Altered acetylation of proteins in patients with rheumatoid arthritis revealed by acetyl-proteomics

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Abstract

Objective

Post-translational modifications (PTMs) are often critical for the function of proteins as well as antigenicity of proteins. We here tried to elucidate alteration of PTMs in Rheumatoid arthritis (RA), focusing on acetylation. We applied acetyl-proteomics to peripheral blood mononuclear cells (PBMCs) to elucidate PTM difference between patients with RA and healthy donors.

Methods

Proteins, extracted from peripheral blood mononuclear cells (PBMCs) of 7 RA patients and 7 healthy donors, were separated by 2-dimansional electrophoresis. Acetylation ratios of each protein spot were estimated by the combination of Sypro Ruby staining and anti-acetylated lysine antibodies. Proteins highly acetylated in the RA group were identified by mass spectrometry. Focusing on α-enolase (ENO1), one of the identified proteins, involvement of histone deacetylases (HDACs) in the high acetylation was investigated. Furthermore, the effects of acetylation on the activity of ENO1 were investigated.

Result

In PBMCs from the patients with RA, 29 acetylated protein spots were detected. One of highly acetylated proteins in the RA patients was identified as ENO1. The acetylation of ENO1 was found to be regulated in part by HDAC1. The enzymatic activity of ENO1 was up-regulated by acetylation.

Conclusion

Highly acetylated ENO1 may play roles in the pathophysiology of RA through the maintenance of activated lymphocytes by increasing glycolysis-derived energy supply.

Key words

rheumatoid arthritis, acetylation, proteomics

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Received on August 27, 2014; accepted in revised form on July 7, 2015.

Funding: this work was supported by MEXT KAKENHI (Grant-in-Aid for Young Scientists (B)), grant no. 24790563.
Competing interests: none declared.

Introduction

Rheumatoid arthritis (RA) is chronic polyarthritis of unknown cause, characterised by proliferation of synoviocytes and infiltration of inflammatory cells into expanded synovial tissue in affected joints (1-3). Although recent therapies with biologics, like anti-TNF-α antibodies and soluble TNF-α receptors, have been found successful (4-8), more specific therapies based on the pathogenesis of RA should be established in the future. Thus, better understanding of the pathogenesis of RA is needed.

In the pathogenesis of RA, the involvement of altered post-translational modifications (PTMs) has been reported. Specifically, abnormal citrullination is detected on synovial proteins and autoantibodies to the citrullinated proteins are generated. In fact, the detection of anti-citrullinated peptides antibodies has been widely used for the diagnosis of RA (9-11).

Another example of the altered PTM would be IgG molecules. IgG in patients with RA is reported to exhibit decreased galactosylation of its conservative N-glycans (Asn-297) in the domains of its heavy chains (12). Similarly as the case of citrullination, autoantibodies to the agalactosyl IgG are frequently detected in RA, which are also used for the diagnosis of RA clinically (13).

Generally speaking, PTMs are often critical for the function of proteins as well as antigenicity of proteins. Thereby it is interesting whether such an aberrant PTM is limited to the citrullination and glycosylation in RA. We hypothesised that proteins in patients with RA possess various kinds of aberrant PTMs that might affect the pathophysiology of RA. Thereby, we here tried to elucidate alteration of PTM, focusing on acetylation.

The expression and activity of histone deacetylase (HDAC) 1 and HDAC2 in synovial tissue were reported to be lower in patients with RA than in patients with osteoarthritis and in healthy donors (14). Increased acetylation of histone proteins has been reported to be associated with the activation of NF-κB and activator protein 1, 2 major transcription factors involved in the pathogenesis of RA (14). Although the HDAC family catalyses the deacetylation of histone proteins (15), it also catalyses the deacetylation of non-histone proteins like p53 (16). This indicates the possibility that acetylation of other various proteins is also promoted in patients with RA. Thus, we here tried to detect proteins predominantly acetylated in peripheral blood mononuclear cells (PBMCs) from patients with RA, compared to PBMCs from healthy donors by proteomic analysis.

We first confirmed that the expression of HDAC1 and HDAC2 were decreased not only in synovial tissues but also in PBMCs of patients with RA compared to healthy donors. We then detected and identified several protein spots which were predominantly acetylated in the RA patients by 2-dimensional electrophoresis (2-DE), one of which was identified as α-Enolase (ENO1). Further, we found that the acetylation of ENO1 was regulated at least in part by HDAC1. Interestingly, the enzymatic activity of ENO1 was found up-regulated by acetylation. The enhanced acetylation of ENO1 may play roles in the pathophysiology of RA through the up-regulation of the ENO activity.

Materials and methods

PBMCs and serum samples

PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden) from blood samples of 7 patients with RA (7 female; mean age 56.7 years old [range 49–80]) and 7 healthy donors (7 female; mean age 56.5 years [range 42–74]). T cells and monocytes were isolated from PBMCs of 3 patients with RA (3 female; mean age 73.6 [range 68–74]) and 3 healthy donors (3 female; mean age 69.3 [range 63-80]). B cells were also isolated from PBMCs of 3 patients with RA (3 female; mean age 66.7 [range 41–89]) and the 3 healthy donors (3 female; mean age 69.3 [range 63–80]). Serum samples were obtained from 14 patients with RA (1 male; age 74 years, 12 female; mean age 53.4 years [range38–77]). The patients were diagnosed according to the criteria of the disease (17). All the samples were obtained with written informed consent

and this study was approved by the institutional ethics committee of Ethics Committee of St. Marianna University Graduate School of Medicine. Proteins were extracted into a lysis buffer (7M urea, 2M thiourea, 4% CHAPS) from each of the PBMC samples. 2-DE was performed as described previously (18, 19). 100 μg of the extracted protein samples were loaded onto 13 cm drystrips (Imobiline, pH range of 3-11, GE Healthcare, Buckinghamshire, UK) and the drystrips were kept at 20 degrees centigrade for 12 hours. Then isoelectric focusing was performed using Ettan IPGphor (GE Healthcare). The proteins separated by the isoelectric focusing were further separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins, stained with SYPRO Ruby Protein Gel Stain (GE Healthcare) to detect total proteins, were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Massachusetts, USA). The membrane was subjected to western blot with anti-acetylated lysine polyclonal antibodies (Abcam, Cambridge, UK) to detect acetylated proteins. Intensity of the protein spots were measured by an image analyser (Typhoon 9400, GE Healthcare). Intensity of acetylation of the protein spots was measured by LAS-3000 LuminoImager (FUJIFILM, Tokyo, Japan).

Isolation of T cells, B cells, and monocytes from PBMCs

T cells, B cells, and monocytes were isolated from PBMCs using EasySep™ NEGATIVE SELECTION Human T Cell Enrichment Kit, Human B Cell Enrichment Kit, and Human Monocyte Enrichment Kit (STEMCELL Technologies Inc., Vancouver, Canada), according to the manufacturer's instructions.

Mass spectrometric analysis

Interest spots excised from the SYPRO Ruby-stained 2-DE gels were digested by trypsin as described previously (20). The obtained peptides were analysed using a MALDI-TOF/TOF mass spectrometer (Ultraflex, Bruker Daltonics, Ettlingen, Germany). For the MALDI-TOF/TOF analysis, a 1 μl aliquot was spotted onto a MALDI target plate using a μ-C18 Zip-Tip (Millipore, Billerica, MA) for desalting and concentration. MS spectra (m/z 500-4,400) were acquired in the positive ion mode. The peptides were fragmented by the laser induced dissociation (LID) for MS/MS analysis. Peak lists were generated by Flex Analysis (v.2.2). Database searching was performed with Mascot (v1.0) against the human Swiss Prot protein database (v.56.7) using the following search parameters: trypsin was selected with 1 missed cleavage, mass tolerance of 100 ppm for MS and 1.0 Da for MS/MS. N-terminal acetylation, lysine acetylation, cysteine carbamidomethylation, and methionine oxidation were set as variable modifications. The acquired MS/MS data were used for peptide identification using a searching program of Mascot MS/MS Ion search (Matrix Science, London, UK) against the human Swiss Prot protein database. Peptide identifications were accepted when the Mascot search results delivered the significant MOWSE scores ($p<0.05$). Peptides with PTM were identified using the Mascot error tolerant search (21).

Cell culture

PBMCs were isolated from the blood samples as described above. The isolated PBMCs were cultured in RPMI1640 (invitrogen/GIBCO, Carlsbad, CA). These media containing 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich), were supplemented with 10% fetal bovine serum (Wako Pure Chemical Industries, Osaka, Japan). The cells were cultured at 37 degrees centigrade in 5% CO_2.

Expression vectors and transfection

The pcDNA3.1-HA-HDAC1 and pcDNA3.1-HA-HDAC2 expression vectors were constructed in our laboratory. These vectors as well as pc DNA3.1 as a negative control vector were used for transfection of PBMCs using Lipofectamine LTX (Invitrogen), according to the manufacturer's instructions.

Western blot

Anti-acetyl lysine and anti-ENO1 polyclonal antibodies (Abcam and LifeSpan Biosciences, Seattle, WA, USA, respectively) were used as the 1st antibodies and HRP-conjugated rabbit anti-mouse IgG antibodies (Invitrogen/Zymed) were used as the 2nd antibody. The bound antibodies were visualised using an enhanced chemiluminescence detection system (GE healthcare).

Analysis of acetylated sites

Peptides were extracted from the ENO1 spot after in-gel digestion by trypsin. Then the extracted peptide solution was diluted at 1:5 with ice-cold 0.1% trifluoroacetic acid (TFA) solution. Then the peptides were analysed using a nano-HPLC system (1200 Series; Agilent Technologies Inc., PaloAlto, CA, USA), connected to the HCT Ultra ion trap mass spectrometer (Bruker Daltonics). The ZORBAX 300SB-C18 trap column (length: 5mm, inside diameter: 300μm, particle diameter: 5μm, pore diameter: 300 ångström, Agilent Technologies) was used to concentrate and desalt the injected samples. Chromatographic separation of the peptides was carried out using the ZORBAX™ 300SB-C18 analytical column (length: 150mm, inside diameter: 100μm, particle diameter: 3.5μm, pore diameter: 300 ångström, Agilent Technologies). Sample elution through the analytical column was obtained at a flow rate of 300 nl/min by starting the following gradient: 0–60% solvent B for 75 min; a step with 80% solvent B for 5 min; and a step with 95% solvent A for 15 min. Solvent A was water containing 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid. The mass spectrometer was operated in the positive ion mode with a maximum accumulation time of 200ms in an m/z range of 350–3000. From a single MS spectrum, the three most intense peaks were selected for MS/MS analysis with an absolute threshold of 10000 and an active ion precursor exclusion after two MS/MS spectra for 0.5 min. The peptides were fragmented in the CID mode. Fragmentation parameters were as follows: width 4.0 m/z and time of fragmentation 200 ms.

The MS/MS data of the peptides, acquired by the ESI-ion trap mass spectrometer, were used for acetylated peptide identification using a search-

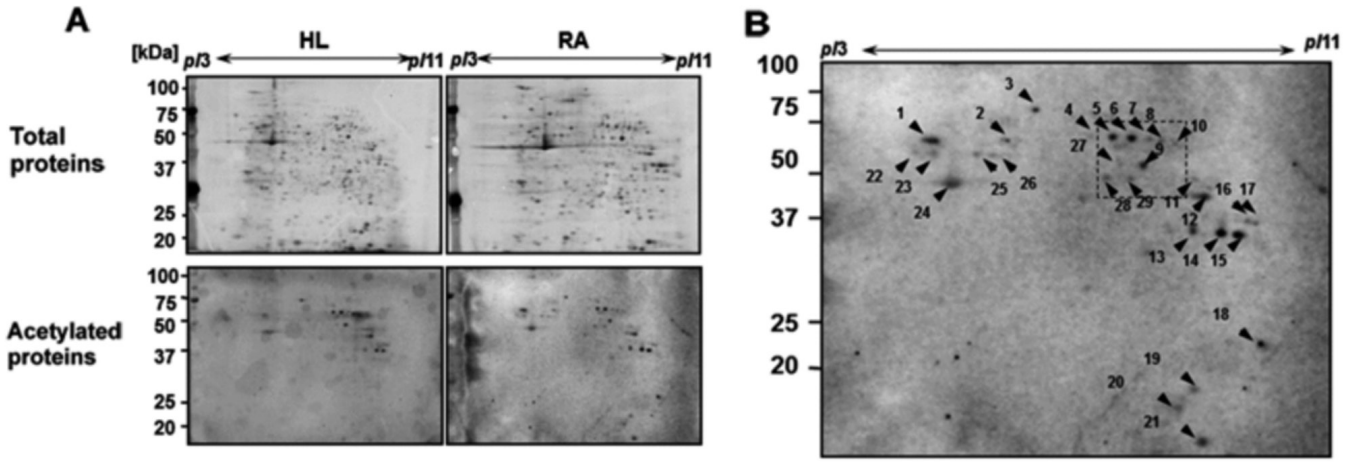

Fig. 1. Detection of total or acetylated proteins in PBMCs from patients with RA and healthy donors .
A. Proteins extracted from PBMCs were separated by 2-DE. The separated proteins, stained with SYPRO Ruby®, were transferred onto PVDF membranes (upper panels) and then were reacted to anti-acetylated lysine polyclonal antibodies (lower panels). Representative results from HL4 and RA6 are shown.
B. In total, 29 acetylated protein spots were detected in the RA group. The 29 acetylated protein spots were indicated by arrow heads in the enlarged image of the right lower panel of Fig. 1A (RA6). HL: healthy.

ing program of the Mascot MS/MS ion search against the Swiss Prot human protein sequence database. Peptide tolerance for precursor ions was set at 0.5Da. MS/MS tolerance for fragment ions was set at 1.0Da. Variable modifications such as acetylation of lysine and carbamidomethylation and oxidation of methionine were taken into consideration. Peptide identification was accepted when the Mascot search results delivered significant MOWSE scores ($p<0.05$).

In vitro acetylation

In vitro acetylation was performed as described previously (22). GST-ENO1 (Abnova) were incubated with acetyl coenzyme A (Sigma) and 0.2 μg of p300 in 50 μl of an acetylation buffer containing 50 mM Tris (pH 8.0), 5% glycerol, 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, and 1 mM PMSF. The acetylation reaction was performed at 30°C for 1 hour.

Measurement of the ENO1 enzymatic activity

The enzymatic activity of ENO1 was measured using the conversion of sodium 2-phospho-glycerate to phosphoenolpyruvate, as described previously (23, 24).

Statistical analysis

All the statistical significance was calculated by Student's *t*-test. All the tests were two-tailed.

Result

Identification of proteins highly acetylated in RA

First, we compared acetylated protein profiles of PBMCs between RA and healthy groups. Specifically, proteins extracted from PBMCs of 7 RA patients and 7 healthy donors were separated by 2-DE. The separated proteins, labelled with SYPRO Ruby®, were transferred onto PVDF membranes. Representative results are shown in the upper panels of Fig. 1A. Then acetylated proteins were detected by anti-acetylated lysine antibodies, as representative results are shown in the lower panels of Fig. 1A. In the both RA and healthy cases, only a small part of the separated proteins were found acetylated. In the RA and healthy groups, we detected 29 acetylated protein spots in total (Fig. 1B).

Next, acetylation levels of the 29 protein spots were compared between the RA and healthy groups. Specifically, we compared the frequency of positive acetylation for each of the 29 acetylated protein spots in the RA and healthy groups (Table I). Interestingly, spot no.28 and no.29 were acetylated only in the RA group and the difference of the frequency was statistically significant. Further, we compared acetylation levels corrected by the spot intensity on the SYPRO Ruby®-stained gel image in each spot between the RA and healthy groups. This comparison revealed that spots no. 9 and no. 27 were acetylated more strongly in the RA group than in the healthy group with statistical significance (Table I). On the other hand, there was no spot that showed higher acetylation in the healthy group than in the RA group. There was also no spot that was acetylated only in the healthy group (Table I). We thus selected these 4 spots of nos. 9, 27, 28, and 29 for identification. The location and the corrected acetylation intensity of the 4 spots are shown in Fig. 2A and 2B, respectively.

We successfully identified all the 4 protein spots by mass spectrometry and protein database searching. Both of the spots no. 9 and no. 27 were identified as ENO1 and both of the spots no. 28 and no. 29 were identified as isocitrate dehydrogenase 1 (IDH1) (Table II).

Expression of ENO1 in PBMCs from the patients with RA

From the above experiments, ENO1, one of the glycolytic enzymes, was found to be highly acetylated in RA. We then asked whether the total amount of ENO1 was increased or not in the RA patients. Western blot with anti-ENO1 antibodies revealed that the total amount of ENO1 was found similar to each other between the RA and healthy groups (Fig. 3A, left). This indicates the increased ratio of the acetylated ENO1 to the total ENO1 in PBMCs from the patient with RA.

Table I. Comparison of the frequency of detection and the relative acetylation levels of acetylated protein spots between the RA and healthy groups.

Spot no.	The frequency of detection (%)		The normalised intensity
	HL	RA	
1	100 (7/7)	100 (7/7)	1.05
2	71 (5/7)	71 (5/7)	1.14
3	86 (6/7)	100 (7/7)	1.06
4	86 (6/7)	100 (7/7)	1.00
5	86 (6/7)	100 (7/7)	1.41
6	86 (6/7)	100 (7/7)	1.40
7	71 (5/7)	71 (5/7)	1.22
8	57 (4/7)	43 (3/7)	0.83
9	100 (7/7)	100 (7/7)	3.19**
10	71 (5/7)	57 (4/7)	1.11
11	29 (2/7)	43 (3/7)	1.15
12	100 (7/7)	100 (7/7)	1.06
13	100 (7/7)	100 (7/7)	1.01
14	100 (7/7)	100 (7/7)	1.21
15	100 (7/7)	100 (7/7)	1.15
16	100 (7/7)	86 (6/7)	1.20
17	14 (1/7)	14 (1/7)	1.10
18	29 (2/7)	14 (1/7)	0.95
19	43 (3/7)	29 (2/7)	1.11
20	29 (2/7)	43 (3/7)	1.05
21	43 (3/7)	29 (2/7)	1.31
22	86 (6/7)	100 (7/7)	1.11
23	86 (6/7)	100 (7/7)	1.23
24	100 (7/7)	100 (7/7)	1.08
25	71 (5/7)	86 (6/7)	1.22
26	71 (5/7)	86 (6/7)	1.21
27	14 (1/7)	71 (5/7)	4.51*
28	0 (0/7)	86 (6/7)	-
29	0 (0/7)	86 (6/7)	-

HL: healthy; RA: rheumatoid arthritis; * $p<0.05$; ** $p<0.01$.

The regulation of deacetylation of ENO1 by HDAC1 in human PBMCs

Recently, Huber *et al*. reported that the expression and activity of both HDAC1 and HDAC2 in synovial tissue was lower in patients with RA than in patients with osteoarthritis and healthy donors (14). The HDAC family was reported to catalyse the deacetylation of histone proteins (15). However, HDACs were reported to deacetylate several non-histone proteins like p53 (16). This indicates the possibility that HDACs are also involved in the increased acetylation of ENO1 in PBMCs from the patient with RA. Thereby, we first investigated the expression of HDAC1 and HDAC2 in PBMCs from the patients with RA and healthy donors. As a result, the protein levels of HDAC1 and HDAC2 were lower in PBMCs from the patients with RA than in those from the healthy donors, like the previous reports on synovial tissue (Fig. 3A, middle and right). Next, to examine the involvement of HDACs in the acetylation of ENO1, we treated the cells from the healthy donors with trichostatin A (TSA) of an HDAC family inhibitor (25) and then measured the degree of

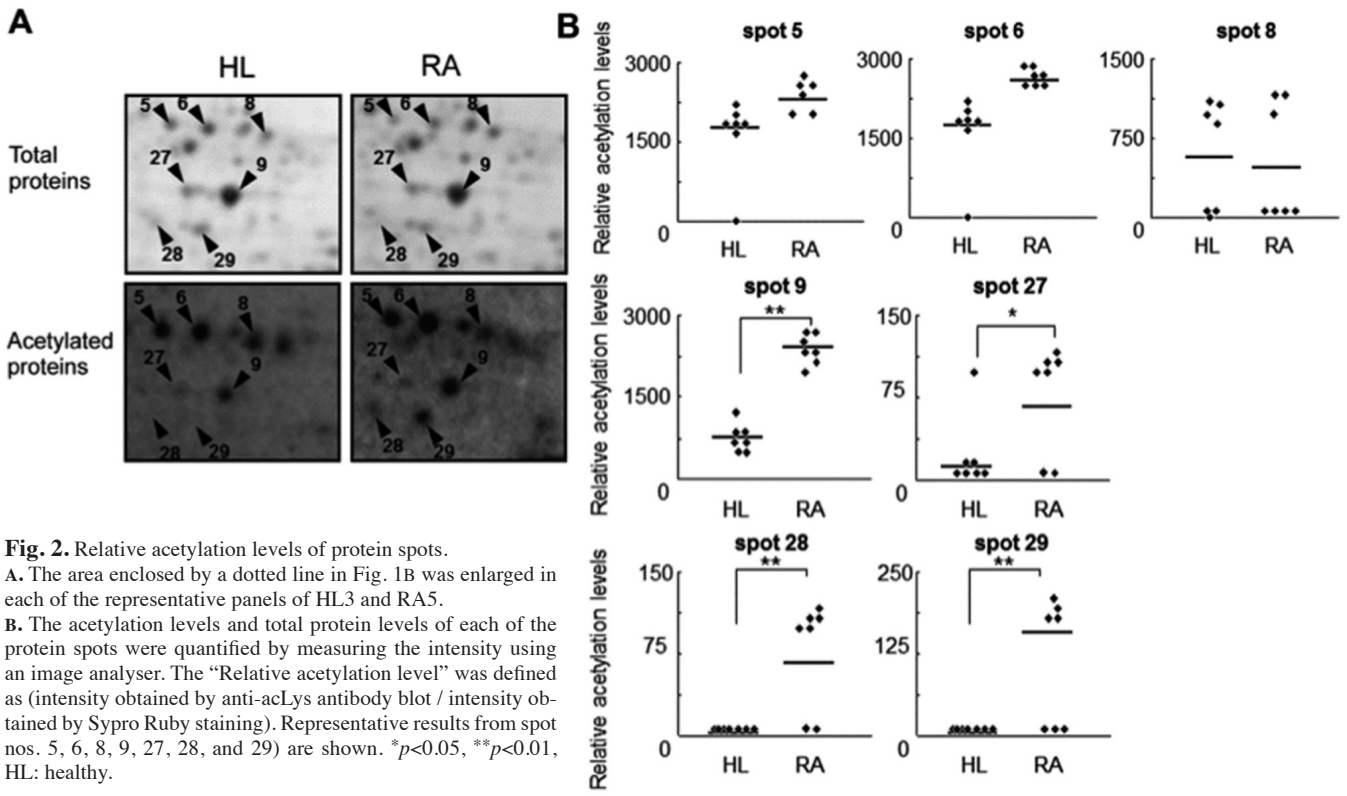

Fig. 2. Relative acetylation levels of protein spots.
A. The area enclosed by a dotted line in Fig. 1B was enlarged in each of the representative panels of HL3 and RA5.
B. The acetylation levels and total protein levels of each of the protein spots were quantified by measuring the intensity using an image analyser. The "Relative acetylation level" was defined as (intensity obtained by anti-acLys antibody blot / intensity obtained by Sypro Ruby staining). Representative results from spot nos. 5, 6, 8, 9, 27, 28, and 29) are shown. *$p<0.05$, **$p<0.01$, HL: healthy.

Table II. Identified highly acetylated proteins in RA.

Spot no.	Observed M.W./pI	Protein name	Accession ID (Accession NO.)	Calculated M.W. (kDa)/pI	Matched / Searched	Seq. Cov. (%)	Score	Sequence confirmed by LID (Mascot ion score)
9	47/8.3	α-enolase	ENOA_HUMAN (gil16507965)	47.1/7.0	26/61	62	392	^{33}AAVPSGASTGIYEALELR^{358} (99) ^{240}VVIGMDVAASEFFR^{253} (56) ^{270}YISPDQLADLYK^{281} (54)
27	47/8.0	α-enolase	ENOA_HUMAN (gil16507965)	47.1/7.0	20/68	49	135	^{16}GNPTVEVDLFTSK^{28} (32) ^{33}AAVPSGASTGIYEALELR^{50} (75) ^{240}VVIGMDVAASEFFR^{253} (64) ^{270}YISPDQLADLYK^{281} (68)
28	50/7.9	isocitrate degydrogenase	IDHC_HUMAN (gil28178824)	46.6/6.5	27/60	68	380	^{223}FKDIFQEIYDK^{233} (35)
29	50/8.1	isocitrate degydrogenase	IDHC_HUMAN (gil28178824)	46.6/6.5	26/69	68	147	^{101}NILGGTVFR^{109} (43) ^{389}SDYLNTFEFMDK^{400} (13)

Acetylation levels of protein spots were compared between the RA and healthy groups. By matching the western blot image and the Sypro Ruby image, we identified the location of the highly acetylated protein spots on the 2D gel. Then the proteins, digested with trypsin in the gel, were identified by MALDI-TOF/TOF mass spectrometry. M.W.; molecular weight, Seq. Cov.; sequence coverage, LID; laser-induced dissociation, M; Oxidised methionine, C; carbaimidomethylated cysteine.

acetylation of ENO1 by western blot. As a result, the acetylation of ENO1 was found significantly increased by the treatment with TSA (Fig. 3B). This indicates that the acetylation of ENO1 is regulated by HDACs. Again, the expression of HDAC1 and HDAC2 in PBMCs was lower in the patients with RA than in the healthy donors. Thus, to further clarify which of HDAC1 and HDAC2 was involved in the deacetylation of ENO1, HDAC1 with a tag of HA (HA-HDAC1) and HDAC2 with a tag of HA (HA-HDAC2) were respectively over-expressed in PBMCs from the patients with RA and then the acetylation degree of ENO1 was measured by western blot. As a result, the acetylation of ENO1 was significantly down-regulated by the over-expression of HDAC1, but not by that of HDAC2 (Fig. 3C). In an additional experiment using PBMCs from a healthy donor, TSA treatment up-regulated the ENO1 acetylation, but did not up-regulate it when HDAC1 was knocked down (data not shown). Collectively, among the HDAC family, HDAC1 is thought to be mainly involved in the regulation of ENO1 acetylation.

Identification of the acetylation sites of ENO1

Since no study has been reported on the acetylation sites of ENO1, it was structurally interesting to identify which lysine residues in ENO1 were acetylated. We thus tried to identify the acetylation sites of the spot no.9 of ENO1, by nanoLC-CID-MS/MS, although ENO1 contained as many as 38 lysine residues. As a result, we found that 3 lysine residues (K120, K126, and K256) of ENO1 were acetylated in the healthy samples (Fig. 3D). In the RA samples, peptides containing the 3 lysine residues were detected but the 3 lysine residues were not acetylated (data not shown). Including these 3 lysine residues, mass spectrometric analysis failed to identify acetylated lysine residues in the RA samples. Again ENO1 possesses 38 lysine residues in total. However, no information has been obtained on more than half of the 38 lysine residues of ENO1 in spite of the extensive analysis (data not shown).

Acetylation levels of ENO1 in T cells, B cells, and monocytes

We then investigated which subsets of PBMCs, T cells, B cells, and monocytes, showed the high ENO1 acetylation. Using pooled lysates of T cells, B cells, and monocytes isolated from PBMCs of 3 patients with RA and 3 healthy donors, we investigated acetylation levels similarly as above. As a result, in the T cells, the acetylated level of ENO1 was higher in the RA sample than in the healthy sample (1.76-fold, Fig. 3E). In contrast, in the B cells and monocytes, the acetylated levels of ENO1 were found similar to each other between the RA and healthy samples (0.91- and 0.84-fold, respectively, Fig. 3E). This data indicates that acetylation of ENO1 was specifically up-regulated in T cells.

The effect of acetylation on the enzymatic activity of ENO1

Next, we examined the effect of acetylation on the function of ENO1. ENO1 catalyses the generation of phosphoenolpyruvic acid from 2-phosphoglyceric acid. Thus, we measured this activity of non-acetylated ENO1 and acetylated ENO1. The acetylated ENO1 was prepared by *in vitro* acetylation of bacterially expressed ENO1, after which the acetylation of ENO1 was confirmed by western blot using anti-acetylated lysine antibodies (Fig. 4A). As a result, the enzymatic activity of the acetylated ENO1 was found significantly higher than the non-acetylated ENO1 (Fig. 4B). This indicates that acetylation up-regulates the enzymatic activity of ENO1.

The effect of acetylation on the antigenicity of ENO1

Finally, we examined whether acetylation affect the antigenecity of ENO1 in patients with RA, since another PTM of citrullination was evidenced to contribute to the acquirement of antiginecity in RA. Specifically, we first checked whether RA serum samples reacted to the acetylated ENO1 or not. We found that 5 out of the tested 14 RA

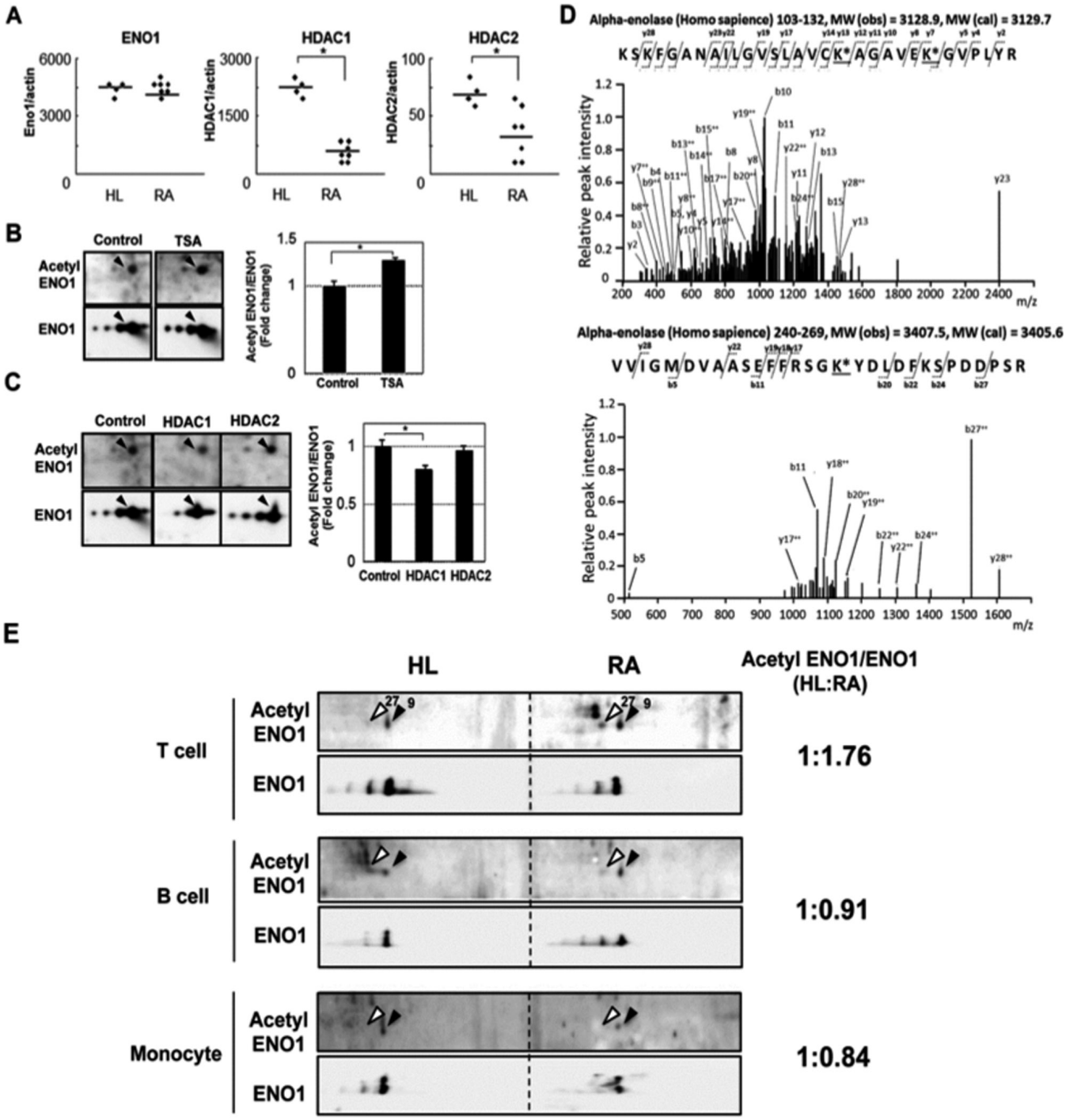

Fig. 3. Effect of HDACs on the acetylation of ENO1.

A. Proteins extracted from PBMCs of 7 patients with RA and 4 healthy donors were separated by 1-DE. The separated proteins were transferred onto PVDF membranes and each membrane was subjected to western blot with anti-ENO1 polyclonal antibodies, anti-HDAC1 polyclonal antibodies, anti-HDAC2 polyclonal antibodies, or anti-α-actin monoclonal antibodies. The relative intensity of ENO1, HDAC1 and HDAC2, corrected by that of α-actin, are shown (ENO1/actin, HDAC1/actin, and HDAC2/actin).

B and **C.** PBMCs of 3 healthy donors were incubated with 330nM TSA for 24 hours (B) and PBMCs of 3 patients with RA, transfected with expression plasmids for HA-HDAC1 and HA-HDAC2 or HA alone, were incubated for 24 hours (C). Each of the whole cell extracts was subjected to 2-DE and western blot with the anti-acetylated lysine polyclonal antibodies and with anti-ENO1 polyclonal antibodies. Representative images of the western blot are shown in the left. Acetylation levels and total protein levels of ENO1 were quantified as described in Fig. 2B. Then the acetylation levels of ENO1 were corrected by the total protein levels of ENO1 (Acetyl ENO1/ENO1 (Fold change)). The relative intensity averages of "control" were defined as 1. *p<0.05, **p<0.01

D. ENO1 peptides from spot no. 9 were subjected to nanoLC-CID-MS/MS analysis. The MS/MS spectrum of acetylated peptides are shown. K*; acetylated lysine, M; oxidised methionine, HL; healthy (**E**) T cells, B cells, and monocytes were isolated from PBMCs of 3 patients with RA and 3 healthy donors. The pooled lysates of T cell, B cell, and monocyte were subjected to 2-DE and western blot with the anti-acetylated lysine polyclonal antibodies and with anti-ENO1 polyclonal antibodies. Acetylation levels and total protein levels of ENO1 were quantified as described in Fig. 2B. The acetylation levels of ENO1, corrected by the total protein levels of ENO1, are shown (Acetyl ENO1/ENO1).

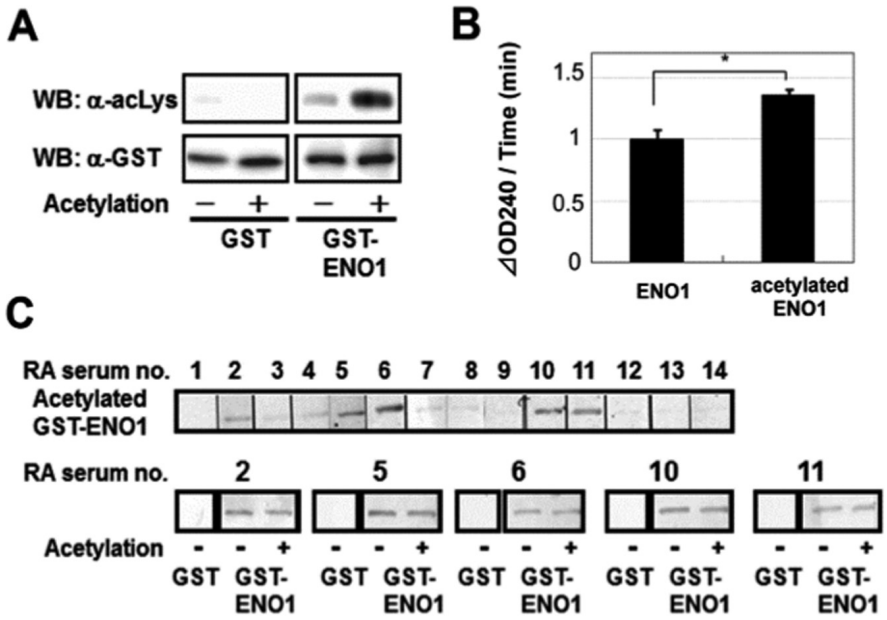

Fig. 4. The effect of the acetylation on the enzymatic activity of ENO1.
A. GST and GST-ENO1 were acetylated *in vitro* by p300 of histone acetyltransferase. Acetylated GST-ENO1 and GST and non-acetylated ones were subjected to western blot with the anti-acetyl lysine polyclonal antibodies (WB: α-acLys) and anti-GST polyclonal antibodies (WB: α-GST).
B. The enzymatic activity of non-acetylated ENO1 and acetylated ENO1 were evaluated by the absorbance of phosphoenolpyruvic acid (wave length=230nm). *$p<0.05$.
C. Recombinant acetylated GST-ENO1 was subjected to western blot with 14 RA serum samples (nos.1~14) (upper panel). Non-acetylated GST, non-acetylated GST-ENO1, and acetylated GST-ENO1 were subjected to western blot with 5 RA serum samples (nos. 2, 5, 6, 10, 11) that positively reacted to the acetylated GST-ENO1 (lower panel). WB; Western blot.

serum samples reacted to the acetylated ENO1 (Fig. 4C upper panel). We then compared the reactivity of the 5 serum sample to the non-acetylated ENO1 and the acetylated ENO1. As a result, the reactivity was not different between the acetylated ENO1 and the non-acetylated ENO1. Thus, we concluded that the acetylation did not contribute to the antigenicity of ENO1 in RA, although autoantibodies to ENO1 were generated in a part of patients with RA.

Discussion

We here tried to elucidate altered PTMs focusing on proteins acetylation in PBMCs of patients with RA by proteomic analysis. We selected PBMCs as a subject, since PBMCs would reflect the immune condition of RA, one of the autoimmune diseases (26). Further, PBMCs are suitable materials when the acetylation detected here is used as a medical examination in the near future.

Our findings are as follows; 1) multiple proteins in PBMCs were highly acetylated in the RA groups compared-to the healthy groups. 2) ENO1 and IDH1 were identified as protein spots highly acetylated in RA. 3) Acetylation of ENO1 was regulated in part by HDAC1. 4) The expression of HDAC1 and HDAC2 was decreased in PBMCs in RA. 5) Three lysine residues (K120, K126, and K256) of ENO1 were acetylated in the healthy samples. 6) Acetylation up-regulated the enzymatic activity of ENO1. 7) The acetylation level of ENO1 was high specifically in T cells in RA. In addition, 8) Acetylation did not affect the antigenicity of ENO1.

On the first and second points, altered acetylation was found in PBMCs of the RA patients. Previously, over-citrullination of proteins and galactose deficiency of IgG were reported as characteristics of RA (8-12). Combining these facts with our data, RA would possess a wide range of PTM dysregulation. The amount of ENO1 in PBMCs of the RA group was similar to that of the healthy group (Fig, 3A). The amount of ENO1 was not changed by the incubation with TSA of a HDAC inhibitor (Fig. 3B and C). These data indicate that acetylation would not affect the stability of ENO1, even though acetylation is known to often alter the stability of proteins (27).

On the third point, the overexpression of HDAC1 decreased the acetylation of ENO1, however, ENO1 was still acetylated at a lower level (Fig. 3C). This indicates that other deacetylases participate in the deacetylation of ENO1. Alternatively, it is possible that activities of acetyltransferases remained in the cells, by which a certain level of acetylation was observed. Such deacetylases and acetyltransferases should be investigated in the future.

On the fourth point, recently, the expression and activity of the HDAC family was reported to be involved in the pathogenesis of RA recently (14, 28-29). However, the results were controversial. It has been reported that TSA, which inhibits the HDAC family, suppressed IL-6 production in fibroblast-like synoviocytes and macrophages by accelerating decay of IL-6 mRNA in RA (28). Furthermore it has been reported that an HDAC3-selective inhibitor reduced IL-6 production in PBMCs of RA patients (29). Thus, acetylation increased by the inhibition of HDACs, in particular, HDAC3, appeared to have anti-inflammatory roles. On the other hand, the expression and activity of HDAC1 and HDAC2 in synovial tissue were reported to be lower in RA than in osteoarthritis and in the healthy condition (14). We here showed that the expression of HDAC1 and HDAC2 in PBMCs was also lower in RA than in the healthy condition. Thus, the ENO1 acetylation increased by the decreased HDAC1 expression may participate in the inflammation of RA. Alternatively, the expression of HDAC1 and HDAC2 may result in compensatory decrease to suppress the inflammation of RA. This point should be investigated in the future.

On the fifth point, to identify acetylated lysine residues, we performed mass spectrometric analysis extensively using MALDI-TOF/TOF and nanoLC-CID-MS/MS, by which we identified in total 3 acetylated lysine residues from the healthy samples. In the RA samples, peptides containing these 3 lysine residues were detected, but the 3 lysine residues were not acetylated. ENO1 possesses 38 lysine residues

in total. However, no information has been obtained on more than half of the 38 lysine residues in spite of the extensive analysis. This would be technical limitation of mass spectrometric analysis at present. Some of the remaining undetected lysine residues would possess acetylation in the RA samples. In the future, it would be needed to establish more effective methods to identify acetylated lysine residues.

On the sixth and seventh point, ENO1, one of the glycolytic enzymes, converts 2-phosphoglyceric acid to phosphoenolpyruvic acid. Our data indicates that acetylation up-regulates the enzymatic activity of ENO1 and that ENO1 is highly acetylated in the patients with RA. Thus, the enzymatic activity of ENO1 would be higher in RA than in the healthy condition in PBMCs, in particular, in T cells. Activated T cells are known to induce production of various inflammatory cytokines in RA (30) and abatacept of a T cell co-stimulation blocker has been shown to be very effective in the treatment of RA (30), indicating critical roles of T cells in the pathology of RA. The supposed high activity of ENO1 would support the activation of T cells in RA, providing much energy through glycolysis. It was reported that acetylation played roles in the intracellular transfer of proteins (31). Thereby, we investigated the effect of acetylation on ENO1 from this viewpoint. ENO1 was localised in not only cytosol but also nuclei. We thus compared the ratios of acetylated ENO1 to the total ENO1 between nuclei and cytosol, however, the ratios were similar to each other in PBMCs (data not shown). Therefore, we concluded that acetylation did not affect the localisation of ENO1.

A recent study reported other new functions of ENO1. In inflamed lung, ENO1 binds with plasminogen at the cell surface and promotes the production of local plasmin, one of the inflammatory agents, and invasion of inflammatory cells are mediated by increased cell-surface expression of ENO1 (32). Further, ENO1 that was expressed on the surfaces of monocytes and macrophages induces synovial inflammation in RA (33). The relation between the high acetylation of ENO1 and the inflammatory process in RA should be investigated in the future.

On the eighth point, it is well known that citrullinated proteins are highly antigenic in RA. In the case of acetylation, the anti-ENO1 positive RA serum samples reacted to acetylated ENO1 and non-acetylated one at similar levels. Thus, acetylation would not contribute to the antigenicity of ENO1. Recently the affinity interaction between DEK and anti-DEK autoantibodies has been reported to be enhanced by acetylation of DEK in juvenile idiopathic arthritis (34). Thus, alteration of antigenicity by acetylation appears to depend on property of each protein.

Besides ENO1 (spot nos. 9 and 27), we identified IDH1 from the other 2 protein spots (spot nos. 28 and 29) that were highly acetylated in RA. IDH1 converts isocitrate to α-ketoglutarate in the citric acid cycle (35). IDH possesses 3 isotypes of IDH1, 2, and 3. IDH2, which shows about 70% homology with IDH1, was reported to be deacetylated by class III HDAC. (36, 37). This indicates that IDH1 might be acetylated similarly as IDH2. In our data, IDH1 (spot nos. 28 and 29) was highly acetylated in the RA samples, but was little acetylated in the healthy condition. Therefore, the acetylated IDH1 may be a new candidate biomarker for RA.

In conclusion, highly acetylated ENO1 may play roles in the pathophysiology of RA through the maintenance of activated lymphocytes by increasing energy supply by glycolysis.

Acknowledgements

The authors thank Ms M. Kanke, Ms K.M. Yokoyama, and Ms. J. Asano for their excellent technical assistance.

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