

Localization of peptidylarginine deiminase 4 (PADI4) and citrullinated protein in synovial tissue of rheumatoid arthritis

X. Chang¹, R. Yamada¹, A. Suzuki¹, T. Sawada², S. Yoshino³, S. Tokuhira¹ and K. Yamamoto^{1,2}

Objectives. Peptidylarginine deiminases (PADIs) convert peptidylarginine into citrulline via post-translational modification. Anti-citrullinated peptide antibodies are highly specific for rheumatoid arthritis (RA). Our genome-wide case–control study of single-nucleotide polymorphisms found that the PADI4 gene polymorphism is closely associated with RA. Here, we localized the expression of PADI4 and the citrullinated protein product in synovial RA tissue.

Methods. We used immunohistochemistry, double immunofluorescent labelling and western blotting.

Results. We found that PADI4 is extensively expressed in T cells, B cells, macrophages, neutrophils, fibroblast-like cells and endothelial cells in the lining and sublining areas of the RA synovium. We also found extracellular and intracellular expression of PADI4 in fibrin deposits with loose tissue structures where apoptosis was widespread. Unlike PADI4, citrullinated protein generally appeared in fibrin deposits that were abundant in the RA synovium. The citrullinated fibrin aggregate was immunoreactive against immunoglobulin (Ig) A and IgM, but not IgG and IgE. Although a little PADI4 was expressed in osteoarthritic and normal synovial tissues, significant citrullination was undetectable.

Conclusions. The results showed that PADI4 is mainly distributed in cells of various haematopoietic lineages and expressed at high levels in the inflamed RA synovium. The co-localization of PADI4, citrullinated protein and apoptotic cells in fibrin deposits suggests that PADI4 is responsible for fibrin citrullination and is involved in apoptosis. The immunoreactivity of citrullinated fibrin with IgA and IgM in the RA synovium supports the notion that citrullinated fibrin is a potential antigen of RA autoimmunity.

KEY WORDS: Rheumatoid arthritis, Peptidylarginine deiminase 4 (PADI4), Citrullination, Fibrin, Synovial tissue.

Rheumatoid arthritis (RA) is a widespread autoimmune disease that is characterized by chronic joint inflammation. Serum from patients with RA contains diverse autoantibodies that constitute one primary outcome of disturbed immunoregulation [1]. In addition to rheumatoid factor (RF), anti-filaggrin autoantibody (AFA), anti-keratin antibody (AKA), anti-perinuclear factor (APF) and anti-cyclic citrullinated peptide antibody (anti-CCP) are highly specific for RA [2–6]. Recent studies indicate that the primary constituent of the B-cell epitope for AFA, AKA, APF and anti-CCP is citrulline, an amino acid metabolite of arginine [7–9]. Because of the specific anti-citrullinated protein antibodies in patients with RA, understanding protein citrullination, the enzymatic conversion of arginine to citrulline, should provide novel insights into RA pathogenesis.

Peptidylarginine deiminases (PADI) post-translationally modify peptidylarginine to citrulline in the presence of calcium ions and can change the conformation and functional properties of target proteins after citrullination [10]. To date, PADI1, PADI2, PADI3 and PADI4 have been identified in the human genome and all of them cluster on chromosome 1p36, a candidate region for RA susceptibility [11–13]. Our large-scale genome-wide case–control study using single-nucleotide polymorphisms found that a PADI4 polymorphism is distinctly associated with RA [14].

PADI4 was originally cloned from human myeloid leukaemia HL-60 cells that were exposed to the granulocyte-inducing agent retinoic acid, dimethyl sulphoxide, or the monocyte-inducing agent 1 α ,25-dihydroxyvitamin D₃. The 2238 base pairs of PADI4 cDNA encode 663 amino acids that have 50–55% sequence identity with the other three known PADIs [15]. Immunohistochemical studies have detected PADI4 in neutrophils and eosinophils of human peripheral blood [16–18]. To understand its role in RA pathogenesis, we investigated the expression of PADI4 and the citrullinated protein in human synovial tissues and peripheral blood using immunohistochemical means. We also discuss here the possible pathway of PADI4 involvement in RA autoimmunity.

Methods and materials

Anti-PADI4 antibody preparation

We raised antisera against human PADI4 by immunizing rabbits with a synthetic oligopeptide (PAKKKSTGSSTWP, the amino acid sequence specific for the N-terminal of PADI4). The antibody was purified by affinity chromatography through a column containing histone-tagged recombinant PADI4.

¹Laboratory for Rheumatic Diseases, SNP Research Center, Institute of Physical and Chemical Research (RIKEN), Kanagawa, ²Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo and ³Department of Joint Disease and Rheumatism, Nippon Medical School, Tokyo, Japan.

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Correspondence to: R. Yamada, Laboratory for Rheumatic Diseases, SNP Research Center, Institute of Physical and Chemical Research (RIKEN), 1-7-22 Suehiro, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan. E-mail: ryamada@src.riken.go.jp

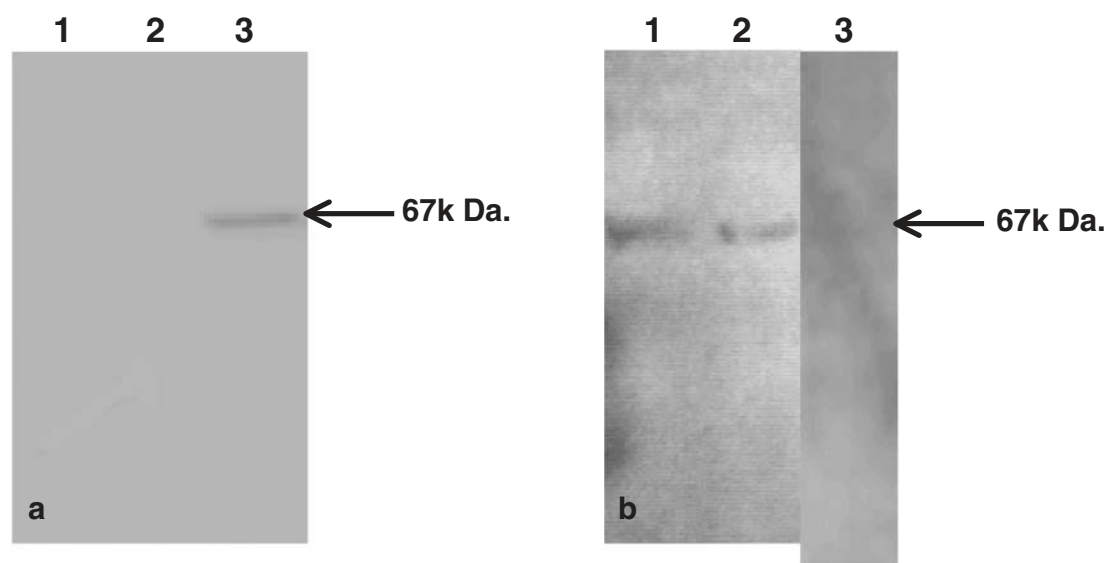


FIG. 1. PADI4 expression determined by western blotting. (a) Cultured HEK293 cells were transfected with expression vector containing coding regions of PADI4 or PADI2 or without inserts. Western blotting with anti-PADI4 antibody detected a 67-kDa band in extracts of cells expressing PADI4 (lane 3). No signals were detected in cells transfected with vector alone (lane 1) or with vector expressing PADI2 (lane 2). (b) Total proteins were extracted from RA synovial tissue (lane 1), leucocyte fraction of peripheral blood from healthy individuals (lane 2) and liver (lane 3). Western blotting shows 67-kDa PADI4 products in synovial samples but not liver samples.

Preparation of peripheral blood

Leucocytes were separated using Monopoly Resolving Medium (DaiNippon) according to the manufacturer's instructions, from samples of fresh blood obtained from 10 RA patients and nine healthy volunteers. The cells were fixed in 4% paraformaldehyde for 2 h at room temperature and then sedimented by centrifugation. Cell pellets were resuspended in phosphate-buffered saline (PBS) and spotted onto Superfrost/Plus microscope slides (Fisher).

Sample preparation of synovial and other tissues

We obtained written informed consent to collect synovial tissue samples from 12 patients with RA and five with osteoarthritis (OA) during arthroplasty. The tissues were fixed in 10% neutral buffered formalin (Sigma) for 12 h at room temperature, embedded in paraffin and sectioned by standard procedures. We also used Tissue Microarray Human Synovitis (Biocat), which includes 14 RA, 12 OA and four normal synovial tissue specimens for comparison. All RA patients met the American College of Rheumatoid Arthritis revised criteria for RA.

We determined the specificity of the PADI4 rabbit antibody and the tissue distribution of PADI4 using Vastarray (InnoGenex), a commercial tissue array slide containing normal human liver, lung, kidney, skin, muscle, brain thymus, spleen, lymph node and tonsil tissues.

Immunohistochemistry

Tissue sections were deparaffinized and rehydrated using standard procedures. Slides spotted with blood cells were processed in the same manner except for deparaffinization.

To increase immunostaining intensity, the sections were heated at 95°C for 20 min with DAKO Target Retrieval Solution (Dako). Sections were incubated with first antibody overnight at 4°C, washed three times, each for 5 min, with PBS, and then incubated with SimpleStain MAX-AP Multi (Nichirei) for 30 min at room temperature. Immunoreactive signals were visualized using the

New Fuchsin Substrate kit (Nichirei) according to the manufacturer's instructions and the cell structure was defined by counterstaining with haematoxylin.

Rabbit polyclonal anti-citrulline antibody (Upstate), monoclonal anti-human fibrin (Monosan), monoclonal anti-human immunoglobulin (Ig) G Fc region, monoclonal anti-human IgM Fc region, monoclonal anti-human IgA heavy chain and monoclonal anti-human IgE Fc region (all from Zymed) were obtained commercially.

Before applying the anti-citrulline antibody, tissue sections were treated using the modification buffer supplied with the kit and then incubated with the first antibody according to the manufacturer's instructions.

Double immunofluorescent immunohistochemistry

The tissue sections were processed as described above. Monoclonal antibodies for various cell surface CD markers (CD3, CD15, CD20, CD34 or CD68) (Zymed), fibroblast-like cell marker [proxyl 4-hydroxylase β (ph β)] (Daiichi Fine Chemicals) or Igs (IgG, IgM, IgA or IgE) (Zymed) were incubated together with rabbit antibody against PADI4 or citrulline at 4°C for 12 h. After three 5-min washes with PBS, sections were incubated with the secondary antibody for 30 min at room temperature. The monoclonal antibody and rabbit antiserum were detected using fluorescein isothiocyanate-goat anti-mouse IgG (H+L) conjugate (Zymed) and CyTM 5-goat anti-rabbit IgG (H+L) conjugate (Zymed), respectively. Immunofluorescent signals were examined using a confocal microscope (Leica). CD3⁺ characterized T cells, CD20⁺ B cells, CD15⁺ neutrophils and CD68⁺ monocytes in peripheral blood or macrophages in tissues. CD34⁺ identified endothelial cells or their precursors in new capillaries.

Detection of apoptosis

We detected apoptotic cells by immunohistochemistry and double immunofluorescent labelling as described above, together with use of the monoclonal antibody M30CytoDeath (Roche). This antibody can recognize a specific caspase cleavage site within

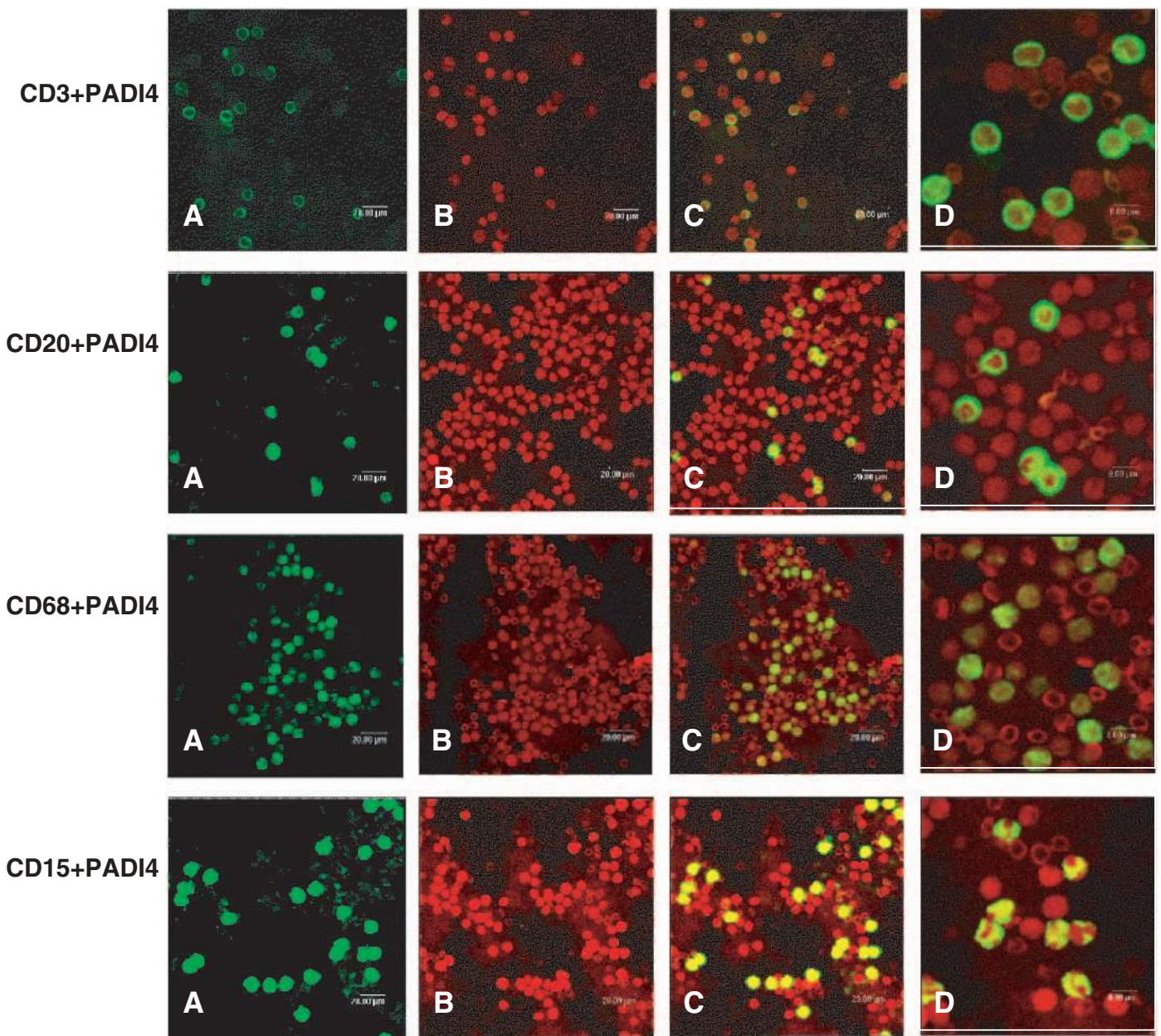


FIG. 2. Immunostaining of PADI4 in peripheral leucocytes. Peripheral leucocytes from healthy individuals were incubated with monoclonal antibodies against various cell surface CD marker and anti-PADI4 antibody. All CD-marked cells (green in A) expressed PADI4 (red in B). C, merged image of A and B; D, magnification of C. Yellow colour in merged images indicates co-localization of two protein targets. Scale bar, 20 μm in A, B and C and 8 μm in D.

cytokeratin 18 (CK18) that is not present in the native CK18 of normal cells. During the very early stage of apoptosis, caspases cleave CK18, an acidic cytokeratin intermediate filament protein of 45 kDa. The ApopDETEK Assay System (Enzo) was used to localize apoptotic cells by labelling fragmented DNA with biotinylated 16dUTP using terminal deoxynucleotide transferase. Biotinylated DNA was then visualized using the Horseradish Peroxidase-DAB *in situ* Detection System (Enzo).

Western blotting

Cultured HEK293 cells were transfected with the pTarget™ mammalian expression vector (Promega) containing the complete PADI4 or PADI2 cDNA coding region. After a 60-h incubation, crude cellular protein was extracted by standard ultrasonic

disruption. The total protein of transfected cells was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transblotted onto nylon membranes and probed with anti-PADI4 antibody. A western blotting kit (KPL) was used to detect signals according to the manufacturer's instructions.

To investigate PADI4 expression in synovial tissue and peripheral leucocytes, we purified total protein of RA synovial tissues and leucocytes using total protein extraction kits (Biochain). Leucocytes were prepared using Monopoly Resolving Medium as described above. The blotted membrane was probed using our anti-PADI4 antibody and the western blot kit. The total protein in a commercial liver sample served as the control (Biochain).

Written consents was obtained from all patients and healthy volunteers according to the Declaration of Helsinki. The design of the work has been approved by the ethical committees of the

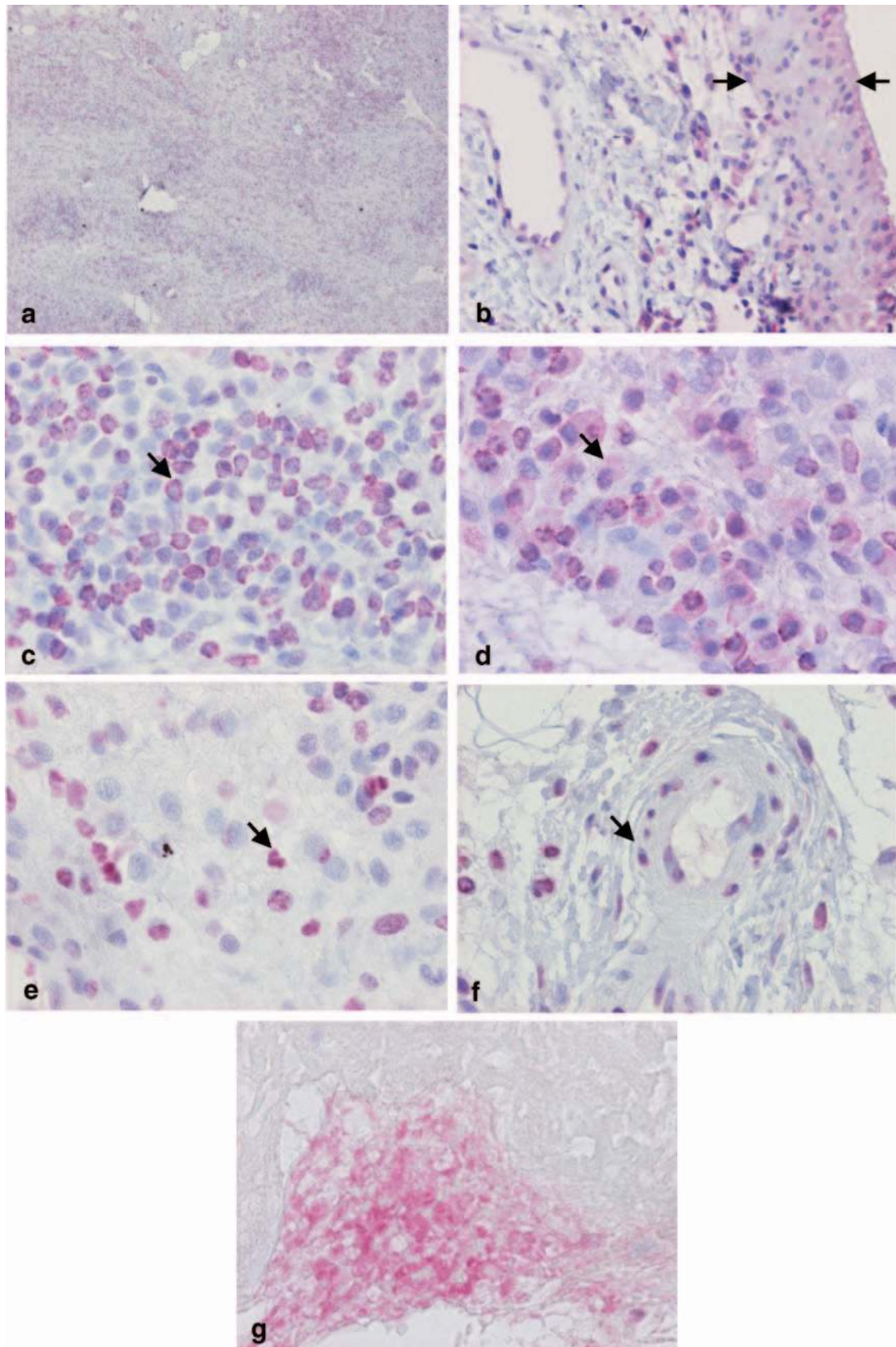


FIG. 3. Immunostaining of PADI4 in synovial RA tissue. (a) Extensive distribution of PADI4 in synovial RA tissue. (b) PADI4 is significantly expressed in the lining area marked with arrows. (c, d, e and f) Expression of PADI4 in potential lymphocytes, macrophages, polymorphic nuclear cells and capillary endothelial cells, respectively (arrows). (g) Intracellular and extracellular expression of PADI4 in loose tissue that was usually close to solid fibrin deposits and which contained cells with apoptotic morphology. Original magnification: a, 40 \times ; b, 200 \times ; c, d, e and f, 400 \times .

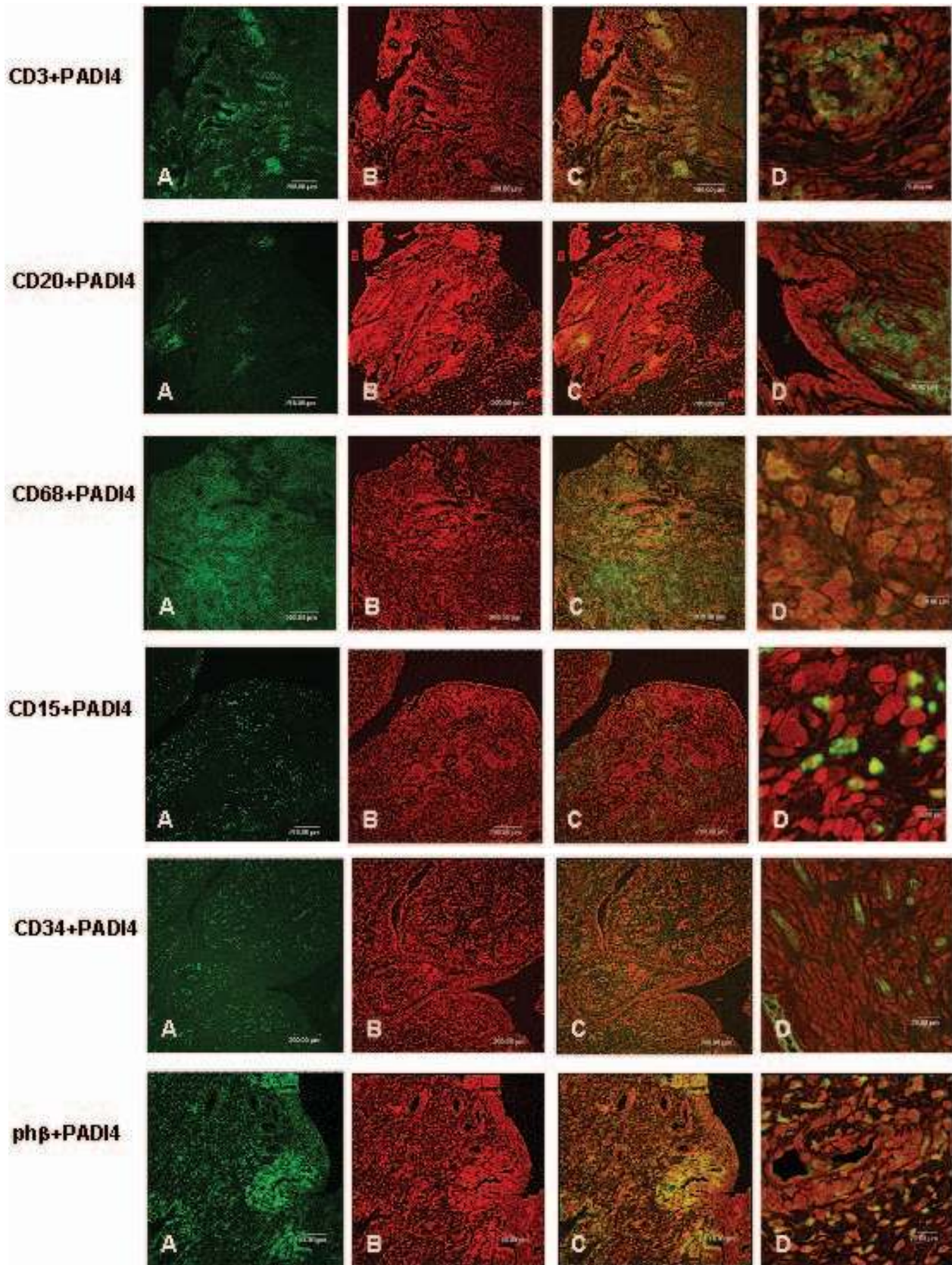


FIG. 4. Cellular distribution of PADI4 in RA synovial tissue. Synovial RA membrane was incubated with anti-PADI4 and monoclonal antibodies against leucocyte cell surface CD markers or $\text{ph}\beta$. All CD3-, CD20-, CD68-, CD15- and $\text{ph}\beta$ -marked cells (green in A) expressed PADI4 (red in B). C is a merged image of A and B. D is magnification of C image. Yellow colour in merged images indicates co-localization of two protein targets. Scale bar, 200 μm in A, B and C, 20 μm in D.

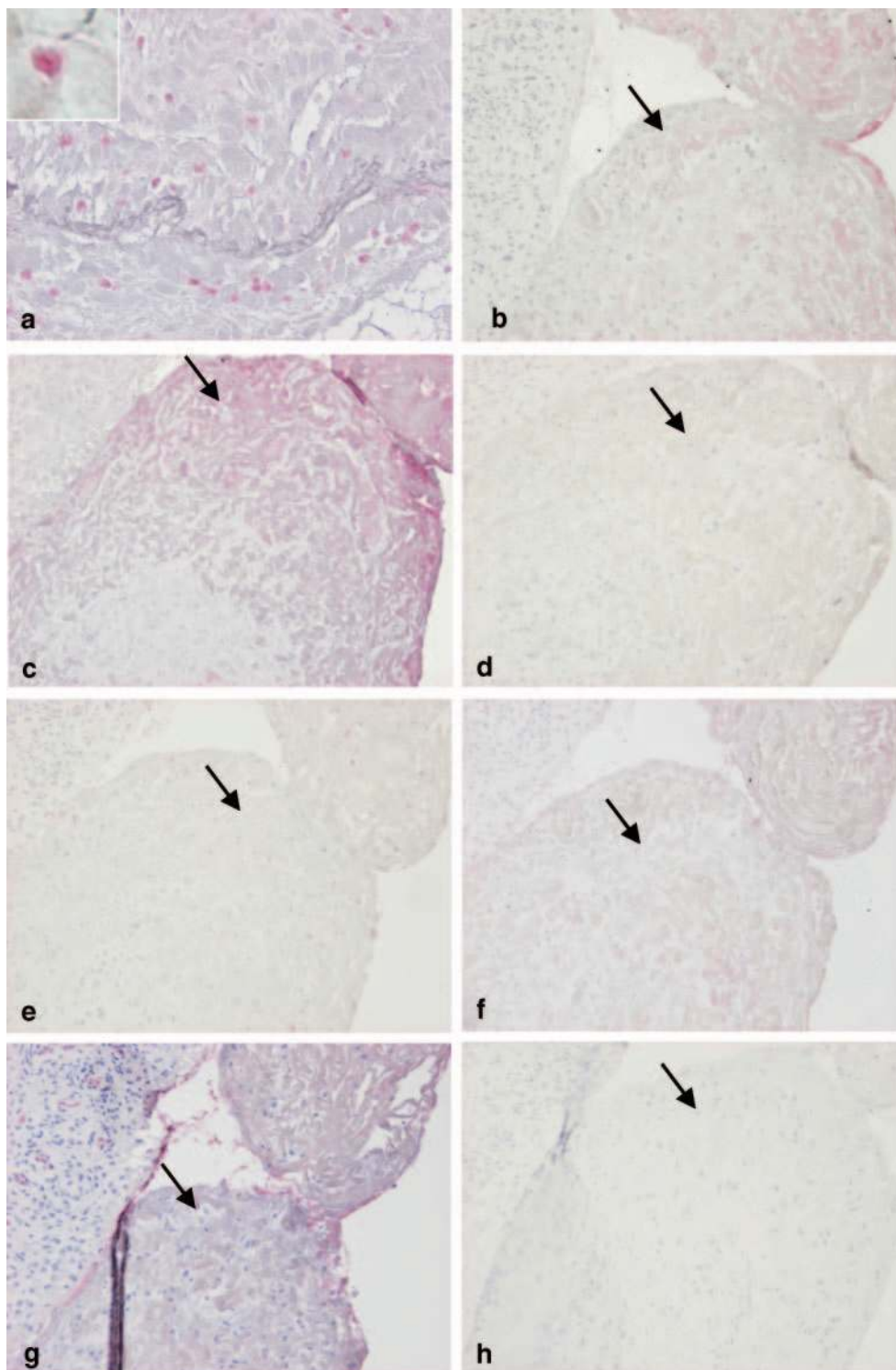


FIG. 5. Immunostaining of fibrin, citrullinated protein, PADI4, IgG, IgM, IgA and IgE in RA synovial tissue. (a) Citrullinated protein is located in some RA synovial cells in the sublining. The left upper corner is a partial magnification, indicating intracellular localization of citrullinated proteins. (b) Fibrin deposit with solid structure. (c) Citrullinated protein is located in the solid fibrin region in a continuous section. PADI4 (d) and IgG (e) were undetectable in the fibrin block. (f) Part of a solid fibrin deposit is lightly stained with anti-IgM antibody. (g) Citrullinated fibrin block was stained intensely with antibody against IgA. Capillary endothelial cells are also immunoreactive to antibody. (h) IgE was undetectable in synovial tissue. Significant IgG (i) and Ig M (j) deposits are obvious in synovial cells in the sublining of the same tissue. Arrow indicates a solid fibrin block. Original magnification: a, 400×; all other sections, 100×. (b), (c), (d), (e), (f), (g) and (h) are continuous sections. The image at the corner is shown at partial magnification.

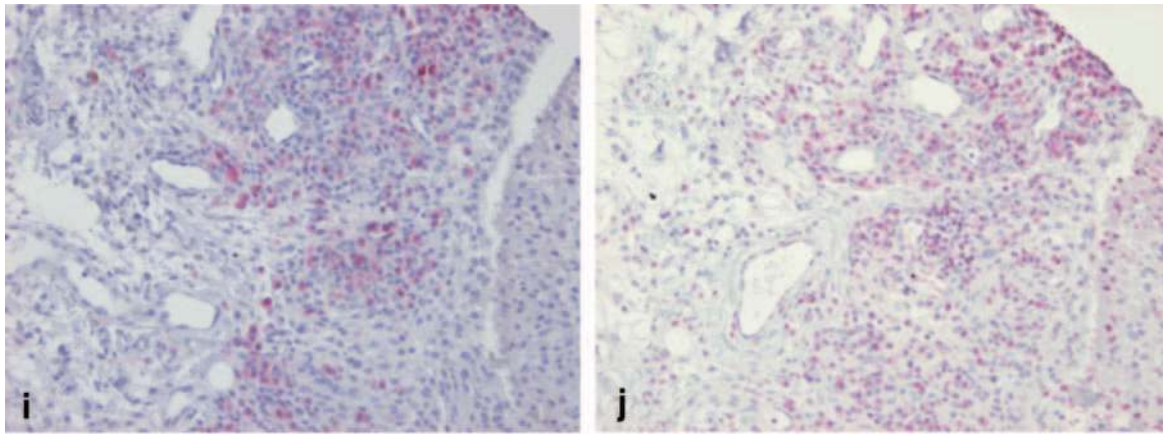


FIG. 5. Continued.

Institute of Physical and Chemical Research (RIKEN) and conforms to standards currently applied in Japan.

Results

Western blotting with PADI4 antibody

The anti-PADI4 antibody detected a band of 67 kDa in lysates of cultured HEK293 cells transfected with PADI4 expression vectors, but not in cells transfected with PADI2 or blank vector (Fig. 1a). Using the same antibody, western blotting detected a 67-kDa band in total proteins extracted from RA synovial tissue and in peripheral leucocytes of both RA patients and healthy individuals, but not in the liver of the healthy individuals (Fig. 1b). These results confirmed the specificity of our anti-PADI4 antibody. Some groups have identified a similarly sized PADI4 product in stimulated HL60 cells and in isolated synovial macrophages using an antibody against recombinant PADI4 protein [15–19].

Immunohistochemistry of PADI4 in peripheral blood cells

Double immunofluorescent labelling using anti-PADI4 antibody and antibodies against various cell surface CD markers showed that all CD3⁺ T, CD20⁺ B cells and CD15⁺ granulocytes of peripheral blood expressed nuclear PADI4. CD68⁺ monocytes also expressed nuclear and cytoplasmic PADI4 (Fig. 2). No differences were evident between samples of blood from patients with RA and healthy controls. The antibody against citrullinated protein did not detect any signals in peripheral leucocytes (data not shown).

Immunohistochemistry of PADI4 in RA synovial tissue

Immunohistochemistry with the anti-PADI4 antibody showed a broad distribution of PADI4 in many types of cells in RA synovial tissue (Fig. 3a). The lining consisted of abundant hyperplastic cells that stained intensely for PADI4 (Fig. 3b). Small mononuclear cells with little cytoplasm formed many clusters of nodular infiltrates. The nuclei of these cells were significantly stained with anti-PADI4 antibody, particularly at the nuclear edge (Fig. 3c). Large mononuclear cells with abundant cytoplasm predominated in RA synovial tissue. These macrophage-like and fibroblast-like cells were clustered in the lining and sublining of RA synovial tissue, or were dispersed in regions with a loose tissue structure. Both the cytoplasm and nuclei of these cells expressed high levels of PADI4 (Fig. 3d). In addition, polymorphonuclear cells that were evenly distributed throughout the sublining distinctly expressed nuclear PADI4 (Fig. 3e). Extensive angiogenesis is a primary feature of RA

synovial tissue. The nuclei of endothelial cells surrounding small capillaries obviously expressed PADI4 peptide (Fig. 3f). PADI4 was also expressed intracellularly and extracellularly in loosely organized tissue in which the cells showed the morphology of apoptosis, having condensed chromatin, cytosol vacuolization and being separated from surrounding tissue [20] (Fig. 3g).

We investigated which type of cells contained PADI4 by double immunofluorescent labelling using an antibody for PADI4 and various CD cell markers (Fig. 4). Both CD3⁺ T cells and CD20⁺ B cells surrounding small capillaries expressed nuclear PADI4. The cytoplasm and nuclei of CD68⁺ macrophages, which constitute one of the key structural components of the inflamed RA synovial membrane, expressed PADI4. CD15⁺ cells were evenly distributed in the tissue and their polymorphic nuclei expressed PADI4. We identified angiogenic regions by detecting CD34⁺ cells that were functional endothelial cells or the active precursors of new capillaries. The nuclei of all CD34⁺ cells in the RA synovium were significantly immunostained with anti-PADI4 antibody. Besides, fibroblast-like cells marked with $\text{ph}\beta$ antibody also expressed PADI4. The double immunofluorescent staining results were consistent with those of standard immunohistochemistry. The results were identical in all 12 of our RA samples and in 14 commercially available RA specimens.

Anti-fibrin antibody identified a significant amount of fibrin deposition in RA synovial tissues. Based on our observations, most of these fibrin deposits formed a solid block and some fibrin appeared as a mesh or spongy structure with loosely organized cells (Figs 5b and 6a). The spongiform structure, which was usually located close to the solid fibrin block, expressed large amounts of intracellular and extracellular PADI4 protein (Fig. 6c).

Immunohistochemistry of citrullinated peptides in RA synovial tissue

We immunolocalized citrullinated protein in the RA synovium using a polyclonal antibody against citrullinated peptide. Unlike the extensive distribution of PADI4, only a few synovial cells were immunostained in the sublining of the tissue (Fig. 5a). Citrullinated protein was primarily located in the solid fibrin block, which was not stained by anti-PADI4 antibody (Fig. 5b–d).

We investigated the deposition of IgG, IgM, IgA and IgE, which are central components of the autoimmune reaction, in continuous sections. Although IgG and IgM were highly immunoreactive in numerous cells at the sublining of the tissue (Fig. 5i and j), only a little fibrin deposit was mildly stained with the antibody against IgM (Fig. 5e and f). However, the citrullinated fibrin blocks in 80% of tested samples stained intensely with the antibody against IgA (Fig. 5g). IgA

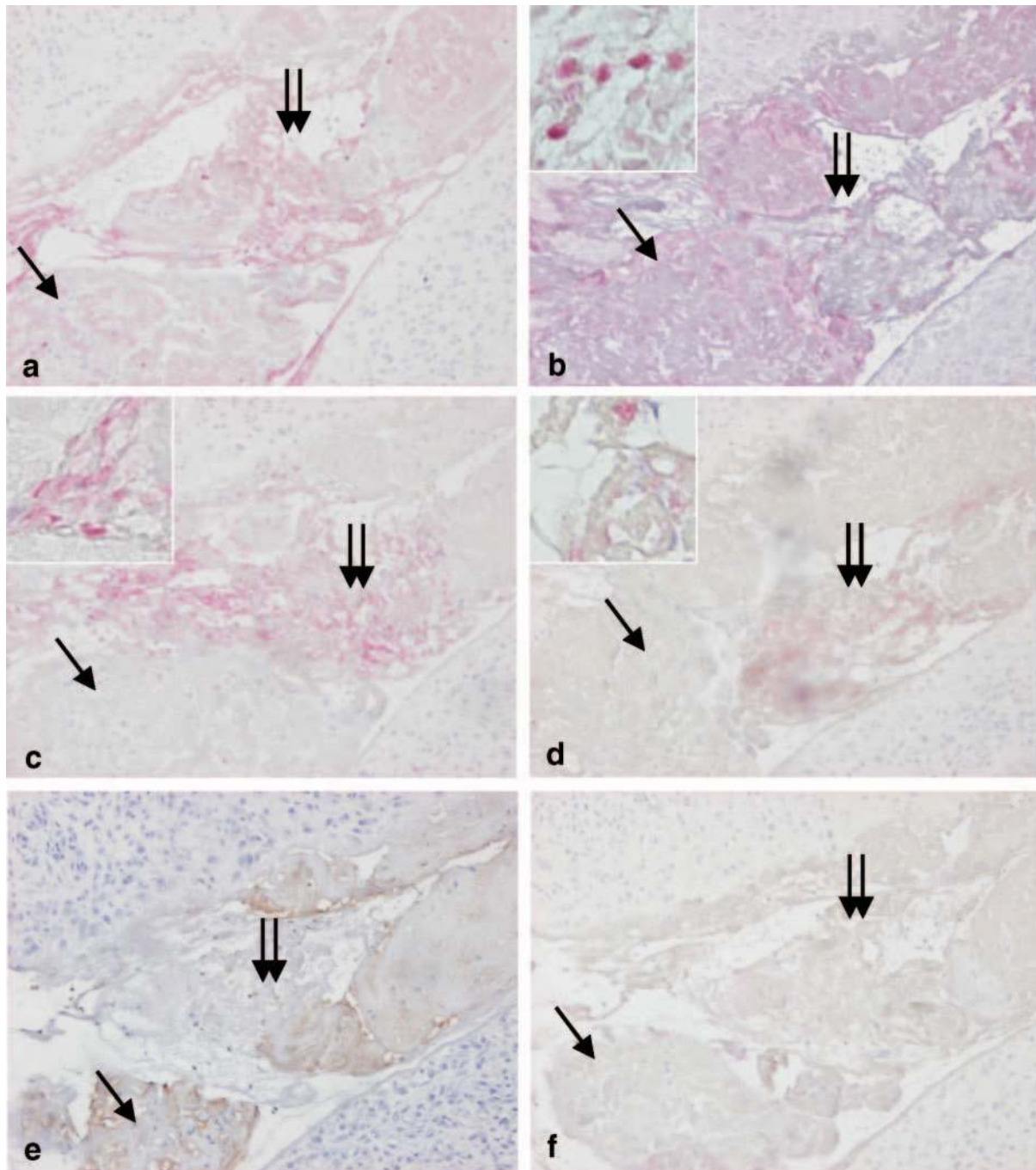


FIG. 6. Immunostaining of citrullinated protein, PADI4, apoptosis, IgG, IgM, IgA and IgE in fibrin deposits in continuous sections of RA synovial tissue. (a) A cell-loose region of RA synovial tissue was immunostained with anti-fibrin antibody. (b) Citrullinated protein is present in both solid (single arrows) and spongy (double arrows) fibrin deposits on continuous sections. (c) PADI4, (d) M30CytoDeath and fragmented DNA (e) are exclusively localized in meshed fibrin deposit. No immunosignals of IgG (f), IgM (g) and IgE (h) were evident in fibrin deposits. (i) The area of fibrin deposits is highly immunoreactive for IgA. (original magnification: (a–i), 100 \times). The inset images are shown at partial magnification.

immunoreaction was also detected in endothelial cells around small capillaries. The RA synovium did not express IgE immunoreactivity (Fig. 5h). These results were reproducible using anti-Ig antibodies from another manufacturer (Biomeda).

Spongiform masses that consisted of about 10% fibrin expressed intracellular citrullinated protein (Fig. 6a and b) that co-localized with PADI4 and apoptotic cells (Fig. 6c–e). Double immunofluorescent labelling also confirmed the co-localization of apoptotic cells and citrullinated protein in some fibrin deposits (results not shown). Most apoptotic cells were localized in the spongiform

fibrin mass with loosely organized cells. Like the solid form, the fibrin mesh was significantly stained with antibody against IgA rather than IgG, IgM or IgE (Fig. 6f–i).

Immunohistochemistry of PADI4 in OA synovial membrane and other tissues

Immunohistochemistry using antibodies against various types of leucocytes and fibrin indicated that OA synovial tissue contains

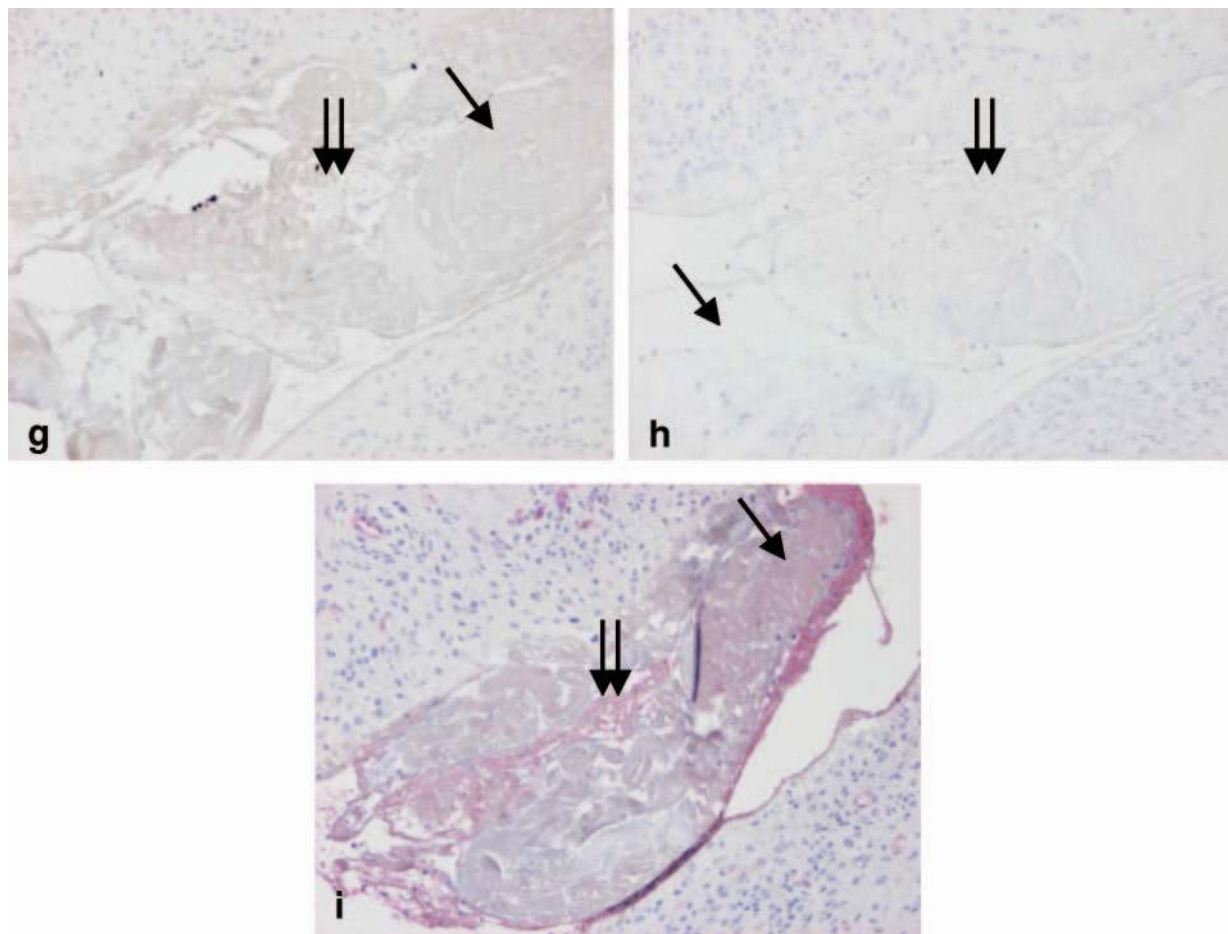


FIG. 6. Continued.

a few CD68⁺ macrophages but lacks lymphocyte infiltrate, a hyperplastic structure and fibrin deposits. Macrophages of OA synovial tissue expressed low levels of PADI4 (Fig. 7a). Because PADI4 is mainly expressed in lymphocytes and macrophages, the loose cell structure and absence of lymphocyte infiltrate contributed to the low density of PADI4 immunostaining in OA synovial tissue. Like the findings in peripheral blood, citrullination was insignificant in OA synovial tissue (Fig. 7b). The results were similar in all 12 OA samples and in four commercially available specimens from healthy individuals.

Immunohistochemistry using the human tissue array showed distinct PADI4 expression in some regions of haematopoietic tissues, including the thymus, spleen and tonsils. The expression of PADI4 was not evident in over 30 other human tissues, including the lungs, stomach, kidneys, liver and brain (results not shown), although capillary endothelial cells and some stroma cells of these organs were stained with the anti-PADI4 antibody. Because the brain, skin and muscle express high levels of PADI1, PADI2 or PADI3 [13], the absence of immunostaining of PADI4 in these tissues further confirmed the specificity of our anti-PADI4 antibody.

In all the above experiments, no immunosignals were detected in the negative controls, which included samples with normal serum instead of the first antibody, as well as those without first or second antibodies.

Discussion

The present study provides evidence that PADI4 is expressed in peripheral blood CD3⁺ T cells, CD20⁺ B cells, CD15⁺ neutrophils

and CD68⁺ monocytes. We also identified PADI4 in the same subtypes of leucocytes, fibroblast-like cells and capillary endothelial cells in RA synovial tissue. Screening over 30 normal human tissues showed selective PADI4 expression in haematopoietic tissues, including the thymus, spleen and bone marrow. Thus, we suggest that the cells expressing PADI4 in the RA synovium are mainly limited to haematopoietic cells or their derivatives. We previously detected PADI4 transcripts by northern hybridization only in haematopoietic tissues, including the spleen, thymus, peripheral blood leucocytes, fetal liver and bone marrow [14]. Mouse and rat PAD4, a homologue of human PADI4, is also expressed at high levels in granulocytes and monocytes [13, 21].

The expression of PADI4 did not differ in peripheral leucocytes from RA patients and healthy individuals. Vossenaar *et al.* also obtained similar results by reverse transcription–polymerase chain reaction and immunoblotting [19]. Moreover, we did not detect citrulline production in blood cells. These findings imply that the expression of PADI4 and its citrullination activity in the synovium both play critical roles in the pathogenesis of RA. The inflamed RA synovial membrane is formed mainly through the abnormal proliferation of macrophages and fibroblast-like cells, as well as by excessive infiltration of lymphocytes from the circulation [22]. These types of cells constitute the main source of PADI4 expression according to the present results and other studies [13, 14, 19]. Therefore, we observed extensive PADI4 expression in RA rather than OA synovial tissue or normal synovium. Vossenaar *et al.* also suggested that inflamed RA joints contain high levels of PADI peptide [19]. In addition, they localized PADI4 mRNA only in monocytes and showed that the PADI4 transcript degrades after the cells differentiate into synovial macrophages,

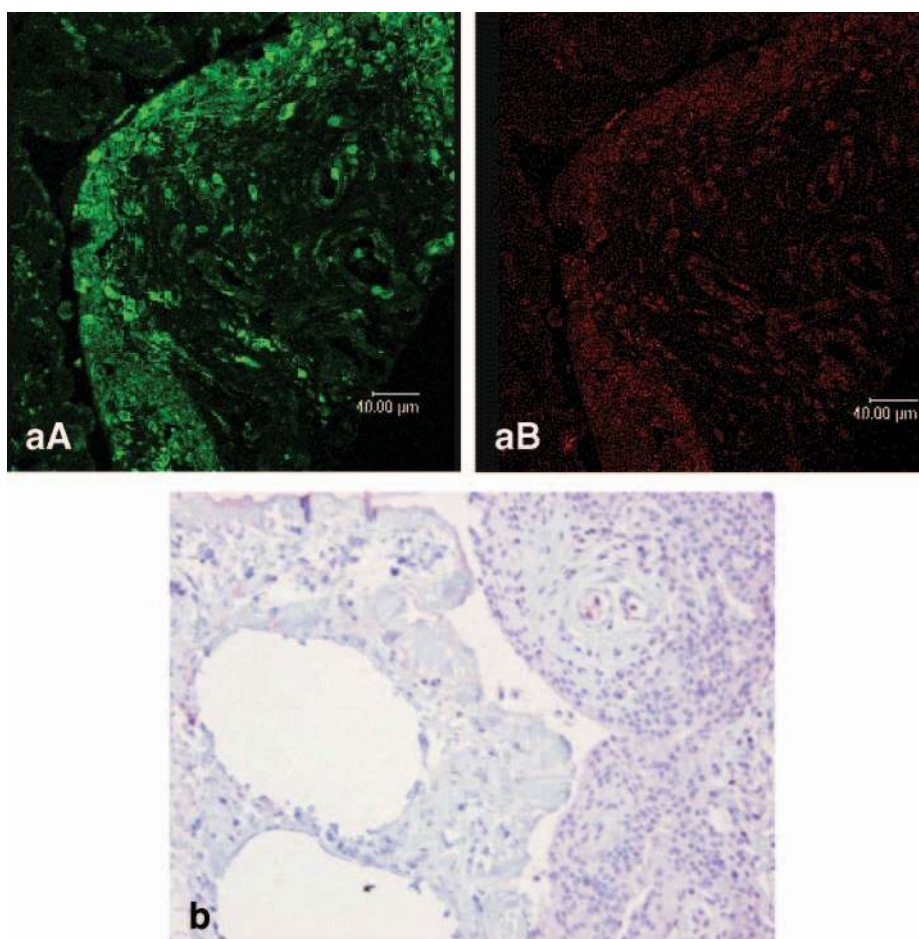


FIG. 7. Immunostaining of PADI4 and citrulline in OA synovial tissue. (a) Double immunofluorescent labelling shows CD68 macrophages (green in aA) and PADI4 signal (red in aB). Expression of PADI4 is relatively weak in macrophages of OA synovial tissue. Scale bar, 40 μm. (b) No significant immunosignals for citrullinated peptide in OA synovial tissues. Original magnification, 100×.

whereas the PADI4 protein level remains unchanged [19]. Because PADI4 transcripts with single-nucleotide polymorphisms conferring RA susceptibility have a longer half-life than non-susceptible mRNA [14], we postulate that mRNA with an RA susceptible-haplotype accumulates and becomes translated into more protein products in the RA inflamed synovium. Therefore, the high abundance of PADI4 in the synovial membrane is a prominent feature of RA pathogenesis.

Excessive fibrin formation is a prominent event of the inflamed RA joint [5, 23–25]. Amorphous fibrin deposits have been detected in the lining and deep layer of RA synovial membrane [23]. Based on our observations, fibrin aggregates appeared in the RA synovium as a solid block or as a loose tissue structure, and both forms were considerably citrullinated. However, only the loose fibrin structure expressed intracellular and extracellular PADI4. In these structures, PADI4-positive cells co-located with protein that contained citrulline, apoptotic cells that contained fragmented DNA and CK18 cleaved by caspase. Furthermore, most apoptotic cells of the RA synovium were localized in the spongiform fibrin mass. The co-localization of PADI4 and apoptotic cells in citrullinated fibrin with a loose tissue structure supports the notion that PADI4 plays a role in apoptosis and locally citrullinates fibrin, possibly by initiating apoptosis as described by Vossenaar *et al.* [19]. Then, the enzyme might leak from dead cells and continually catalyse extracellular fibrin protein. As a result, the spongy fibrin develops into a solid block after citrullination and the PADI4

enzyme is degraded extracellularly. This could explain why PADI4 expression was essentially undetectable in the solid fibrin block. In the rabbit model of antigen-induced arthritis, Sanchez-Pernaute *et al.* observed meshed fibrin at the initial stage of joint inflammation. They postulated that citrullination facilitates proteolytic fibrin cleavage and that fibrin after structural transformation activates the autoimmune reaction of RA [5, 25].

In most of the RA synovial tissue samples we tested, an antibody against IgA rather than other immunoglobulins recognized citrullinated fibrin, although some fibrin clots were also mildly immunostained with anti-IgM antibody. The IgA autoantibody has been broadly identified in rheumatoid disease. Among diverse types of RF, IgA RF is more frequently detected than IgM RF or IgG RF in the sera of individuals before a diagnosis of RA [26]. Berthelot *et al.* found the IgA class of APF in RA sera, though IgA APF was less sensitive than its classical IgG isotype [27]. Therefore, RA patients might develop an IgA class of anti-citrullinated protein antibody in response to a high concentration of citrullinated fibrin protein in the synovium. In fact, Masson-Bessiere *et al.* have reported that some fibrin in the RA synovial membrane is citrullinated and that the α and β chains of fibrin are the major targets of AFA [23]. The present study supports the notion that citrullinated fibrin triggers citrulline-specific B-cell maturation and thereby leads to RA autoimmunity [5, 23].

Although PADI4 was widely distributed in the lining and sublining of the RA synovial membrane, only a few cells of the

tissue expressed citrullinated protein. Baeten *et al.* obtained similar results using two commercially available anti-citrulline antibodies (Upstate and Biogenesis) [28]. This observation implies that the PADI4 enzyme is inactive in most RA synovial cells as well as in peripheral blood. The activation of PADI requires a high concentration of Ca^{2+} (10^{-5} mol/l) [29]. Under normal physiological conditions, the cytosolic and nucleoplasmic Ca^{2+} concentration of 10^{-7} mol/l is too low to trigger PADI enzymatic activity [19, 29]. Vossenaar *et al.* recently found that a high concentration of calcium ions induced by ionomycin could stimulate PADI and the subsequent citrullination of intracellular protein in RA synovial macrophages [19]. Ionomycin is a calcium ionophore that facilitates a sustained Ca^{2+} influx [30]. Thus, an altered level of calcium ions should explain the disparate expression of PADI4 enzyme and citrullinated peptides. However, exactly how Ca^{2+} leads to apoptosis involving PADI4 and subsequent fibrin citrullination remains unknown.

In summary, we demonstrated extensive PADI4 expression in diverse leucocyte subtypes of RA synovial tissue. We also observed significant citrullination of fibrin, as well as the co-location of PADI4, citrullinated protein and apoptosis in some fibrin deposits of the tissue. These findings might be helpful in understanding the close association of the PADI4 haplotype with RA and the important role of PADI4 in RA pathogenesis.

The authors have declared no conflicts of interest.

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