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Synovial detection and autoantibody reactivity of processed citrullinated isoforms of vimentin in inflammatory arthritides

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Objectives. To investigate the presence and characteristics of citrullinated vimentin in protein extracts of inflamed synovial tissue.
Methods. Cytosolic protein extracts obtained from RA ($n=14$) and SpA patients ($n=14$) were analysed by gel electrophoresis and western blotting. Citrullinated vimentin isoforms were visualized by a combination of anti-modified citrulline (AMC) staining and anti-vimentin detections (V9, H-84). This was subsequently confirmed by immunoprecipitation. Autoantibody detection was verified using sera obtained form RA ($n=6$) and SpA ($n=6$) patients.
Results. A specific cluster of spots displayed on the 2D gel images of cytosolic synovial tissue extracts, was identified by mass spectrometry as vimentin. Interestingly, our results suggested that these isoforms could be the result of caspase cleavage. In addition, these cleaved forms of vimentin were found to be citrullinated in synovial cytosolic protein extracts of inflammatory arthritides, mainly in RA patients. Caspase-3 is able to cleave vimentin at amino acid 85. Western blot analysis with a specific antibody against amino acids 1–84 of vimentin (H-84) confirmed that the citrullinated isoforms of vimentin were lacking this part of the protein. These results were also confirmed by immunoprecipitation of vimentin derived from cytosolic protein extracts of RA and SpA patients. Furthermore, the presence of autoantibodies against these citrullinated processed forms of vimentin was found to be predominantly associated with RA patients.
Conclusions. These findings show the presence of processed citrullinated vimentin in inflammatory arthritides, mainly in RA and suggest a possible origin of the ACPA immune response in RA.

Key words: Rheumatoid arthritis, Autoantibodies, Inflammation, Human, Citrullinated vimentin.

Introduction

RA and SpA are two frequent forms of inflammatory arthritis that are both characterized by synovitis, the chronic inflammation of the SM. As a result, synovial hyperplasia, neovascularization and the infiltration of lymphocytes and macrophages eventually leads to the transformation of the SM into an invasive and destructive tissue, called the pannus [1]. Although there are some differences in synovial histopathology between RA and SpA [2], the molecular differences between RA and SpA synovial inflammation remain poorly understood.

The presence of autoantibodies in the sera of patients diagnosed with inflammatory arthritis reflects the humoral autoimmune processes occurring during these pathologies. Many autoantibodies directed against a variety of autoantigens have been described [3], amongst them the Sa antigen (named after the patients name, which began with 'Sa', where specific autoimmunity was described using this patient's serum) [4]. Although, Vossenaar *et al.* [5] showed that anti-Sa antibodies targeted citrullinated epitopes of vimentin, information on *in vivo* citrullinated vimentin, in particular, in synovial tissue extracts, is scarce.

Vimentin, an intermediate filament which is abundantly expressed in synovial fibroblasts [6], has long been thought to solely play a role in the stability of the cytoplasmic architecture [7]. It is now known that vimentin is a highly dynamic protein whose assembly and disassembly is regulated by phosphorylation [8, 9]. In addition, its function stretches far beyond being part of the cytoskeleton. This was elucidated by Eckes *et al.* [10] who observed impaired wound healing in vimentin$^{-/-}$ mice by a failure of mesenchymal contraction.

More recently, studies indicate a possible role for vimentin in inflammation, since fragments of this protein can be secreted by activated macrophages during inflammation [11]. Secretion of vimentin was induced by TNF-α and this extracellular vimentin seemed necessary for efficient killing of bacteria [11]. Cell surface expression of vimentin peptides has also been observed by neutrophils undergoing spontaneous apoptosis [12]. The possible consequence of secreting or presenting vimentin or fragments of this protein is the development of autoantibodies against this intermediate filament. Indeed, the presence of anti-vimentin autoantibodies in autoimmune diseases like RA has been demonstrated. Citrullinated vimentin has been identified to be the Sa antigen in RA [5]. Although the presence of citrullinated vimentin has been reported in monocytes, macrophages and SF mononuclear cells [13], there are no reports that indicate that the antigen itself is present in protein extracts of synovial tissue.

In the present study, we wanted to identify and explore the characteristics of vimentin in synovial tissue protein extracts of inflammatory arthritides. Our data show that vimentin extracted from inflamed synovial tissue is displayed as a specific cluster of spots on 2D images. This cluster contains vimentin fragments possibly due to the result of caspase-3 cleavage. In addition, evidence is shown for the presence of processed citrullinated vimentin in cytosolic protein extracts obtained from synovial tissue biopsy samples derived from patients with inflammatory arthritis. Moreover, autoantibodies against these modified vimentin fragments are predominantly associated with RA.

Patients and methods

Patients

Synovial tissue biopsy samples were obtained from patients undergoing needle arthroscopy of the knee [14] for diagnostic work-up or for therapeutic reasons. Patients with RA fulfilling the ACR criteria [15], patients with SpA fulfilling the ESSG criteria [16] and patients with knee OA fulfilling the ACR criteria [17] were included in this study. All patients undergoing needle arthroscopy had active synovitis (RA and SpA) or joint effusions (OA) of the knee. Serum samples were collected from 10 RA and 10 SpA patients.

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The clinical characteristics of these patients are summarized in Table S1 (see Supplementary Table S1, available as supplementary data at *Rheumatology* Online). The study was conducted after approval by the local ethics committee. Written informed consent was obtained from all participating patients. Detailed information on the samples used in each of the experiments is given in Table S1 (see Supplementary Table S1, available as supplementary data at *Rheumatology* Online).

Protein extraction

The cytosolic proteins (hereafter also referred to as the soluble proteins) were extracted from the synovial tissue samples using the ReadyPrep Sequential Extraction Kit from Biorad (Hercules, CA, USA) according to the manufacturer's instructions. A cocktail of phosphatase inhibitors containing cantharidin, bromotetramisole, microcystin LR, sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole (Sigma, Steinheim, Germany) was added to each sample. Endonucleases (Sigma, Steinheim, Germany), protease inhibitors for a broad range of serine, cysteine and metalloproteases, as well as calpain (Roche Diagnostics, Mannheim, Germany) were also added to each sample. Before protein extraction, synovial biopsy samples were transferred to an Eppendorf tube, washed with phosphate-buffered saline (PBS) and homogenized for 2 min using a Turrax homogenizer (Ika-Werke, Stanfer, Germany) in buffer I of the ReadyPrep Sequential Extraction kit (Biorad) containing 40 mM Trisbase. Protein concentrations were measured using the Coomassie Protein Reagent assay from Pierce (Rockford, IL, USA) according to the instructions provided by the manufacturer.

Gel electrophoresis

1D gel electrophoresis. Ten micrograms of protein extract was diluted with sample buffer containing 0.5 M Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol and incubated at 95°C for 5 min. The samples were subsequently subjected to SDS-PAGE (10%, Laemmli) at 150 V for 30 min followed by 200V for 1 h. For visualization of citrullinated proteins, 50 μg of soluble protein extract was loaded on the SDS-PAGE. 1D gels were further prepared for western blotting or specific bands were cut out for mass spectrometric identification.

2D gel electrophoresis. For 2D analysis, 50 μg of soluble synovial protein extract was precipitated with cold acetone overnight at −20°C. After centrifugation at 20 000*g* for 5 min, the pellet was air dried. 2DE was performed as described before [IPG 3–10 (17 cm) + 12% SDS-PAGE (Laemmli)] [18]. The gels were stained with Sypro Ruby (Molecular Probes, Eugene, OR, USA) and analysed by PD Quest software (Biorad).

For 2D immunoblotting experiments, 40 μg soluble protein extract was prepared as described earlier and incorporated in a linear IPG pH 4–7 (11 cm) strip (Biorad) and rehydrated overnight. After in-gel rehydration, the strips were isoelectrically focused on the Protean IEF cell (Biorad) at 18°C, using 100 V for 30 min (linear ramping), 250 V for 30 min (linear ramping), 500 V for 1 h (linear ramping), 1 kV for 1 h (linear ramping), rapid ramping to 8 kV in 2 h and steady state at 8 kV for 25 kVh. After IEF, the IPG strips were equilibrated as described before [18], and placed on a Laemmli 10% (29 : 1) resolving polyacrylamide gel and run in sets of two at 150 V for 30 min followed by 60 min at 200 V.

Western blotting

Protein transfer onto nitrocellulose membranes (Biorad, Hercules, CA, USA), was performed by tank blotting using the Trans-Blot Cell (Biorad) at 50 V for 3 h. Prior to tank blotting, both the polyacrylamide gels and nitrocellulose membranes were incubated in 1 × 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11 for at least 15 min.

Ponceau S visualization of the membranes was conducted to check the blotting efficiency, and to verify if the 2DE was done successfully. Blocking was performed for 1 h, using PBS/0.3% Tween-20 and followed by probing the membrane with patient sera (1/100 in PBS/0.3% Tween-20) overnight. Immune reactive spots were detected using horseradish peroxidase (HRP)-labelled goat anti-human IgG (Pierce) (1/5000 in PBS/0.3%Tween-20) and visualized by enhanced chemiluminescence (ECL) using the Supersignal West Dura Extended Duration Substrate from Pierce. Blots were stripped using the Restore Western Blot Stripping Buffer (Pierce) at 37°C for 30 min and re-incubated with blocking buffer for 1 h. Sufficient stripping was confirmed by re-probing the membrane with secondary antibody and subsequent detection by ECL.

Vimentin was visualized using two antibodies: the clone V9 (Sigma, St Louis, MI, USA): an antibody raised against the C-terminal end of vimentin, the H-84 polyclonal antibody (Santa Cruz Biotechnology inc. CA, USA): an antibody raised against the N-terminus of vimentin (against amino acids 1–84). Both antibodies were diluted in PBS/0.3% Tween-20 at 1/400 for V9 and 1/200 for H-84 and incubated overnight. The vimentin V9 cluster and the head of vimentin (H-84) were visualized by ECL following incubation with HRP-labelled rabbit anti-mouse IgG (Pierce) (1/1000 dilution) and HRP-labelled goat anti-rabbit (1/1000 dilution), respectively, in PBS/0.3% Tween-20 for 1 h.

For fibrinogen-β detection, the blots were incubated with rabbit anti-human fibrinogen B (Cambio, Cambridge, UK) (1/5000 in blocking solution) after 1 h blocking (5% milk, 0.1% Tween-20 in PBS) and visualized by ECL using an HRP-labelled goat anti-rabbit IgG (Pierce) (1/1000 in PBS 0.3% Tween-20) for 1 h.

For the detection of citrullinated proteins, blots were first stripped as described earlier and subsequently chemically modified prior to immunostaining using the anti-modified citrulline (AMC) detection kit (Upstate, Charlottesville, VA, USA) as indicated by the manufacturer's instructions.

Protein patterns were scanned and digitized using the VersaDoc Imaging System (Biorad) and band detection analysis was performed using Quantity One Analysis Software (Biorad). Bands were represented as Gaussian models where the Gaussian model trace, representing the area under the profile curve calculated in pixel intensity × millimetres (INT × mm), was used to quantify the bands.

For all western blot experiments, negative controls were included in which the nitrocellulose membranes were incubated solely with the secondary antibody. In addition, to determine disease-specific autoantibody reactivity, blots were incubated with a pool of sera obtained from four healthy individuals.

Immunoprecipitation of vimentin from synovial biopsy tissue

Cytosolic protein extracts of synovial tissue of RA and SpA patients were pooled to obtain a total amount of 2 mg protein material. The cytosolic protein extract pool of RA and SpA was concentrated to a volume of ~500 μl using Vivaspin 2 (hydrosart) spin columns (Sartorius, Goettingen, Germany). The concentrated protein extracts were pre-cleared by incubating them with protein G slurry (100 μl) (Pierce) for 5 min. After centrifugation at 10 000*g* for 10 min at 4°C, the supernatant was transferred to another Eppendorf tube and 100 μg of anti-vimentin V9 was transferred to the pre-cleared protein extracts and incubated for 1 h on ice. Subsequently, the 100 μl washed protein G slurry was added and incubated while rotating for 2 h at 4°C. After centrifugation at 10 000*g* for 1 min followed by five washes with PBS, the protein G slurry pellet was incubated with reducing Laemmli sample buffer at 95°C for 10 min. The immunoprecipitated proteins were stored at −20°C until SDS-PAGE and western blotting.

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Protein identification by mass spectrometry

Spots or bands of interest were excised from the 2D gel or 1D gel, respectively and digested with modified sequence grade trypsin (porcine) (Promega, Madison WI, USA) or Lys-C (Sigma, Steinheim, Germany) and analysed on a Quadrupole Time of flight (Q-TOF) Ultima mass spectrometer (Waters, Milford, MA, USA) using matrix assisted laser desorption ionisation (MALDI) or electrospray ionisation (ESI) as previously described [18]. Processing and database searching of the data against the Swiss-Prot database was performed by ProteinLynx Global Server v2.2.5 software (PLGS) (Waters) or with MASCOT daemon and identified with the MASCOT in house search engine (http://www.matrixscience.com). During the database search, several variable modifications were accounted for, including carbamidomethyl (cys), oxidation (Met) and citrullination (Arg).

In vitro *caspase-3 cleavage of vimentin*

Human recombinant vimentin (Progen Biotechnik, Heidelberg, Germany) was cleaved by human recombinant caspase-3 (Chemicon International, Temecula, CA, USA) overnight at 37°C at 1 U/1 μg vimentin.

Results

Vimentin is observed as a specific pattern on 2D images of cytosolic protein extracts of inflammatory synovial tissue

Analysis of the soluble synovial tissue proteome of inflammatory arthritides revealed a rather specific pattern on the acidic side of the 2D gel image. This figure consisted of vimentin isoforms, as confirmed by mass spectrometry (Table 1, Fig. 1).

We focused on two vimentin protein isoform spots, further referred to as VIM_1 and VIM_2 and indicated on Fig. 1. Detailed examination of the mass spectrometric data revealed a clear difference between the higher MW forms VIM_1 and VIM_2, which was found at a lower MW. For the identification by ESI-Q-TOF of VIM_2, no peptides were retrieved within the first 100 amino acids of the protein sequence (see Supplementary Figure S1A, available as supplementary data at *Rheumatology* Online), in contrast with the identification of VIM_1 (see Supplementary Figure S1B, available as supplementary data at *Rheumatology* Online). In addition, MALDI-Q-TOF identification of these isoforms confirmed this data. The peptide mass fingerprint (PMF) responsible for the identification of VIM_1 showed peptides derived from the first 100 amino acids with high ion count intensity (>50% of total ion count), contrary to the PMF for VIM_2, where these peptides had very low ion count intensity (<20% of total ion count) or were absent from the mass spectrum in multiple analyses (data not shown).

Vimentin has a defined structure consisting of a head, starting from the second amino acid up to amino acid 95, a body containing the coils 1A, 1B and coil 2 and a tail (amino acids 408–466) (see Supplementary Figure S1C, available as supplementary data at *Rheumatology* Online) [19]. The mass spectrometric data clearly showed that the N-terminal head (comprising the first 95 amino acids) of vimentin form VIM_2 was absent (see Supplementary Figure S1A, available as supplementary data at *Rheumatology* Online).

We reasoned that this may reflect protein processing. It is known that vimentin is a substrate for caspase cleavage [20]. Indeed, caspase-3 can cleave vimentin at amino acid 85, cleaving the head from the body of the protein resulting in a cleaved form of vimentin with theoretical MW = 44.5 kDa and pI = 4.75. This form would appear ± 9 kDa lower than the native form, which has a MW of 53.5 kDa and would shift towards the acidic side on a 2D gel as the native form has a pI of 5.06. When comparing these theoretical characteristics with the experimental data obtained from the 2D gel (Table 2, Fig. 1), in addition to the mass spectrometric data, we can assume that VIM_2 is a caspase-3 cleaved form of vimentin.

TABLE 1. Identification of processed vimentin isoforms obtained from soluble synovial tissue extracts analysed by 2D gel electrophoresis

Spot (Fig. 1)	Name	Peptide sequence matches by ESI-Q-TOF	Sequence coverage (%) by MALDI-Q-TOF
a	VIME_human	(K)VELQELNDR(F) (R)EEAENTLQSFR(Q) (R)EYQDLLNVK(M) (R)ISLPLPNFSSLNLR(E)	21.1%
b	VIME_human	(K)ILLAELEQLK(G)	–
c	VIME_human	(K)VELQELNDR(F) (K)MALDIEIATYR(K)	14.6%
d	VIME_human	(K)FADLSEAANR(N) (K)VELQELNDR(F) (R)EYQDLLNVK(M) (K)ILLAELEQLK(G) (R)ISLPLPNFSSLNLR(E (R)SLYASSPGGVYATR(S) (R)EEAENTLQSFR(Q)	38.5%
e	VIME_human	(K)VELQELNDR(F) (K)ILLAELEQLK(G) (R)QVDQLTNDK(A) (R)EEAENTLQSFR(Q) (R)QDVDNASLAR(L) (R)ISLPLPNFSSLNLR(E) (K)FADLSEAANR(N) (R)EYQDLLNVK(M) (K)MALDIEIATYR(K) (R)KVESLQEEIAFLK(K)	–
f	VIME_human	(K)VELQELNDR(F) (R)EEAENTLQSFR(Q) (K)ILLAELEQLK(G) (R)QDVDNASLAR(L) (R)ISLPLPNFSSLNLR(E) (K)FADLSEAANR(N) (R)EYQDLLNVK(M) (K)MALDIEIATYR(K) (K)VESLQEEIAFLK(K)	–
g	VIME_human	(K)VELQELNDR(F) (R)EEAENTLQSFR(Q) (R)EYQDLLNVK(M) (K)MALDIEIATYR(K) (R)ISLPLPNFSSLNLR(E)	16.3%
h	VIME_human	(K)VELQELNDR(F) (K)ILLAELEQLK(G) (R)QVDQLTNDK(A) (R)EEAENTLQSFR(Q) (R)QDVDNASLAR(L) (R)ISLPLPNFSSLNLR(E) (K)FADLSEAANR(N) (R)EYQDLLNVK(M) (K)MALDIEIATYR(K) (R)KVESLQEEIAFLK(K)	52.04%
i	VIME_human	(K)MALDIEIATYR(K) (R)EYQDLLNVK(M) (R)DNLAEDIMR(L)	–

'Spot' represents the letter indicated on Fig. 1; 'Name' indicates the protein name of vimentin described in the protein identification format of the Swiss-Prot database (http://www.expasy.org/); Peptides identified by ESI tandem mass spectrometry and protein coverage obtained by peptide mass fingerprinting are shown; protein spots that were not analysed by MALDI are indicated as '-'.

In vitro *caspase-3 cleavage of vimentin*

In order to confirm these data, *in vitro* cleavage of vimentin with caspase-3 was analysed by 1DE and 2DE. It is known that vimentin cleavage by caspase-3 gives rise to a fragment at ~48 kDa on SDS-PAGE [20]. 1DE analysis confirmed the caspase-3 cleavage and indeed showed the appearance of a vimentin isoform at MW = 48 kDa [Fig. 3A (*in vitro* cleaved vimentin)].

The 2D image showed several cleavage products. However, they did not show a dramatic shift towards the acidic pI of the IPG strip (data not shown). Nevertheless, the fragments were located at the correct MW, at ~48 kDa. An explanation could be that the *in vivo* cleavage forms of vimentin bear an additional

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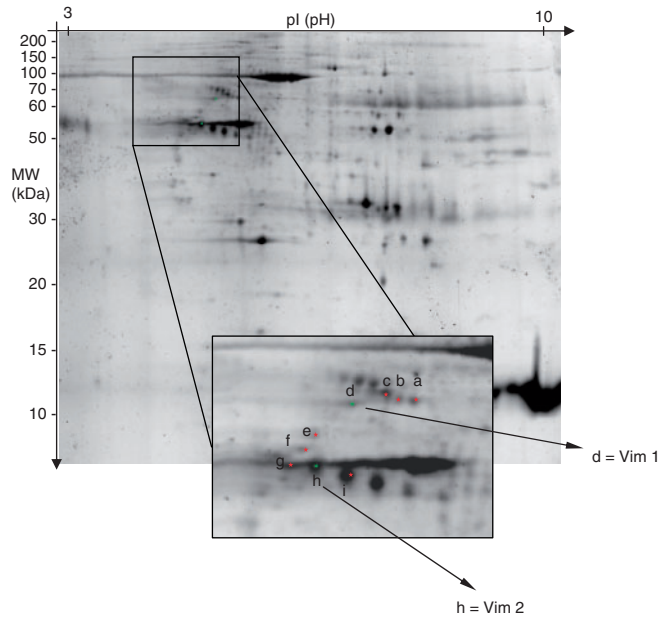

FIG. 1. Raw 2D image of the cytosolic proteome of synovial tissue obtained from a knee biopsy sample of a patient diagnosed with SpA. The vimentin cluster is organized in a specific figure on the 2D image of the synovial tissue soluble protein extract. Spots that were analysed by mass spectrometry are indicated from (a) to (i). The green spots represent VIM_1 and VIM_2.

TABLE 2. Theoretical and experimental 2D characteristics of vimentin isoforms

	Full length (Vim_1)	Cleaved (Vim_2)	$\Delta_{(VIM_1–VIM_2)}$
pI_{th} (pH)	5.06	4.75	0.31
MW_{th} (Da)	53 520.19	44 534.56	8985.63
pI_{exp} (pH)	5.00	4.63	0.37
MW_{exp} (Da)	57 300	49 300	8000

'pI_{th}' and 'MW_{th}': the theoretical isoelectric point and molecular weight calculated from the amino acid sequence of the full length protein as indicated in the Swiss-Prot database; 'pI_{exp}' and 'MW_{exp}': the experimental isoelectric point and molecular weight obtained from the position on the 2D gel; '$\Delta_{(VIM_1–VIM_2)}$': difference in characteristics between VIM_1 and VIM_3

modification, making these products more acidic. Citrullination, a modification that changes an arginine to citrulline, is known to shift proteins towards a more acidic pI [21]. This possibility was explored by AMC staining of the soluble synovial proteome.

Citrullinated vimentin in soluble protein extracts of inflammatory arthritides

Pooled synovial tissue extract of four RA patients ($RAp^1 = 658–882–966–947$) and four SpA patients ($SpAp^1 = 713–524–511–350$) was analysed by 2DE. The proteins were transferred to nitrocellulose membranes and the vimentin cluster was first visualized. Protein citrullination was subsequently analysed by AMC staining. The vimentin cluster (MW 40–60 kDa, pI 4.7–5.5) was nicely visualized in both RA and SpA [Fig. 2, Vimentin (V9)]. We observed citrullination of vimentin in these pooled cytosolic protein extracts of both RA and SpA patients (Fig. 2, AMC). Besides the vimentin cluster (MW 40–60 kDa, pI 4.7–5.3), two other spot clusters were found to be citrullinated in cytosolic protein extracts of inflammatory arthritides (data not shown). These spot trains were observed at MW 60 kDa covering pI 5.1–5.6 and MW 50 kDa between pI 5.8–6.2 and were identified by western blot as fibrinogen-β chain (Fig. 2, FIBB). The fibrinogen-β chain at MW 60 kDa and pI 5.1–5.6 overlaps partially with the high MW forms of vimentin. Since there is some minor citrullination staining in this area, this could be the result of fibrinogen-β citrullination rather than vimentin citrullination. However, there is no distinct AMC staining in this particular area on the 2D image. In addition, it is clear that the citrulline containing protein spots between MW 40 and 50 kDa are derived from processed vimentin isoforms as will be shown by additional experiments described subsequently.

In order to verify patient variability in protein citrullination, cytosolic protein extracts of RA ($n = 14$), SpA ($n = 14$) and OA ($n = 8$) patients were subjected separately to SDS-PAGE followed by western blotting. The vimentin fragments were visualized by western blotting in all pathologies [Fig. 3A, Vimentin (V9)_individual patients]. Processed isoforms of vimentin were present in all pathologies.

Strong AMC staining (Gaussian model trace of most intense band >1000 INT × mm) was observed in 43% of the RA patients, a weak signal was detected in soluble protein extracts of SpA patients (Gaussian model trace of most intense band >300 INT × mm) and none in OA patients (Gaussian model trace of most intense band <100 INT × mm) (Fig. 3B, AMC_individual patients).

The strong positive AMC bands corresponded to bands at MW between 40 and 50 kDa. We wanted to ascertain that the AMC staining in 1D-PAGE matched vimentin. Therefore, the region between MW 40 and 60 kDa of the cytosolic protein extract of synovial tissue of RA882 was cut out from the SDS-PAGE gel, partitioned in six slices, digested with trypsin or Lys-C and analysed by mass spectrometry. The slices, between 40 and 50 kDa contained vimentin as primary identified protein (see Supplementary Figure S2, available as supplementary data at *Rheumatology* Online). Again, no peptides were identified in the first 100 amino acids of the vimentin sequence, indicating that the identified isoforms of vimentin were processed forms. Additionally, western blotting with anti-human vimentin (H-84), an antibody raised against the first 84 amino acids of vimentin, showed that when vimentin is cleaved and loses the first part of its amino acid sequence, no bands appear under the MW of 50 kDa (Fig. 3A, VIM H-84).

Finally, vimentin was immunoprecipitated from pooled protein extracts of RA, SpA and OA. AMC staining confirmed that the positive AMC reactivity seen in the 1D-PAGE of RA and SpA patients is derived from vimentin (Fig. 3B, IP vimentin). AMC staining of immunoprecipitated vimentin showed bands at MW 60 kDa, and two bands between MW 40 and 50 kDa.

The results of the immunoprecipitations showed that the citrullinated processed forms of vimentin are present both in RA and SpA. However, the intensity of the bands is lower in SpA patients than RA patients suggesting an enrichment of citrullinated vimentin in RA.

This immunoprecipitation experiment was confirmed in another pool of RA and SpA patients and in addition, no citrullinated vimentin could be immunoprecipitated from synovial tissue extracts of OA patients (data not shown).

Autoantibody reactivity of in vitro *synovial citrullinated vimentin fragments*

Citrullinated vimentin has been described as a potential autoantigen in RA; therefore, autoantibody reactivity was examined by immunoblotting.

Incubations with individual serum samples ($n = 12$) (see Supplementary Table S1, available as supplementary data at *Rheumatology* Online) were analysed on 2D blots containing a pool of soluble synovial protein extracts of RA (969–882–966–947). In order to verify the autoantibody reactivity to the exact forms of cleaved vimentin, the blots were landmarked with narrow lines. After serum incubation, the nitrocellulose membranes were stripped and re-probed with anti-human vimentin in order to visualize the vimentin cluster. Using the landmarks, exact positioning of the autoimmune spots within the vimentin cluster was possible. These spots were indicated by '+' on the image. For easy interpretation of the data, the position of the

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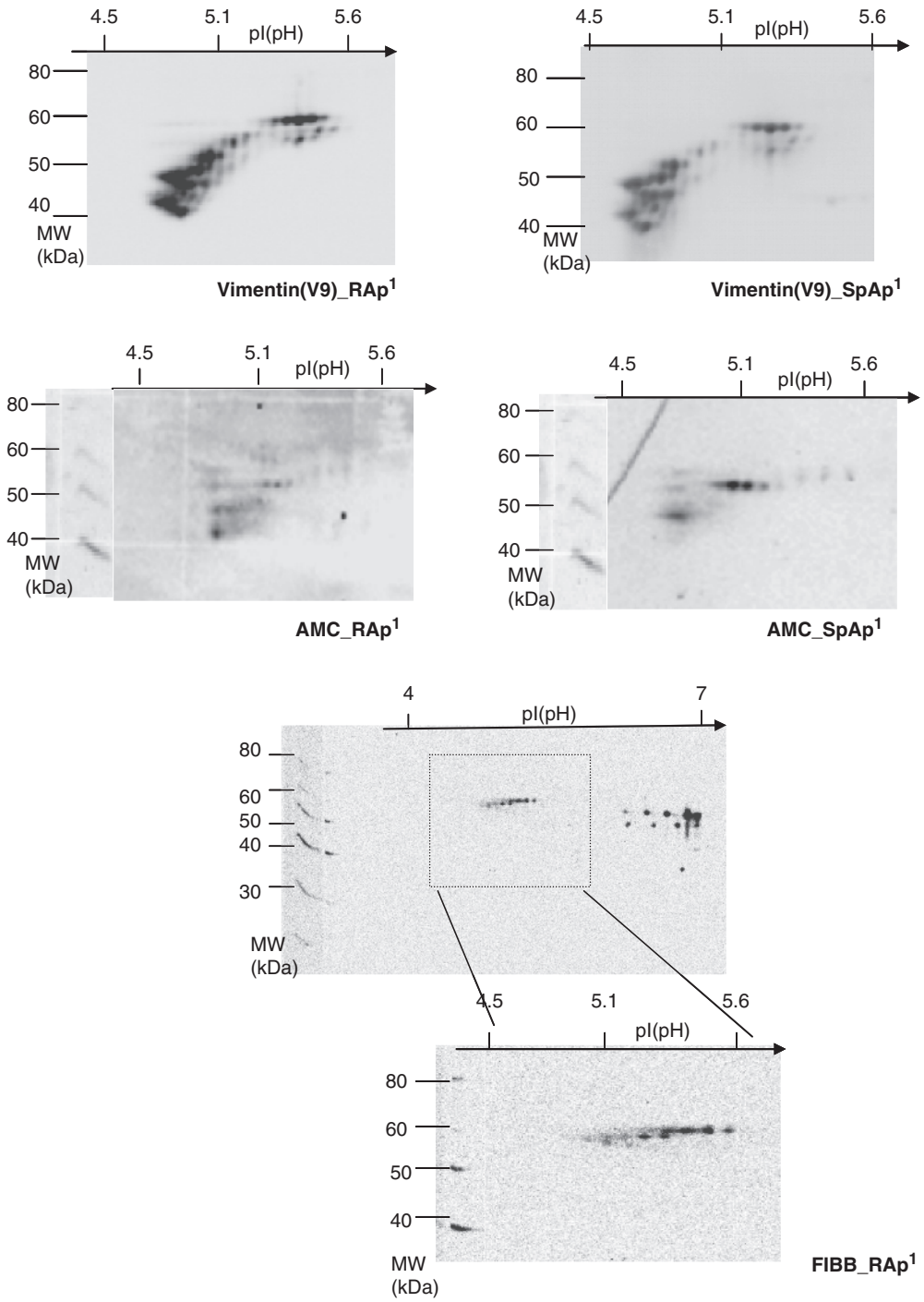

FIG. 2. Detection of processed citrullinated vimentin in inflammatory arthritides by 2DE. Forty micrograms of a pool of soluble synovial tissue protein extract was subjected to 2DE (IPG 4–7, 10% SDS-PAGE) and transferred to nitrocellulose membrane. Detection of vimentin [Vimentin (V9)], AMC staining (AMC) and fibrinogen-β (FIBB) are shown.

vimentin cluster is indicated on each blot displaying the autoimmune reactivity of the patient's sera (Fig. 4).

As a control for disease-specific autoantibody reactivity, blots were incubated with a serum pool obtained from four healthy individuals, in which minor immune reactivity against the high MW forms of vimentin was observed (Fig. 4). This reactivity was considered not disease specific and is indicated in blue on the blots.

In the 2D immunoblotting experiment, we observed a different immune reactive pattern in serum derived from RA CCP^- patients in comparison to RA CCP^+ (cyclic citrullinated peptides) patients. In contrast to RA CCP^- patients (0/3 patients showed autoantibody reactivity against vimentin), there was a distinct reactivity of RA CCP^+ sera against processed forms of synovial vimentin (3/3 showed positive autoantibody reactivity). When blots were probed with individual SpA serum, only the serum of one SpA patient out of six was considered as positive autoantibody reactivity against vimentin fragments. The blots probed with serum of other patients were negative or the signal was only slightly above the background noise level.

The data shown in Fig. 4 are representative for each group.

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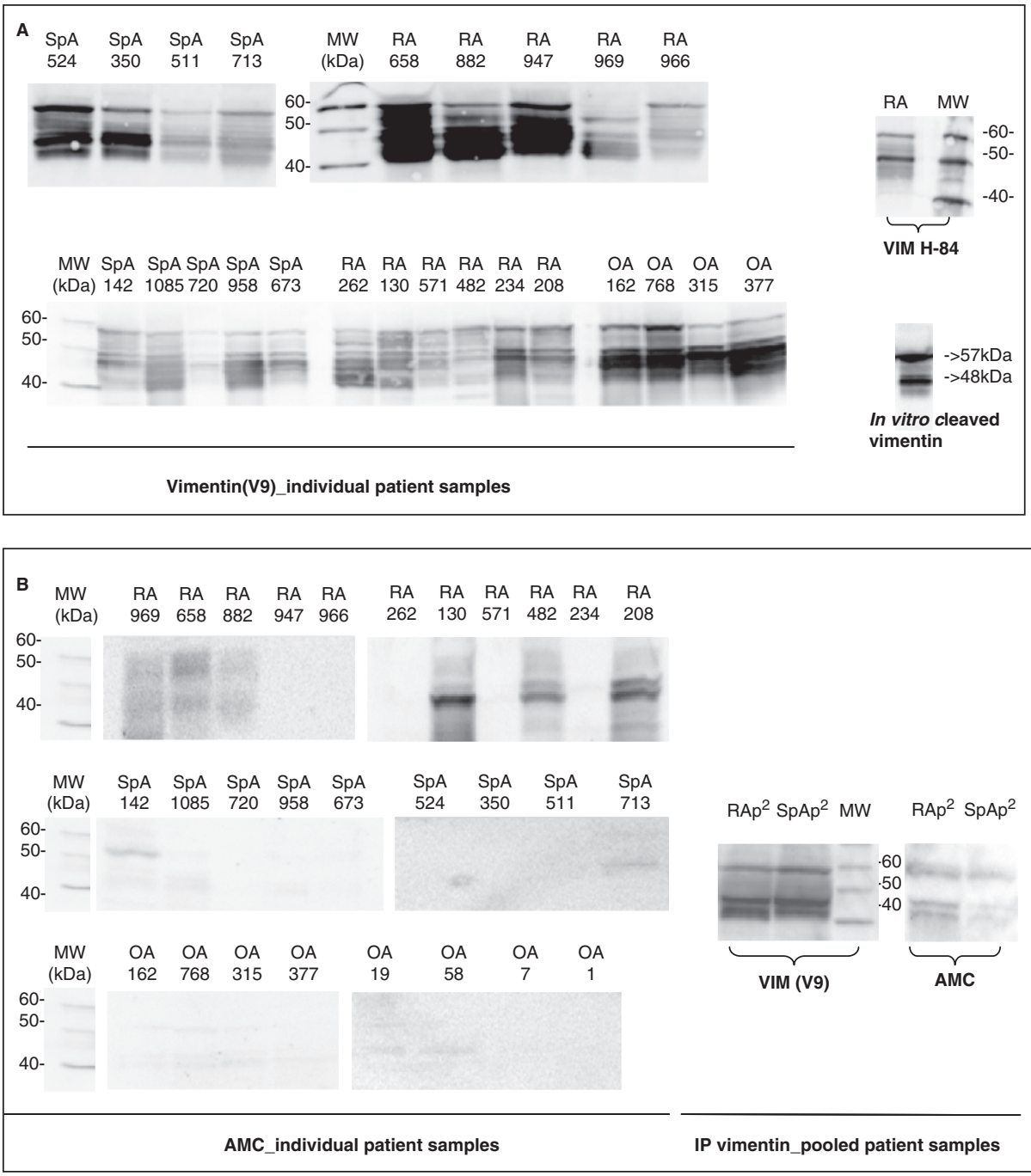

FIG. 3. Detection of processed citrullinated vimentin inflammatory arthritides by SDS-PAGE and immunoprecipitation. (**A**) Individual patient protein extracts were analysed by western blot on which vimentin was detected with two different antibodies [(V9) or (H-84)]; *in vitro* capase-3 cleavage of vimentin was run as a positive control for the appearance of the 48 kDa fragment. (**B**) Individual AMC staining of RA, SpA and OA patients (AMC_individual) is analysed and citrullination of processed vimentin is confirmed by immunoprecipitation in RA and SpA (IP vimentin_pooled).

Discussion

We investigated the properties of vimentin in inflammatory synovial tissue and showed that citrullinated vimentin was present in cytosolic protein extracts of knee synovial tissue obtained from patients with inflammatory arthritides. It is known that upon *in vitro* citrullination, vimentin is irreversibly disassembled resulting in an accumulation of soluble vimentin oligomers [22]. This could explain in part the lack of previous observations of citrullinated vimentin in synovial protein extracts as most extracts described in literature are ureum–dithiothreitol (DTT) extracts [23]. These ureum–DTT extracts are enriched in deiminated fibrin, the most frequently studied deiminated protein in synovial tissue [23].

In addition, the synovial cytosolic citrullinated vimentin appeared to be a collection of processed fragments of the protein. The processing of vimentin can occur by caspase cleavage during the event of apoptosis [20]. It is known that within 2 h of apoptosis induction, when the majority of the cells are still viable, a 48 kDa fragment of vimentin appears as the result of a caspase-3 like cleavage releasing the N-terminal head of the protein [20]. In our proteome study, the low MW isoform of vimentin appeared

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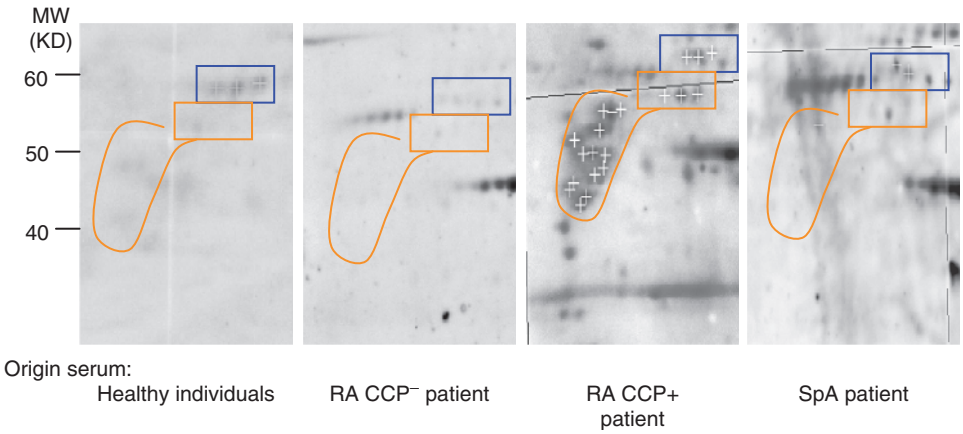

FIG. 4. Presence of autoantibodies against vimentin in inflammatory arthritides. Soluble synovial tissue extracts of a pool of RA patients (969–882–966–947) was subjected to 2D immunoblotting. In order to verify that the autoantibody reactivity was against vimentin, the blots were stripped and vimentin was detected. Immune reactive spots indicated by (+) correlated to vimentin isoforms subsequently detected on the same membranes. Reactivity of healthy sera is indicated in blue and should be regarded as non-disease specific. CCP(−) sera showed no reactivity for vimentin in contrast with CCP(+) RA sera in which strong positive detection of vimentin isoforms was observed. In SpA patients, autoantibody reactivity against cleaved products of vimentin was rather weak to absent. Serum samples of six RA and six SpA patients were individually incubated and the displayed figures are results that are representative for sera of the same pathology.

at ~48 kDa. In addition, careful interpretation of the mass spectrometric data revealed the absence of peptides, which originated from the head of the protein (consisting of amino acids 1–95). We therefore concluded that the N-terminal head of vimentin was lacking, possibly by caspase-3 cleavage. When *in vitro* caspase-3 cleavage of vimentin was performed and subjected to 2DE, we observed multiple cleavage fragments. However, we could not observe any fragments shifting towards the acidic pI. As this was in contrast with the *in vivo* 2D image of synovial citrullinated vimentin, where fragments of vimentin were observed shifting towards the acidic side of the IPG strip, we investigated the presence of citrullinated residues in these fragments by AMC staining.

Vimentin fragments did appear to be citrullinated in cytosolic synovial protein extracts of inflammatory arthritis patients. Immunoprecipitation of synovial soluble vimentin confirmed these results. It has been indicated that citrullination of proteins is not specific for RA, but inherent to chronic inflammation of the synovial tissue. Previous reports showed that deiminated fibrin can be found in synovial protein extracts of other arthritides [24]. In agreement with these studies, this report demonstrates citrullinated vimentin in synovial soluble protein extracts of both RA and SpA patients, although there appears to be an enrichment of these modified vimentin isoforms in RA. Studies on larger patient cohorts are needed in to order to confirm this observation. A specific enrichment of citrullinated vimentin could be the result of a less effective clearing of apoptotic cells. Indeed, citrullination of proteins has been associated with apoptosis [25–27] and more specifically, it has been shown that vimentin is selectively and rapidly citrullinated in macrophages undergoing apoptosis [27]. Additionally, activated macrophages secrete fragments of vimentin [11] and also neutrophils undergoing spontaneous apoptosis express fragments of the C-terminal tail of vimentin on their surface [12]. Whether these vimentin fragments are citrullinated has not been determined yet. It is possible that citrullination of vimentin is the only way towards a direct and irreversible disassembly of vimentin filaments resulting in cytoskeleton disruption leading to cell shrinkage in the process of apoptosis [20]. Therefore, citrullination of vimentin could be an early event in the processing of the protein as the disassembly of the intermediate filament by citrullination could be necessary for further processing of the protein by caspases or other proteases. The exact sequence of events, however, is not known.

The specific autoantibody reactivity against processed citrullinated vimentin was also investigated. Autoantibody reactivity against the processed and modified forms of vimentin could be detected in RA. RA CCP^+ sera reacted strongly with low MW citrullinated vimentin isoforms, whereas in RA CCP^- and SpA, this reactivity was absent or extremely weak. Additional studies on large-scale cohorts of RA and control groups including SpA still need to be conducted to determine the sensitivity and specificity of the autoantibodies against processed forms of vimentin. Recently, the development of the anti-mutated citrullinated vimentin (anti-MCV) ELISA [28] has made it possible to analyse the specificity and sensitivity of the anti-Sa antibodies. Several groups have evaluated the clinical relevance of this test and conclude that the performance of the anti-MCV test in the diagnosis of RA is similar to that of the anti-CCP2 test [29–31]. Antibodies against citrullinated vimentin have been shown to be significantly associated with erosive disease outcome, whereas anti-CCP antibodies were not significant predictors [32]; however, these results need further confirmation in larger patient cohorts.

It is well known that antibodies against citrullinated proteins (ACPA) are highly specific for RA [33, 34]. The presence of ACPA is significantly related to HLA-DR shared epitope (SE) [35, 36] and this can be explained by the fact that MHC with SE has a high affinity for negatively charged or uncharged polar amino acids, while positively charged amino acids inhibit peptide binding [37]. Therefore, when arginine is deiminated, the positive charge is converted to a polar but uncharged residue increasing the affinity in the binding pocket. This was shown by Hill *et al.* [38] where conversion of an arginine into citrulline in the N-terminal head of vimentin increased peptide affinity and resulted in CD4+ T-cell activation in HLA-DR4-IE transgenic mice.

In conclusion, we reported on processed vimentin isoforms in the cytosolic proteome of inflammatory arthritides. Our data suggest that these isoforms are probably the result of caspase cleavages. The enrichment of citrullinated vimentin isoforms in the synovium could serve as a possible origin of the ACPA immune response in RA.

Rheumatology key messages

- Processed vimentin is citrullinated in synovial cytosolic protein extracts of inflammatory arthritides.
- The presence of citrullinated vimentin isoforms indicates a possible origin of the ACPA immune response in RA.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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