix,

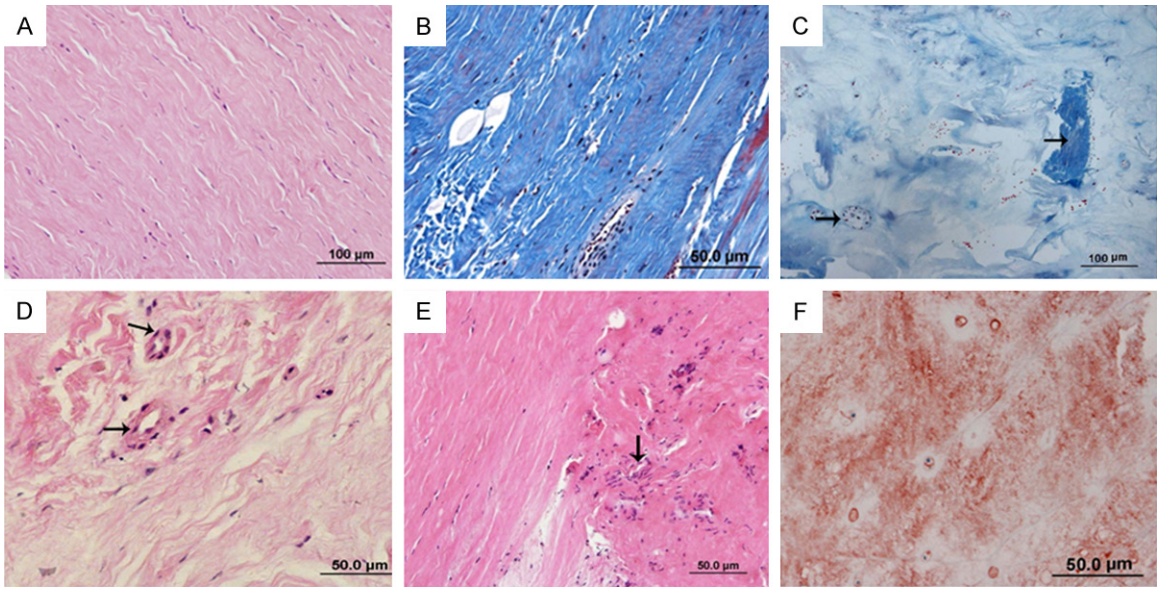


**Figure 1.** Pathological characteristics of degenerated PLL. A: HE staining of the PLL in control group; B: Masson's trichrome staining of the PLL in control group; C: In the PLL of patients cases, the fibers showed disordered arrangement, patchy calcification (dashed arrows) and increase in small vessels (solid arrow) (Masson's trichrome staining); D: In the PLL of patients cases, a plenty of newly formed small vessels was observed (HE staining); E: Apparent infiltration of cells in the PLL of patients cases (HE staining); F: Immunohistochemistry for collagen II in the PLL of patients cases.

but there was no marked difference between two group overall (**Figure 1F**).

*Infiltration of cells in the degenerated PLL*

A plenty of $CD68^+$ macrophages and a few $CD3^+$ T lymphocytes were found in the degenerated PLL. Results showed most $CD3^+$ cells mainly localized around vessels (**Figure 2A**). In degenerated PLL, $CD68^+$ cells showed different morphologies and were widely distributed (**Figure 2B**). In addition, $CD68^+$ cells were found in both degenerated ligaments and control ligaments. Formation of small vessels was another phenomenon found in the degenerated PLL. In PLL with severe degeneration, the newly generated vessels formed clusters, and the vascular wall was positive for CD31, a marker of vascular endothelial cells (**Figure 2C**). Above cells were not observed in PLL of control group (**Figure 2D**).

*TGF-β1 expressions in degenerated PLL*

The TGF-β1 expression was significantly higher in degenerated PLL than in control group. TGF-β1 expression was identified in macrophages, chondrocytes and fibroblasts, and TGF-β1 positive cells had yellow-brown granules in cytoplasm (**Figure 3A-C**). TGF-β1 was mostly expressed in macrophages, followed by chondrocytes, and only a few TGF-β1 fibroblasts were found along the edge of ligaments.

*Biological detection*

Real-time PCR indicated that Col1A1 mRNA expression in CSM group was significantly lower than that in control group. The mean Col1A1 expression was 26.1 ± 21.2 and 100.0 ± 92.2 in CSM group and control group, respectively ($P = 0.004$) (**Figure 4**).

## Discussion

*Pathological and biochemical characteristics of degenerated PLL*

PLL is a group of serrated, long, thin and tough dense connective tissues, locates at the anterior wall of the spinal canal and extends along the both sides of spinal canal. PLL is constituted of collagenous fibers and elastic fibers in a specific order and plays important physiological roles in the maintenance of spine stability and the prevention of excess flexion of the spine and kyphosis of intervertebral discs.

In degenerated PLL, fibrous tissues fractured, and had disordered arrangement. In PLL with severe degeneration, a plenty of small vessels

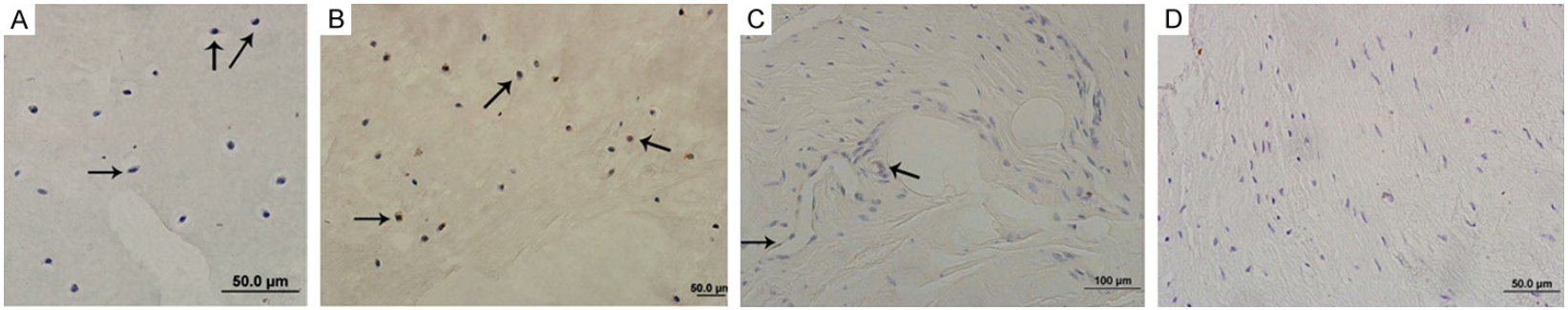


**Figure 2.** Infiltration of cells in PLL (IHC). A: $CD3^+$ cells in the PLL of patients cases had brown granules; B: $CD68^+$ cells in the PLL of patients cases had yellow or brown granules in the cytoplasm; C: $CD31^+$ cells in the PLL of patients cases had yellow granuels; D: $CD3^+$, $CD68^+$ and $CD31^+$ cells were not observed in the PLL of autopsy cases.

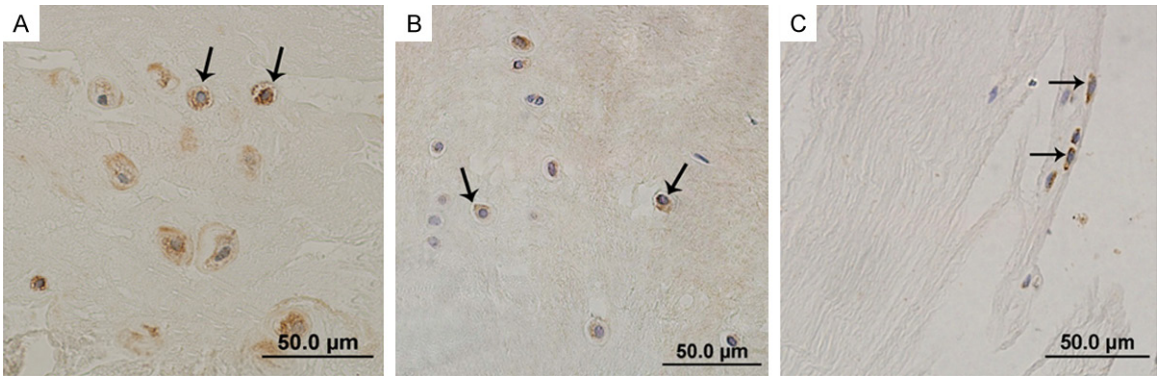


**Figure 3.** Immunohistochemistry for TGF-β1 in the PLL of patients. TGF-β1 positive granules were mainly found in the cytoplasm and yellow (DAB). A: TGF-β1 positive macrophages; B: TGF-β1 positive chondrocytes; C: TGF-β1 positive fibroblasts.

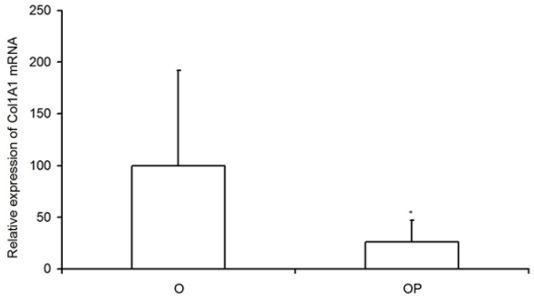


**Figure 4.** mRNA expression in PLL of two groups. Col1A1 mRNA expression was down-regulated in the patients group (OP) when compared with control group (O). The mRNA expression of Col1A1 was detected by RT-PCR and normalized to that of CybB. Data are shown as mean ± SD (n = 16). *P < 0.01.

formed, accompanied by patchy calcification. Immunohistochemistry showed the increase in collagen II expression in PLL, suggesting cartilaginous metaplasia. Type I collagen is a major support of ligaments and may provide tensions for ligaments. RT-PCR showed the collagen I expression is decreased in the PLL of patients group. Above changes may cause the reduction of strength.

*Chronic inflammation involves in the pathological processes of cervical PLL degeneration*

Traumatic inflammatory responses may occur when abnormal stresses are loaded on the cervical PLL. COX-2 is an inflammation related cytokine, and the inhibitor of COX-2 is capable of reducing scar formation during wound healing [12, 13]. Previous studies indicated a correlation between the PLL degeneration and inflammation [11]. However, the cell types and cytokines participating in such inflammatory responses are still not identified.

Our results showed that the numbers of macrophage and T lymphocytes infiltrating in PLL of patients were higher than those in control group, and the increase in macrophages was more obvious. Macrophages are the important inflammatory cells, and capable of secreting a great amount of cytokines such as TGF-β1, basic Fibroblast Growth Factor (bFGF), interleukin-1 (IL-1) and tumor necrosis factor (TNF). These cytokines may significantly stimulate cell proliferation, differentiation and angiogenesis [14]. In the present study, findings revealed that most macrophages were distributed at the edge of degenerated PLL and in the areas with severe degeneration. Their morphologies were also diverse as their locations vary, which might be ascribed to the difference in the functions of macrophages at different sites. T lymphocytes are important cells participating immune responses. Most of them were mainly distributed around vessels and adjacent tissue space, and a few of them were also found at the edge and other sites.

Vascular endothelial cells are a group of nonspecific phagocytes, participate in immune responses and play important roles in the repair process of inflammation. Our results showed a lot of $CD31^+$ endothelial cells were found in the areas with severe degeneration, and mainly distributed within small vascular walls and their adjacent tissue space. However, in control group, $CD31^+$ endothelial cells were not found. The above findings suggest chronic

and non-specific inflammatory responses in degenerated PLL.

*Correlation between abnormal TGF-β1 expression and PLL degeneration*

A lot of cytokines are involved in the inflammatory responses. TGF-β1 is a cytokine with multiple biological activities. It can simulate the fibroblast proliferation and increase the synthesis of extracellular matrix (especially collagenous fibers). TGF-β1 plays important roles in the organ hypertrophy and fibrosis [15-18]. However, the relation between PLL degeneration and TGF-β1 has not been identified.

Immunohistochemistry was performed to detect TGF-β1 in degenerated PLL. Results showed TGF-β1 expression in the PLL of patients group was higher than that in control group. TGF-β1 was mainly expressed in macrophages, and only a few TGF-β1 fibroblasts were observed. These findings suggest that TGF-β1 is related to the PLL degeneration, and macrophages are the potential cells secreting TGF-β1 to induce PLL degeneration.

Based on the above results, we speculated that cervical PLL are damaged under stresses, causing fracture and disordered arrangement of collagenous fibers and elastic fibers and subsequent chronic inflammatory responses [19]. Macrophages infiltrate the inflammatory tissues and secrete a large amount of TGF-β1 which may stimulate fibroblasts to secrete collagens for compensatory repair of injured fiber tissues. These processes progress repeatedly, and finally cause formation of scar tissues, leading to PLL fibrosis. In addition, the degenerated PLL also form chondrification and calcification. Above changes in PLL may eventually cause PLL degeneration and reduction in the PLL strength. Thus, the degenerated PLL may compress the spinal cord and cause symptoms of nerve injuries. Such an "inflammation-fibrosis link" is already known from studies in adult skin wound healing, in which macrophages also are the major, sustained source of TGF-β [20].

On the basis of the correlation between PLL degeneration and inflammation, anti-inflammation may be able to inhibit the inflammatory responses of cervical PLL degeneration, and thereby prevent or relieve the thickening of PLL. This may become a new approach for the prevention and non-surgical treatment of PLL degeneration.

## Acknowledgements

The Study Sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry. The authors thank Prof. M. Mokry in the Department of Neurosurgery, and Prof. M. Scarpatti in the Department of Pathology at Medical University of Graz, Austria, for the experimental supports.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Hong-Xing Song, Department of Orthopaedics, Beijing Shijitan Hospital, Capital Medical University, Beijing 100038, China. Tel: 010-63926159; E-mail: shx2@medmail.com.cn

## References

[1] Karadimas SK, Erwin WM, Ely CG, Dettori JR and Fehlings MG. Pathophysiology and natural history of cervical spondylotic myelopathy. Spine 2013; 38: S21-S36.

[2] Yamazaki A, Homma T, Ishikawa S and Okumura H. Magnetic resonance imaging and histologic study of hypertrophic cervical posterior longitudinal ligament. Spine 1991; 16: 1262-1266.

[3] Mizuno J, Nakagawa H and Hashizume Y. Cervical amyotrophy caused by hypertrophy of the posterior longitudinal ligament. Spinal Cord 2002; 40: 484-488.

[4] Matsunaga S and Sakou T. Ossification of the posterior longitudinal ligament of the cervical spine: etiology and natural history. Spine 2012; 37: E309-E314.

[5] Wang X, Chen Y, Chen D, Yuan W, Zhao J, Jia L and Zhao D. Removal of posterior longitudinal ligament in anterior decompression for cervical spondylotic myelopathy. J Spinal Disord Tech 2009; 22: 404-407.

[6] Sato R, Uchida K, Kobayashi S, Yayama T, Kokubo Y, Nakajima H, Takamura T, Bangirana A, Itoh H and Baba H. Ossification of the posterior longitudinal ligament of the cervical spine: histopathological findings around the calcification and ossification front. J Neurosurg Spine 2007; 7: 174-183.

[7] Smith ZA, Buchanan CC, Raphael D and Khoo LT. Ossification of the posterior longitudinal ligament: pathogenesis, management, and current surgical approaches: a review. Neurosurg Focus 2011; 30: E10.

[8] Song J, Mizuno J, Hashizume Y and Nakagawa H. Immunohistochemistry of symptomatic hypertrophy of the posterior longitudinal ligament with special reference to ligamentous ossification. Spinal Cord 2006; 44: 576-581.
[9] Mizuno J, Nakagawa H and Hashizume Y. Analysis of hypertrophy of the posterior longitudinal ligament of the cervical spine, on the basis of clinical and experimental studies. Neurosurgery 2001; 49: 1091-1098.
[10] Motegi H, Yamazaki M, Goto S, Mikata A and Moriya H. Proliferating cell nuclear antigen in hypertrophied spinal ligaments: Immunohistochemical localization of proliferating cell nuclear antigen in hypertrophied posterior longitudinal ligament of the cervical spine. Spine 1998; 23: 305-310.
[11] Song HX, Scarpatetti M, Kreil W, Shen HL, Bodo K, Ebner B, Schröttner H and Mokry M. Quantitative analysis of cyclooxygenase 2 in the posterior longitudinal ligament of cervical spondylotic myelopathy. Chin Med J 2011; 124: 2480-2484.
[12] Wilgus TA, Vodovotz Y, Vittadini E, Clubbs EA and Oberyszyn TM. Reduction of scar formation in full-thickness wounds with topical celecoxib treatment. Wound Repair Regen 2003; 11: 25-34.
[13] Wilgus TA, Bergdall VK, Tober KL, Hill KJ, Mitra S, Flavahan NA and Oberyszyn TM. The impact of cyclooxygenase-2 mediated inflammation on scarless fetal wound healing. Am J Pathol 2004; 165: 753-761.
[14] Freemont AJ, Jeziorska M, Hoyland JA, Rooney P and Kumar S. Mast cells in the pathogenesis of chronic back pain: a hypothesis. J Pathol 2002; 197: 281-285.
[15] Cutroneo KR. TGF-β-induced fibrosis and SMAD signaling: oligo decoys as natural therapeutics for inhibition of tissue fibrosis and scarring. Wound Repair Regen 2007; 15: S54-S60.
[16] Rhett JM, Ghatnekar GS, Palatinus JA, O'Quinn M, Yost MJ and Gourdie RG. Novel therapies for scar reduction and regenerative healing of skin wounds. Trends Biotechnol 2008; 26: 173-180.
[17] Barrientos S, Stojadinovic O, Golinko MS, Brem H and Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen 2008; 16: 585-601.
[18] Zhang Y, Chen J, Zhong ZM, Yang D and Zhu Q. Is platelet-derived growth factor-BB expression proportional to fibrosis in the hypertrophied lumber ligamentum flavum? Spine 2010; 35: E1479-E1486.
[19] Adair-Kirk TL and Senior RM. Fragments of extracellular matrix as mediators of inflammation. Int J Biochem Cell Biol 2008; 40: 1101-1110.
[20] Stramer BM, Mori R and Martin P. The inflammation-fibrosis link? A Jekyll and Hyde role for blood cells during wound repair. J Invest Dermatol 2007; 127: 1009-1017.