Proteomic analysis of synovial fibroblast-like synoviocytes from rheumatoid arthritis

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Abstract

Objectives

The purpose of this study was to identity protein expression patterns of fibroblast-like synoviocytes (FLSs) derived from the synovial tissue of patients with rheumatoid arthritis (RA) and osteoarthritis.

Methods

Two-dimensional gel electrophoresis (2-DE) in combination with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) was used to visualise and identify differential cellular protein expression profiles in FLSs between RA and control groups. Western-blot analysis was performed to further verify selected differentially-expressed proteins.

Results

A total of 1633 and 1603 protein spots were examined in synovial FLSs of RA patients and controls, respectively. Ninety-two spots in the RA group were statistically over- or under-expressed compared with controls. Among them, 33 proteins over-expressed by more than 3-fold were then identified by MALDI-TOF-MS analysis. These proteins included enzymatic and structural proteins (e.g. PKM1/M2, α-enolase, ERp60, lamin-A/C), signal transduction proteins (e.g. annexin 11, peroxiredoxin 1, TrpRS), heat-shock/chaperone proteins (e.g. TCP-1, GRP75, HspB5, Bip) and some unknown protein species. Three proteins, namely α-enolase, GRP75 and PKM2, were verified by Western blot and the results were found to be consistent with proteomic analysis.

Conclusion

The differentially expressed proteins identified in RA synovial FLSs might be candidate RA-associated proteins and may prove to be promising diagnostic indicators or new therapeutic targets for RA.

Key words

rheumatoid arthritis, fibroblast-like synoviocytes, proteomic analysis, matrix assisted laser desorption/ionisation time-of-flight mass spectrometry

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Received on November 20, 2012; accepted in revised form on January 28, 2013.

Funding: this study was supported by a grant from National Natural Science Foundation of China (no. 81071419).

Competing interests: none declared.

Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease that is associated with chronic inflammation, progressive disability and systemic complications. In RA, synovial fibroblast-like synoviocytes (FLSs) assume a semiautonomous phenotype and participate in complex activation networks with macrophages, lymphocytes and dendritic cells. FLSs also play a role in blood vessel growth, which is necessary for pannus formation and arthritis development (1). FLSs therefore contribute directly to local cartilage destruction and chronic synovial inflammation, and promote a permissive microenvironment that sustains T-cell and B-cell survival and adaptive immune organisation. Considering the important role of synovial FLSs, the identification of a biomarker or a panel of biomarkers related to RA synovial FLSs would be of great clinical significance not only for tracking the process of inflammation in detail, but also for the development of experimental and clinical therapeutic trials and promoting a further understanding of RA. Moreover, the identified differential expression proteins might also hold an important value as joint-specific disease biomarkers.

Two-dimensional gel electrophoresis (2-DE) coupled to mass spectrometry (MS) and bioinformatics has been shown to be a powerful tool for protein profiling in target cells or tissues and has been widely used for large-scale identification of complex protein mixtures (2-4). Proteins digested with a known cleavage pattern can be accurately predicted based on the location and masses of peptides, and employing statistical-based bioinformatic approaches, it is possible to identity proteins with high confidence. Since gene expression at the mRNA level does not necessarily correspond 1:1 to the protein level, and proteins can be post-translationally modified in various ways (5), it is important to confirm gene expression data by direct quantitation of protein levels. Previous proteomic investigations of arthritis diseases (6-8), including rheumatoid arthritis, juvenile arthritis and osteoarthritis, have focused on the comparative analysis of synovial and plasma protein expression patterns, and have identified a number of proteins that may contribute to inflammation or invasion events (9, 10). In contrast, work on the proteome of synovial FLSs is still in its infancy, necessitating further study to define the molecular basis of RA and select possibly specific or significant proteins.

The present study was initiated to determine the specific protein expression patterns of FLSs derived from the synovial of RA patients. We carried out this study using 2-DE to compare protein expression profiles in FLSs between RA patients and controls. Subsequent identification of proteins by MS identified several novel protein species that may participate in the immune response in RA.

Materials and methods

Isolation and culture of FLSs

Human synovial tissue samples were obtained from three RA patients and three osteoarthritis patients undergoing total knee replacement or synovectomy. All RA patients fulfilled the American College of Rheumatology (ACR) criteria and the serologic profile and clinical phenotype of RA patients were shown in Table I. Approval from the local Ethics Committee (Nanjing, China) was obtained in advance of the study and all subjects gave their informed consent for the study.

FLS cells were isolated as described elsewhere (10). Briefly, synovial tissue was dissected and digested for 4–5 hours at 37°C with collagenase (0.2%). Cells were collected by centrifugation at 210×g for 10 min after filtration, then resuspended with DMEM containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 U/ml of streptomycin and cultured at 37°C in a humidified 5% CO_2 environment overnight. When confluence was reached, cells were trypsinised and subcultured. Cells from passages 4–7 passages were used for experiments.

Sample preparation and 2D-PAGE analysis

For extraction of proteins, FLSs were lysed in 7 M urea containing 2 M thiourea, 4% CHAPS, 1% DTT and protease

inhibitor cocktail, then the mixture was vortexed for 30 seconds, put on ice for 1 min, vortexed again for 30 seconds and then snap-frozen in liquid nitrogen. Two-DE was performed following the manufacturer's instructions (Amersham Biosciences, Sweden). Briefly, up to 150 μg of the extracted proteins were applied on linear immobiline dry strips (24 cm, pH 3–10) by which the strips were rehydrated overnight at room temperature. First dimension separation of proteins by isoelectric focusing (IEF) was performed for a total of 80.000 Vh including a final 8000 V step for 10 h to obtain high quality resolution. Prior to analysis in the second dimension, the strips were equilibrated in equilibration buffer for two 15-minute steps. Second dimension electrophoresis was carried on 12.5% SDS-PAGE gels and sealed in with 1% agarose (w/v) in running buffer. Samples were run at 5 W/gel for the first 45 min and then at 15 W/gel until the bromophenol blue dye reached the bottom of the gel. Finally, gels were stained with silver nitrate for matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS).

Image analysis

Silver-stained gels were analysed with Image Master 2D (Amersham Pharmacia), software comprising gel warping, DIGE normalisation and comparison modules. A single reference gel was assigned to the gel image with the most spots detected, and all remaining gel images were aligned to this reference. Proteomic differences in each gel were analysed by comparison with the standard image containing the matched information of all the gels created by the software itself. Protein spots with a p-value <0.05 for the paired t-test and having a greater than three-fold change in expression between the two groups were recognised as differential spots.

Mass spectrometry and database search

Thirty-three spots over-expressed by more than 3-fold in the RA group were manually excised from silver-stained 2D gels, and then digested overnight with trypsin. The samples were prepared on a MALDI target by applying the dried droplet method with HCCA as matrix (Bruker Daltonik, Bremen, Germany). Peptide mixtures were operated in positive ion reflector mode using an ion acceleration voltage of 19 kV. Measurements were externally calibrated with a standard peptide mixture. Database searches (IPI Human database) were performed using the Mascot software 1.8 (Matrix Science, London, UK), identification of protein was based on the following criteria: (1) carbamidomethyl cysteine fixed modifications; (2) oxidation methionine variable modifications; (3) up to 1 missed cleavage permitted; (4) peptide tolerance set at 100 ppm for the precursor ions. The acceptance criteria for peptide mass finger print (PMF) based identifications was a minimum Mascot score of 61, 20% sequence coverage of matched theoretical sequences, using a 95% confidence interval threshold (p<0.05).

Differential proteins verified by Western blot analysis

SDS-PAGE was carried out on precast 12% Bis-Tris gels according to the manufacturer's recommendations. For immunoblotting, proteins were electro-transferred onto polyvinylidene difluoride (PVDF) membranes. The primary antibodies used were rabbit polyclonal anti-pyruvate kinase isozymes M2 (PKM2), anti-α-enolase, anti-stress-70 protein (GRP75). Goat anti-rabbit conjugated with horseradish peroxidase was used as a secondary antibody. Optimal concentrations for each antibody were determined empirically. Densitometric analysis of the band intensities was performed using Quantity one Version 4.5.0 software. The statistical significance of these results was determined using the SPSS 17.0 statistical package.

Table I. Clinical and laboratory characteristics of the rheumatoid arthritis (RA) patients.

Sample	Sex	Age (years)	Age history (years)	Tender joint counts	Swollen joint counts	ESR (mm/h)	CRP (mg/dL)	Anti-CCP (IU/mL)	RF (IU/mL)
RA1	Female	61	29	9	12	28.9	36.8	125.8	265.2
RA2	Female	73	34	11	16	40.5	49.5	190.7	137.6
RA3	Female	79	45s	14	23	87.4	70.3	287.0	186.1

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; CCP: cyclic-citrullinated peptides; RF: rheumatoid factor.

Results

Proteome profiles of RA and controls

Approximately 1633 and 1603 protein spots were visualised on pH 3–10/12.5% 2-DE gels after silver staining in the RA and control groups, respectively (Fig. 1). Ninety-two spots were statistically under- or over-expressed in the gels of the two groups. Among them, 33 spots were at least 3-fold over expressed in the RA samples. The two-dimensional patterns for FLSs presented in this study were obtained from three representative results, and the patterns were found to be highly reproducible.

Mass spectrometric identification of selected proteins

The identification of the above-mentioned 33 spots after in-gel digestion relied on a peptide mass fingerprinting (PMF) approach using MALDI-TOF-MS peptide mapping analysis combined with IPI Human Database search (Table II). This approach is illustrated here with the identification of isoform C of Lamin-A/C (spot 218) and α-enolase (spot 486). Representative MALDI mass spectrometry spectra are provided in Figure 2.

Of the 33 spots, 30 spots were successfully identified. Of these 30 proteins, that with the highest apparent molecular mass was myomesin 1 isoform b, with a calculated molecular mass of 179.0 kDa, while the lowest was PSME4, with approximately 12.0 kDa. There was a high match between the theoretical and apparent values in the inspected cases.

The selected identified protein spots were broadly classified into several functional categories. Prominent spots representing protein species were identified to be enzymes, including pyruvate kinase isozymes M1/M2 (PKM2), α-enolase (NNE), protein disulfide-

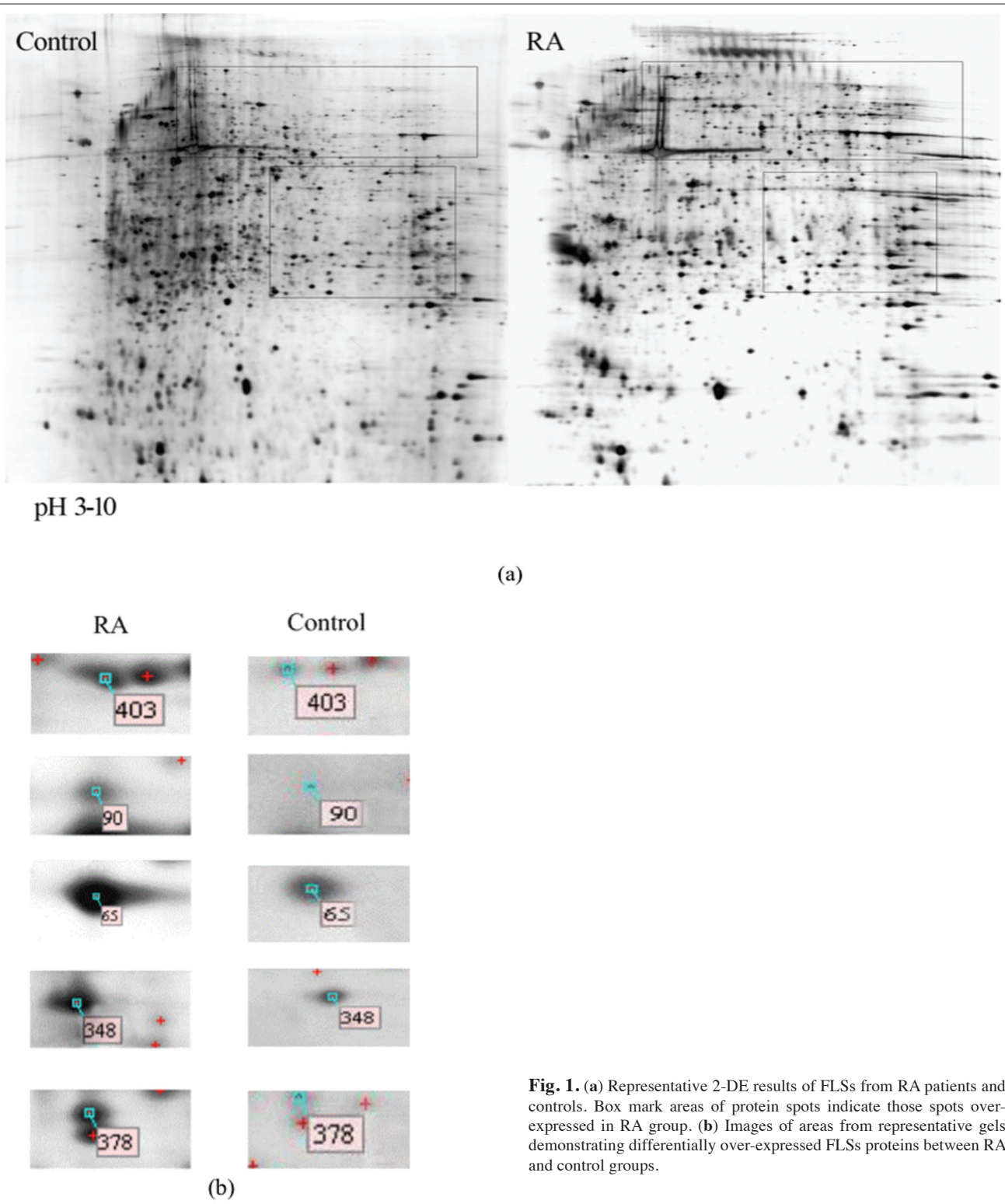

Fig. 1. (**a**) Representative 2-DE results of FLSs from RA patients and controls. Box mark areas of protein spots indicate those spots over-expressed in RA group. (**b**) Images of areas from representative gels demonstrating differentially over-expressed FLSs proteins between RA and control groups.

isomerase A3 precursor (ERp60), glutathione S-transferase (GSTM2), ubiquitin-conjugating enzyme E2K (E2-25K), platelet-activating factor acetylhydrolase B (PAF-AH-β), dihydropyrimidinase-related protein 2 (CRMP-2), adenosine kinase (AK), transaldolase (TAL), δ-δ-dienoyl-CoA isomerase/mitochondrial precursor (ECH1) and 26S protease regulatory subunit 6A (TBP). Many other spots contained protein species of cytoskeletal origin or cytoskeletal-associated proteins. Examples include lamin-A/C, myomesin-1 isoform b and ruvB-like 1(NMP238). Another group of proteins well represented on the 2-DE gel were the heat-shock/chaperone proteins, including T-complex protein 1 (TCP-1), Stress-70 protein/mitochondrial precursor (GRP75), α-crystalline B chain

Table II. Protein species identified on 2-DE gels by MALDI mass fingerprinting.

Spot n.	Accession n.	Protein name	Mascot score	Sequence coverage (%)	pI	MW (kDa)
65	IPI00021369	αB-crystallin (HspB5)	145	69	6.76	20.1
90	IPI00640741	peroxiredoxin1,19 000 protein (PRDX1)	121	55	6.41	19.1
140	IPI00007765	stress-70 protein, mitochondrial precursor (GRP75)	128	29	5.87	73.9
150	IPI00894476	PSME4	72	43	7.82	12.0
151	IPI00021370	ubiquitin-conjugating enzyme E2K (E2-25K)	84	36	5.33	22.5
218	IPI00216952	isoform C of Lamin-A/C	304	58	6.4	65.2
242	IPI00290566	T-complex protein 1 subunit α (TCP-1-α)	224	45	5.8	60.8
319	IPI00414320	annexin A11	79	24	7.53	54.7
323	IPI00021439	actin, cytoplasmic 1 (β-actin)	120	41	5.29	42.1
348	IPI00290279	adenosine kinase (AK)	139	37	6.24	40.9
350	IPI0041273	tryptophanyl-tRNA synthetase isoform b (TrpRS)	135	38	6.03	49.2
354	IPI00026546	platelet-activating factor acetylhyd- rolase IB subunit β (PAF-AH IB)	68	37	5.57	25.7
360	IPI00297779	T-complex protein 1 subunit β (TCP-1-β)	287	69	6.01	57.8
378	IPI00220644	isoform M1 of Pyruvate kinase isozymes M1/M2 (PKM1)	218	38	7.60	58.5
382	IPI00018398	26S protease regulatory subunit 6A (TBP-1)	158	43	5.13	49.5
401	IPI00021187	isoform 1 of RuvB-like 1 (NMP238)	132	36	6.02	50.5
403	IPI00220644	isoform M1 of Pyruvate kinase isozymes M1/M2 (M2-PK)	86	31	7.6	58.5
406	IPI00745729	SELENBP1, 54 kDa protein	115	30	6.04	54.7
424	IPI00025252	protein disulfide-isomerase A3 precursor (ERp60)	130	38	5.98	57.1
425	IPI00479186	isoform M2 of Pyruvate kinase isozymes M1/M2 (PKM2)	286	51	7.96	58.5
429	IPI00257508	dihydropyrimidinase-related protein 2 (CRMP-2)	209	47	5.97	62.7
456	IPI00479390	myomesin 1 isoform b	75	12	6.38	179.0
486	IPI00465248	α-enolase (NNE)	219	57	7.01	47.5
557	IPI00003362	78 kD glucose-regulated protein (GRP78, Bip)	110	23	5.07	72.5
809	IPI00744692	Transaldolase(TAL)	70	24	6.36	37.7
840	IPI00257508	dihydropyrimidinase-related protein 2 (CRMP-2)	64	17	5.97	62.7
1045	IPI00011416	δ-δ-dienoyl-CoA isomerase, mitochondrial precursor (ECH1)	93	32	8.16	36.1
1303	IPI00645711	GSTM2,Uncharacterized protein (GSTM2)	89	28	6.00	27.8
1484	IPI00021369	αB-crystallin (HspB5)	107	53	6.76	20.1
1499	IPI00021369	αB-crystallin (HspB5)	83	58	6.76	20.1

pI: isoelectric point; MW: molecular weight.

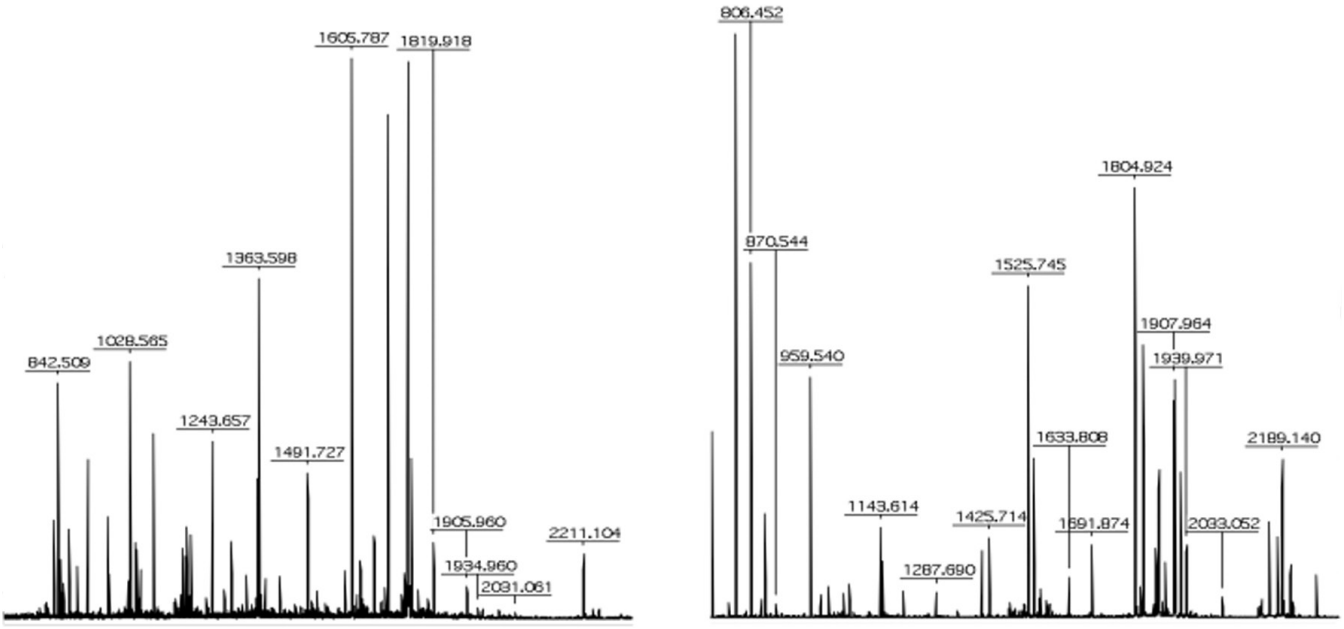

Fig. 2. Representative examples of MALDI-TOF mass spectrometry spectra of samples digested in gel with trypsin: (left) isoform C of Lamin-A/C and (right) α-enolase. Numbers in the mass spectra give precise m/z values for the detected peptide ion signals.

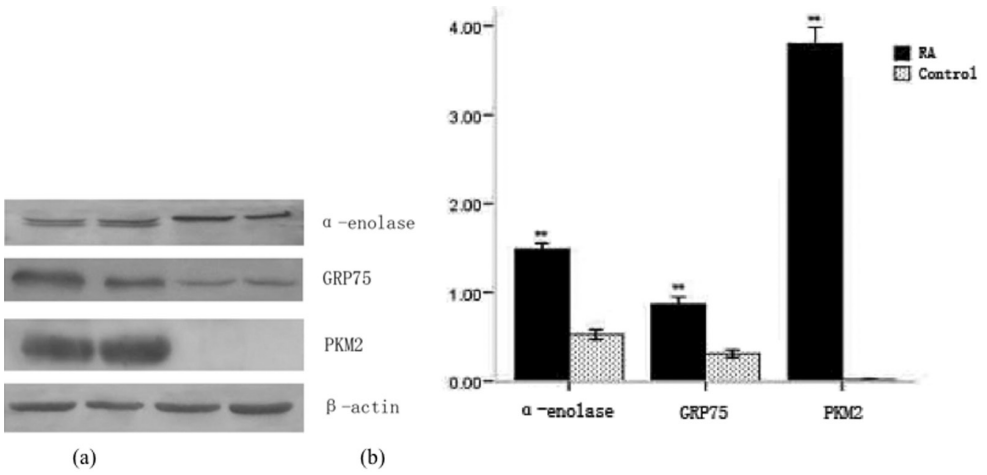

Fig. 3. Western blot analysis of α-enolase, GRP75, PKM2 within FLSs from RA and controls.
(**a**) Densitometry of the blots. The left two filters represent RA patients while the right two were controls.
(**b**) Semi-quantitative values obtained by densitometric analysis of corresponding protein expression in the two groups. The significance of the differences between the two groups is indicated by **(p<0.01).

(HspB5) and 78.000 glucose-regulated protein (Bip). Signal transduction proteins identified in the complex extract included annexin A11 (AnxA11), peroxiredoxin 1 (PRDX 1) and tryptophanyl-tRNA synthetase isoform b (TrpRS). In addition, there were several unknown protein species, such as PSME4 and SELENBP1.

Verification of protein expression in FLSs

Western blotting was employed to further verify differential protein expression in order to ensure the reliability of proteomic results from 2-DE. The results indicated that expression levels of α-enolase, GRP75, PKM2 were significantly higher in RA FLSs compared with controls, which was consistent with proteomic analysis (Fig. 3).

Discussion

Our approach of analysing FLSs protein expression in RA synovial tissue was based on the fact that synovial FLS cells have emerged as key pro-inflammatory cells promoting disease, in addition to immune cell accumulation (1, 10). Moreover, synovial FLSs also contribute to angiogenesis, which is essential for pannus formation and the arthritic process. It is clear that no single agent or process is wholly responsible for RA, but rather the interaction of these processes results in a characteristic protein profile with a particular inflammatory nature (11). The 30 over-expressed proteins identified by MS after 2-DE separation constitute an effort to describe the proteome of RA synovial FLSs on a global scale. These proteins include cytoskeletal components, chaperones and enzymes, signal transduction molecules, in addition to some unknown protein species.

A number of chaperones were identified within the RA synovial FLSs, in particular BiP and GRP75, members of stress-inducible heat-shock protein 70 (Hsp70) family (12). Hsp70s are known to be determinants of cell death and elevated expression of Hsp70s has been detected in tumour cells (13). Hsp70 family members also interact with key molecules of the cell cycle control systems, including p53, Cdk4, Wee-1, c-Myc, pRb and p27/Kip1. Recent studies have reported on the ability of Hsp70s to negatively regulate various stages of the p53-dependent or independent apoptotic pathways. While it has been estimated that p53 is overexpressed in RA synovium and is associated with joint damage, loss of p53 function through somatic mutation can occur in long-standing disease (14, 15). Therefore, it is reasonable to consider that BiP and GRP75 participate in the aggressive proliferation or defective apoptosis of RA synovial FLSs.

Several studies of RA have demonstrated that BiP can function as a potential biomarker in RA patients, since BiP was found to stimulate synovial T-cell proliferation and anti-BiP antibodies are present in the serum of RA patients (16, 17). Furthermore, down-regulation of BiP increases the rate of apoptosis of RA synovial FLSs and, conversely, overexpression of BiP prevents apoptosis in FLSs (18). Based on the present results, it appears that there may be some association between BiP responses and RA. GRP75 is relevant to human carcinogenesis since it interacts with p53 and inactivates its transcriptional activity and apoptotic functions (12, 13). GRP75 is elevated in many human tumours, as well as in many tumour-derived and *in vitro* immortalised cell lines. These results raise the possibility that subcellular localisation and expression levels of GRP75 could correlate with the proliferation of RA synovial FLSs.

Pyruvate kinase M2 (PKM2) is an isoform of pyruvate kinase, whose expression in cancer cells results in aerobic glycolysis and is suggested to confer a selective growth advantage (19). Knockdown of PKM2 results in decreased rates of glucose metabolism, increased apoptosis in multiple cancer cell lines and reduced cell proliferation. In contrast, rescue of PKM2 enhances tumour growth. Furthermore, it has been reported that plasma concentrations of PKM2 are increased in patients with rheumatic diseases (20), including RA, systemic lupus erythematosus (SLE), spondyloarthritis and others. In previous studies conducted in our laboratory, we also found increased levels of PKM2 in the sera of RA patients (data not shown). Determining the relevance of these findings to the pathology of synovial FLS proliferation in RA, however, requires future investigation.

Elevated levels of Annexin 11 (AnxA11), a member of the annexin superfamily of structurally related Ca^{2+}-dependent phospholipid-binding proteins, participate in a variety of in-

tracellular processes, ranging from the regulation of membrane dynamics to cell migration, proliferation and apoptosis (21-23). Previous studies suggested that AnxA11 may play a role in cellular DNA synthesis and in cell proliferation (24). Consistent with these observations, knockdown of annexin A11 expression results in a slower rate of cell growth (25). Our observation of increased levels of AnxA11 in RA synovial FLSs reflects the possibility that the association between AnxA11 and extensive FLS hyperplasia may be mediated through alterations in cell cycling/proliferation. The exact mechanism underlying this phenomenon remains to be further investigated.

Although the current analysis identified previously unidentified differential expressed protein species, important limitations remain (26), including: the relatively small number of samples per groups; 2-DE gels of the pH 3-10 range do not display protein species with isoelectric points outside this range; and 2-DE gels in general have intrinsic difficulties in resolving large and hydrophobic proteins. Future studies will employ affinity depletion to enrich low abundance proteins, thereby narrowing the high dynamic range of RA synovial FLSs proteins. These limitations indicate the need for larger validation studies and prospective synovial studies in groups where larger samples are available.

Conclusion

In summary, we carried out semi-quantitative analysis of the protein content of synovial FLSs, comparing samples from RA patients and controls. We demonstrated that (a) Bip and GRP75 may interact with cell cycle key molecules such as tumour suppressor p53 to stimulate synovial FLSs proliferation or inactivate FLSs apoptotic function; (b) PKM2 may play an important role in the growth advantage of FLS and might be a promising diagnostic indicator; and (c) AnxA11 may be a new therapeutic target for RA. We concluded that the newly identified protein biomarkers presented in this study will promote the understanding of the molecular biology of RA and facilitate future investigations on searching for markers of diagnosis and new therapeutic agents.

Acknowledgements

We thank International Science Editing for language editing the manuscript.

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