Vimentin fragments are potential markers of rheumatoid synovial fibroblasts

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Abstract

Objective

To study the protein expression differences between primary fibroblasts explanted from synovial membranes of patients with rheumatoid arthritis (RA) and osteoarthritis (OA).

Methods

Fibroblast cultures were obtained from 10 patients with RA and 5 patients with OA. After two-dimensional gel electrophoresis, proteins were excised and identified using peptide mass fingerprint. Expression of selected proteins was subsequently examined by immunoblot. Furthermore, we examined the cellular lysates for the presence of citrullinated proteins.

Results

The study was designed to compare expression changes of the common proteins detected in all studied fibroblast cultures (i.e. detected in all patients samples). We totally identified 191 shared proteins between RA and OA fibroblasts. A significant difference was defined as at least 2-fold upregulation or 0.6-fold downregulation of protein expression. The most obvious alteration observed in RA was the appearance of several vimentin fragments not present in OA. We did not detect citrullinated proteins in lysates from RA fibroblasts. This corroborates the current assumption that fibroblasts are not able to citrullinate proteins by themselves and that invading macrophages play a central role in this process.

Conclusion

We demonstrated that fibroblasts from patients with RA, despite being grown under identical conditions, preserve a particular feature and generate vimentin fragments not present in fibroblasts from OA. Elevated levels of different vimentin fragments have been recently reported in several rheumatic conditions. Further studies are needed to elucidate the pathogenic mechanisms induced by vimentin fragments in RA.

Key words

rheumatoid arthritis, osteoarthritis, proteomics, citrullination, vimentin fragments.

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Introduction

Rheumatoid arthritis (RA) is the most common type of chronic autoimmune inflammatory arthritis. Hereditary, immunological and environmental factors contribute to the development and modulate the progression of the disease. Normally, synovial fibroblasts form a thin cellular layer, the synovial membrane. In RA, the chronic inflammation of the synovial membrane results in hyperplasia, mononuclear cell infiltration and development of an invasive tissue pannus (1). Rheumatoid synovial fibroblasts acquire an aggressive phenotype, release pro-inflammatory cytokines and hence actively contribute to the inflammatory joint injury (2). In contrast, osteoarthritis (OA) is a primarily degenerative disease with destruction of the articular cartilage, affecting predominantly weight-bearing joints in older patients. However, there is now increasing evidence that at least in a small subset of patients with osteoarthritis, inflammatory pathways may be more involved than previously thought (3). The differences in the pathogenesis of the typical inflammatory RA and non-inflammatory OA have been reflected in the diagnostic approach to patients with undifferentiated arthritis. The traditional inflammatory markers and C-reactive protein have been complemented by testing for rheumatoid factor and anti-cyclic citrullinated peptide antibodies (ACPA). In recent years, a number of studies have provided new evidence on the role of specific cytokines in RA and led to the development of cytokine-targeted therapeutic strategies. Nevertheless, we still encounter patients with arthritis who are difficult to categorise because of uncharacteristic presentation and/or absence of the typical laboratory findings. In the present work, using clinical proteomic approach, we studied expression differences in shared proteins (*i.e.* expressed in both groups) between primary synovial fibroblasts isolated from patients with rheumatoid arthritis or osteoarthritis.

Material and methods

Isolation and culture of synovial fibroblasts

Synovial specimens were obtained at the time of total joint replacement surgery or arthroplasty from ten patients with rheumatoid arthritis (RA) and five patients with osteoarthritis. Patients with RA fulfilled the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for RA (4). The study was approved by the local Ethics Committee of the University Medical Center in Gottingen. Tissue samples were placed into cold sterile phosphate buffered saline (PBS) immediately after removal. To remove blood contamination, the tissue was washed several times in sterile PBS. Then, the synovial membrane was microsurgically separated from the fibrous membrane, and digested in 35 ml of collagenase (0.05%)/dispase (0.05%) solution for 3 hours at 37°C. Next, the suspension was filtered through a sterile 100 μm cell sieve, the filtrate washed with PBS and centrifuged at 400 g for 7 minutes at 4°C two times. The resulting cell pellet was resuspended in 20 ml of Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Germany) culture medium and seeded in 75cm^2 tissue culture flasks. Cells were maintained in DMEM medium supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine and 1% penicillin/streptomycin. Culture media were changed twice weekly and cells microscopically inspected for contamination. Cells were subcultured at 80% confluence.

Cell lysis and sample preparation

Cells were trypsinised in 0.25% trypsin, washed with 25 ml PBS and centrifuged at 400 g for 5 minutes at 4°C twice. The ensuing cell pellet was dissolved in 0.5 ml lysis buffer containing 9.5 M urea, 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate CHAPS (w/v), 50 mM DTT, 10 mM phenylmethylsulfonyl fluoride (PMSF) and Complete™ Protease Inhibitor Cocktail (Roche Diagnostic) for 30 min at 4°C. The lysate was centrifuged at 21000 g for 30 min at 4°C, and the supernatant stored in aliquots at -80°C.

Protein precipitation

In order to reduce salt contamination and to enrich the protein fraction,

chloroform-methanol precipitation according to Wessel and Fluge was performed (5). Briefly, 400 µL of ice-cold methanol (100%) was added to 100 µL of protein sample and mixed together. Then, 100 µL of chloroform was added and the mixture was vortexed. Subsequently, 300 µL of ultrapure water was added and the solution was vortexed and centrifuged at 14000 g for 1 min at 4°C. The aqueous layer was removed, and another 600 µL of methanol (100%) was added to the rest of the chloroform and the interphase with the precipitated proteins. The sample was mixed and centrifuged at 14000 g for 1 min at 4°C, and the supernatant was removed. The resulting pellet was vacuum-dried and dissolved in lysis buffer. Total protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA), with bovine serum albumin used as a standard.

Two-dimensional gel electrophoresis

2D-gel electrophoresis was performed as described previously (6). Prior to SDS-PAGE, the IPG strips were reduced in SDS equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 0.05 M Tris-HCl, and 2% DTT) for 20 min at room temperature on a rocking table. The strips were subsequently alkylated in the same solution with 2.5% iodoacetamide substituted for DTT. For SDS-PAGE, 12% Bis-Tris Criterion precast gels (Bio-Rad, Hercules, CA) were used according to the manufacturer's instructions. The gels were run at 150 V for 10 min and then by 200 V until the bromophenol blue dye front had reached the bottom of the gels.

Gel staining

For image analysis, 2-DE gels were fixed in a solution containing 50% methanol and 12% acetic acid overnight and then stained with Flamingo fluorescent stain (Bio-Rad, Hercules, CA) for a minimum of 5 hours. After staining, gels were scanned at 50 µm resolution on a Fuji FLA-5100 fluorescence laser scanner. The digitalised images were analysed using Delta 2D 3.4 software (Decodon, Braunschweig, Germany). For protein identification, 2-DE gels were additionally stained with colloidal Coomassie blue (Roti-Blue, Roth, Karlsruhe, Germany) overnight.

MALDI-TOF MS identification of protein spots

Analysis was performed as described previously (7). The peptide samples were extracted with different concentrations of ACN and trifluoroacetic acid (TFA), then co-crystallised with matrix (α-cyano-4-hydroxycinnamic acid) on a stainless steel target using 1 µl matrix and 1 µl sample. Applied Biosystems Voyager-DE STR MALDI-TOF mass spectrometer, operating in delayed reflector mode with an accelerated voltage of 20 kV was used to generate peptide mass maps. Mass spectra were obtained by averaging 50 individual laser shots. All samples were externally calibrated with a peptide mix of des-Arg-bradykinin ([M+H]+ 904.46), angiotensin I ([M+H]+ 1296.68), Glu1-fibrinopeptide B ([M+H]+ 1570.67), ACTH (1-17) ([M+H]+ 2093.08), ACTH (18-39) ([M+H]+ 2465.19) and the resulting mass spectra were internally calibrated with trypsin autolysis products (m/z 842.50 and m/z 2211.10). Monoisotopic peptide masses were assigned and then used in Mascot database searches.

Database search

A database search with the peptide masses was performed against the MSDB or NCBInr database using the Mascot peptide mass fingerprint software provided by Matrix Science (Oxford, UK). Carboxamidomethylation and methionine oxidation were considered as variable modifications. A database search was performed so that after identification each hit was inspected visually to match as much spectral information as possible. The quality criteria encompassed: optimised mass accuracy (ca. 50 ppm), minimal mass deviation (in the mDa range), maximised sequence coverage, highest possible probability score, and maximal number of intense ion signals had to be assigned to the identified protein.

Western blotting

To verify the differences in protein expression observed in proteomic studies, we examined the expression of selected proteins by Western blot. Briefly, equal amounts of total cellular protein (40 µg) from RASF and OASF were loaded on a 12.5% polyacrylamide gel (Tris-HCl, Mini-Protean, Biorad). After electrophoresis, the proteins were transferred to a PVDF membrane (Immobilon-P^{SQ}, Milipore), and incubated with primary antibodies (anti-HSPB1, R&D; 1:500; anti-vimentin, Sigma Aldrich, 1:2000) overnight at 4°C. After several washings, membranes were incubated with HRP-conjugated secondary antibodies (Amersham Biosciences, Germany) for 1 h at room temperature. Visualisation was performed using a chemiluminescence detection kit (ECL Plus; Amersham) according to the manufacturer's instructions.

Statistical analysis

In 2D-gel electrophoresis experiments, the digitalised images were analysed; spot matching across gels and normalisation were performed using Delta2D 3.4 software (Decodon, Braunschweig, Germany). Delta2D computes a 'spot quality' value for every detected spot. This value shows how closely a spot represents the 'ideal' 3D Gaussian bell shape. Based on average spot volume ratio, spots whose relative expression is changed at least 2-fold (increase or decrease) between the compared samples were considered to be significant. To analyse the significance of protein regulation, Student's *t*-test was performed, and statistical significance was assumed for *p*-values less than 0.05.

Results

Patients

Fifteen synovial membrane samples were obtained from 10 patients with RA and 5 patients with OA. In order to obtain sufficient protein, synovial tissue material dissected from knee, hip and shoulder joints was examined. We did not examine pooled samples from a group of patients but studied differences between the individual fibroblast cultures isolated from the individual patients.

Primary fibroblasts explanted and cultured from synovial membrane obtained of patients with RA were named "rheumatoid arthritis synovial fibroblasts

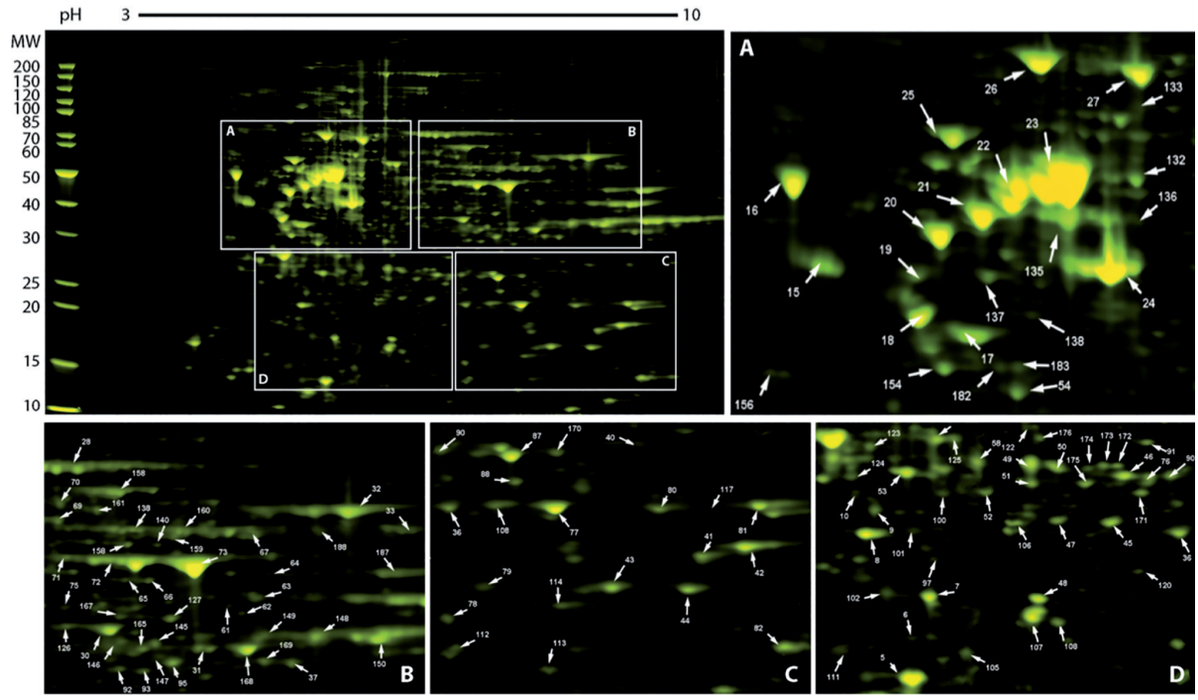

Fig. 1. Representative 2D-gel-electrophoresis image of protein lysate from cultured synovial fibroblasts. Synovial fibroblasts were explanted after collagenase and dispase digestion of microsurgically dissected synovial membrane. After three passages, fibroblasts were harvested, lysed and the proteome examined with two-dimensional gel electrophoresis. 150 µg protein was loaded on the IPG strip pH 3-10 and isoelectrically focused using the Biorad PROTEAN IEF Cell. Criterion precast 12 % Bis-Tris gel was used for SDS-PAGE. The protein spots were visualised with Flamingo® gel stain and identified by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOF). The molecular weight of the proteins is indicated on the left, Page Ruler® Prestained Protein Ladder was used as a marker. The complete list of the identified proteins is presented in Supplementary Table I.

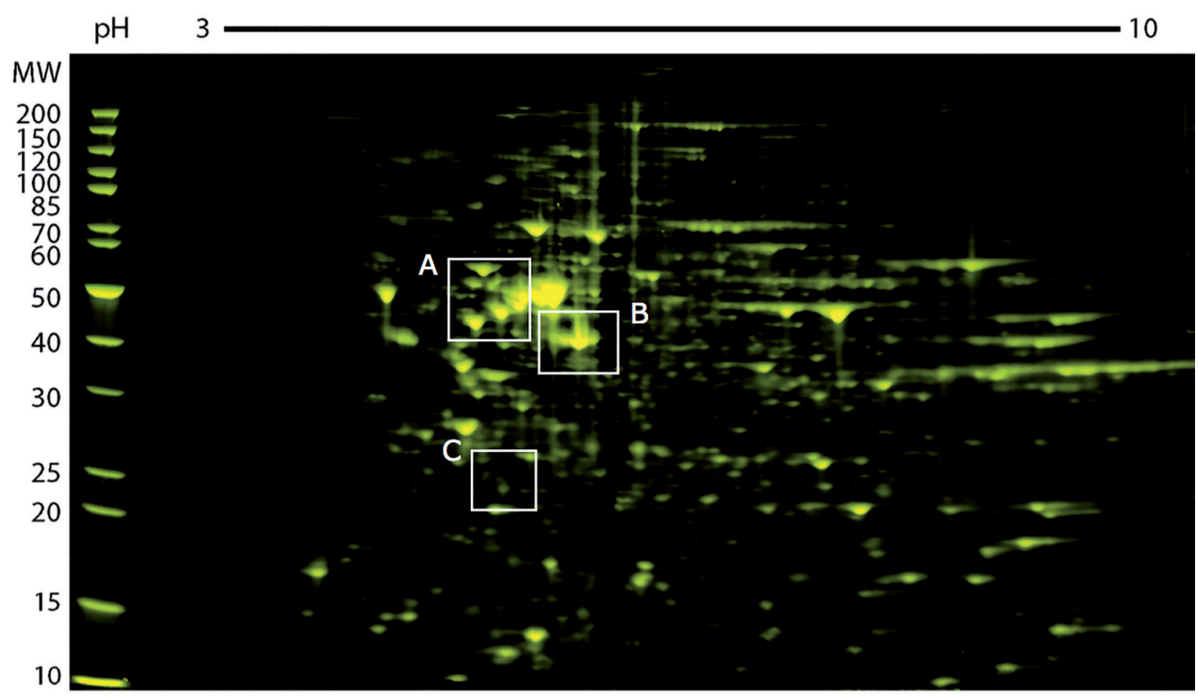

Fig. 2. Representative 2D-gel-electrophoresis image of protein lysate from explanted primary synovial fibroblasts. Synovial fibroblasts were explanted after collagenase and dispase digestion of microsurgically dissected synovial membrane. After three passages, fibroblasts were harvested, lysed and the proteome examined with two-dimensional gel electrophoresis. 150 µg protein was loaded on the IPG strip pH 3-10 and isoelectrically focused using the Biorad PROTEAN IEF Cell. Criterion precast 12% Bis-Tris gel was used for SDS-PAGE. The protein spots were visualised with Flamingo® gel stain and identified by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOF). The molecular weight of the proteins is indicated on the left, Page Ruler® Prestained Protein Ladder was used as a marker. The frames show gel sections with differentially regulated proteins, which are in detail presented in Figure 3.

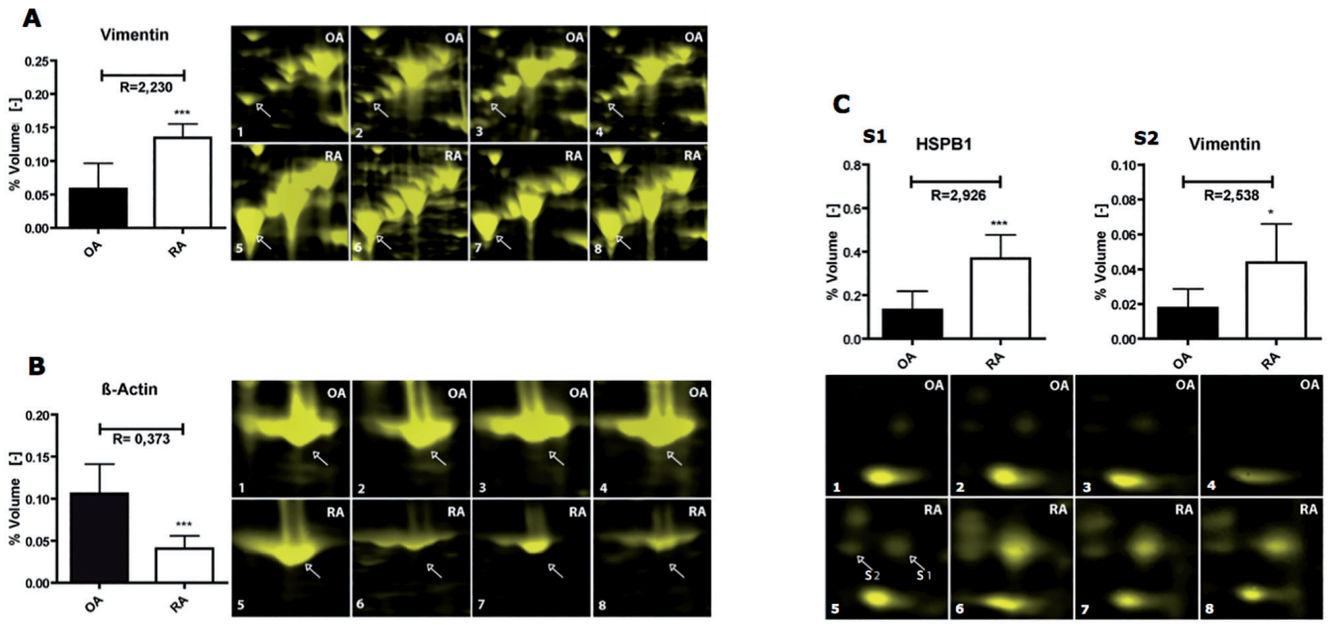

Fig. 3. Expression of vimentin fragments, β-actin und HSPB1 primary synovial fibroblasts examined by two-dimensional gel electrophoresis. Synovial fibroblasts were explanted after collagenase and dispase digestion of microsurgically dissected synovial membrane. After three passages, fibroblasts were harvested, lysed and the proteome examined with two-dimensional gel electrophoresis. 150 µg protein was loaded on the IPG strip pH 3-10 and isoelectrically focused using the Biorad PROTEAN IEF Cell. Criterion precast 12 % Bis-Tris gel was used for SDS-PAGE. The protein spots were visualised with Flamingo® gel stain and identified by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOF). **(A)** The levels of 44 kDa vimentin fragment were upregulated in fibroblasts from patients with rheumatoid arthritis (RA). **(B)** The expression levels of β-actin were downregulated in RASF. **(C)** The levels of HSPB1 (Spot 1, S1) and 25 kDa vimentin fragment (Spot 2, S2) were upregulated in RASF from patients with rheumatoid arthritis (RA). R represents the expression ratio calculated from mean values of the spots volumes computed by Delta2D software. (*) p=0,05, (***) p=0,001.

(RASF)", and fibroblasts obtained from patients with OA "osteoarthritis synovial fibroblasts (OASF)". After digestion of the fresh synovial membrane, numerous mononuclear cells were still present in the fibroblasts cultures. Zimmermann *et al*. showed that the proportion of mononuclear cells in cultures of synovial fibroblasts decreases below 3% after the third passage (8). For this reason, fibroblasts were subcultured and during the first three passages, all cultures were inspected for morphology and growth characteristics. Cultures that fulfilled the criteria for homogeneous population were used in further experiments. Five groups of RASF and three groups of OASF were selected and explored (Supplementary Fig. 1). The mean culture period (three passages) was 22.2 days for RASF and 22.5 days for OASF. Both, RASF and OASF were cultured under identical conditions.

After 2D gel electrophoresis of lysates from RASF and OASF, the protein spots were excised and identified using peptide mass fingerprint. We purposely designed the study to examine only common protein spots present on all gels (*i.e*. detected in all RASF and all OASF cultures) as evidenced by their overlapping in 2D gels. We totally identified 191 common proteins present on all studied gels (Fig. 1 and Supplementary Table I). The spot matching across gels and normalisation were performed using Delta2D software, protein expression level was quantified based on average spot volume ratio. A significant difference was defined as at least 2-fold upregulation or 0.6-fold downregulation of protein expression simultaneously present in all studied gels. Using this preselection, significant differences between RASF and OASF were detected for vimentin, beta-actin and heat shock protein beta-1 (Fig. 2). RASF demonstrated a 2.23-fold increase of the 44-kDa (p=0.001, Fig. 3A) vimentin fragment, a 2.54-fold increase of the 25-kDa (p=0.05, Fig. 3C) vimentin fragment, and a 3-fold upregulation of the heat shock protein beta-1 (HSPB1, p=0.001, Fig. 3C) compared to OASF. The levels of β-actin were decreased in RASF (0.37-fold, p=0.001, Fig. 3B).

Western blot

The expression of vimentin and HSPB1 were examined additionally by Western blot. Immunoblotting confirmed the presence of specific vimentin fragments between 25 and 37 kDa, and one additional vimentin fragment at 44 kDa in RASF (Fig. 4). None of these vimentin fragments was present in OASF. We did not see an upregulation of HSPB1 in RASF using immunoblot. This might be explained by the phosphorylation of HSPB1 and subsequent pI shift under stress, which can be only detected by 2D gel electrophoresis but not by immunoblotting (9).

Citrullination of proteins

Next, we examined the proteomes of RASF for the presence of citrullinated residues. To accomplish this, we implemented two methods. Firstly, RASF proteomes were examined for the presence of unmodified citrulline residues using an anti-citrulline antibody (Biomol, Hamburg, Germany, 1:1000). Secondly, we used an anti-citrulline detection kit (Biomol) that also identifies modified citrulline residues. Re-

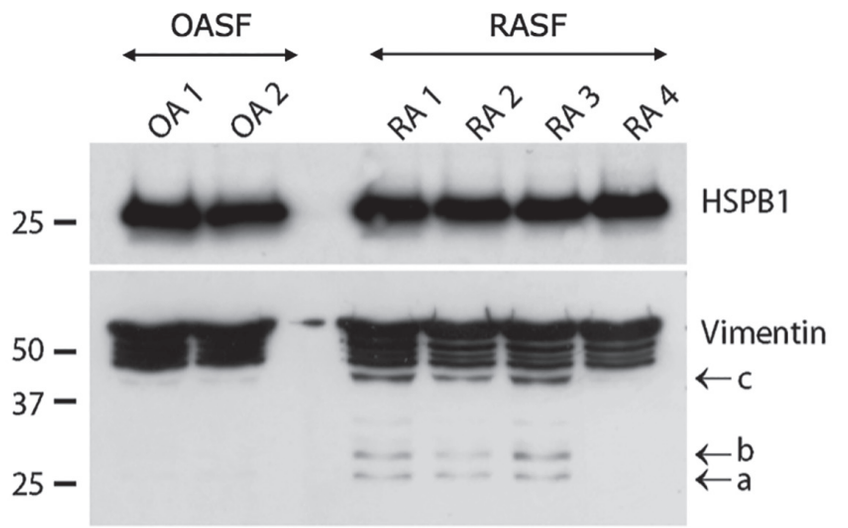

Fig. 4. Western blot of protein lysates from rheumatoid arthritis synovial fibroblasts (RASF) and osteoarthritis synovial fibroblasts (OASF) isolated from patients with rheumatoid arthritis (RA) or osteoarthritis (OA), respectively. Immunoblotting confirmed the presence of vimentin fragments between **(a)** 25 kDa and **(b)** 37 kDa, and one additional vimentin fragment around **(c)** 44 kDa in RASF. None of these vimentin fragments were present in OASF. 40 µg of protein lysate from synovial fibroblasts was separated by SDS-PAGE using 12,5% Tris-HCl precast gels and Biorad Mini-Protean® System. After transfer onto a PVDF membrane, incubation with primary antibodies against HSPB1 (R&D; 1:500), vimentin (Sigma Aldrich; 1:2000), and HRP-conjugated secondary antibodies, protein bands were visualised using a ECL Plus chemiluminescence detection kit.

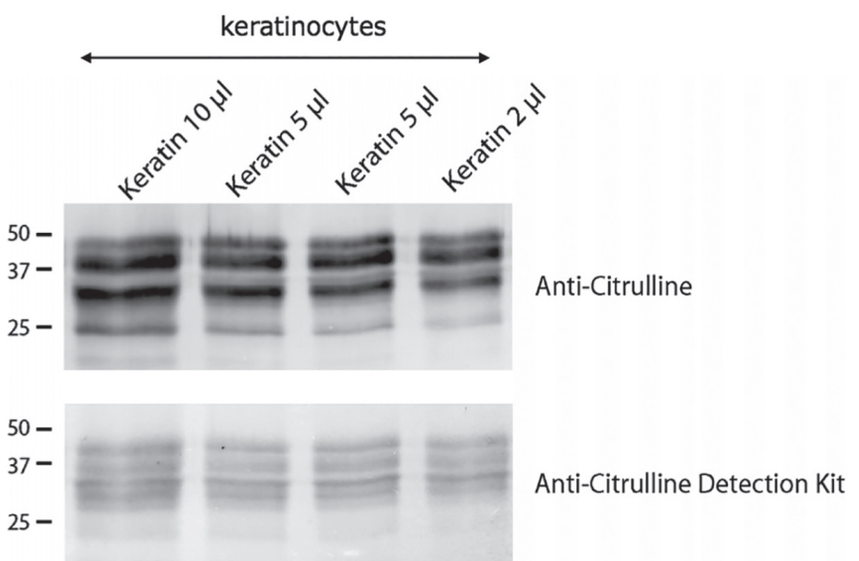

Fig. 5. Detection of citrullination. Western blot of protein lysates from human skin keratinocytes. 2 µl, 5 µl and 10 µl of protein lysates from human keratinocytes were separated by SDS-PAGE (12,5% Tris-HCl precast gel, BioRad Mini-Protean® System) and transferred onto a PVDF membrane. The unmodified citrulline residues were detected using an anti-citrulline antibody (Biomol, Germany, 1:1000), the modified citrulline residues were examined with an anti-citrulline detection kit (Biomol). Both methods confirmed correct functioning and proper detection of citrulline residues in cellular protein lysates.

gardless of the numerous modifications of the experimental settings, we did not detect citrullinated residues in protein lysates from RASF (or OASF). The correct functioning of both methods was confirmed using control lysates prepared from human epidermal cells, which gave constantly positive results (Fig. 5). (*In vivo*, citrullination naturally occurs during terminal differentiation of keratinocytes (10)).

Discussion

There is a high demand for additional biomarkers to improve the diagnostics of RA. The "-omics" techniques that have emerged in recent years have allowed us to improve the understanding of the pathogenesis of diseases. At the same time, advancements in technology offer an excellent opportunity to identify new putative biomarkers valuable for early diagnosis and stratification of RA patients, and even for prediction of response to a specific therapy (11, 12). In the present work, we compared the proteomes of synovial fibroblasts explanted from patients with RA and OA. This is a substantial difference compared to studies exploring lysates of the whole synovial tissue containing blood vessels and immune cells. Prolonged e*x vivo* culture of isolated synovial fibroblasts reduces the number of contaminant mononuclear cells. Therefore, we were interested in exploring the differences between RASF and OASF that persist after a prolonged culture under identical conditions.

We identified 191 shared proteins between RASF and OASF. By applying a stringent preselection, significant differences were found in only few common proteins. RASF presented increased levels of HSPB1, a member of small heat shock proteins, molecules with important chaperone function and participation in native and adaptive immunity. Sera from patients with Behçet's disease and RA exhibited increased reactivity against HSPB1 (13, 14). Treatment of dendritic cells with recombinant HSPB1 induced an inflammatory phenotype with upregulation of IL-1, IL-6 and TNF-α (15). Precise mechanisms underlying these observations are not clear, even though the role of HSPB1 in antigen presentation implies an immunomodulatory function. Moreover, HSPB1 binds to HSP70 and hinders its ability to refold denatured proteins and inhibit apoptosis (14).

RASF showed decreased expression of β-actin, which has also been previously reported in inflammatory diseases, and consequently questions its reliability as housekeeping gene/protein under these conditions (16). The most noticeable change observed in RASF was the appearance of several vimentin fragments not present in OASF. Almost twenty

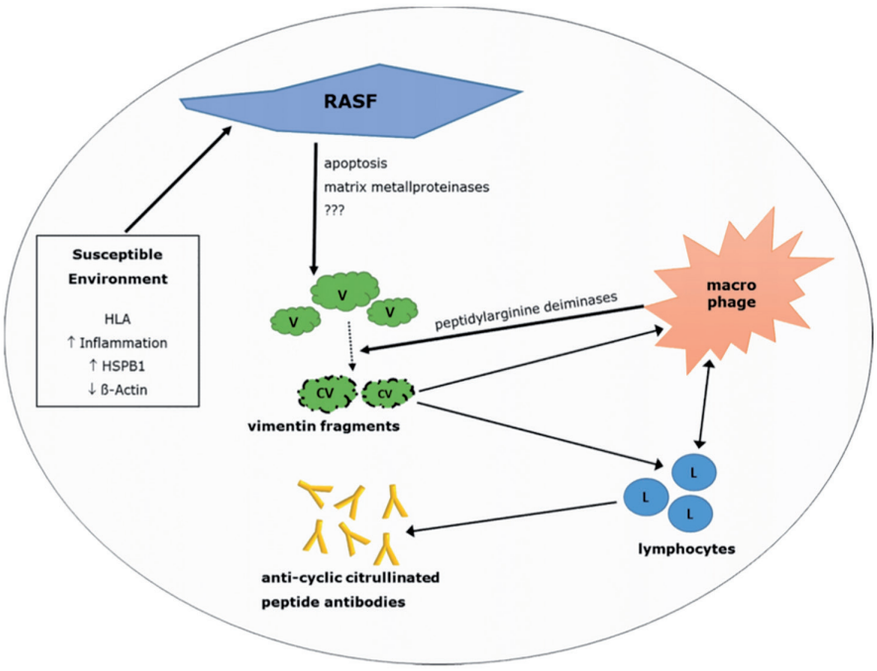

Fig. 6. Schematic presentation of the study results. Rheumatoid arthritis synovial fibroblasts (RASF) generate vimentin fragments (apoptotic cleavage by caspases, cleavage by matrix metalloproteinases or by other unknown mechanism). Vimentin fragments become citrullinated by peptidylarginine deiminases from invading macrophages. Citrullinated vimentin fragments are recognised by mononuclear cells and in susceptible environment and/or individuals, they may induce the production of anti-cyclic citrullinated peptide antibodies.

years ago, Despres *et al*. described reactivity of the serum from one patient with RA to an approximately 50 kDa protein band in immunoblot using protein lysates from placenta (17). This band was named after the patient as "Sa" antigen, and the antibodies anti-Sa antibodies. The Sa antigen was later identified in immunoprecipitation studies by Vossenaar as citrullinated vimentin (18). High specificity of anti-Sa antibodies for RA was confirmed by the group of Huber (19). Supplementary Figure 2 shows a side by side comparison of immunoblots from the work by Vossenaar (18), Hueber (19) and the one from our present study. The size of the Sa antigen corresponds approximately to the 44 kDa vimentin fragment seen in RASF. The common size of vimentin fragments reported in the literature varies between 30 and 50 kDa (20). These minor variations are probably a consequence of different tissue samples and dissimilar experimental conditions used in these studies.

During inflammation, many antigens become modified, thereby creating cryptic epitopes which may trigger autoimmunity (21). A well-recognised phenomenon in RA is the formation of citrullinated proteins (22-24). Citrullination is a post-translational modification (deimination) of arginine residues catalysed by peptidylarginine deiminases (PAD). Positively charged arginine residues, important for the tertiary structure of proteins are converted to uncharged citrulline residues, which may result in conformational and functional alterations. Among the numerous citrullinated proteins described in RA synovium, the best characterised are vimentin, fibrin, collagen type II and α-enolase. Van Beers created the term 'citrullinome' referring to the whole ensemble of citrullinated proteins identified in sera and synovial fluid of RA patients (20). Citrullination has also been reported in other, mostly inflammatory conditions, suggesting an inflammation-dependent rather than a RA-specific process (25). Vimentin represents the principal substrate of PAD expressed in macrophages invading the synovial tissue in RA (26). It was generally assumed that fibroblasts do not possess PAD. A recent study demonstrated a weak to moderate mRNA expression of PAD2 and PAD4 in synovial fibroblasts isolated from patients with RA. The authors showed that the extent of synovial citrullination (examined in whole synovial homogenates) significantly fluctuates between individual RA patients and some of them exhibit only minimal or even no citrullination (27).

Contrary to citrullination, the generation of ACPA seems to be RA-specific. However, the occurrence of citrullinated proteins is not inevitably associated with the induction of ACPA. In the early studies by Despres, citrullinated vimentin was identified in placental extracts, although pregnant women do not synthetise ACPA (17). Therefore, the immunological recognition and response to (citrullinated) vimentin (fragments) seems to be specific for patients with RA (28).

RASF generated vimentin fragments that were not present in OASF cultured under identical conditions. The origin of vimentin fragments in RA has been mainly explained by the secretion from infiltrating macrophages and/or cleavage by activated caspases in RA synovium (29, 30). The pathogenic significance of vimentin fragments in RA is not known. In a recent work, a 45 kDa vimentin fragment was found to be the dominant autoantigen and target of immune response in lupus tubulointerstitial nephritis (31). Endothelial cells injured during ischaemia/reperfusion after solid organ transplantation release vimentin fragments into circulation, which become recognised by host lymphocytes and stimulate production of anti-vimentin antibodies (32). Our group described the occurrence of different vimentin fragments in renal tubular cells during osmotic stress (33). Elevated levels of fragmented vimentin were reported in patients with ankylosing spondylitis, and patients with high levels presented more severe disease with accelerated radiographic progression (34).

It seems that the generation of vimentin fragments is associated with increased cellular stress. We showed that fibroblasts from patients with RA, despite being grown under identical conditions, preserve this particular feature

and generate vimentin fragments, a process not observed in fibroblasts from OA (Fig. 6).

The 2DE used in our study may have several limitations as protein separation technique. Among others, hydrophobic proteins hardly enter the gel and are often lost during the separation, limiting its use in the analysis of membrane proteins. Very high or very low molecular weight proteins, highly acidic or highly basic proteins may also be lost during 2DE. 2DE requires relatively large amounts of protein, encompasses many manual-processing steps and is therefore not easily automated. Despite the above-mentioned disadvantages, compared to other techniques, 2DE allows the identification of post-transcriptional and post-translational modifications, which play important role in the pathogenesis of diseases, and increase the diversity of proteins that can be synthesised from a fixed number of genes. Because of this advantage, we could identify vimentin fragments as a potential indicator of RA. Further studies are needed to elucidate the potential pathogenic properties and mechanisms induced by vimentin fragments in RA.

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