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Dynamic Expression of Peptidylarginine Deiminase 2 in Human Monocytic Leukaemia THP-1 Cells During Macrophage Differentiation

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Peptidylarginine deiminases (PADs) consist of five enzymes which are widely distributed in human and rodent tissues. The two types of enzymes are found in human peripheral blood cells; PAD4 mainly in granulocytes and monocytes and PAD2 in lymphocytes and macrophages. Little is known about the regulation of PAD expression in macrophages. Here, we report that PAD2 is expressed in human monocytic leukaemia THP-1 cells during differentiation into macrophages by 12-*O*-tetradecanoylphorbol-13-acetate. During this differentiation, the levels of PAD2 mRNA and protein increased concomitantly, indicating the transcriptional regulation of PAD2 gene expression in the cells. The treatment of THP-1-derived macrophages with calcium ionophore A23187 generated vimentin deimination and resulted in the disruption of vimentin filament organization. We discuss the possible role of vimentin deimination in cell physiology.

Key words: citrullinated proteins, monocytes, protein deimination, rheumatoid arthritis, vimentin.

Abbreviations: AMCA, anti-modified citrulline antibody; BA, N^{α}-benzoyl-L-arginine; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; DIG, digoxigenin; DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; kDa, kilodalton; knt, kilonucleotide; PAD, Peptidylarginine deiminase; PBS(-), Mg^{2+} and Ca^{2+}-free phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; RT-PCR, reverse transcription-polymerase chain reaction; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 1α, 25-$(OH)_2$ D_3, 1α, 25-dihydroxyvitamin D_3.

A large variety of post-translational modifications regulate functions of various proteins in living cells by changing protein structure. Protein deimination/citrullination, which is the conversion of arginine residues in protein into citrulline residues, is catalysed by peptidylarginine deiminase (PAD) (EC 3.5.3.15) in a Ca^{2+}-dependent manner (*1–4*). There are at least four types of enzymatically active PADs (PAD1–PAD4), which are widely distributed in human and rodent tissues (*1*, *3*, *4*). Among these enzymes, PAD2 and PAD4 occur in peripheral blood cells. PAD4 occurs mostly in granulocytes and monocytes and PAD2 in lymphocytes and macrophages (*5–7*). PAD(s) in these cells have been implicated in rheumatoid arthritis (RA), a chronic inflammatory disease (*8–11*). The citrulline residue of proteins is an essential constituent of an antigenic epitope recognized by autoantibodies specific for RA patients (*8*,*9*); a haplotype of the PAD4 gene *PADI4* has been identified as an allele associated with susceptibility to RA in Asian and North American populations (*10*, *11*). However, the expression and function of PADs in immune cells remain unclear.

PAD4 is expressed during the differentiation of HL-60 cells into granulocytes or monocytes and in peripheral blood granulocytes (*7*, *12*, *13*). In these granulocytes, histone deimination and release of deiminated histones into the extracellular space occur in response to proinflammatory cytokines or endotoxins and seem to be involved in bactericidal action and inflammation (*14*, *15*). A PAD in mouse peritoneal macrophages is activated to generate cellular vimentin deimination that is accompanied by cell apoptosis (*16*). Recently, the expression of PAD2 and PAD4 has been explored in peripheral blood mononuclear cells, primary *ex vivo* macrophages and accumulating cells in synovial tissue (*5*, *6*). However, the pattern and regulation of expression of these PADs in monocytes and macrophages is not particularly clear. The difficulty in assessing the expressions of PADs arises from the high monocyte and macrophage heterogeneity during their differentiation (*6*, *17*).

It is well known that THP-1 cells, a human monocytic leukaemia cell line, differentiate into macrophages in response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (*18–20*). The differentiated THP-1 cells have most morphological and functional characteristics similar to primary *ex vivo* macrophages (*19*). In this study,

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we assessed the expression of PADs in THP-1 cells during the monocyte–macrophage differentiation. We found that expression of PAD2 was up-regulated during differentiation, thereby deiminating cellular vimentin in response to calcium influx. We will also discuss deimination of vimentin in THP-1-derived macrophages in relation to vimentin functions.

MATERIALS AND METHODS

Chemicals—N^{α}-benzoyl-L-arginine ethyl ester (BAEE) was purchased from the Peptide Institute, Inc. (Osaka, Japan). N^{α}-benzoyl-L-arginine (BA) was from Sigma-Aldrich (St. Louis, MO, USA). The 1α, 25-dihydroxyvitamin D_3 (1α, 25-$(OH)_2$ D_3) was from Wako (Osaka, Japan). TPA was from Midland Corp. (Brewster, NY, USA). Anti-modified citrulline antibody (AMCA) was a gift from Tatsuo Senshu (*21*, *22*). A23187 was from Merck (Whitehouse Station, NJ, USA).

Cell Culture—THP-1 cells were obtained from the RIKEN cell bank. THP-1 cells were grown in RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated foetal bovine serum (Bioproducts, Inc., Boston, MA, USA) and kanamycin sulphate (50 mg/l) at 37°C in 5% CO_2 under air. For macrophage differentiation, cells were seeded at a density of 3×10^5 cells/ml and cultured in the presence of 10 ng/ml TPA. Adherent cells were briefly digested with trypsin, suspended and used for cell counting and the PAD activity assay. HL-60 cell culture and its monocyte differentiation were as described previously (*12*).

Assay of PAD Activity—The harvested THP-1 cells were suspended in cell lysis buffer containing 20 mM Tris–HCl (pH 7.6), 1 mM EDTA, 0.1% Triton X-100 and 1 mM phenylmethanesulphonyl fluoride (PMSF) and lysed 10 times by a 10 s sonication on ice. The reaction mixture (50 μl) contained 0.1 M Tris–HCl (pH 7.6), 10 mM $CaCl_2$ and 5 mM dithiothreitol with or without 10 mM BAEE and a 25 μl aliquot of the cell lysate. The mixture was incubated at 37°C for 1 h. The reaction was stopped by addition of 12.5 μl of 5 M perchloric acid and the mixture was centrifuged at 15,000*g* for 5 min at 4°C. The supernatant was used for the colorimetric reaction with citrulline as a standard (*23*). One unit of the enzyme was defined as the amount of enzyme catalysing the formation of 1 μmol of citrulline derivative in 1 h under the conditions. Protein concentrations were determined by the method of Bradford with bovine serum albumin (BSA) as a standard (*24*).

Northern Blotting—Total RNAs were extracted by the method of acid guanidinium thiocyanate–phenol–chloroform extraction from THP-1 cells treated with or without TPA for 3 days (*25*). Poly(A)$^+$ RNA was purified from total RNA by oligotex-dT 30 (Roche Applied Science, Mannheim, Germany). Poly(A)$^+$ RNA (2 μg/lane) was subjected to 0.8% agarose gel electrophoresis containing 2.2 M formaldehyde, transferred to a nylon membrane and fixed by UV irradiation (*26*). Probes of PADs 2, 4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labelled with digoxigenin (DIG)-dUTP using a DNA labelling and detection kit (Roche Applied Science). The RNA blots were incubated in a hybridization buffer [5 × SSPE, 2% blocking reagent/no fat dry milk fraction, 0.1% *N*-lauroylsarcosine, 2% sodium dodecyl sulphate (SDS), 50 μg/ml salmon sperm DNA) containing 50% formamide and 25 ng/ml of a probe at 42°C for 20 h. The bound probe was detected as a chemiluminescent signal using a DIG-alkaline phosphatase system with CDP-Star (Roche Applied Science). For reuse, the blots were microwaved five times in a 0.1% SDS solution for 2 min.

Reverse Transcription–Polymerase Chain Reaction—Total RNA was treated with DNase I (NIPPON GENE, Tokyo, Japan) in the presence of RNase inhibitor (GE Healthcare Bio-Science, Uppsala, Sweden). Reverse transcription–polymerase chain reaction (RT–PCR) was performed using the Titan One Tube RT-PCR system (Roche Applied Science) with total RNA (500 ng) and a pair of 21-nt forward and reverse primers according to the supplier's manual.

Preparations of Recombinant PADs and an N-terminal Part of PAD2—Human recombinant (hr) PADs and PAD2-N of the N-terminal PAD2 mutant (1–261) were expressed as glutathione *S*-transferase (GST)-fusion proteins in BL-21 cells. These were purified by glutathione–Sepharose affinity chromatography as described previously (*12*). The GST portions were removed by digestion with PreScission Protease (GE Healthcare Bio-Science).

Preparation of Anti-PAD2 IgG—rPAD2 (300 μg) was mixed with complete Freund's adjuvant and injected subcutaneously into female New Zealand rabbits. After 3 weeks they were given a booster injection of the same antigen in incomplete Freund's adjuvant. After 3 weeks, blood was collected. IgG was precipitated by mixing the serum with an equal volume of 34% (w/v) sodium sulphate. It was purified by a passage through a DEAE-Sephacel column (3 ml) (GE Healthcare Bio-Science) equilibrated with 17.5 mM phosphate buffer pH 6.3, and a passage through a GST–Sepharose column (2 ml) to remove anti-GST IgG. The unadsorbed fraction was bound to a PAD2-N Sepharose column (1.5 mg PAD2-N covalently linked with 1.2 ml gel). The bound IgG was eluted with 0.1 M glycine–HCl (pH 3.0). The eluate was neutralized with 1 M Tris–HCl (pH 8.0) and dialysed against Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline [PBS(−)]. The purified antibody was referred to as anti-PAD2 IgG.

Immunoprecipitation—Immunoprecipitation was performed as described (*27*). The 10^7 THP-1 cells cultured with or without TPA for 4 days were lysed with 1 ml of ice-cold non-denaturing lysis buffer [1% Triton X-100, 50 mM Tris–HCl (pH 7.4), 300 mM NaCl, 5 mM EDTA, 0.02% sodium azide, 1 mM PMSF and 2 μg/ml leupeptin]. The lysate was pre-cleared by mixing with 30 μl of 50% protein A Sepharose CL-4B bead slurry (GE Healthcare Bio-Science) for 30 min at 4°C. The pre-cleared cell lysate (400 μl) was mixed with 10 μl of 10% BSA and incubated for 2 h at 4°C with anti-PAD2 IgG (1 μg) and normal rabbit IgG (1 μg) which had been bound to 30 μl of the protein A Sepharose bead slurry. The mixture was centrifuged at 16,000*g* at 4°C for 5 s. The bead pellet was washed successively with 1 ml of wash buffer [0.1% Triton X-100, 50 mM Tris–HCl (pH 7.4), 300 mM NaCl, 5 mM EDTA and 0.02% sodium azide] four times, 1 ml of

wash buffer containing 0.1% SDS and 1 ml of PBS(−). The bead-bound proteins were eluted with 15 μl of SDS sample buffer, and the eluate was subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting.

Protein Deimination in Cells and Immunoblotting—Protein deimination by A23187 and immunoblotting of citrullinated proteins were as described (*13*, *16*, *22*, *28*). TPA-treated adherent cells were washed and immersed with Locke's solution (0.15 M NaCl, 5 mM KCl, 5 mM HEPES, 2 mM $CaCl_2$, 0.1% glucose, pH 7.3), and then incubated with 4 μM A23187 for 15 min at 37°C. The cells were rinsed and fixed with 10% cold trichloroacetic acid for 30 min on ice, and were washed successively with cold methanol containing 0.02 M HCl and cold methanol. The cells on the dish were scrapped in methanol, collected and washed twice with cold acetone. The cell pellet was dissolved in 1× SDS–PAGE sample buffer containing 5% 2-mercaptoethanol at a concentration of 2×10^7 cells/ml by heating to 95°C for 5 min. The samples were subjected to SDS–10% PAGE. The resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was incubated with citrulline modification solution at 37°C for 3 h and washed three times with PBS(−) for 10 min, and then blocked with 5% skimmed milk at 37°C for 1 h, and further incubated with rabbit AMCA (0.125 μg/ml) (*22*) or mouse anti-vimentin monoclonal antibody V9 (0.335 μg/ml, DAKO, Glostrup, Denmark) in 5% skimmed milk at 4°C overnight. The membrane was washed twice with PBS-T for 15 min and then incubated with 5% skimmed milk containing goat anti-rabbit IgG (H + L) HRP conjugate (Bio-Rad, Hercules, CA, USA) or goat anti-mouse IgG HRP conjugate for 1 h at room temperature. The bound antibodies were visualized by an enhanced chemiluminescence system (Perkin Elmer, Waltham, MA, USA).

2D gel electrophoresis of citrullinated proteins—The A23187-treated cells were lysed in a buffer containing 10 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM PMSF and 50 μg/ml of RNase by 10 times of a 2 s sonication. The lysate was treated with 50 μg/ml of DNase I for 30 min on ice and was brought into a sample solution further containing 9.5 M urea, 2% Triton X-100, 2% Ampholines pH 3.5–10 and 5% 2-mercaptethanol. The sample was subjected to 2D gel electrophoresis by the method of O'Farrell (*29*); isoelectric focusing electrophoresis in the first dimension and SDS–10% PAGE in the second dimension and then was immunoblotted as described above. To reprobe the blot, the blot was treated with 0.2 M NaOH for 5 min.

Double Immunofluorescence Cytostaining—Double immunofluoresence staining of citrullinated protein and vimentin in cells was as described (*13*). THP-1 cells were cultured with TPA for 4 days on glass coverslips and stimulated by 2 μM A23187 for 15 min in Lock's solution. The cells were fixed using 4% paraformaldehyde in PBS(−) for 30 min at 4°C. They were incubated with medium for chemically modifying citrulline residues for 3 h at 37°C. The medium consisted of one part of solution A (0.0416% $FeCl_3$-$6H_2O$, 4.6 M H_2SO_4 and 3 M H_3PO_4) and one part of solution B (1% diacetylmonoxime, 0.5% antipyrine and 0.5 M acetic acid). After three washes with PBS(−), the cells were treated successively with 2 M Tris–HCl (pH 7.4) for 15 min, PBS(−) containing 0.1% Triton X-100 for 10 min and PBS(−) containing 2% BSA, 2% normal goat serum and 0.1% Triton X-100 for 30 min. The coverslip cells were incubated with a mixture of rabbit AMCA (0.625 μg/ml) and mouse anti-vimentin monoclonal antibody V9 (0.42 μg/ml) at 4°C overnight. The bound IgGs were detected by incubation with a mixture of FITC-conjugated goat anti-rabbit IgG (Wako) (1/200) and TRITC-conjugated anti-mouse IgG (Sigma) (1/200) for 30 min.

RESULTS

Increased PAD Activity in THP-1 Cells During the Differentiation into Macrophages—First, we investigated whether PADs were expressed during the differentiation of THP-1 cells into macrophages in the presence of TPA (10 ng/ml). Without TPA, cells did not alter their cell morphology and proliferated in suspension for 4 days (Fig. 1Aa and Ba). In contrast, with TPA, the proliferation of cells ceased, and the cells adhered to the culture dish within 1 day (Fig. 1Ba). Thereafter, they spread on the dish with a characteristic appearance as a variety of oblong cell and irregular nuclear shapes (Fig. 1Ab). The cells started to express vimentin 1 day after, whose amount further increased for 2 days, as assessed by immunoblotting (Fig. 1C). In the absence of TPA, cells showed low levels of vimentin. Figure 1Bb shows changes in PAD activity during those periods of cultivation. In the presence of TPA, PAD activity was initially detected at 2 days of culture, which increased in the following 2 days (open circles). In the absence of TPA, no PAD activity was detected throughout the culture period (closed circles). The enzymatic activity occurred concomitantly with adhesion, changes in morphology and levels of vimentin. These results indicate that differentiation of THP-1 cells into macrophages triggers an increase in the expression and enzymatic activity of cells.

It is known that the PAD activity towards the two synthetic substrates of BAEE and BA differs in different PAD types (*12*, *23*). Table 1 shows that the BA/BAEE ratio for THP-1 macrophage PAD was 0.21, similar to the ratio (0.14) for rPAD2. The ratio for HL-60 monocyte PAD was 1.3, similar to the ratio (1.5) for rPAD4. The kinetic parameters for rPAD2 were estimated from Lineweaver–Burk plots (Table 2). These data indicate that THP-1 macrophage PAD is perhaps PAD2.

Regulation of PAD Expression in THP-1 Cells During Macrophage Differentiation—Next, we studied the expression of PADs in THP-1 cells during differentiation by immunoprecipitation and RNA analysis. First, we prepared antibodies specific for the PAD2 N-terminal portion (1–261) by an affinity purification of rabbit anti-serum against rPAD2 and examined specificity for rPADs 1–4. The purified antibody IgG specifically recognized the GFP–PAD2 fusion protein (97.4 kDa) produced in HeLa cells, as tested by immunoblotting (Fig. 2A). This antibody also recognized purified PAD2 produced by *Escherichia coli* (Fig. 2B). Second, we tested whether the antibody can precipitate PAD2 from lysates of

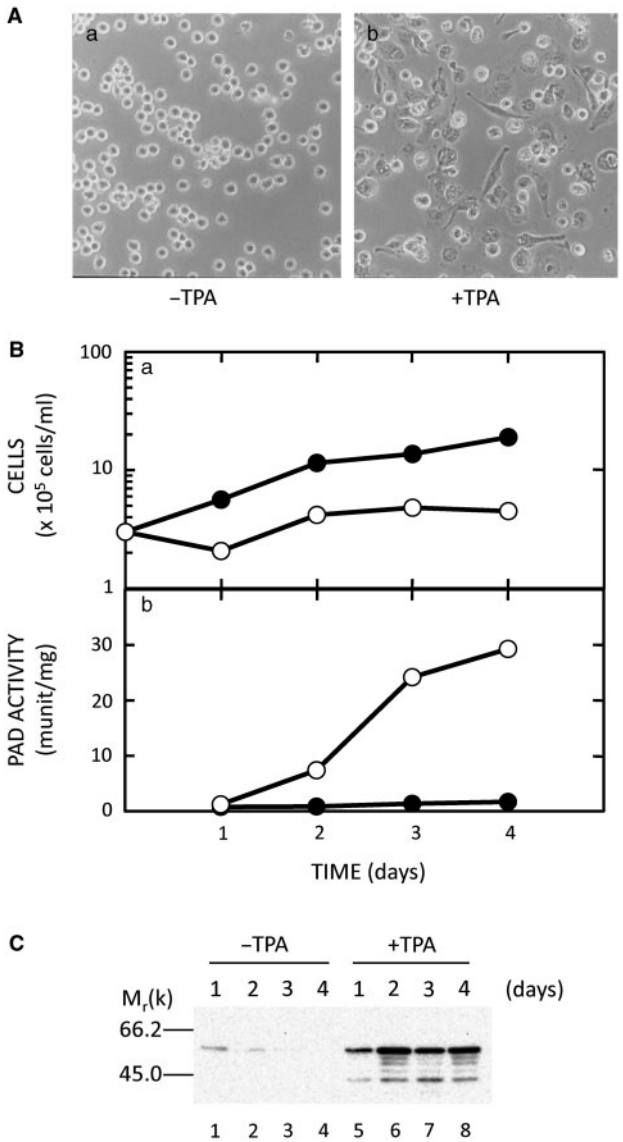

Fig. 1. **Changes in PAD activity during differentiation of THP-1 cells to macrophages by TPA.** (A) Gross morphology of THP-1 cells. THP-1 cells were cultured for 3 days without (a) or with (b) 10 ng/ml TPA. (B) Time courses of cell number (a) and PAD activities (b) of THP-1 cells in culture. THP-1 cells were grown with (open circles) or without TPA (filled circles) for the indicated times. PAD activity of the cells was determined as described in the text. (C) Expression of vimentin in THP-1 cells. Vimentin in the above cells was studied by immunoblotting using anti-vimentin. The tops of lanes show the cultured periods with or without TPA.

THP-1 cells. Figure 2C shows that from the lysate of TPA-treated THP-1 cells, the antibody precipitated an ~72 kDa protein that co-migrated with rPAD2 (lanes 4 and 5). However, the control IgG did not precipitate a similar protein from the same lysate (lane 3). However, lysate of TPA-untreated THP-1 cells with the antibody or non-immune IgG did not give a specific protein precipitate (lanes 1 and 2). These results indicated that PAD2 was produced in THP-1 cells following macrophage differentiation.

Table 1. **Substrate specificities of THP-1 macrophage PAD and HL-60 monocyte PAD towards BAEE and BA.**

Cell lysates and rPADs	Activity (U/mg)		BA/BAEE ratio
	BAEE	BA	
THP-1 macrophages	0.024	0.005	0.21
HL-60 monocytes	0.015	0.019	1.3
rPAD2	135.4	19.5	0.14
rPAD4	222.4	324.8	1.5

THP-1 cells and HL-60 cells were cultured for 3 days with 10 ng/ml TPA and with 0.1 μM 1α, 25(OH)$_2$D$_3$, respectively, to differentiate into macrophages and monocytes. PAD activities of the cell lysates were determined using BAEE and BA as a substrate. rPADs 2 and 4 were included for comparison.

Table 2. **Kinetic parameters of PAD2 for synthetic substrates.**

Substrates	V_{max} (pmol/s)	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($mM^{-1}s^{-1}$)
BAEE	25.5	2.12	3.21	1.51
BA	9.39	5.21	1.18	0.23

The reaction mixture was as described under MATERIALS AND METHODS section, except that the mixture contained 0.6 μg of rPAD2 and indicated substrates and was incubated at 37°C for 1 h.

Then, we studied the expression of PAD mRNAs in THP-1 cells during macrophage differentiation compared with monocyte differentiation of HL-60 cells. Figure 3A shows a northern blot of the Poly(A)$^+$ RNA with a PAD2 or PAD4 probe. PAD2 mRNA (~6.4 knt) was found in TPA-treated THP-1 cells, but not in TPA-untreated cells (left panel, lanes 1 and 2). However, this mRNA was not found in either 1α, 25-(OH)$_2$ D$_3$-untreated or treated HL-60 cells (lanes 3–4). No PAD4 mRNA was detected in both TPA-untreated and -treated THP-1 cells by probing the same blots with a PAD4 probe (right panel, lanes 1–2). However, PAD4 mRNA (~3 knt) was detected in 1α, 25-(OH)$_2$ D$_3$-treated but not untreated HL-60 cells (lanes 3–4).

To determine whether there is a small amount of PAD mRNAs in THP-1 cells, we amplified mRNAs by RT–PCR using total RNA of THP-1 macrophages and primer pairs specific for PADs 1, 2, 3 or 4. Figure 3B shows that PAD2 mRNA, but not that of PADs 1, 3 or 4, was observed in the THP-1 cells. Thus, PAD2 is the sole expressing PAD at the transcriptional level in THP-1 macrophages.

Vimentin Deimination in situ *in THP-1 Macrophage*—To identify the target of PAD2 in THP-1 cells, we challenged THP-1 macrophages with calcium ionophore A23187 in a 2 mM $CaCl_2$ milieu for 15 min (*5*, *16*, *28*) and searched citrullinated proteins via immunoblotting and immunocytochemistry by using an antibody against modified citrulline residue (AMCA). As shown in Fig. 4A, two apparent citrullinated proteins were observed after treating the cells with various concentrations of A23187; the major one was ~58 kDa in size and the other was ~50 kDa. The 58 kDa protein was detected at low levels even without A23187 stimulation (left panel, lanes 1 and 2). Its amount increased in a dose-dependent fashion

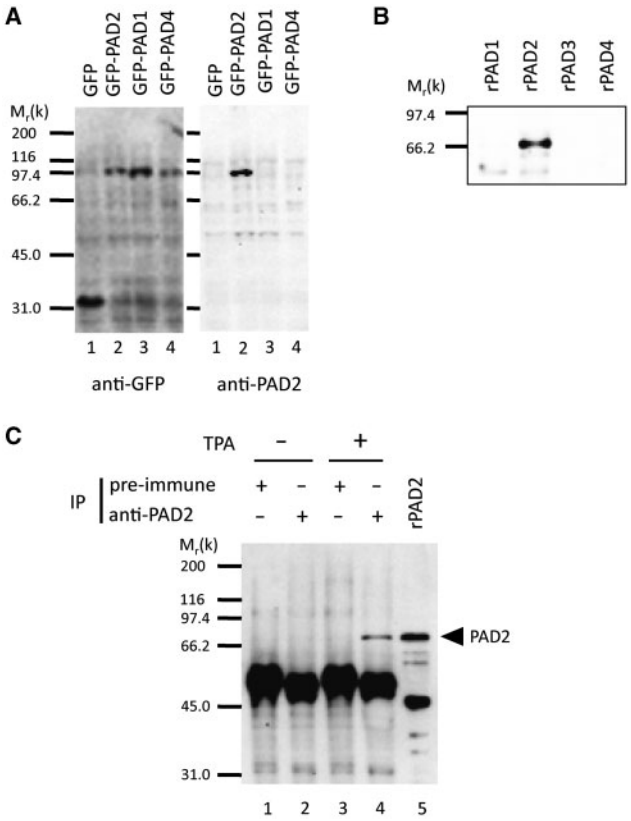

Fig. 2. **Immunoprecipitation of THP-1 cell lysate with anti-PAD2 antibody.** (A) The specificity of anti-PAD2 antibody. PADs 1, 2 and 4 were expressed as EGFP-tagged proteins in HeLa cells. EGFP was expressed as a control. The cell lysates (20 μg) shown at the tops of lanes were subjected to SDS–PAGE. The protein blots were probed using anti-GFP (left panel) and then reprobed using anti-PAD2 IgG (right panel). Intensities of 97.4 kDa bands stained by anti-GFP on the left panel indicate the levels of EGFP-tagged PADs in HeLa cells and serve as internal standards on protein blotting. The numbers on the left indicate molecular weight standard proteins. (B) The anti-PAD2 antibody specificity for purified rPADs. rPADs were expressed in *E. coli* and purified. The SDS–PAGE protein blots of rPADs 1, 2, 3 and 4 (10 ng) shown at the tops of lanes were probed using anti-PAD2 IgG. Only parts in question in the blots are shown. (C) Immunoprecipitation of THP-1 cell PAD with anti-PAD2 antibody. The cell lysates of THP-1 cells cultured with or without TPA for 4 days were immunoprecipitated with anti-PAD2 IgG (663 ng/ml) or with non-immune IgG as described in the text. The immunoprecipitates were subjected to SDS–PAGE and their protein blots were probed using anti-PAD2. IP; immunoprecipitation. Lanes 1 and 2, cells without TPA; lanes 3 and 4, cells with TPA; and lane 5, rPAD2 (10 ng) without IP. The arrow indicates 72 kDa PAD2. The heavy stained 55 kDa bands were rabbit IgG heavy chains.

(lanes 3–5). Reprobing the blots with an anti-vimentin monoclonal antibody showed the co-migration of the 58 kDa protein with vimentin (right panel). The 50 kDa protein did not react with the anti-vimentin. In the presence of 4 μM A23187, the amount of the 58 kDa protein reached its highest level at 15 min, and thereafter decreased gradually (Fig. 4B). Furthermore, we analysed the citrullinated proteins by 2D gel electrophoresis (Fig. 4C). Reasonable protein resolution was achieved on the 2D gel (lower panel, protein staining). As assessed by immunoblotting using AMCA, the major 58 kDa protein focused to pI 5.6 (upper panel arrowhead), and the minor 58 kDa proteins migrated towards more acidic regions seen as very faint signals of consecutive dots (bar with a star). Reprobing the protein blot with the anti-vimentin showed that the major signal of the 58 kDa citrullinated protein coincided with the vimentin signal (upper and middle panels, large arrowheads).

Next, we studied co-localization of the citrullinated proteins and the vimentin intermediate filament within cells. We treated THP-1 macrophages on a glass coverslip with 2 μM A23187 for 15 min, and double immunostained the cells using a mixture of AMCA and anti-vimentin. Figure 5B shows that citrullinated protein was densely found in the perinuclear region of a cell and was also distributed as filaments over the whole cell extending to the cellular margin. As seen in Fig. 5A, vimentin distribution in the same cell was almost confined to the perinuclear region, most likely citrullinated proteins. However, in other citrullinated protein-negative cells, vimentin filaments were broadly distributed over the cytoplasm. Citrullinated cells amounted to ~17% of the vimentin-positive cells (Fig. 5C and D). Apparently, citrullinated protein signals were mostly observed in the nucleus (Fig. 5D). These results suggested that vimentin deimination might affect and reorganize the intracellular distribution of vimentin filaments to condense into the nucleus.

DISCUSSION

In this study, we found that PAD2 but not PAD4 was expressed in THP-1 cells differentiated into macrophages by TPA. During THP-1 macrophage differentiation, PAD2 mRNA and protein were concurrently expressed. Also during HL-60 monocyte differentiation, PAD4 mRNA and protein were simultaneously expressed, as previously found (*12*). These findings indicated that PAD expression in these cells is regulated at the transcriptional level. However, in peripheral blood monocytes and *ex vivo* macrophages, the protein abundance of PAD2 and PAD4 does not always depend on the amount of their mRNAs (*5*, *6*). Foulquier *et al*. (*6*) found that PAD2 is a sole expressing PAD protein in macrophages, even though the cells have both PAD2 and PAD4 mRNAs. However, Vossenaar *et al*. (*5*) observed that in macrophages prepared in a similar but different way, PAD2 and PAD4 proteins are both found, even in the absence of PAD4 mRNA. This discrepancy in the PAD expression among macrophages may originate from differences in the methods used for monocyte preparation and subsequent macrophage differentiation. Moreover, in monocytes, only PAD4 protein is expressed, even though both PAD2 and PAD4 mRNAs exist (*5*). Thus, a translational regulation of PAD2 and PAD4 mRNAs in monocytes and macrophages has been suggested. However, the difference in the regulation of the expression among peripheral blood-derived cells and THP-1 cells remain unsolved. Importantly, both transcriptional and translational regulation may govern PAD2 and PAD4 expression in monocytes and macrophages, possibly depending on

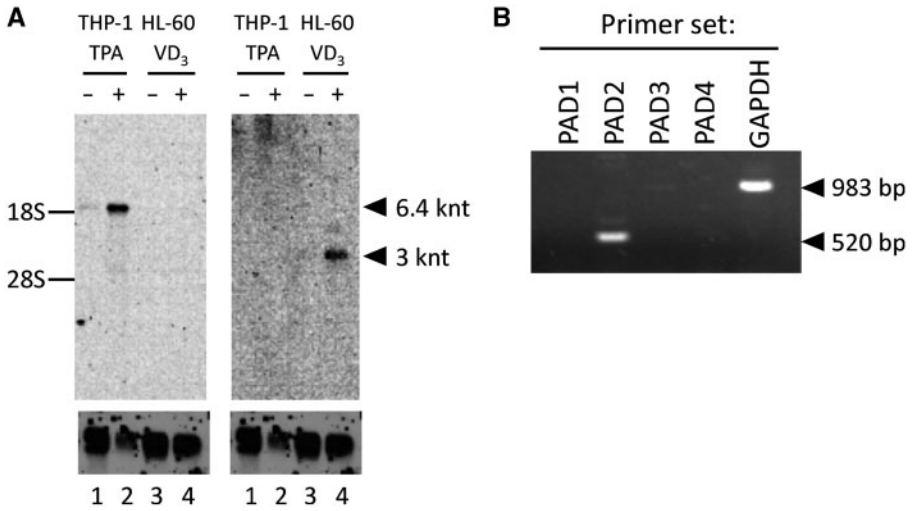

Fig. 3. **Expression of PAD2 mRNA in THP-1 cells.** (A) Northern blot of THP-1 cell RNA and HL-60 cell RNA. THP-1 cells and HL-60 cells were cultured with or without TPA and with or without 1α, 25-$(OH)_2$ D_3, respectively, for 3 days. Poly $(A)^+$ RNAs (2 μg) from these cells were subjected to northern blotting. The blots were probed with DIG-labelled PAD2 cDNA (500 bp) (left panel) and then reprobed with DIG-labelled PAD4 cDNA (500 bp) (right panel). GAPDH was probed as an internal control for integrity and membrane transfer of loaded samples (lower panels). VD_3; 1α, 25-$(OH)_2$ D_3. Lanes: 1, untreated THP-1 cells; 2, TPA-treated THP-1 cells; 3, untreated HL-60 cells; 4, VD_3-treated HL-60 cells. The arrowheads on the right show the approximate sizes of 6.4 knt PAD2 and 3 knt PAD4. (B) PAD2 mRNA expression in THP-1 cells. Total RNA from TPA-treated THP-1 cells was amplified by RT–PCR with the primer sets shown at the tops of lanes. GAPDH was a control for RT–PCR.

various stages of their development and cytokine environment (*17*). In addition, the presence of four plausible Sp1 binding sites in a *PADI2* promoter region is indicative that the expression of PAD2 may be transcriptionally regulated by TPA (*30*, *31*). Such a regulation of the expression of PADs in mononuclear phagocytes might also be associated with the disease activity of RA (*4–6*).

Vimentin was a target protein of PAD2 in THP-1 cells, as observed in mouse peritoneal macrophages and human *ex vivo* macrophages (*5*, *16*). Vimentin was the major citrullinated protein that co-migrated with uncitrullinated vimentin on a 2D gel. Thus, the number and sites of deiminated residues in a molecule are limited. This limited deimination caused a clearly visible change in vimentin filament organization from a fine filament network in the whole cell to an amorphous aggregate clumped at the nuclear periphery, similar to that found previously (*16*). This filament disorganization may occur through deimination of the non-α-helical N-terminal head domain, which is rich in arginine, serine and threonine, and is prone to post-translational modification such as phosphorylation (*32*, *33*). In fact, PAD2 *in vitro* deiminates predominantly the N-terminal head domain and results in the disassembly of vimentin filaments and incompetence of the vimentin assembly (*32*). This vimentin deimination competes for protein kinase A- or C-mediated phosphorylation which affects vimentin assembly (*32*, *34*). Moreover, the aggregated vimentin seen on the nucleus after filament disruption could be accounted for by high affinity of the vimentin head domain for specific DNA regions, namely AT-rich nuclear matrix attachment regions (*35*).

Vimentin participates in various cellular signalling as well as cell architecture (*33*, *36–38*). Possibly, the observed condensation of citrullinated vimentin around the nucleus may trigger the breakdown of higher order chromatin structures, and eventually result in cell apoptosis (*16*). However, whether vimentin deimination affects THP-1 macrophage functions such as phagocytosis and cell adhesion is largely unknown.

In RA, various kinds of cells such as mononuclear cells, plasma cells and neutrophils are infiltrated into synovial tissue, and their cellular activities are dependent on the cytokine network (*39*). Tumor necrosis factor α-activated *ex vivo* macrophages secrete vimentin into the extracellular space (*40*). Possibly, citrullinated vimentin is also secreted from the activated macrophages by calcium signalling or by cytokines, and is further released by cell death. Indeed, citrullinated histones are extruded from neutrophils in response to various pro-inflammatory cytokines (*14*, *15*). In inflamed tissues, apoptotic cells may release PADs to deiminate extracellular fluid proteins such as a fibrin (*41*). Various citrullinated proteins produced in such multiple ways will be a candidate of autoantigen generating anti-citrullinated protein antibodies in RA, and will also be associated with pathophysiology of RA (*8*, *9*, *41*, *42*). For studies on the possible role of PAD in macrophages, THP-1 macrophages will be useful.

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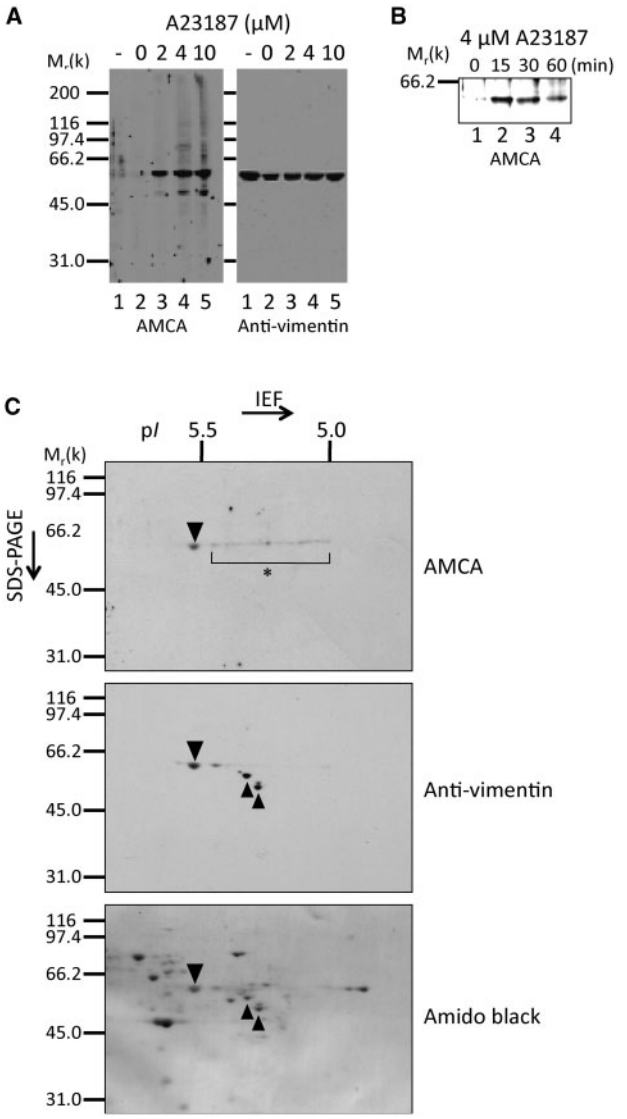

Fig. 4. **Protein deimination in THP-1 cells triggered by A23187.** (A) Effect of A23187 concentration on protein deimination. THP-1 cells cultured with TPA for 3 days were exposed to A23187 at the various concentrations shown at the top of the lanes for 15 min. The cell lysates were subjected to SDS–PAGE and citrullinated protein on the protein blots were probed using AMCA (left panel) and then the blots were reprobed using anti-vimentin antibody (right panel). Lanes: 1, cells unexposed; 2–5, cells exposed to 0, 2, 4 or 10 μM A23187, respectively. (B) Effect of incubation time on vimentin deimination. The TPA-treated THP-1 cells were incubated in the presence of 4 μM A23187 for the indicated times. Vimentin deimination in the cells was assessed by immunoblotting as above. (C) Identification of citrullinated protein as vimentin by 2D gel electrophoresis. THP-1 cells cultured with TPA for 4 days were exposed to 4 μM A23187 for 15 min. The cell lysate was prepared and subjected to 2D gel electrophoresis as described in the text. The protein blot was probed using AMCA (upper panel), followed by protein staining (lower panel), and then reprobed using anti-vimentin antibody (middle panel). IEF, isoelectric focusing; p*I*, isoelectric point. The arrowhead in the upper panel shows citrullinated protein (58 kDa, p*I* 5.6). The large arrowheads in the middle and lower panels show the same position as in the upper panel and the two small arrowheads show degradation products of vimentin. The asterisk indicates the regions with faint dots in the upper panel.

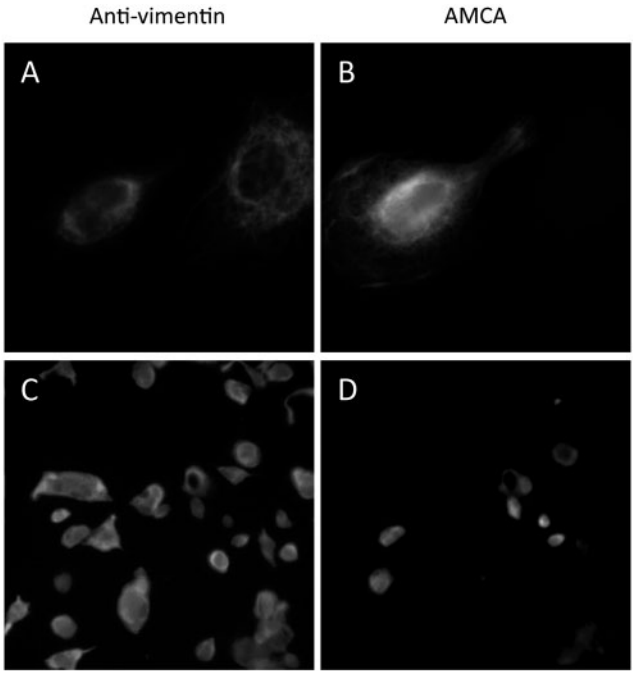

Fig. 5. **Double immunofluorescence staining of citrullinated protein and vimentin in TPA-treated THP-1 cells.** THP-1 cells cultured with TPA on a glass coverslip were incubated with 2 μM A23187 for 15 min. The cells were stained with mouse anti-vimentin antibody (A and C) and rabbit AMCA (B and D) as described in the text. (A and B) Note the difference in vimentin distribution between citrullinated protein-positive and -negative cells. Panels (A) and (B) show the same view. (C and D) Gross appearances of cells displaying vimentin and citrullinated protein. Panels (C) and (D) show the same view.

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CONFLICT OF INTEREST

None declared.

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