Research Article

Biomedical Chromatography

Received 2 February 2010, Revised 26 February 2010, Accepted 1 March 2010 Published online in Wiley Online Library: 7 June 2010

(wileyonlinelibrary.com) DOI 10.1002/bmc.1436

Proteomic analysis of chondrocytes exposed to pressure

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ABSTRACT: Chondrocytes are the only cell type present in mature articular cartilage (2–5% of total tissue). The biological activities of the chondrocyte population are regulated by genetic, biologic and biochemical factors, as well as environmental factors (stress, flow and electric field). Although compressive forces within joint articular cartilage are required for maintenance of the normal composition of articular cartilage, there is a lack of knowledge about the number of pressure-related proteins expressed in articular cartilage. Two-dimensional gel electrophoresis (2-DE) and high-performance liquid chromatography–electrospray/tandem mass spectrometry (HPLC/ESI-MS/MS) were used to identify the levels of pressure-related proteins expressed by chondrocytes grown in the presence or absence of hydrostatic pressure. A total of 266 spots were excised from the gels and analyzed by HPLC/ESI-MS/MS. Functional classification of up-regulated proteins indicated that energy and protein fate were the main biological processes occurring in pressurized chondrocytes. Furthermore, membrane-bound transferrin-like protein p97, a marker of chondrocyte differentiation, was only expressed in chondrocytes under hydrostatic pressure. These data suggest that hydrostatic pressure can induce cell differentiation by increasing the expression level of energy metabolism- and protein fate-related proteins, indicating that hydrostatic pressure may be needed for normal biosynthesis and differentiation of articular chondrocytes. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: mass spectrometry; chondrocytes; proteomic; 2D electrophoresis; Pressure

Introduction

The three major types of cartilage in the human body are hyaline cartilage, elastic cartilage and fibrocartilage. Hyaline or articular cartilage is a smooth, resilient, load-bearing connective tissue that covers the articular surface of synovial joints. The main functions of articular cartilage are to provide a shock-absorbing structure that can withstand compression, tension and shearing forces and to dissipate excessive loading forces. Under normal conditions, the compressive forces within joint articular cartilage can rise to 20 MPa on standing (Muir, 1995). Nevertheless, joint loading caused by normal physical activity or moderate exercise is required for maintenance of the normal composition of articular cartilage (Tammi *et al*., 1987; Palmoski *et al*., 1979; Jurvelin *et al*., 1990). On the other hand, strenuous exercise can induce significant alterations in the characteristics of the collagen network of articular cartilage (Arokoski *et al*., 1993). During exercise, bearing regions of the articular surface undergo cycles of compressive loading. The function of the extracellular matrix (ECM) is to carry out the compressed cartilage deforms and recovers which results from the combined properties of the collagen fibril network and large proteoglycans within it (Bruckner and van der Rest, 1994).

Chondrocytes are the only cell type in mature articular cartilage and comprise 2–5% of total cartilage volume. They are important in the control of cartilage integrity by activating two distinct functional programs: catabolic and anabolic processes. The catabolic program responds to pro-inflammatory stimuli and is characterized by the secretion of proteases, suppression of matrix synthesis and induction of chondrocyte apoptosis. The anabolic program is associated with the secretion of antagonistic

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Abbreviations used: 2-DE, two-dimensional gel electrophoresis; DTT, dithiothreitol; ECM, extracellular matrix; EDTA, ethylenediamine tetraacetic acid; HPLC-MS/MS, high-performance liquid chromatography–tandem mass spectrometry; MS, mass spectrometry; PBS, phosphate buffer saline; Q-TOF, quadrupole-time of flight; RT-PCR, reverse transcription and polymerase chain reaction; SDS, sodium dodecyl sulfate; Tris, trishydroxymethyl aminomethane.

cytokines of the catabolic program, synthesis of protease inhibitors, production of ECM and cell replication (Lotz, 1995). *In-vitro* studies have shown that the biological activities of chondrocytes are regulated by genetic, biologic and biochemical factors, and environmental factors (stress, flow and electric field; Guilak *et al*., 1997; Mow *et al*., 1999). Several studies have discussed the effects of mechanical and/or hydrostatic/osmotic pressure loading on cartilage explant metabolism (Guilak and Mow, 2000; Lai *et al*., 2002). Mechanical compression of the cartilage extracellular matrix has a significant effect on the metabolic activity of the chondrocytes. Our previous work also suggests that hydrostatic pressure significantly increases cell numbers and biosynthesis of cultured chondrocytes (Lee *et al*., 2005).

In the post-genomic era, the combination of two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) has provided an alternative approach to examine the complex structure of protein expression by chondrocytes under stress (Haglund *et al*., 2008; Ruiz-Romero *et al*., 2005, 2008; Garcia *et al*., 2006; Schreiweis *et al*., 2007). In this study, we conducted a comparative proteome analysis of rabbit chondrocytes grown in the presence or absence of hydrostatic stress. In order to identify the cellular mechanisms involved in articular cartilage under pressure, we globally surveyed the differential protein expression to identify pressure-related proteins.

Materials and Methods

In this study, rabbit chondrocytes were cultured *in vitro* for 3 weeks. Subsequently, the cartilage-like membrane was weighed and the numbers of chondrocytes were counted. The RNA was isolated and reverse transcription and polymerase chain reaction (RT-PCR) was performed to measure the mRNA expression of collagen II. In addition, the differential protein expressions in pressurized cells were profiled by 2-DE and then identified by nanoLC-MS/MS.

Chemicals and Reagents

Culture media, TRIzol reagent and Hank's buffer saline solution were purchased from Gibco BRL (Paisley, UK). Culture plates were purchased from Costar (Cambridge, MA, USA). Collagenase II and CHAPS were obtained from Sigma (St Louis, MO, USA). Protein quantitative reagent was purchased from Bio-Rad (Hercules, CA, USA). Ammonium bicarbonate and isopropanol were purchased from J. T. Baker (Phillipsburg, NJ, USA). 2-DE materials (IPG buffer, strips, etc.) were from GE Healthcare (Uppsala, Sweden). Monomeric acrylamide solution (40%, 37.5:1), agarose I, ammonium persulfate (APS), sodium dedecyl sulfate (SDS), TEMED, urea, bromophenol blue and Tris-HCl were purchased from Amresco (Solon, OH, USA). Proteinase inhibitors (Complete™ Mini) were purchased from Roche (Mannheim, Germany). Modified trypsins for in-gel digestion were purchased from Promega (Madison, WI, USA). Dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Amersham Phrmacia (Piscataway, NJ, USA). Wide-range molecular marker standard proteins were obtained from Invitrogen (San Diego, CA, USA). Acetonitrile (ACN) was obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) and formic acid was obtained from Fluka (Steinheim, Germany).

Chondrocyte Isolation

Rabbit chondrocytes were obtained from the patellofemoral joints of rabbits. The cartilage was carefully separated from the rabbits and was rinsed three times in PBS. Subsequently, the cartilage was immersed in 10 mL Dulbecco's modified Eagle medium (DMEM) with 0.2% collagenase II, 100 U/mL penicillin and 100 µg/mL streptomycin and kept at 37°C in 5% CO_2 for 24 h. The solution was centrifuged for 10 min at 100***g***. The cells were re-suspended with 10 mL DMEM with 10% fetal bovine serum (FBS) to halt the enzyme reaction. Cells were filtered through a nylon mesh filter and collected by centrifugation at 100***g*** for 10 min, and then washed three times with 10 mL PBS. After re-suspension in 10 mL DMEM, 0.5 mL of the cell suspension was stained with 0.1 mL 0.4% trypan blue, and cell density was measured with a hemocytometer.

Cell Culture Protocol

Chondrocytes were then cultured in a medium containing DMEM with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 50 mg/mL ascorbic acid. Initial cell seeding was $5 \times 10^4/cm^2$. Chondrocytes were divided into two groups: the control, cultured in a standard 24-well culture plate (1.9 cm^2) with a medium height of 0.5 cm (equivalent to a hydrostatic pressure of 50 Pa), and the loading group, cultured in a modified 24-well culture plate with a medium height of 2.5 cm (equivalent to a hydrostatic pressure of 250 Pa) × 7.5 cm (equivalent to a hydrostatic pressure of 750 Pa) and 15 cm (equivalent to a hydrostatic pressure of 1500 Pa), respectively. The cultured cells were maintained at 37°C in a humidity incubator at 5% CO_2 and 95% air.

Weight Measurement of Formed Cartilage and Cell Number Counting

After the 3-week culture period, the culture medium was removed and a tissue-like membrane was visualized in each well. Each membrane was collected from the surface of the well with a needle. Membranes from six wells in each group were allowed to dry at 37°C for 4 h. The dry weight of each sample was measured with a balance. Cultured chondrocytes were then isolated from the undried membrane by digesting them with 0.05% trypsin and 0.2% collagenase II in 5 mL DMEM. After the cells were completely free in suspension, 5 mL DMEM with 10% FBS was added to halt enzyme reaction. Centrifugation was performed at 1500***g*** for 5 min. The supernatant was discarded and the cells were resuspended with 10 mL Hank's buffer saline solution. Next, 0.5 mL of the cell suspension was placed in a screw cap test tube and 0.1 mL of 0.4% trypan blue stain was added. The cells were mixed thoroughly and stood for 5 min at room temperature. A hemocytometer was used for cell counting. Under a microscope, nonviable cells were stained and viable cells that resisted the stain were counted. The cells within the suspension were prepared for RNA isolation.

RNA Isolation

RNA was isolated from both fresh cartilage and cultured chondrocytes. Total cellular RNA was extracted from chondrocytes after dissolving in TRIzol reagent according to the manufacturer's instruction. Then, 1 mL TRIzol reagent was added to each culture well. After the cells were dissolved, the samples were disrupted with a sonic dismembrator (Model F60, Fisher Scientific, Pittsburgh, PA, USA). Next, 0.2 mL chloroform was added and the samples were centrifuged at 12,000***g*** for 15 min at 4°C. The aqueous phase was collected and RNA pellets were obtained by

Table 1. Sequences of primers used for RT-PCR

Target genge	Forward primer	Reverse primer	Amplicon size (bp)
Collagen II	CCAAGAAGAACTGGTGGAGC	ATCCTGCAGCACGGTATAGG	314
GAPDH	GCTTCTTCTCGTGCAGTGCT	CATCACCCCACTTGATGTTG	301

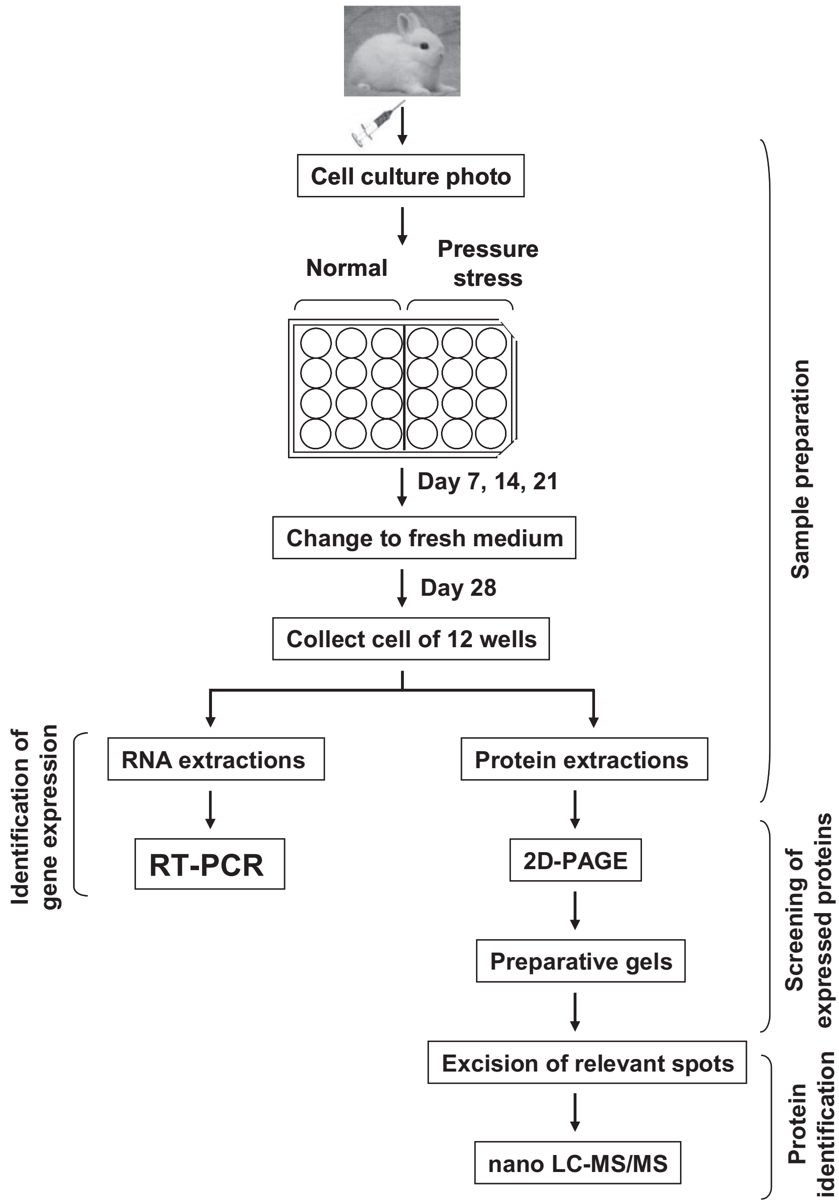

Figure 1. Schematic representation of the procedures used for screening differential expression of proteins in pressurized chondrocytes.

precipitation with isopropanol. After washing with 75% ethanol, the RNA pellet was dissolved with 50 µL water with 0.01% DEPC and stored at –80°C.

RT-PCR

A total of 1 µg of total RNA was used as a template for reverse transcription (RT) and polymerase chain reaction (PCR) amplification using the Superscript One-Step RT-PCR System (Gibco BRL, New York, USA), according to the manufacturer's instructions. Amplification was performed within a thermocycler (PTC-200, MJ Research,Watertown, MA, USA). The cyclic parameters for PCR were 48°C for 30 min to reversely transcribe RNA to cDNA, 95°C for 2 min to activate the *Taq* DNA polymerase, followed by 20 cycles of 30 s at 94°C for denaturing, 30 s at 60°C for annealing, 60 s at 72°C for extension, and a final cycle of 7 min

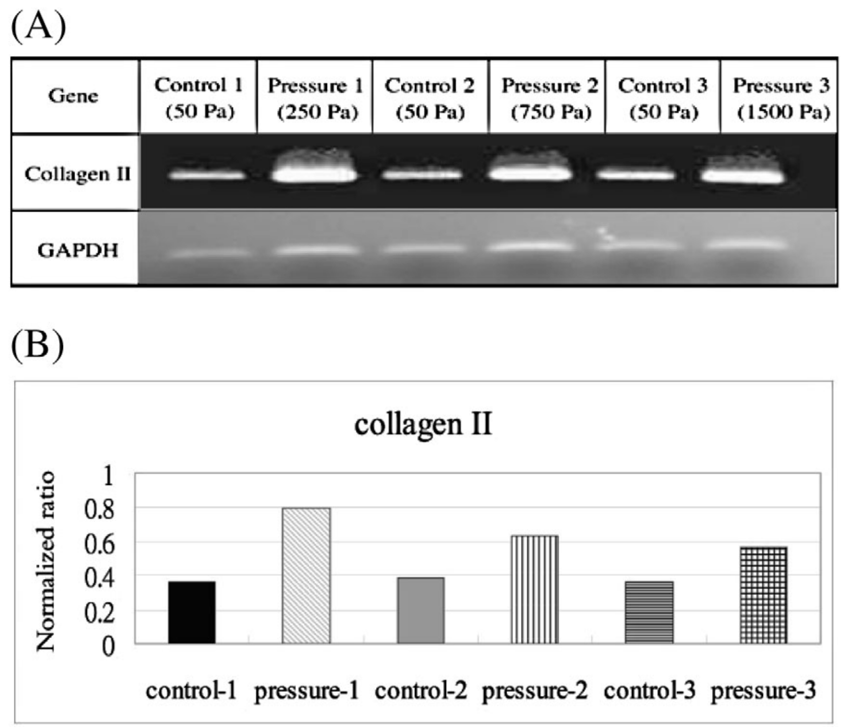

Figure 2. The mRNA expression of type II collagen and GAPDH under various pressures detected on agarose gel (A) and the normalized ratio (to GAPDH) by software AlphaEase™ (B).

at 72°C for extension. The amplification cycle number was carefully chosen to be within the linear zone of each gene. The primers were derived from Genbank (http://www.ncbi.nih.gov) sequences. GAPDH was used as an internal standard. Primers specific for collagen II and GAPDH are listed in Table 1. Aliquots of 20 µL of the PCR products were electrophoresed in 3% agarose gels stained with ethidium bromide. The signals were quantified using image analysis software AlphaEase™ (Alpha Innotech Corp., San Leandro, CA, USA) and were normalized to the expression of the GAPDH.

2-DE and Protein Spot Analysis

For 2-DE, control chondrocytes and pressurized chondrocytes were harvested, washed twice with ice-cold PBS, and then extracted with lysis buffer containing 8 M urea, 4% CHAPS, 2% pH 3–10 non-linear (NL) IPG buffer, and the Complete, Mini, EDTA-free protease inhibitor mixture. After a 3 h incubation at 4°C, the cell lysates were centrifuged for 15 min at 16,000***g***. The protein concentration of the resulting supernatants was measured using the Bio-Rad Protein Assay. Protein sample (200 µg) was diluted with 350 µL of rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer pH 3–10 NL, 18 mM DTT, 0.002% bromophenol blue), and then applied to the nonlinear Immobiline DryStrips (17 cm, pH 3–10). After the run of 1-D IEF on a Multiphor II system (GE Healthcare), the gel strips were incubated for 30 min in the equilibration solution I (6 M urea, 2% SDS, 30% glycerol, 1% DTT, 0.002% bromophenol blue, 50 mM Tris–HCl, pH 8.8), and for another 30 min in the equilibration solution II (6 M urea, 2% SDS, 30% glycerol, 2.5% iodoacetamide, 0.002% bromophenol blue, 50 mM Tris–HCl, pH 8.8). Subsequently, the IPG gels were transferred to the top of 12% polyacrylamide gels (20 × 20 cm × 1.0 mm) for the secondary dimensional run at 15 mA, 300 V for 14 h. Separated protein spots were fixed in the fixing solution (40% ethanol and 10% glacial acetic acid) for 30 min, stained on the gel with silver nitrate solution for 20 min, and then scanned by GS-800 imaging densitometer with PDQuest software version 7.1.1 (Bio-Rad). Data from three independently stained gels of each sample were exported to Microsoft Excel for creation of the correction graphs, spot intensity graphs and statistical analysis.

In-gel Digestion

The modified in-gel digestion method based on previous reports (Gharahdaghi *et al*., 1999; Terry *et al*., 2004) was performed for nanoelectrospray MS. Briefly, each spot of interest in the silver-stained gel was sliced and put into the microtube, and then washed twice with 50% ACN in 100 mM ammonium bicarbonate buffer (pH 8.0) for 10 min at room temperature. Subsequently, the excised-gel pieces were soaked in 100% ACN for 5 min, dried in a lyophilizer for 30 min and rehydrated in 50 mM ammonium bicarbonate buffer (pH 8.0) containing 10 µg/mL trypsin at 30°C for 16 h. After digestion, the peptides were extracted from the supernatant of the gel elution solution (50% ACN in 5.0% TFA), and dried in a vacuum centrifuge.

Nanoelectrospray MS and Database Search

The proteins were identified using an Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled to a QSTAR^{XL} quadrupole-time of flight (Q-TOF) mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA, USA). The peptides were separated using an RP C_{18} capillary column (15 cm × 75 µm id) with a flow rate of 200 nL/min, and eluted with a linear ACN gradient from 10 to 50% ACN in 0.1% formic acid for 60 min. The eluted peptides from the capillary column were sprayed into

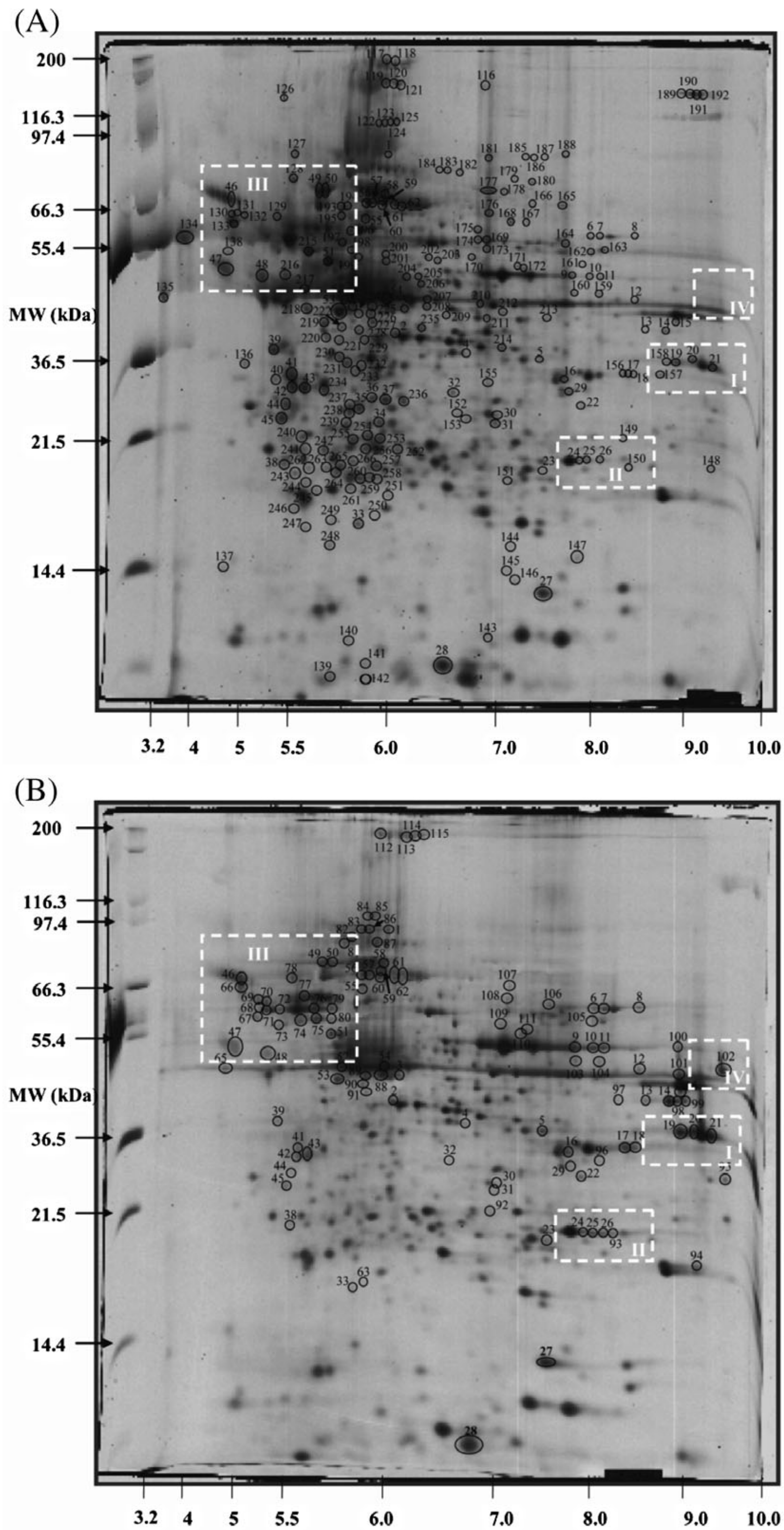

Figure 3. 2-DE images of total cell extracts from rabbit articular chondrocyte cells (A) and pressurized cells (B). Protein sample (200 µg) was applied to the nonlinear Immobiline DryStrip (17 cm, pH 3–10). After incubation in the equilibration solutions, the IPG gels were transferred to the top of a 12% polyacrylamide gel. The 2-DE gels were then stained with silver nitrate solution. Protein size markers are shown at the left of each gel (in kDa). Relevant differences are enlarged in (C). [I, glyceraldehyde 3-phosphate dehydrogenase (G3P2); II, triosephosphate isomerase (TPIS); III, protein disulfide–isomerase precursor (PDI); IV, heat shock protein 47 (HS47).] The protein spot ID numbers are consistent with those in Table 2.

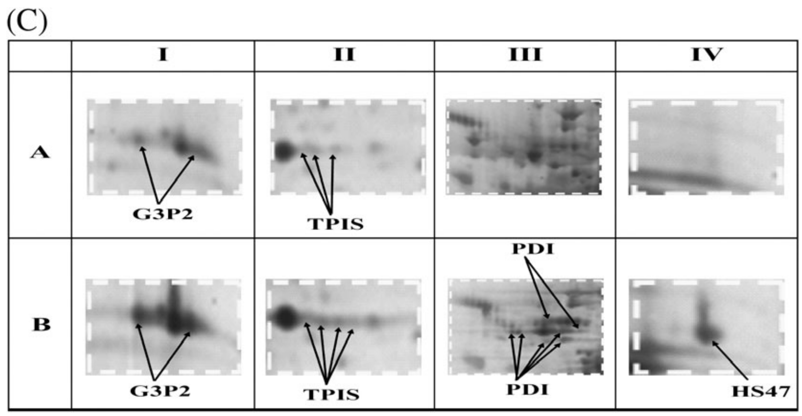

Figure 3. *Continued.*

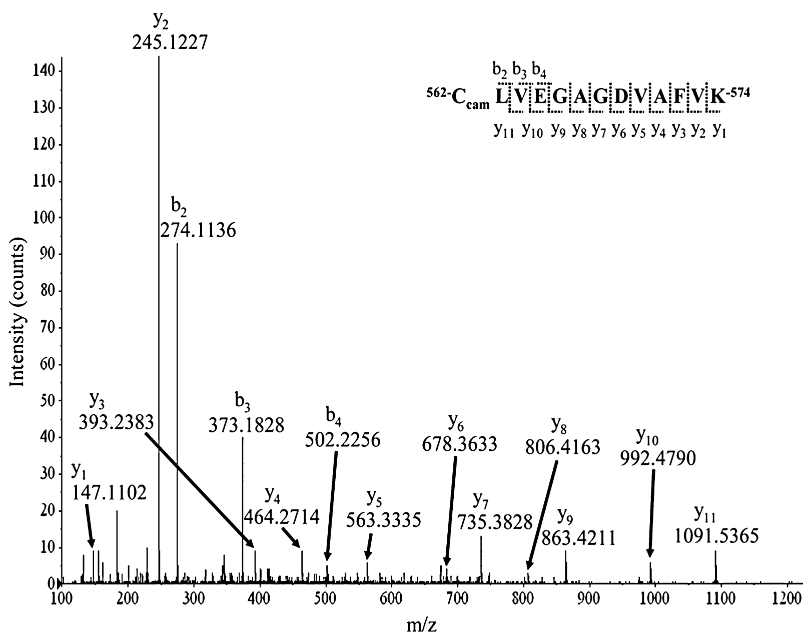

Figure 4. Identification of membrane-bound transferrin-like protein p97 (Spot ID 87). The MS/MS spectrum of the doubly charged ion *m/z* 682.86 for Spot ID 87 is shown. The amino acid sequence CLVEGAGDVAFVK was determined from mass differences in the y- and b-fragment ions series and matched residues 562–574 of membrane-bound transferrin-like protein p97. The abbreviation 'cam' denotes carbamidomethylated cysteine.

the MS by a PicoTip electrospray tip (FS360-20-10-D-20; New Objective, Cambridge, MA, USA). Data acquisition from Q-TOF was performed using the automatic Information Dependent Acquisition (IDA; Applied Biosystem/MDS Sciex). Proteins were identified by the nanoLC-MS/MS spectra by searching against NCBI databases for exact matches using the MASCOT search program (http://www.matrixscience.com; Hirosawa *et al*., 1993). An in-house *Oryctolagus cuniculus* taxonomy restriction was used and the mass tolerance of both precursor ion and fragment ions was set to ±0.3 Da. Carbamidomethyl cysteine was set as a fixed modification. The protein function was annotated using the Swiss-Prot (http://us.expasy.org/sprot/). The proteins were also categorized according to their biological process and pathway using the PANTHER classification system (http://www.pantherdb.org) as described in the previous studies (Thomas *et al*., 2003; Lazareva-Ulitsky *et al*., 2005; Mi *et al*., 2005).

Results and Discussion

Chondrocytes under Hydrostatic Pressure

Experimental evidence suggests that appropriate hydrostatic pressure is necessary to maintain the normal composition and biological properties of articular cartilage (Tammi *et al*., 1987; Palmoski *et al*., 1979; Jurvelin *et al*., 1990). Moreover, in our previous study we found that the cell numbers and biosynthesis of cultured chondrocytes increased when cultured under a hydrostatic pressure of 250 Pa (Lee *et al*., 2005). Currently, however, it is not clear which pressure-related proteins are expressed in chondrocytes under pressure.

In this study, we conducted a comparative proteome analysis of rabbit chondrocytes grown in the presence (loading group) or absense (control group) of hydrostatic pressure. A flow chart of

Table 2. Functional classification of the differentially expressed proteins in pressurized chondrocytes

Spot no.[a]	Protein ID	Access. no.[b]	M_r experimental[c] (kDa)	pI experimental[c]	M_r predicted[d] (kDa)	pI predicted[d]	No. peptides[e]	Sequence coverage[f] (%)
Metabolism								
Lipid and fatty acid								
5	Aldose reductase	gi|537593	36.5	7.44	35.7	6.46	3	10
Other groups								
88	Creatine kinase B-type	gi|125295	47.2	5.95	42.6	5.34	14	58
89	Creatine kinase B-type	gi|125295	47.2	5.87	42.6	5.34	3	14
Energy								
Glycolysis and gluconeogenesis								
6	Pyruvate kinase	gi|2623945	57.2	7.97	57.7	7.96	15	37
7	Pyruvate kinase	gi|2623945	57.2	8.07	57.7	7.96	13	34
8	Pyruvate kinase	gi|2623945	57.2	8.44	57.7	7.96	11	30
9	$\beta\beta$ enolase	gi|14141143	49.6	7.69	47.0	7.63	7	23
10	$\beta\beta$ enolase	gi|14141143	49.6	7.96	47.0	7.63	7	24
11	$\beta\beta$ enolase	gi|14141143	49.6	8.07	47.0	7.63	5	18
14	Chain A, Fructose 1,6-Bisphosphate Aldolase From Rabbit Muscle	gi|157874604	42.0	8.78	39.2	8.55	9	42
17	Fructose-bisphosphate aldolase A (muscle-type aldolase)	gi|113608	33.6	8.35	39.3	8.31	3	12
19	Glyceraldehyde-3-phosphate dehydrogenase	gi|406107	36.5	8.90	35.8	8.51	6	23
20	Mitochondrial malate dehydrogenase 2	gi|89574123	36.8	9.08	31.1	8.44	12	60
21	Glyceraldehyde-3-phosphate dehydrogenase	gi|406107	35.2	9.35	35.8	8.51	8	31
24	Triosephosphate isomerase	gi|136066	20.2	7.85	26.6	7.10	11	66
25	Triosephosphate isomerase	gi|136066	20.2	7.95	26.6	7.10	12	66
26	Triosephosphate isomerase	gi|136066	20.2	8.07	26.6	7.10	10	60
69	Pyruvate kinase	gi|1177221	63.5	5.17	57.9	7.60	6	15
93	Triosephosphate isomerase	gi|136066	20.2	8.18	26.6	7.10	11	66
97	Fructose-bisphosphate aldolase A	gi|113608	42.5	8.24	39.3	8.31	4	16
98	Chain A, fructose 1,6-bisphosphate Aldolase from rabbit muscle	gi|157874604	42.0	8.87	39.2	8.55	9	36
99	Chain a, fructose 1,6-bisphosphate Aldolase from rabbit muscle	gi|157874604	42.0	8.95	39.2	8.55	10	43
106	Pyruvate kinase	gi|2623945	62.4	7.51	57.7	7.96	16	41
109	Pyruvate kinase	gi|2623945	58.4	7.00	57.7	7.96	11	31
110	$\beta\beta$ Enolase	gi|14141143	56.1	7.21	47.0	7.63	4	11
111	$\beta\beta$ Enolase	gi|14141143	57.0	7.27	47.0	7.63	5	16
156	Fructose-bisphosphate aldolase A (muscle-type aldolase)	gi|113608	33.9	8.28	39.3	8.31	4	9
157	Mitochondrial malate dehydrogenase 2	gi|89574123	33.6	8.72	31.1	8.44	6	22
254	$\beta\beta$ Enolase	gi|14141143	21.9	5.88	47.0	7.63	4	10
Anaerobic glycolysis								
18	L-Lactate dehydrogenase A chain	gi|126050	33.6	8.41	36.5	8.17	7	22
Respiration and fermentation								
51	Mitochondrial ATP synthase, H^+ transporting F1 complex β subunit	gi|89574025	52.2	5.68	45.6	5.21	19	62
80	Mitochondrial ATP synthase, H^+ transporting F1 complex β subunit	gi|89574025	59.5	5.70	45.6	5.21	12	40
Transcription, protein synthesis and turnover								
230	Elongation factor 1 δ	gi|1134985	37.2	5.74	31.1	5.06	4	20
Protein fate (folding, modification, destination)								
47	Cardiac calumenin	gi|37904869	51.0	4.61	37.0	4.42	10	36
66	Calreticulin precursor	gi|117504	66.8	4.98	48.2	4.33	9	30
68	Protein disulfide-isomerase precursor	gi|129730	61.4	5.17	56.8	4.77	5	13
71	Protein disulfide-isomerase precursor	gi|129730	61.4	5.24	56.8	4.77	10	29
72	Protein disulfide-isomerase precursor	gi|129730	61.4	5.38	56.8	4.77	17	44
74	Protein disulfide-isomerase precursor	gi|129730	59.3	5.54	56.8	4.77	18	41
75	Protein disulfide-isomerase precursor	gi|129730	59.5	5.62	56.8	4.77	10	27
76	Protein disulfide-isomerase precursor	gi|129730	61.5	5.61	56.8	4.77	18	49
77	Protein disulfide-isomerase precursor	gi|129730	64.4	5.56	56.8	4.77	14	36
78	Protein disulfide-isomerase precursor	gi|129730	70.5	5.50	56.8	4.77	7	22
79	Protein disulfide-isomerase precursor	gi|129730	61.7	5.70	56.8	4.77	18	50
83	Glucose-regulated protein GRP94	gi|2581793	93.1	5.86	82.6	4.90	4	6
86	Glucose-regulated protein GRP94	gi|2581793	93.1	5.89	82.6	4.90	9	14
102	Heat shock protein 47	gi|8698691	48.2	9.43	14.4	9.13	7	65
108	Chaperonin Cct6	gi|3201994	64.1	7.08	58.0	6.46	10	27
136	Calreticulin	gi|237420	36.2	5.02	46.6	4.33	6	14
137	Calreticulin precursor	gi|117504	14.6	4.59	48.2	4.33	3	7
169	Protein disulfide-isomerase precursor	gi|129730	56.5	6.89	56.8	4.77	16	39
Signal transduction								
22	G-protein β subunit like-protein	gi|30025862	27.3	7.86	34.5	8.09	9	38
Cellular Organization								
Cytoskeleton and Microtubules								
39	Tropomyosin β	gi|223122	38.3	5.31	32.8	4.66	7	17
41	α-Tropomyosin	gi|1042003	33.6	5.50	32.7	4.69	14	36
44	Tropomyosin β	gi|223122	27.4	5.43	32.8	4.66	8	19
52	γ Non-muscle actin	gi|1703	45.8	5.76	41.7	5.30	8	30
53	γ Non-muscle actin	gi|1703	43.6	5.74	41.7	5.30	6	21

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Table 2. *Continued*

Spot no.[a]	Protein ID	Access. no.[b]	M_r experimental[c] (kDa)	pI experimental[c]	M_r predicted[d] (kDa)	pI predicted[d]	No. peptides[e]	Sequence coverage[f] (%)
54	γNon-muscle actin	gi|1703	45.6	5.98	41.7	5.30	10	32
65	γNon-muscle actin	gi|1703	48.8	4.63	41.7	5.30	7	24
101	γNon-muscle actin	gi|1703	47.4	8.89	41.7	5.30	9	32
103	γNon-muscle actin	gi|1703	50.2	7.79	41.7	5.30	3	9
189	Type II Collagen	gi|2190238	146.9	8.96	24.2	6.64	4	22
190	Type II Collagen	gi|2190238	146.9	9.06	24.2	6.64	6	28
191	Type II Collagen	gi|2190238	146.9	9.13	24.2	6.64	9	44
192	Type II Collagen	gi|2190238	146.9	9.21	24.2	6.64	8	34
198	γNon-muscle actin	gi|1703	53.6	5.80	41.7	5.30	7	23
199	γNon-muscle actin	gi|1703	52.0	5.84	41.7	5.30	3	10
207	γNon-muscle actin	gi|1703	45.7	6.31	41.7	5.30	6	17
214	LIM and SH3 protein 1	gi|3319229	29.3	7.03	29.9	6.61	9	29
217	γNon-muscle actin	gi|1703	47.3	5.56	41.7	5.30	5	20
Cell cycle								
87	Membrane-bound transferrin-like protein p97	gi|3786308	86.3	5.93	80.1	5.81	22	40
Annexin family								
16	Annexin VIII	gi|4102576	32.5	7.69	36.7	5.53	6	20
243	Annexin I	gi|1052873	19.4	5.52	38.7	6.28	7	32
263	Annexin I	gi|1052873	19.7	5.67	38.7	6.28	5	20
265	Annexin I	gi|1052873	20.6	5.74	38.7	6.28	6	24
266	Annexin I	gi|1052873	20.0	5.80	38.7	6.28	6	21
266	Annexin I	gi|1052873	20.0	5.80	38.7	6.28	6	21
Intermediate filament family								
112	Unknown (Filamin-B [Fragment])	gi|9664286 (Q9MZD2)[g)]	192.5	5.95	30.9	8.22	6	27
197	Vimentin [Fragment]	gi|39545951 (Q6S5G2)[g)]	55.6	5.7	6.9	4.46	3	38
Cell rescue, defense and stress								
206	Crystallin αA2	gi|229521	48.1	6.27	19.8	5.78	3	21
253	β A3-crystallin	gi|14285307	21.6	5.95	25.2	5.98	3	19
255	β A3-crystallin	gi|14285307	21.5	5.81	25.2	5.98	3	19
256	β A1-crystallin	gi|14285308	20.9	5.86	23.2	6.38	3	21
257	β A3-crystallin	gi|14285307	19.8	5.95	25.2	5.98	3	19
259	Crystallin αA2	gi|229521	19.2	5.89	19.8	5.78	4	17

[a] Numbers refer to spots depicted in Fig. 3.
[b] Protein accession number according to NCBI databases.
[c] Experimental Mr and pI calculated by analysis of the gel images with PDQuest 7.1.1 software.
[d] Predicted Mr and pI according to protein sequence and Swiss 2-D PAGE database.
[e] Number of peptide masses matching the top hit from MASCOT.
[f] Amino acid sequence coverage for the identified proteins.
[g] Protein accession number according to Swiss-Prot and TrEMBL databases.

the methods used in this work is depicted in Fig. 1. Initially, the chondrocytes were divided into four groups with pressure stress of 50 Pa (control group), 250 Pa, 750 Pa, and 1500 Pa. After the 3-week culture period, the mRNA expression of collagen II was studied (Fig. 2A). The mRNA expression of collagen II in the control group was 0.38 ± 0.20 (normalized ratio to GAPDH). In agreement with our previous study (Lee *et al.*, 2005), the level was significantly lower than in the pressurized groups (0.79 for 250 Pa, 0.63 for 750 Pa and 0.56 for 1500 Pa, respectively; Fig. 2B). Chondrocytes exposed to 250 Pa (loading group) were selected as the representative model for proteomic analysis in this study.

Identification of Differentially Regulated Proteins with Two-Dimensional Electrophoresis and LC/MS/MS

Identification of differential protein expression by proteomics currently relies on 2-DE technology, the excision of protein gel spots, enzyme digestion and sequencing by MS. In the present study, we quantified the types and levels of proteins expressed in rabbit-derived chondrocytes grown under atmospheric pressure (control) and in rabbit-derived chondrocytes grown under pressure (loading group). The protein expression level of chondrocytes in the control group (Fig. 3A) and in the loading group (Fig. 3B) were profiled by 2-DE and then analyzed by PDQuest software version 7.1.1 as described in the Methods section. In this study, a total of 266 spots were excised from the gels and analyzed with LC-MS/MS (Fig. 3). Details of the spot numbers from each gel are described in the next section. These selected protein spots were excised from the stained gel, subjected to in-gel tryptic digestion, and then subjected to nanoLC/MS/MS analysis using a nanoLC/Q-TOF MS system (Table 2). The representative peptide peaks from Q-TOF MS/MS analysis were detected, such as membrane-bound transferrin-like protein p97 (Spot ID 87) (Fig. 4), resulting in confident protein identification by MASCOT searching. The amino acid sequence coverage of the identified proteins varied from 6 to 66%. For example, membrane-bound transferrin-like protein p97 (Spot ID 87) had sequence coverage of 40% among 22 matched peptides.

Functional Classification of the Identified Proteins

All of the proteins identified in this work are listed in Table 2 and numbered on the 2-DE image (Fig. 3). Protein spots 1–28 and 29–62 were identified as being up-regulated and down-regulated proteins, respectively, in the loading group. Protein

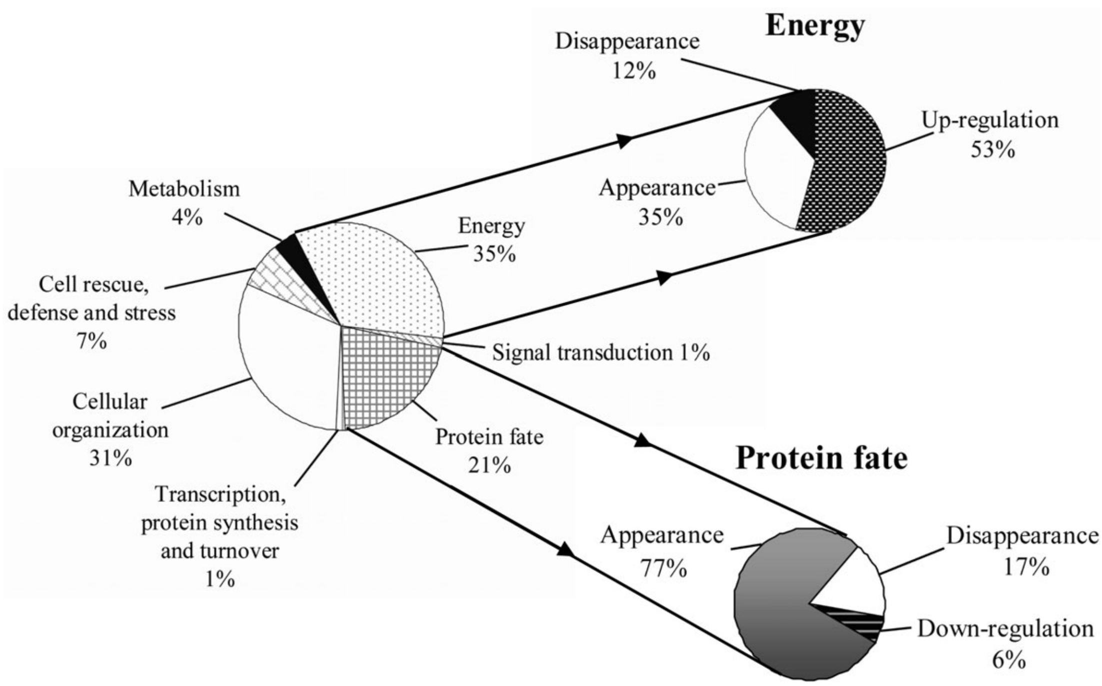

Figure 5. Functional distribution of the differentially expressed proteins identified by 2-DE and LC-MS/MS in rabbit articular chondrocytes under pressure stress. The up-regulated proteins in the pressurized cells (loading group) were labeled as 'up-regulation'. The down-regulated proteins in the loading group were labeled as 'down-regulation'. The proteins expressed only in the loading group were labeled as 'appearance'. The proteins expressed only in the control group were labeled as 'disappearance'.

spots 63–115 were identified as appearance only in the loading group and spots 116–266 were identified as appearance only in the control group. The up-regulated and down-regulated proteins were further categorized according to their biological functions using the PANTHER classification system. Functional distributions of the proportion of proteins are listed in Fig. 5. These proteins were associated with a variety of biological processes such as cellular organization (tropomyosin α and β, γ non-muscle actin, type II collagen, LIM and SH3 protein 1, membrane-bound transferrin-like protein p97, annexin I and VIII, filamin-B and vimentin), energy (pyruvate kinase, $\beta\beta$ enolase, fructose 1,6-bisphosphate aldolase A, glyceraldehyde-3-phosphate dehydrogenase, mitochondrial malate dehydrogenase 2, triosephosphate isomerase, L-lactate dehydrogenase A chain and mitochondrial ATP synthase), and protein fate (cardiac calumenin, calreticulin, calreticulin precursor, protein disulfide-isomerase precursor, glucose-regulated protein GRP94, heat shock protein 47 and chaperonin Cct6).

A significant proportion of proteins was associated with cellular organization (31%), energy (35%) and protein fate (21%) (Fig. 5). In addition, among the proteins involved in energy metabolism, 53% were up-regulated and 35% were expressed only (labeled as appearance) in the loading group. Among the proteins related to protein fate, 77% were expressed only (labeled as appearance) in the loading group.

It was interesting to find that chondrocytes in the loading group expressed an abundance of proteins related to metabolism and protein fate. Some of the metabolism-related proteins that were expressed included glyceraldehyde-3-phosphate dehydrogenase (Fig. 3C, panel I), triosephosphate isomerase (Fig. 3C, panel II), and mitochondrial ATP synthase (H+ transporting F1 complex β subunit), molecules that are involved in cellular respiration. Some of the protein fate-related proteins included protein disulfide-isomerase (Fig. 3C, panel III), heat shock protein 47 (Hsp47) (Fig. 3C, panel IV), glucose-regulated protein GRP94, and calreticulin, molecules that are involved in protein folding or degradation. Glucose-regulated protein GRP94, protein disulfide-isomerase and calreticulin also play a role in the processing and transport of wild-type cartilage oligomeric matrix protein (COMP) in normal chondrocytes, and in the retention of mutant COMP in pseudoachondroplasia (PSACH) chondrocytes (Hecht *et al*., 1998).

In this study we found that the mRNA level of type II collagen increased under pressure stress, whereas the expression level of the protein decreased. Colligin 1 (Hsp47), however, was only detected in the pressurized group. Hsp 47 is expressed exclusively in the endoplasmic reticulum and plays a vital role in procollagen processing (Masuda *et al*., 1998; Hattori *et al*., 2005).

Membrane-bound transferrin-like protein p97 was only expressed in the loading group. Protein p97 is a major concanavalin-A-binding protein in the chondrocyte plasma membrane, and plays a mediatory role in the chondrogenesis-promoting action of concanavalin A (Yan *et al*., 1990). Furthermore, p97 is a marker of chondrocyte differentiation and is involved in maintaining the cell surface characteristics of chondrocytes (Kawamoto *et al*., 1998).

Conclusion

Proteomic analysis of cellular responses to hydrostatic pressure demonstrated that pressure up-regulates the expression of proteins involved in energy metabolism and fate. Protein p97 was expressed only in pressurized cells. These data suggest that hydrostatic pressure can induce cell differentiation by increasing the expression level of energy metabolism- and protein fate-related proteins, indicating that hydrostatic pressure may be needed for normal biosynthesis and differentiation of articular chondrocytes.

Acknowledgements

The study was funded by a grant from the National Research Council of the Republic of China and the China Medical University Hospital (DMR 93-017).

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