Citrullination is the post-translational modification of arginine residues to citrulline residues in proteins. This process is catalysed by Ca²⁺-dependent peptidylarginine deiminase (PAD) [1]. To date, five isoforms of PAD (PAD1, 2, 3, 4 and 6) have been identified on human chromosome 1p36, a region with susceptibility for rheumatoid arthritis (RA) [1, 2]. Our large-scale genome-wide case–control study using single nucleotide polymorphisms (SNPs) has demonstrated that the gene for PADI4 (also termed PAD4 or PAD5, 1) in some references) is closely associated with RA in the Japanese population [3].

We recently investigated the expression of PADI4 and protein containing citrulline in RA synovial tissue [4]. PADI4 was abundantly expressed in the inflamed synovial membrane and mainly distributed among T cells, B cells, granulocytes, endothelial cells and macrophages. In contrast, the citrullinated protein product of PAD was detected in few synovial cells of the tissue. Citrullination in the RA synovium was mainly detected in extracellular fibrin deposits. Extensive fibrin deposition and high coagulating activity are important features of the diseased synovium [5, 6]. Scott et al. and Sanchez-Pernaute et al. [6–8] reported that most fibrin aggregates in the RA synovium contain fibrinectin (Fn). They suggested that Fn cross-linked with fibrin matrices participates in fibrin deposition and promotes the invasiveness of fibroblasts.

Fn comprises a large family of isomeric glycoproteins characterized by repeated amino acid units that form domains [including fibrin, gelatin, collagen, cell, vascular endothelial growth factor (VEGF) and heparin-binding domains]. These domains interact with various components of extracellular matrix (ECM), cell-surface Fn integrin receptor and growth factors. A single gene encodes Fn, but alternative splicing of pre-mRNA allows the generation of many isoforms that play critical roles in various physiological activities, including cell adhesion, migration, proliferation and differentiation, as well as wound healing, fibrosis and haemostasis [9]. Plasma Fn is mainly produced in the liver and distributed in the plasma, whereas cellular Fn is primarily located on the surface of fibroblasts and myoblasts and is associated with the basement membrane. The expression level, distribution and
molecular heterogeneity of Fn in inflammatory synovial fluid and synovial tissue have been thoroughly investigated [10, 11]. Fn plays important roles in pro-inflammation, pannus invasion and other events associated with disease progression. The present study investigates whether Fn in RA synovium is citrullinated as fibrin and how Fn is involved in the pathogenic processes of RA.

Materials and methods

In vitro citrullination of fibronectin and its detection by enzyme-linked immunosorbent assay

Cellular fibronectin (Upstate, USA) and plasma fibronectin (Sigma) at a final concentration of 0.5 mg/ml was incubated with 5 units/ml of PAD from rabbit skeletal muscle (Sigma) in working buffer (100 mM Tris-HCl, 5 mM CaCl₂, pH 7.4) for 24 h at 37°C. Rabbit PAD is a counterpart of human PADI2 and is highly homologous to human PAD4. The enzyme was omitted from control reactions. Reaction mixtures were diluted 50-fold in 0.05 M carbonate/bicarbonate buffer (pH 9.6) and coated onto EIA/RIA microplates (Costar) overnight at 4°C. Thereafter, the plates were washed with phosphate-buffered saline (PBS) buffer (8 g NaCl, 0.2 g KCl, 1.15 g NaHPO₄ and 0.2 g KH₂PO₄ per litre, pH 7.4–7.6) containing 0.1% Tween 20 (PBS-Tween) and then non-specific binding was blocked by incubation with 5% non-fat dry milk for 1 h at room temperature. The plates were then incubated with 5000-fold diluted anticitrulline antibody (raised with citrulline-glutaraldehyde carriers) (Biogenesis, UK) for 2 h at room temperature, washed with PBS-Tween, and incubated with alkaline phosphatase-conjugated goat antirabbit immunoglobulin G (IgG) (Sigma) diluted 5000-fold in PBS buffer at room temperature, washed with PBS-Tween, and incubated with alkaline phosphatase-conjugated goat antirabbit immunoglobulin G (IgG) (Sigma) diluted to 10 μg/ml with binding buffer (PBS containing 0.5 mg/ml with binding buffer (PBS containing 0.5 mg/ml with binding buffer consisting of 2% BSA, 150 mM NaCl, 0.05% NaN₃, 25 mM Tris-HCl pH 7.4 and 1 mM MnCl₂. The protocol was based on the studies of Mould et al. and Nishiuchi et al. [12, 13].

We prepared controls including (1) incubating Fn in the absence of PAD and (2) incubating Fn with PAD in Tris-HCl buffer without Ca²⁺ to estimate the effect of PAD protein on VEGF and integrin β₁.

Effect of citrullinated fibronectin on apoptosis determined by cell culture

The human promyelocytic leukaemia cell line HL-60 was seeded at an initial density of 3 × 10⁵ cells/ml in serum-free DMEM/F12 medium (Gibco, Japan) containing 10% FCS (Wako, Japan) and cultured for 3 days in the presence of either 12,13-dibutyrate (Sigma) or 1 μM all-trans retinoic acid (Wako, Japan), which respectively induce the cells to become monocytes or granulocytes. Differentiated cells were further incubated with 100 μg/ml of cellular or plasma Fn (citrullinated or not) for 24 h. Controls included cultures without (1) extra additives and with (2) BSA, (3) BSA treated with rabbit PAD (prepared as citrullination of Fn) and (4) the control with PAD enzyme but without Fn. The cells were collected by centrifugation at 200 g for 5 min, fixed with cold methanol for 30 min and transferred to 96-well microplates. Apoptotic cells were assayed using the ApoStrand ELISA apoptosis detection kit (Mol, USA) according to the manufacturer’s instructions. Fragmented DNA of apoptotic cells was detected using a peroxidase-conjugated antisingle-strand DNA antibody in the kit and reactive single-strand DNA exposed by the peroxidase substrate was quantified at 405 nm.

Tissue sample preparation

Blood samples obtained by standard venepuncture from patients with RA (n = 39) and with systemic lupus erythematosus (SLE) (n = 16) as well as healthy volunteers (n = 14) were collected into Monovette tubes containing 3.8% sodium citrate (Terumo, Japan). Following centrifugation at 1000 g for 30 min, the supernatant of anticoagulated plasma was collected and stored at −80°C.

We collected synovial tissue samples from 12 patients with RA and from 5 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. All RA patients met the American College of Rheumatology revised criteria.

All patients and healthy volunteers provided written, informed consent for providing blood and tissue samples. The Ethics Committee of the Institute of Physical and Chemical Research (RIKEN) approved the research protocol.
Determining levels of fibronectin citrullination in plasma by sandwich ELISA

Antihuman fibronectin monoclonal antibody (Abcam) was diluted 4000-fold in 0.05 M carbonate-bicarbonate buffer (pH 9.6) according to the manufacturer’s instructions and coated onto 96-well EIA/RIA microplates overnight at room temperature. The antibody is specific for cellular and plasma Fn and recognizes an epitope within the first 158 amino acids of the heparin-binding domain of the protein. After a brief wash with PBS-Tween, non-specific binding was blocked with PBS containing 5% non-fat dry milk for 1 h at room temperature. Plasma samples diluted 4000-fold with the 5% non-fat milk were then added to the plate and then incubated for 2 h at room temperature. Following another wash with PBS-Tween, the plates were incubated with 5000-fold diluted anticitrulline antibody (Biogenes) for 1 h at room temperature. The rabbit anticitrulline antibody was labelled with alkaline phosphatase using the AP labelling kit (Roche) according to the manufacturer’s instructions. After washing with PBS-Tween, citrulline signals were developed in buffered p-nitrophenyl phosphate as described above and absorbance was measured at 405 nm. Negative controls contained carbonate-bicarbonate buffer instead of anti-Fn antibody for each plasma sample (plasma non-specific binding) or the blocking milk instead of plasma (antibody non-specific binding). Experimental data were corrected by subtracting the sum of the corresponding non-specific plasma and antibody binding values.

Data were collected from three independent tests. The citrullination level of Fn in blood is expressed as median and range. The Mann–Whitney U-test statistically assessed differences between groups. A level of $P < 0.05$ was considered significant.

Immunolocalizing fibronectin expression in arthritic synovial tissues

Sections of RA and osteoarthritis (OA) synovial tissues were deparaffinized and rehydrated using standard procedures. To increase immunostaining intensity the sections were heated at 95°C for 20 min with DAKO Target Retrieval Solution (Dako, Japan). Sections were incubated with the first antibody overnight at 4°C, washed three times each for 5 min with PBS (7.75 g NaCl, 1.5 g K$_2$HPO$_4$ and 0.2 g KH$_2$PO$_4$ in 1 ml of distilled water, pH 7.4–7.6), and then incubated with SimpleStain MAX-AP multi (Histofine, Japan) for 30 min at room temperature. After another PBS wash, immunoreactive signals were visualized using the New Fuchsin substrate kit (Histofine, Japan) according to the manufacturer’s instructions and the tissue structure was defined by counterstaining with haematoxylin.

Rabbit polyclonal anti-MC, monoclonal antihuman fibrin (Monosan, The Netherlands) and monoclonal antihuman fibronectin (Abcam) antibodies were commercially obtained. The anti-MC antibody that was also applied to western blotting specially recognizes protein containing citrulline. Before incubation with the antibody, citrulline residues of proteins with 2,3-butanedione monoxime and antipyrine moieties in tissue sections were modified using the buffer supplied with the kit. The buffer forms ureido group adducts in strong acids, which ensures detection of citrulline-containing protein regardless of neighbouring amino acid sequences [14].

We prepared a series of controls without first antibody, secondary antibody or modification buffer.

Total protein extraction and immunoprecipitation of fibronectin

We purified total protein from RA ($n = 5$) and OA synovial tissues ($n = 4$) using the Total Protein Extraction Kit (Biochain, USA) according to the manufacturer’s instructions. The controls were commercial total protein from placenta and ovary, parotid, prostate and thymus adenocarcinoma (Biochain). Fn in the total proteins was precipitated using a Protein G Immunoprecipitation Kit (Sigma) as follows. Tissue lysates were incubated with the monoclonal antifibronectin antibody overnight at 4°C. Protein G beads provided with the kit were added to the mixtures and the incubation was continued for 2 h at 4°C. After a thorough wash, purified Fn was eluted with $1\times$ Laemmli sample buffer (Sigma). Concentration of the immunoprecipitates was determined using the BCA protein assay kit (Pierce, USA). Protein samples ($5 \mu$g) were analysed by western blotting as described above. The blotted membranes were probed with anti-MC or the antifibronectin antibody.

Results

Citrullination of fibronectin in vitro

Our ELISA indicated that the contents of citrulline in cellular and plasma Fn were significantly increased after incubation with rabbit PAD (Figs 1A and B). Western blotting also detected citrulline production in the two Fn forms after the incubation (Figs 1C and D). The above results demonstrated citrullination of both cellular Fn and plasma Fn by rabbit PAD in vitro.

Binding of citrullinated fibronectin to VEGF and integrin $\beta_1$

The binding of Fn to VEGF and integrin $\beta_1$ was determined using a solid-phase receptor binding assay. Following citrullination in vitro, plasma Fn binding to VEGF was significantly elevated. The binding of cellular Fn was also increased, but to a lesser extent than that of plasma Fn (Figs 2A and B). In contrast, PAD treatment obviously reduced the affinity of both cellular Fn and plasma Fn for integrin $\beta_1$ (Figs 2C and D). The controls indicated that rabbit PAD protein had no direct effect on the binding activities.

The effect of citrullinated fibronectin on apoptosis of HL-60 cells

The apoptosis of differentiated HL-60 cells was determined by a quantitative DNA fragmentation assay. Compared with cultures incubated with BSA, citrullinated BSA, rabbit PAD protein or in the absence of extra additives, Fn without citrullination significantly induced the apoptosis of monocytes but not of granulocytes. However, Fn after citrullination considerably lost its stimulatory effect on the programmed cell death of monocytes. These results also indicated that rabbit PAD enzyme had no direct effect on apoptosis of the cultured cells (Fig. 3).

Levels of citrullinated fibronectin in RA plasma

After extraction using antifibronectin antibody and detection using anticitrulline antibody, sandwich ELISA showed that the absorbance of citrullinated Fn was significantly higher in the plasma of RA than of SLE patients or of healthy individuals (Fig. 4). We replaced antifibronectin antibody with other antibodies to determine the effect of rheumatoid factor (RF). The citrulline signals did not obviously differ between the RA group and controls, indicating that RF did not interfere with our protocol (results not shown).
Expression of fibronectin in arthritic synovial tissue

Continuous sections of RA and OA synovial tissue were immunostained with antibodies against fibronectin, citrullinated protein containing and fibrin. As reported by others [15, 16], Fn immunostaining was mainly detected at the outer surface of the RA synovial membrane where Fn formed a tight block containing rich fibrous materials but not synoviocytes (Fig. 5, panels 1 and 2). The majority of such extracellular masses was also immunopositive for fibrin on continuous sections (Fig. 5, panel l), although fibrin was immunonegative in a few Fn aggregates (Fig. 5, panels 2 and 3). The observation indicated that most Fn was incorporated into the fibrin deposits that were extensively expressed in RA synovial tissue. Immunostaining for Fn was not obvious in the sublining area of the diseased membrane. On the continuous sections, almost all of the Fn mass was significantly immunostained by anti-MC antibody, regardless of co-location with fibrin (Fig. 5, panels l and 2). The Fn and fibrin matrix mainly consists of an amorphous fibrous mass and lacks distinct cell structure, thus rendering the figures a little vague. Besides, tissue sections could not be counterstained after exposure to the modification buffer to immunostain anti-MC. These fibrin/Fn aggregates were also immunonegative for PADI4 protein, various cell surface cluster designation markers and IgE [4], indicating specificity of the current immunostaining. Extracellular accumulation of fibrin /Fn and a significant citrullination signal were undetectable in OA synovial tissue except for the deep sublining region where some fibrous materials and endothelial cells were immunostained by anti-MC antibody (Fig. 5, panel 4). Control slides incubated with normal serum, PBS buffer or anti-MC antibody without treatment of the modification buffer were never positive.

After immunoprecipitation using anti-Fn heparin-binding domain antibody, Fn ranging from 45 to about 230 kDa was detected in the extracts of RA and OA synovial tissue as well as placenta and several tumour tissues (see Figs 6A and B). The 230 kDa Fn was the dominant form in the tissues. In another blotted membrane prepared in the same manner, anti-MC antibody detected an intense signal at 230 kDa and several relatively weak signals at smaller sizes in RA synovial tissues. No immunosignal of citrullinated protein was detectable in Fn extracted from OA synovial tissues, placenta or the tumour tissues (Figs 6C and D). The results from all synovial tissue samples tested were identical. Because some weak Fn bands that were detected in RA synovial tissues by western blotting emitted obvious citrulline signals, we speculated that small fragmented Fn is more easily citrullinated than large molecules or Fn is destabilized after citrullination. Fn was undetectable in total protein samples without immunoprecipitation (data not shown).

Discussion

Immunohistochemistry indicated the accumulation of extracellular Fn in RA synovial tissue. Furthermore, almost all Fn aggregates were significantly citrullinated regardless of incorporation into fibrin deposits. Synovial tissues from patients with OA did not contain extensive Fn deposits. Other groups have also reported the extracellular expression of Fn as well its co-location with fibrin.
Absorbance at 405 nm represents the binding activity.

FIG. 2. Binding of citrullinated Fn to VEGF and integrin β1. Cellular and plasma Fn were incubated with rabbit PAD (lane 3) or not (lane 1). A and B: cellular and plasma Fn binding to VEGF. C and D: cellular and plasma Fn binding to integrin β1. Fn in lane 2 was incubated with PAD in the absence of Ca²⁺. Absorbance at 405 nm represents the binding activity.

in tissues from rheumatic joints [15, 16]. The PADI4 enzyme is extensively distributed in RA synovial membrane [4]. Vossenaar et al. [17] also reported that PADI2 and PADI4 are extensively expressed in macrophages from synovial fluid of RA patients. By injecting citrullinated collagen II into rats, Lundberg et al. [18] recently demonstrated that the amount of citrullinated proteins and PADI4 enzyme correlated with the severity of inflammation, and that neither are detectable in healthy joints. Thus, we propose that PADI4 and/or PADI2 locally catalyse Fn in the diseased tissue. The present study found that some citrullinated fibrin deposits were Fn immunonegative. Studies by others have also provided direct evidence of citrullination of fibrin [19]. We therefore suggest that both extracellular fibrin and Fn are citrullinated in RA synovial tissue. Moreover, our previous and present immunohistochemical investigations revealed that citrullination in RA synovial tissue mainly occurs in extracellular aggregates of fibrin and Fn rather than inside synovial cells. Lundberg et al. [18] also confirmed that extracellular deposits constitute the major source of citrullinated proteins in inflamed joints of a rat model.

Vossenaar et al. [20] showed by immunohistochemical means that the presence of citrullinated proteins is not specific to the RA synovium but is related to inflammation. We also found some citrulline immunosignals in the middle of OA synovial tissue that consisted of an amorphous fibrous mass with little cell structure. In the present study we discovered that Fn immunoprecipitated from RA synovial tissue was citrullinated, whereas that from OA synovial tissue, placenta and tumour tissue was not. This observation corresponded with the immunohistochemical detection of citrullinated extracellular fibrin/Fn deposits in RA synovial membrane. These findings imply that the citrullinated Fn instead of total citrullinated protein is strongly associated with RA pathogenesis. Peters et al. [21] detected various Fn forms ranging from about 47 to over 200 kDa in both RA and OA synovial fluid by gelatin affinity isolation and 2D western blotting. High levels of Fn with an extra domain A (ED-A) or B (ED-B) have been found in RA synovial fluid and Fn containing the 25-amino-acid CS1 sequence was exclusively localized in the capillary endothelium of rheumatic joints [22–25]. However, which types of Fn were citrullinated remains obscure.

We detected more citrullinated Fn in RA plasma than in samples from patients with SLE and healthy controls. The citrullinated Fn could be diffused from the inflamed RA synovium rich in PADI4 enzyme. On the other hand, other groups found similar total Fn levels in plasma from RA patients and in healthy individuals as well as an elevated concentration of Fn in SLE plasma [26–28]. Together with our immunohistochemical data and western blot results, we speculate that Fn citrullination is a process specific to the RA synovium, although Fn expression is also increased in other inflammatory arthropathies [28–30].
FIG. 5. Immunohistochemistry of arthritic synovial tissue. Continuous sections of RA (panels 1–3) and OA (panel 4) synovial tissue were probed with antibodies against Fn (A), citrulline-containing protein (B) and fibrin (C), respectively. Negative controls include incubation of normal serum instead of the first antibody (D1), PBS buffer instead of the first antibody (D2) or anti-citrullinated protein antibody without treatment of the modification buffer (D3). D4 shows immunostaining of citrullinated protein in a deep sublining area of OA synovial tissue. Original magnification ×100.

FIG. 4. Scatter plots of citrullinated Fn levels in plasma from RA and SLE patients and from healthy controls determined by sandwich ELISA.
Besides, anti-Fn autoantibody has been identified in RA patients [31, 32]. However, whether the B-cell epitope of the antibody is reactive against citrulline like that of other RA-specific autoantibodies including antiflaggrin autoantibody (AFA), antikeratin antibody (AKA), antiperinuclear factor (APF) and anticyclic citrullinated peptide antibody (anti-CCP) remains unknown [33].

Excessive angiogenesis is a primary feature of RA synovial tissue [34] and VEGF is the main mediator that stimulates vasculogenesis and angiogenesis via its receptors on endothelial cells or their progenitors. Levels of VEGF are elevated in the synovial fluid and serum of patients with RA and they correlate with disease severity [35]. Using solid-phase assays and surface plasmon resonance analysis, Wijelath et al. [36] identified two novel VEGF-binding domains on the N- and C-terminus of Fn. They found that an association between Fn and VEGF, which bridges VEGF to VEGF receptor and Fn receptor, integrin α5β1, is necessary for the full effects of VEGF-induced endothelial cell migration and proliferation. Using a similar protocol, we found that the binding of citrullinated Fn to VEGF was significantly increased. We surmise that altering the arginine residue to citrulline in the VEGF-binding domain changes the molecular conformation of Fn and thereby increases the affinity of Fn for VEGF. As a result, the integration of citrullinated Fn–VEGF complex with integrin α5β1 and VEGF receptor might be strengthened along with signal amplification. As described above, PAD4 is abundant in the inflamed RA synovial membrane. Thus, citrullination of Fn can profoundly affect the biological activity of VEGF and drive neovascularization and progressive joint destruction of the RA synovium.

In contrast to its increased affinity for VEGF, the binding activity of citrullinated Fn to integrin β1 decreased. The altered molecular conformation possibly loosens the binding between integrin β1 and Fn. Integrin β1, also known as CD29, is a 130 kDa transmembrane glycoprotein that forms non-covalent complexes with various integrin α subunits to bind molecules of the ECM such as Fn and collagen. The interaction of Fn with integrin β1 mediates cell adhesion, migration and signal transduction and consequently plays a crucial role in various important biological processes including wound repair, haemostasis, the immune response and programmed cell death [37]. Integrin β1 is expressed in various types of cells in the rheumatoid synovial membrane [38]. The aggregation of integrin complex, mainly α5β1, with Fn and collagen or other ECM proteins obviously up-regulates the expression of various cytokines, intercellular adhesion molecule 1 (ICAM-1) and Fas as well as the Fas-mediated apoptosis of synovial cells [39, 40]. How citrullinated Fn and its dissociation to integrin β1 affect the RA pathogenic process remains unknown. Sarkissian and Lafyatis [41] reported that collagenase expression in cultured fibroblast-like synoviocytes increases after the cells lose adhesion to Fn. Metalloproteinase as well as collagenase produced in the RA synovium contributes to destruction of the cartilage and bone of arthritic joints. The interaction of Fn and integrins is also involved in tumourigenesis. Loosened cell adhesion allows tumour cells to detach from their original position in tissues and grow in an anchorage-independent fashion, contributing to tumour-directed angiogenesis, cell growth and metastasis [42]. Thus, the disaggregation of integrin β1 from citrullinated Fn might drive the destruction, inflammation and abnormal cell proliferation associated with the RA synovium.

Terui et al. [43] found that Fn enhances the apoptosis of monocytes from peripheral blood and cultured HL-60 cells exposed to phorbol 12,13-dibutyrate. We induced the HL-60 cell line to generate monocytes using the same protocol and then measured apoptosis in the presence of citrullinated and untreated Fn. We found that the ability of Fn to promote apoptosis of the induced monocytes was considerably reduced after citrullination. Because Fn binding to integrin β1 declined after citrullination, we postulated that the intracellular signal cascade altered by the interaction of citrullinated Fn with integrin affects cell survival, although the exact mechanism remains obscure. Although the induced cell culture and above binding assays were all performed in vitro, the data should help to clarify the contribution of citrullinated Fn to RA pathogenesis under physiopathological conditions.

Fn also interacts with other cell-surface and extracellular ligands such as fibrin, heparin, collagen, gelatin, IgG and CD44 through its binding sites [9]. Yasuda et al. [44–46] found that the COOH-terminal of the Fn fragment induced nitric oxide production and matrix metalloproteinase production in rheumatic cartilage and synovial cells through CD44, a cell-surface glycoprotein that is up-regulated during the progress of the disease. Both nitric oxide and matrix metalloproteinases principally activate pro-inflammatory and tissue destruction of inflamed joints. In addition, Fn induces degradation of type II collagen by an interleukin-1-mediated pathway and cooperates with tumour necrosis factor (TNF)-α and hyaluronic acid to enhance the expression of granulocyte/macrophage-colony stimulating factor (GM-CSF) to inhibit the apoptosis of airway and blood eosinophils [47–49]. Because PAD4 is abundantly expressed in RA synovium, citrullinated Fn could be implicated in RA pathogenesis through altered interactions with various mediators.
We detected large amounts of citrullinated human antithrombin III in RA plasma and suggested that inactivation of antithrombin by citrullination increases thrombin activity in RA synovium and thus initiates excessive fibrin deposition and angiogenesis [50]. Here, we found significant citrullination of Fn in RA synovial tissue and high levels of citrullinated Fn in RA plasma. Thus, the citrullination of Fn is another potential pathway through which PADI4 contributes to the progression of RA. Because our protocol can detect citrullinated Fn much more sensitively than citrullinated antithrombin, our findings might be useful for diagnosing RA.

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