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Candidate Molecules Identified by Proteomic Study of Synovial Cells in Experimental Post-Traumatic Arthritis of Knee in Swine

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Abstract: This research was done to study the protein expression profile of synovial cells of knee using proteome analysis during the development of experimental Post-Traumatic Arthritis (PTA) in swine. PTA was induced by transection of the Anterior Cruciate Ligament (ACL) of left knee in three piglets. Articular cartilage and synovial tissues were obtained after 0, 2, 5 and 8 weeks for histopathologic examination. After sacrificing the piglets at 8 weeks, synovial tissues were collected for 2-dimensional electrophoresis and mass spectrometric analysis. Histopathologic examination at 8 weeks showed overt chronic inflammation indicating the development of ETA. Through proteome analyses more than 1,500 protein spots were identified in which 7 differentially expressed protein spots were observed in ACL-transacted synovial tissue. Five proteins were down-regulated (cytoskeletal β actin, cofilin-1, destrin, Rho GDP dissociation inhibitor α and an unnamed protein product) and two proteins were up-regulated (α-B crystalline and Smooth Muscle Protein (SMP) 22α). These results showed that proteins that are related to cellular organization and signal transduction are down-regulated and those that are related to cell rescue, defense and stress are up-regulated. Therefore, the proteome analysis of synovial tissue provided us new candidate molecules which may be useful to understand the pathogenesis for diagnostic and therapeutic studies of Post Traumatic Arthritis (PTA).

Key words: Arthritis, 2D electrophoresis, Biomarker, protein expression

INTRODUCTION

Post Traumatic Arthritis (PTA) develops sometime after articular injury is one of the most common causes of secondary osteoarthritis (D'Lima *et al*., 2001). Primary osteoarthritis predominantly affects the elderly, but PTA usually affects younger people, especially athletes who are likely to have joint injury due to vigorous physical activities (Buckwalter and Brown, 2004; Buckwalter and Martin, 2004). Older patients of osteoarthritis can often be treated effectively with reconstructive procedures and restriction of activities but these approaches are not acceptable for young and athletic people and also have poor long-term outcomes (Ahlberg and Henricson, 1981; Coester *et al*., 2001; Ellingsen and Rand, 1994; Sullivan *et al*., 1994). Therefore, management of these cases of PTA is a difficult clinical problem.

The innermost structures of synovium consist of one to three layers of cells generally identified as Fibroblast like Synovial (FLS) cells. Any kind of erosion, trauma, injury and destruction of cartilage, bone and peri-articular structures drives both inflammation and autoimmunity. The pathogenesis of PTA has not been fully understood yet. However, there have been numerous clinical studies and scientific experiments trying to show how articular injuries could cause joint degeneration. Clinical experiences show that joint injuries including intra-articular fracture may induce considerable incongruities and ligament tears that may lead to unstable joint, increase the risk of progressive joint degeneration that causes PTA (Buckwalter and Lane, 1997; Gelber *et al*., 2000; Lohmander *et al*., 2004; Porat *et al*., 2004).

Direct impact damage at the time of injury (Atkinson and Haut, 2001; Ewers *et al*., 2001), increase of cartilage contact stress resulting from residual articular incongruity (D'Lima *et al*., 2001; McKinley *et al*., 2004) and pathologic loading resulting from articular instability (Delamarter *et al*., 1990; Lovasz *et al*., 2001) are the three considerations that have been thought as the major etiologic factors of PTA. For the prevention and treatment of PTA after an initial joint injury, it is important to restore joint congruity and stability for the purpose of proper healing of the articular cartilage and decreasing the rate of progression of PTA. Incongruity and instability are likely

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to be important determinants of PTA, but their relative contribution to the development of PTA is not well-known. Although many experiments have shown evidences linking incongruity to PTA, there has been little experimental study on the role of joint instability in the development of articular cartilage degeneration (Buckwalter and Brown, 2004). There is no method for prediction or an early diagnosis of PTA incases of trauma or injury of joints.

Recently, proteome analysis, which refers to large-scale study of protein expression and function (Pandey and Man, 2000) has gained great interest. This is used for the determination of biochemical processes involved in diseases (Fung *et al*., 2000; Sinz *et al*., 2002). The comparative characterization of protein patterns in tissues has the potential to serve as the basis for new diagnostic tools and in designing of disease specific therapies (Sinz *et al*., 2002). Two-dimensional gel electrophoresis (Klose, 1975; O'Farrell, 1975) followed by in-gel proteolytic digestion and mass spectrometric analysis (Lottspeich, 1999; Mann and Talbo, 1996) has become a powerful method for the identification of proteins present in specific tissues or organs (Wang and Chait, 1994). Proteomics is gaining popularity in the research on arthritis such as rheumatoid arthritis, spondyloarthropathy and osteoarthritis (Sinz *et al*., 2002; Lorenz *et al*., 2003; Tielleman *et al*., 2005; Xiang *et al*., 2004). Particularly, rheumatoid arthritis has been investigated by proteomics with the use of synovial tissues or body fluids such as plasma, synovial fluid (Sinz *et al*., 2002; Lorenz *et al*., 2003; Tilleman *et al*., 2005). However, no detailed study on proteome analysis of joint tissue from PTA has been done. Therefore, we have studied the protein expression profiles of synovial tissue using proteome analysis and evaluated the histological characteristics of the knee joint in experimental traumatic arthritis (ETA) in swine.

MATERIALS AND METHODS

Inducing PTA: This study was conducted at School of Biotechnology, Hankyong National University, South Korea during 2006. Three male hybrid piglet, the first generation of a female Yorkshire and male Landrace (weighing 6.6 kg) were used in this study. Surgery was done under sterile conditions using atropine (subcutaneously) 0.1 mg kg^{-1} and ketamine (intramuscularly) 5 mg kg^{-1}. Longitudinal skin and fascial incisions were made over the anterolateral side of the left knee. The Anterior Cruciate Ligament (ACL) was exposed by a capsulotomy, then it was transacted at its tibial insertion area with a surgical blade and a cartilage sample including synovial tissue was obtained (Fig. 1). The joint capsule and skin incision were closed with nylon sutures and a dressing of povidone-iodine was applied to the wound. After surgery, penicillin 500 KIU was injected intramuscularly daily for a week to prevent post-operative bacterial infection and the piglet was allowed unrestricted activities in a clean cage.

Follow-up experiments: At 2 and 5 weeks after surgery, cartilage tissues of the ACL-transacted knee were obtained by needle biopsy, which was performed under the same anesthesia as described earlier, using ultrasonographic guidance (Fig. 1). The piglet was sacrificed at 8 weeks, normal and experimental knee joints were disarticulated to take tissue samples for proteome analysis and to confirm the development of PTA by histopathologic examination.

Tissue preparation for histopathology: All samples taken from synovium and cartilage during the experimental period from both knee were immediately placed in 70% ethanol, then specimens were fixed in 10% neutral buffered formalin at room temperature for 24 h and embedded in paraffin. Thin sections were cut from the paraffin blocks, mounted on the slide and stained with Hematoxylin and Eosin as per standard procedure.

Tissue preparation and protein extraction from synovial tissues: Samples for proteome analysis were obtained from the synovial tissue of normal and PTA knee joints under sterile condition to avoid contamination of tissues and used as pooled sample. Sample from the non-operated normal knee served as a control. Synovial cells were cultured in RPMI-1640 medium supplemented with 10 ml L^{-1} Fetal Bovine Serum (FBS) and antibiotics. The cells were maintained in an incubator at 37°C in 5% CO_2 humidified atmosphere. The cells grown at the exponential growth phase were harvested with trypsinization. After washing in Hanks' solution and ice-cold phosphate buffered saline (PBS), the cells were counted. Cell lysis was done in a cocktail of 9 mol L^{-1} urea, 40 g L^{-1} CHAPS, 40 mM Tris and 40 mM DTT and centrifuged at 12,000 g for 1 h at 4°C (Avanti^{R} J-E, Beckman Coulter, USA). The pellet was washed with ice-cold acetone five times and lyophilized prior to 2-DE analysis.

Isoelectric Focusing (IEF) and 2 dimensional electrophoresis: Dried samples (250 μg) were dissolved in rehydration buffer (8 M urea, 2% CHAPS, 60 mM DTT, 0.5% IPG buffer). The samples were applied on 18 cm immobilized pH gradient (IPG) strip gel (pH 3-10) for Iso-Electric Focusing (IEF) using IPGphor system (Amersham

Biosciences, Uppsala, Sweden). In the first dimension, the IEF of proteins were performed in five steps (rehydration for 12 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h and 8000 V for 9 h). The strip gels were equilibrated with the series of DTT, iodoacetamide and the SDS-PAGE running buffer containing disequilibrium buffer for 30-40 min. In the second dimension electrophoresis, the equilibrated strip gels were run on 12% polyacrylamide SDS gel using a PROTEAN II electrophoresis kit (BioRad, Hercules, USA). Silver staining was carried out according to the method of Heukeshoven and Dernick (1988) using a silver-staining kit (Amersham Biosciences). The 2 DE was repeated three times.

Image analysis: Silver-stained gels were scanned using a PowerLook III image scanner (UMAX data system, Hsinchu, Taiwan). Image treatment, spot detection and protein quantification were carried out using Image Master 2D Elite software (Amersham Biosciences). The molecular weights of proteins on the gel were determined by comparing with the standard markers (Sigma, St. Louis, MO, USA) run in parallel to the sample. The pI of the proteins were determined by comparing the migration of protein spots on 18 cm IPG strips (pH 3-10). Spot volumes and intensity were determined from more than three gels by repeating the experiments.

In-gel digestion: Spots of interest were excised manually with clean tips, cut off into fine slices with a razor blade and then transferred to micro-centrifuge tubes. Gel slices were washed with distilled water and kept frozen at -20°C till further use. Silver stained gel slices were destained in microtubes with 15 mM potassium ferricyanide and 50 mM sodium thiosulfate. When the brownish color disappeared, the gel slices were rinsed with distilled water, then kept in 200 mM ammonium bicarbonate for 20 min, after that the slices were crushed using micro-pestles (Eppendorf, Hamburg). For rehydration, the gel pieces were incubated in 100 mM ammonium bicarbonate and 10 mM DTT for 1 hour at 56°C. Alkylation was performed in 100 mM ammonium bicarbonate and 55 mM iodoacetamide for 40 min in the dark, at room temperature. After dehydration in acetonitrile, the gel pieces were dried under vacuum (Thermo Savant SpeedVac Plus, Savant, Holbrook, NY). Samples were digested with sequencing grade trypsin (Promega, Madison, WI, enzyme:substrate ratio >1:20) at 37°C, overnight, in 50 mM ammonium bicarbonate. Trypsinized gel pieces were extracted through a repeated process of hydration-dehydration and sonication. Supernatants were transferred into new tubes and dried completely under vacuum for 6 h.

MALDI-TOF-MS and MALDI-TOF/TOF: The resulting tryptic peptides were dissolved in 0.5% trifluoroacetic acid (TFA) solution and then desalted using the ZipTipC18 (Millipore, Bedford, MA) tip. Peptides were eluted directly onto MALDI target by a-cyano-4-hydroxy-cinnamic acid (CHCA) matrix solution [10 mg mL^{-1} CHCA in 0.5% TFA/50% acetonitrile (1:1, v/v)]. All mass spectra were acquired at a reflection mode by a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA). External calibration was performed using a standard peptide mixture of des-Arg bradykinin, angiotensin-I, glu-fibrino-peptide-B, adrenocorticotrophic hormone (ACTH) clip 1-17, ACTH clip 18-39 and ACTH clip 7-38. Internal calibration was also performed using two autolysis peaks of trypsin [$(M+H)^+$ = 842.5099 and 2211.1046]. When the protein spots were not identified by peptide mass fingerprinting (PMF), fragmentation patterns of tryptic peptide molecular ions $(M+H]^+$) were analyzed by MS/MS (tandem mass spectrometry) methods for obtaining their partial sequences using MALDI-TOF/TOF technique. All samples were irradiated with UV light (355 nm) of an Nd:YAG laser with a repetition rate of 200 Hz. Approximately 1,000 and 3,000 laser shots were averaged to normal mass spectra and MS/MS spectra, respectively. The samples were analyzed at 25 kV of source acceleration voltage with two-stage reflection in the MS mode. In the MS/MS experiment, collision energy, which was defined by the potential difference between the source acceleration voltage (8 kV) and the floating collision cell (7 kV), was set to 1 kV.

Search of database and identification of proteins: The proteins were identified by searching NCBI non-redundant database using MASCOT PMF (Matrix Science, London) and MS-Fit (Protein Prospector; UCSF, San Francisco, CA) softwares. All mass spectra were searched in the database of Mus musculus. The search parameters were considered to allow the modifications of N-terminal Gln to pyroGlu, oxidation of methionine, acetylation of protein N-terminus, carbamidomethylation of cysteine and acrylamide-modified cysteine. The criteria for positive identification of proteins were set as follows: (i) minimum of matching peptide masses, (ii) 50 ppm mass accuracy and (iii) molecular weight and pI obtained from image analysis. For MS/MS search, fragmentation of selected peptide molecular ion peak was used to identify the protein in the same manner by searching NCBI nonredundant database using MASCOT MS/MS ion search program.

Isolation of RNA from synovial cells: To isolate RNA, the FLS cells from normal and ETA knee joint were used in this study after five passage. One ml of Trizol reagent was added to a 10 cm diameter dish with cells and the cells were passed through a pipette several times. The

Table 1: The primers used for RT-PCR for Cofilin-1 and smooth muscle protein (SMP) 22- α synthesized by BIONEER Corporation, South Korea.

Gene symbol	Primer sequence 5'→3'	Expected fragment size (bp)
SMP-22 α	5'-CCTGTATCCTGACGGCTCCAAACC-3'	412
	5'-TGCCCCACCCCCTCTAACTGATGA-3'	
Cofilin-1	5'-CTGGCTCCTTGCTATCTCCTTTTC-3'	420
	5'-CAGCTTCTTCTTGATGGCGTCCTT-3'	

homogenized sample was incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. The sample was mixed with 0.2 mL of chloroform per 1 mL of Trizol reagent by shaking vigorously by hand for 15 sec and incubated for 2-3 min. Then, the samples were centrifuged at 12000 rpm for 15 min at 4°C. Following centrifugation, the mixture was separated into a lower red phenol-chloroform phase, an interphase and a colorless upper aqueous phase. The aqueous phase was transferred to a fresh tube and precipitated the RNA from the aqueous phase by mixing with isopropyl alcohol. Then 0.5 mL isopropyl alcohol was added per 1 mL Trizol reagent used for the initial homogenization and samples were incubated at room temperature for 10 min and centrifuged at 12000 rpm for 10 min at 4°C. Then the supernatant was removed and the RNA pellet was washed by adding at least 1 mL of 75% ethanol (100% EtOH 35 mL + DEPC water 15 mL) per 1 mL of Trizol reagent. The samples were mixed by vortexing and centrifuged at 7500 rpm for 5 min at 4°C. At the end of the procedure, the RNA pellet was briefly dried and 20 μL of RNase-free water was added and incubated for 10 min at 56°C. Finally, the RNA concentration was measured at the UV spectrophotometer by the 100 times.

Polymerase Chain Reaction (PCR): To determine whether the down-regulation of Cofilin-1 and the up-regulation of SMP22-α expression occurred at the mRNA level, we performed semi-quantitative RT-PCR analysis using Cofilin-1 and SMP22-α specific primers (Table 1). The thermocycler profile was 5 min 94°C (initial denaturation) and then 22-30 cycles of 40 sec at 94°C (denaturation), 30 sec at 60-65°C (annealing), 40 sec at 72°C (extension) and followed by a 10 min final extension at 72°C. The PCR products were analyzed by electrophoresis (1.5% agarose gels) in 40 mM Tris-acetate containing 1 mM EDTA (1 x TAE).

RESULTS

Experimental traumatic arthritis was induced in 3 piglets by anterior cruciate ligament transection and studied the protein expression by 2 D electrophoresis analysis. The results are described below.

Ultra sonographic finding: Ultrasonography was performed at 2 and 5 weeks post transection of ACL to observe the pathologic changes in the joint and to confirm the arthritis. The results has shown that there was no changes after 2 weeks, however, inflammatory changes were observed at 5 weeks which confirmed the development of arthritis due to ACL transaction (data not shown).

Histopathology: Histology of initial articular cartilage (Fig. 1A) showed the normal structure of hyaline cartilage. Chondrocytes near the trabecular bone were round-shaped and there was no regular direction or orientation among them. Chondrocytes near the articular surface, however, were flat-shaped and were arranged relatively parallel with the articular surface. Briefly, the arrangement of chondrocytes within the articular cartilage was different in trabecular and articular portion and this difference might be due to the weight-bearing effect on the articular cartilage.

Articular cartilage at 2 weeks post-ACL transection (Fig. 1C) showed patterns similar to the initial articular cartilage and no definite abnormal findings were observed at light microscopy. However, articular cartilage at 5 weeks post-ACL transection (Fig. 1D) showed apparently different findings compared to initial and at 2 weeks articular cartilages. Nuclei were mostly enlarged and cytoplasms were also thickened. Arrangement of chondrocytes were different, a layering within articular cartilage disappeared and the entire cartilage layer was evenly arranged. These findings indicated that articular cartilage was in a proliferative state and this change might be due to the response of articular chondrocytes to some kind of abnormal stimulations such as mechanical overload, inflammatory process etc. Articular cartilage at 8 weeks post-ACL transection (Fig. 1E) showed findings similar to that of cartilage at 5 weeks and chondrocytes were relatively stabilized. Initial joint synovium (Fig. 1B) showed normal structure and did not exhibit any inflammatory process. Joint synovium at 8 weeks post-ACL transection (Fig. 1F) showed overt chronic inflammation that supported the development of ETA.

2DE map of protein expression patterns: More than 1,500 protein spots on silver-stained 2-DE gels were identified with the pH range from 3 to 10 and molecular weight range from 7 to 200 kDa (Fig. 2). In three repeated experiments, 7 differently expressed protein spots were identified. As described in the method, down or up-regulated protein expressions between normal and ACL-transacted synovial cells were evaluated using Image Master 2D Elite software. In comparison with the software quantification, the differentially expressed spots that were statistically significant ($p<0.05$) were selected, the spots

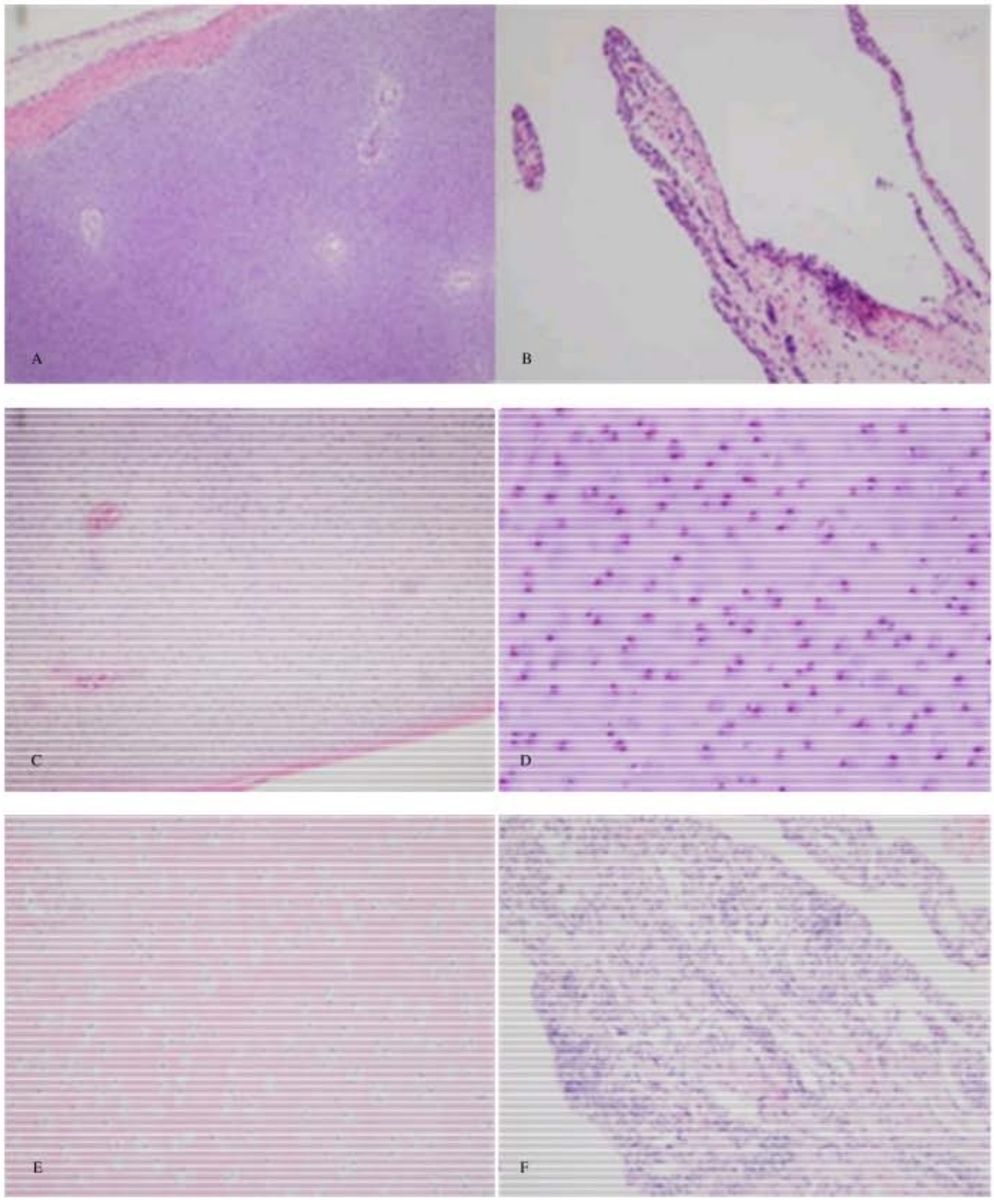

Fig. 1: Articular cartilage of femoral condyle and joint synovium with H and E stain from 0, 3, 5 and 8 weeks post-ACL transected swine knee. (A) Normal cartilage (initial, x100), (B) Normal synovium (initial, x100), (C) Post-ACL transection cartilage (2 weeks x100), (D) Post-ACL transection cartilage (5 weeks x400), (E) Post-ACL transection cartilage (8 weeks x200) and (F) Post-ACL transection synovium (8 weeks x100)

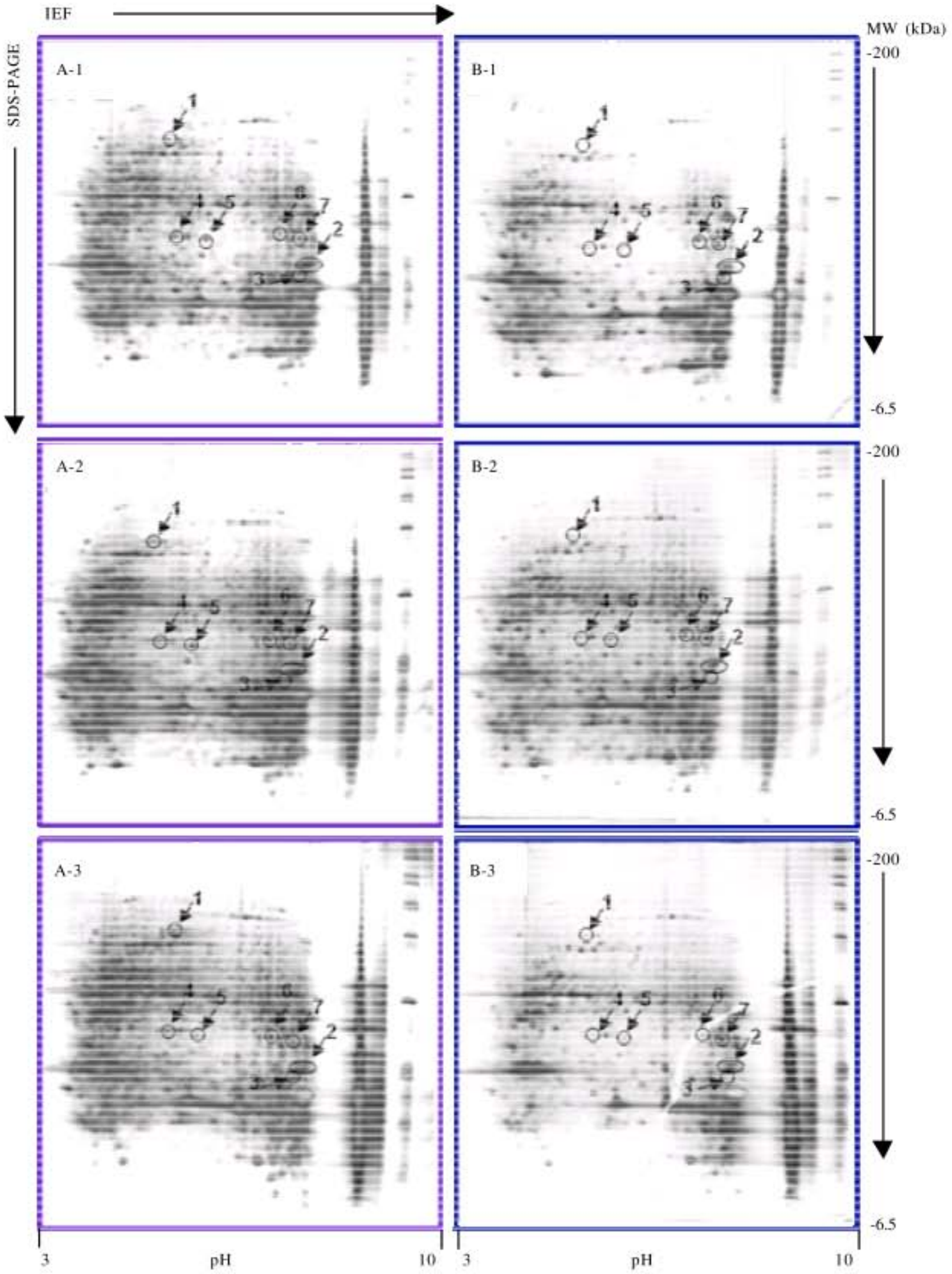

Fig. 2: Comparison of proteome by two-dimensional gel electrophoresis on normal (A) and PTA (B) knee joint FLS cells. Total proteins were separated by 2-DE using IPG phor system (pH 3-10) in the first and 12% SDS-polyacrylamide gel in the second dimension. Identified protein spots are indicated by numbers. M = molecular weight markers (kDa). (1-3 are the replicates)

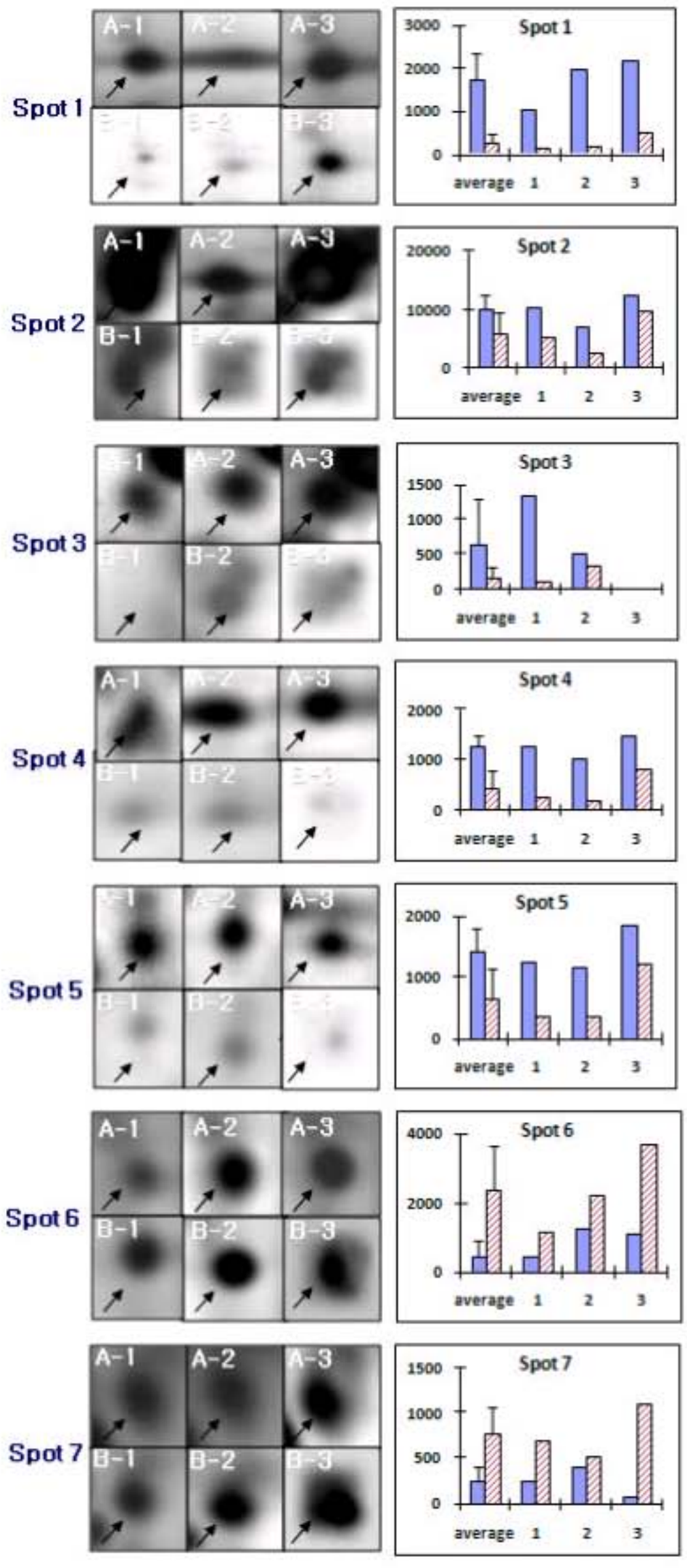

Fig. 3: Protein expression comparisons of normal (A) and PTA (B) knee joint synovial cells. The expression difference was statistically significant ($p<0.05$) from the Image Master 2D Elite software. Down and up regulated proteins in ACL-transected synovial tissue are listed here. (1-3 are the replicates)

Table 2: Protein spots with differential expression in 2 DE of synovial cells of knee cell from experimental PTA in swine identified by mass spectrometry

Spot	Protein Identity	MW (kDa)	pI	Accession No.*	Difference
1	Cytoskeletal β actin	44.76	5.55	AAS55927	Down
2	Cofilin-1 (cofilin, non-muscle isoform)	18.51	8.16	Q6B7M7	Down
3	Destrin, Nmr, Minimized average structure	19.34	8.05	1AK6	Down
4	Unnamed protein	47.21	6.37	CAA32409	Down
5	Rho GDP dissociation inhibitor (GDI) α	23.41	5.12	NP_788823	Down
6	α-B crystallin	20.17	6.76	JC5971	Up
7	Smooth muscle protein 22-α	10.10	4.93	AAC08010	Up

Table 3: The fold change in RT-PCR analysis of the mRNA of Cofilin-1 and SMP-22-α proteins

		Fold change	
No.	Protein identity	2DE	RT-PCR
1	Cofilin-1 (cofilin, non-muscle isoform)	-1.4	-0.98
2	Smooth muscle protein 22-α	+2.5	+0.60

that showed more than a 50% increase or decrease in intensity were defined as up or down-regulated spots. According to this definition, among 7 protein spots, 5 proteins were down-regulated and 2 proteins were up-regulated in ETA FLS cells (Fig. 3).

Identification of differently expressed proteins: The 7 differently expressed protein spots were identified by MALDI-TOF-MS based on PMF after in-gel digestion with trypsin. The spots could not be recognized with the information available in the PMF were identified by MS/MS using MALDI-TOF/TOF technology. In the final analysis amino acid sequences of 7 spots were identified, 6 of which represent different unambiguous proteins and one ambiguous protein product. All the proteins identified are listed in Table 2 and numbered on the 2-DE map shown in Fig. 2. The five down-regulated proteins in ACL-transacted synovial tissues compared to normal tissues were cytoskeletal β actin, cofilin-1, destrin, Rho GDP dissociation inhibitor α and an unnamed protein product. The two up-regulated proteins in ETA synovial tissue compared to normal tissues were α-B crystalline and SMP-22α.

Semi-quantitative RT-PCR: Total RNA were isolated from the normal and experimental synovial cells after 5 passage for RT-PCR. The SDS PAGE of the total RNA sample from normal and experimental synovial cells has shown a single band. Normal and experimental synovial cells RNA sample quality were also measured with the Bioanalyzer which has also shown good quality with a low baseline and sharp ribosomal peaks for 18S and 28S (data not shown).

The results of RT-PCR for Cofilin-1 and SMP-22α genes (Table 3, Fig. 4) confirmed that the mRNAs of SMP-22α was expressed at higher levels and mRNAs of cofilin 1 was expressed at lower level in the pathological synovial cells. These results substantiated the specific up-regulation of SMP-22α and down regulation of cofilin 1 protein expression in the pathological synovial cells.

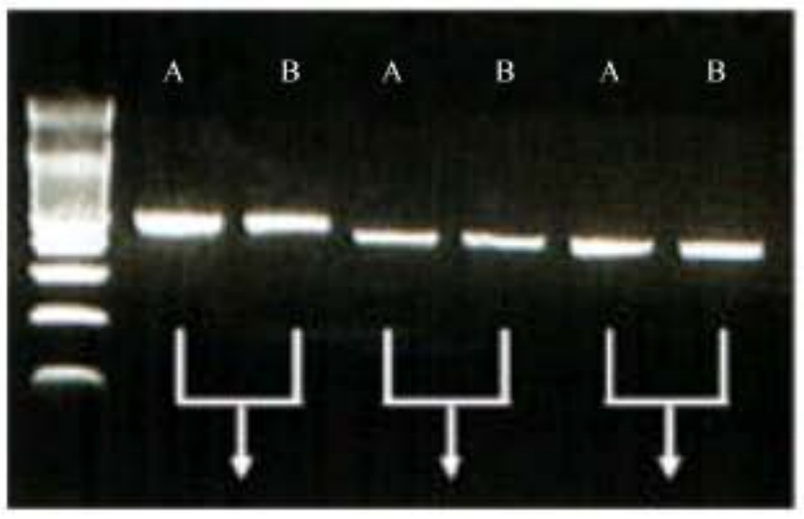

Fig. 4: RT-PCR analysis of the mRNA of Cofilin-1 and SMP-22-α proteins. Primers specific for SMP-22-α and Cofilin-1 were used to amplify the transcripts from total RNA isolated from normal (A) and PTA (B) knee joint synovial cells. RT-PCR of the housekeeping gene GAPDH was used as control for RNA variation

DISCUSSION

Histopathology: Pathogenesis of synovial joint degeneration after injury and subsequent development of PTA is not properly understood. In our experiment we have successfully developed PTA by ACL transection which was confirmed in hispathological findings. There has been a consistently observed association of instability with PTA (Delamarter *et al*., 1990; Honkonen, 1995; Stevens *et al*., 2001) and the presence of instability and incongruity. Patients sustaining ligament injuries in the knee also had a significant incidence of PTA (Daniel *et al*., 1994; Gillquist and Messner, 1999; Kannus and Jarvinen, 1989). Patients sustaining ACL tears, followed up for more than 5 years, had a significantly increased incidence of PTA (Daniel *et al*., 1994). In a review of ACL tears, injured knees had a 10-fold increase of degenerative changes, compared with uninjured limbs (Gillquist and Messner, 1999).

Protein expression: The identification of proteins with distinct expression in a diseased can improve our understanding of the pathogenesis of the disease and identification of novel diagnostic technique and therapeutic targets (Tilleman *et al*., 2005). The search for

differently expressed genes is an important means to find markers of disease for diagnosis and starting points for evaluation of pathophysiological pathways (Lorenz *et al*., 2003). In this study, we compared protein expression profiles of normal and PTA synovial cells and found differential expression of 7 proteins in PTA. Five proteins (cytoskeletal β actin, cofilin-1, destrin, Rho GDP dissociation inhibitor α and unnamed protein product) were down-regulated while two proteins (α-B crystallin, SMP-22α) were up-regulated.

Cytoskeletal $ actin: There are six known actin proteins in mammalian cells, two sarcomeric muscle actins, two smooth muscle actins and two nonmuscle, cytoskeletal actins (Kedes *et al*., 1985). Mammalian cytoplasmic actins are the products of two different genes and differ by many amino acids from muscle actin (Vandekerckhove and Weber, 1978). The cytoskeletal β actin is a structural protein, the down-regulated expression of it might be in response to the inflammation though it has no significant function in cellular defence mechanism.

Cofilin 1 and destrin: Cofilin is a widely distributed intracellular actin-modulating protein, depolymerizes filamentous F-actin and inhibits the polymerization of monomeric G-actin in a pH-dependent manner. It is involved in the translocation of actin-cofilin complex from cytoplasm to nucleus. The two cofilin isoforms that have been identified in mouse are muscle or M-type and nonmuscle or NM-type (Ono *et al*., 1994). The mouse M-type cofilin is expressed in the heart, skeletal muscle and testis, whereas, the NM-type is found in a wide variety of tissues, including the heart and testis. Cofilin exhibits actin-depolymerizing activity as a result of its phosphorylation by LIM kinase. Maekawa *et al*. (1999) demonstrated that cofilin was phosphorylated during lysophosphatidic acid-induced, Rho-mediated neurite retraction. They concluded that phosphorylation of LIM kinase by ROCK, consequently, increased phosphorylation of cofilin by LIM kinase which contribute to Rho-induced reorganization of the actin cytoskeleton.

Destrin is an essential actin regulatory protein belonging to the Actin-Depolymerizing Factor (ADF)/cofilin family. This family of proteins is responsible for enhancing the turnover rate of actin *in vivo*. ADF was first isolated from chick embryo brains as a protein that promoted the disassembly of actin filaments (Bamburg *et al*., 1980). The reason for down regulation of cofilin and destrin might be in response to the arthritis which led to stress on movement in which the muscle contraction get affected. The body might be responding to that stress by reducing depolymerization of actin fibre by the activity of cofilin-1 and destrin through down regulation and this two molecule may be useful as candidate gene for PTA.

GDP dissociation inhibitors-" (GDIAs): GDP dissociation inhibitors α (GDIAs) are proteins that regulate the GDP-GTP exchange reaction of members of the rab family, small GTP-binding proteins of the RAS superfamily, that are involved in vesicular trafficking of molecules between cellular organelles. Aplysia Ras-related Homologs (ARHs), also called Rho genes, belong to the RAS gene superfamily encoding small guanine nucleotide exchange (GTP/GDP) factors. The ARH proteins may be kept in inactive GDP-bound state by interaction with GDP dissociation inhibitors (GDIAs). By screening a transformed amnion cell library with an ARHGDIB cDNA, Leffers *et al*. (1993) isolated cDNAs encoding ARHGDIA. Down regulation of GDIA might be a cellular response of survival in inflammation of synovial tissue, which needs further studies for confirmation.

"-B Crystallins: The crystallins include three major families of ubiquitously expressed crystallins: α, β and γ. α-B-crystallin is a member of the small heat-shock (HSP) protein family (Dubin *et al*., 1990) which promote cell survival under adverse environmental conditions. The α-crystallin subunits, α-A and α-B, can form an oligomer by itself or with the other. Fu and Liang (2002) used a 2-hybrid system to study heterogeneous interactions among crystallins of different classes and found the intensity of interaction was one-third that of α-A-α-B interactions. The up-regulation of α-B crystallin must be a cellular response mechanism for survival in the adverse environment induced by ligament injury and may be useful a candidate molecule for PTA.

SMP-22": SMP 22-α gene product is expressed exclusively in smooth muscle-containing tissues of adult animals and is one of the earliest markers of differentiated smooth muscle (Bae *et al*., 2006). Smooth muscle plays important role in the healing process for the formation of new blood vessels. Up-regulation of SMP 22-α may be the result of increased vascular endothelium for the increased neovascularization in arthritic synovial tissue (Shahrara *et al*., 2002) which also may be useful as a biomarker for PTA.

RT-PCR of cofilin-1 and SMP-22" genes: The results of RT-PCR substantiated the specific up-regulation of SMP-22α and down regulation of cofilin-1 gene expression in the mRNA of pathological synovial cells.

Therefore, the results we have reported for the differential expression of seven proteins through proteomics study are further confirmed by the similar expression of their respective genes at mRNA level of the synovial cells.

CONCLUSION

Considering the results of present study the proteome analyses of the synovial tissue by 2-DE is a useful approach to investigate the inflammation of the joint after injury and porcine model can be used to understand the pathogenesis of PTA. The identified proteins viz. cofilin-1, destrin, α-B crystalline and SMP-22α may play an important role as candidate molecule in the future studies to better understand the pathogenesis of PTA for diagnostic and therapeutic approach for PTA.

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