

Adenosine Deaminase in Synovial Fluid at Rheumatoid Arthritis is Citrullinated

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ABSTRACT

AIM: The objective of this article is to compare accumulation and citrullination of small (SADA) and large (LADA) isoforms of adenosine deaminase (ADA) in the synovial fluids (SFs) of patients with rheumatoid arthritis (RA), ankylosing spondylitis, gout, juvenile idiopathic and reactive arthritis.

MATERIALS AND METHODS: The SFs of patients, diagnosed in the Medical Center "Muratsan", were taken from knee joints. The methods of ion-exchange and gel-chromatography were applied for separation and purification of ADA isoforms from the SFs of above

mentioned five arthritis types. The activities and citrullination state of ADA isoforms were studied using appropriate colorimetric assays.

RESULTS: This work revealed accumulation of SADA in RA SFs and of LADA in the SFs of four other arthritis types. The average protein citrullination, measured as A_{530}/A_{280} ratio, was of 0.07 ± 0.04 ($n = 5$) in SADA purified from RA SFs with the initial ADA activity between 35-60 U/L. In SADA samples from RA SFs with the initial ADA activity of 70-190 U/L, the average value of protein citrullination was higher by an order (0.83 ± 0.28 , $n = 8$). The citrullination of LADA was not registered in any of all five studied arthritis types.

CONCLUSION: In SFs of RA, the citrullinated SADA is accumulated. In four other studied arthritis types, LADA is accumulated. One can suggest: in RA the citrullination of SADA prevents its binding to dipeptidyl peptidase IV and transformation to LADA. The obtained data permit the assumption of being SADA as a new citrulline containing auto-antigen applicable in RA diagnosis.

Key words: Adenosine deaminase; Arthritis; Protein citrullination; Synovial fluids

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INTRODUCTION

The protein citrullination is a post-translational irreversible modification of arginine residues to citrulline by peptidyl arginine deiminase (PAD) family enzymes. This process causes loss of protein positive charge, changes its conformation, influences on interaction with other proteins and eventually disrupts functions. In extracellular medium these changes were registered in different pathologies. Increasing of PAD expression and protein citrullination are observed in Alzheimer's and Parkinson's diseases, multiple sclerosis, glaucoma, psoriasis, cancer, rheumatoid arthritis, etc.^[1].

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by chronic inflammation, progressive articular damage,

swelling of joints and appearance of autoantibodies^[2]. In synovial tissue of RA patients, the expression of PAD2 and PAD4 isoenzymes and the level of citrullinated proteins are up-regulated^[3,4]. Anti-citrullinated protein antibodies (ACPA) are considered as specific serological markers for RA. This boosted the clinical interest in ACPA as a new diagnostic tool^[5].

Adenosine deaminase (ADA, EC 3.5.4.4) is a purine metabolizing enzyme^[6]. Its small isoform (SADA) is a catalytically active protein with a molecular mass of 36-40 kDa. Large isoform of ADA (LADA, Mm of 280-300 kDa) represents a non-covalent complex of SADA and an ADA-binding cell membrane enzyme dipeptidyl peptidase IV (DPPiV)^[7].

Adenosine, the substrate of ADA, is known as anti-inflammatory agent. It suppresses the inflammation by decreasing pro-inflammatory and increasing anti-inflammatory cytokines, regulates the inflammatory function of endothelial cells^[8]. The enhanced of ADA activity in extracellular medium was registered in different diseases (reviewed in^[6]). Down-regulation of adenosine concentration in the physiological fluids due to enhanced ADA activity, released from the injured tissues, contributes to the development of inflammation.

There are several studies on the ADA activity application for differential diagnosis of joint diseases. Researchers from different countries have proposed to apply the level of ADA activity in synovial fluid (SF) in the differential diagnosis of RA and osteoarthritis (OA)^[9-11]. For the population of Iran, India and Armenia the using of the threshold values of ADA activity equal to 42, 20 and 12 U/L, respectively, were recommended^[10-12].

Our studies in RA SFs have demonstrated increasing the ratio activity of SADA to LADA (SADA/LADA) in accordance with rising of initial ADA activity^[13]. In SF from RA, this ratio was significantly higher than in the SFs from four other arthritis types with the similar ADA activities^[14].

In this connection it is intriguing that one of 17 Arg residues in SADA molecule^[15], Arg-142, participates in DPPiV binding^[16]. Hence, it can be suggested that the citrullination of this residue may hinder this binding preventing the SADA transformation to LADA and resulting in accumulation of SADA in RA SF. Indeed, the preliminary study has showed that SADA, purified from SFs of RA patients, was citrullinated^[17].

The aim of the present study is to compare the citrullination degree of two isoforms of ADA purified from SFs of five arthritis types.

MATERIALS AND METHODS

1. Materials

Diacetylmonoxime (DAMO), thiosemicarbazide (TSC) and adenosine were purchased from Sigma (USA). Sephadexes were from Pharmacia Biotech (Sweden) and DEAE-cellulose - from Whatman (England). All the used reagents were of the highest available purity.

SF samples were taken in the Medical Center "Muratsan" from knee joints of 82 patients with RA ($n = 40$), reactive arthritis (ReA, 22), ankylosing spondylitis (AS, 9), juvenile idiopathic arthritis (JIA, 3) and gout (G, 8). The SFs stored at -20° before the use.

2. Determination of ADA activity

The ADA activity was assayed by the phenol-hypochlorite colorimetric method, evaluating the amount of ammonia, liberated in the catalyzed deamination of adenosine, from the absorbance of the assay mixture at 630 nm^[12,18].

3. Purification of ADA isoforms

The SADA and LADA isoforms from SFs of various types of arthritis were separated by gel-filtration on the Sephadex G-200 containing columns. The isolated fractions were purified to an electrophoretically homogenous state, using gel-filtration and ion-exchange chromatography procedures as described earlier^[18].

4. Gel filtration procedures

The gel filtration of native SF samples and the solutions of SF proteins precipitated in trichloroacetic acid (TCA) and re-dissolved in buffer were performed on Sephadex G-200sf columns equilibrated in 0.01 M K-Na-phosphate buffer, pH 7.4, containing 0.1 M KCl. In the cases of the native SF samples (for separation of SADA and LADA isoforms), 1-1.2 ml of SF solution was injected to the 100-120 ml column. In the cases of precipitated in TCA proteins, 0.15 - 0.2 ml aliquot of re-dissolved protein solution was injected to the 15-20 ml column. The columns were calibrated with standard proteins (mol. mass, kDa): trypsin (24), pepsin (35.5), ovalbumin (45), BSA monomer (67) and dimer (134), catalase (230). The protein quantity in eluates was expressed by the absorbance at 280 nm.

5. Citrulline determination

The presence of citrulline in proteins was shown using the colorimetric assay based on the specific reaction of DAMO with ureido group under highly acidic conditions^[19]. The protein sample was subjected to acidic hydrolysis by incubating in the presence of 3 M HCl in a water bath for 1.5 hours. To 0.5 ml of hydrolyzed protein, 1.5 ml of freshly prepared chromogenic solution was added. The chromogenic solution in 30 ml contained 1 mg TSC, 10 ml 0.5% (w/v) DAMO in water and 20 ml 0.025% (w/v) FeCl₃ solution in 15% H₂SO₄ (v/v). The mixture of hydrolyzed protein and chromogenic solution in a capped test tube was incubated for 15 min in a water bath. After cooling, the quantity of citrulline was calculated based on the intensity of the absorbance at 530 nm. The extent of citrullination was expressed as the A_{530}/A_{280} ratio.

The distribution of citrullinated proteins in the SF samples by molecular mass was studied after precipitation of proteins in 5% (w/v) TCA. The precipitate was washed twice with water, re-dissolved in 0.1 M Tris-HCl buffer, pH 8.2 and subjected to gel-filtration on Sephadex G-200sf column (see previous section 4).

6. Equipment

The spectral measurements were carried out at 25°C on the spectrophotometer Cary 60 (USA).

7. Statistical analyses

All data were analyzed using InStat software, version 3, for Windows (GraphPadSoftware, Inc., SanDiego, CA, USA). The unpaired two-tailed t-test with Welch correction was applied. Results are expressed as mean \pm SEM (n , number of independent experiments).

RESULTS

At rheumatoid arthritis SADA accumulates in synovial fluid

As it was described earlier^[12], the activity of ADA in SFs from RA patients is statistically higher compared with the SFs from OA patients ($p < 0.0001$). However, the high ADA activity was registered also in some SFs of four other studied arthritis types: reactive arthritis (ReA), ankylosing spondylitis (AS), juvenile idiopathic arthritis (JIA) and gout (G). Consequently, the high activity of ADA is not

characteristic to RA exclusively.

The present work compares the ratios of molecular forms of ADA in the SFs from above mentioned five arthritis types. The SADA and LADA isoforms were separated by gel-filtration of SFs through Sephadex G-200sf containing columns. Figure 1 shows two examples of elution diagrams registered at gel-filtration of SFs from ReA and AS with ADA activities of 63.5 and 56.9 U/L, respectively. These diagrams evidence very low, even negligible presence of SADA isoform in these cases.

The same was observed with G and JIA SFs with the initial ADA activities of 53.7 and 50 U/L, respectively. When the measurable SADA activity was registered, the statistics have shown the average ratio of SADA to LADA in the SFs from 30 cases of RA was 0.76 ± 0.1 . In the 32 samples of SF from four other arthritis types, the average SADA/LADA ratio (0.24 ± 0.02) was significantly (≈ 3.1 times, $p < 0.0001$) lower than in RA SFs. The increasing of this ratio in RA SFs indicates SADA accumulation in contrary to SFs of other studied arthritis types.

To confirm the accumulation of SADA in SFs of RA patients, unlike SFs of different types of arthritis, for instance, ReA, the following comparative calculations were carried out. Per 8 SF samples were selected from 40 patients with RA and 22 patients with ReA: 4 with the greatest and 4 with the lowest initial ADA activity. SADA and LADA were separated by the Sephadex columns and the means of the isoforms and their ratios were compared. The results are shown in Table 1.

The comparison of the data in lines 1 and 2 evidences that the threefold increasing of the initial ADA activity in RA SFs ($p = 0.011$), LADA activities are differing slightly ($p = 0.757$), but in the SFs with high ADA activity (line 2), SADA is 2.6-fold higher than in the SFs with low ADA activity (line 1, $p = 0.023$). As a result, the obtained SADA/LADA ratio increases by 3 times.

In the case of ReA (lines 3 and 4), the differing of the initial ADA activity by 2.7 times ($p < 0.0001$) is accompanied with the rising of LADA activity by 1.9 times ($p = 0.016$). The activity of SADA actually is not changed. So, the SADA/LADA ratio halved.

These calculations confirm the previous conclusion that increasing of initial ADA activity in the SFs of RA patients is accompanied with SADA accumulation. It is worth of notice that in the SFs of ReA patients LADA accumulated.

The protein citrullination in the SFs of various arthritis types

The analysis was implemented to determine the molecular distribution of citrullinated proteins in the SFs of various arthritis types. The SF samples were subjected to protein precipitation in TCA and re-dissolved in the 0.1 M Tris-HCl buffer, pH 8.2 (see section 5 in MATERIALS AND METHODS). The aliquots from these solutions were passed through the Sephadex containing columns and citrulline was measured in the fractions.

In Figure 2, two examples of the obtained diagrams are presented for SFs from RA (a) and AS (b). The diagram in Figure 2a evidences that, besides the low-molecular fractions, the significant protein citrullination was found in the fractions with molecular masses in the region of 35-40 kDa. It should be noted that this region matches to the molecular mass of SADA. Figure 2b shows negligible (on the level of the analysis error) protein citrullination in the fractions of SF from AS patient. The results for the SFs from ReA were similar to those for AS SF.

These data indicate that in RA SFs, the protein fractions with molecular masses close to that of SADA are citrullinated. This is not observed in the other arthritis types.

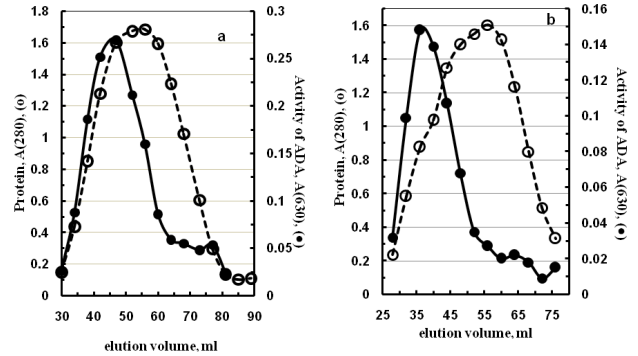


Figure 1 Gel-filtration on Sephadex G-200sf of synovial fluids. a - from patients with reactive arthritis; b - from patients with ankylosing spondylitis. Protein concentration (o) and activity of ADA (●) are expressed by absorbance at 280 and 630 nm, respectively

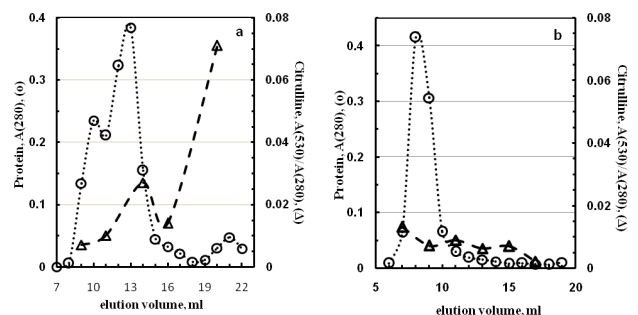


Figure 2 Gel-filtration on Sephadex G-200sf of synovial fluid proteins re-dissolved after precipitation in TCA. a - from patients with rheumatoid arthritis; b - from patients with ankylosing spondylitis. The protein concentration (o) and citrullination (Δ) are expressed by absorbance at 280 nm and by absorbance ratio A(530)/A(280), respectively.

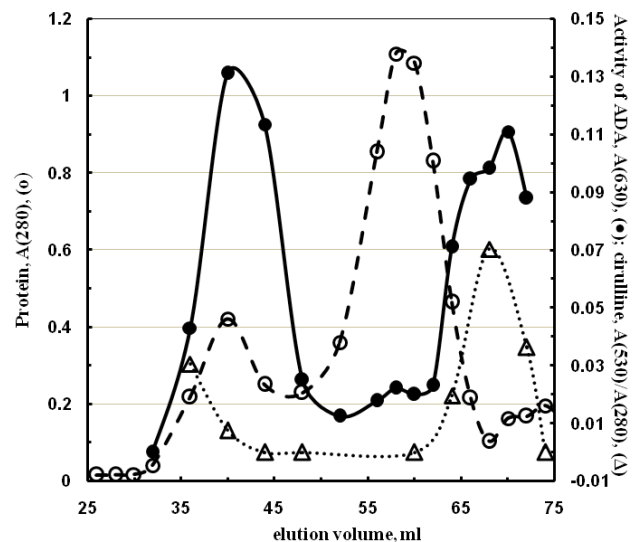


Figure 3 Gel-filtration on Sephadex G-200sf of native synovial fluid from rheumatoid arthritis. The protein concentration (o), ADA activity (●), and citrullination (Δ) are expressed by absorbance at 280 nm, 630 nm and the absorbance ratio A(530)/A(280), respectively.

SADA in synovial fluids of RA patients is citrullinated

An aliquot of the native SF from RA was subjected to gel-filtration through Sephadex G-200sf. In the obtained fractions, the ADA activity and the protein citrullination were evaluated. Figure 3 shows the diagram of this gel-filtration. This picture evidences protein citrullination in fractions preceding the LADA (Mm > 300 kDa), and the noticeable citrullination in the protein fractions in the region of

SADA activity (Mm ~ 35-40 kDa). This result is in accordance with the above described observation concerning SADA citrullination in RA SF.

The procedures of separation and purification of SADA and LADA isoforms by ion-exchange chromatography on DEAE-cellulose and gel-filtration on Sephadex G-200 columns were applied to several RA SFs with the initial ADA activity in the interval of 35-190 U/L. The SADA content in the samples with the activity lower of this interval was negligible. In the samples with the initial ADA activity between 35-60 U/L, the portion of SADA was about 35 % of the total ADA. The samples of SADA isoform, purified from these SFs shown the level of protein citrullination, expressed as A_{530}/A_{280} ratio, near to 0.07 ± 0.04 ($n = 5$). In the SFs with high initial ADA activity (70-190 U/L), the portion of SADA was about 52 % of the total ADA activity. The samples of SADA isoform, purified from these SFs, shown more pronounced citrullination degree: the A_{530}/A_{280} ratio was around of 0.83 ± 0.28 ($n = 8$). Hence, these analyses revealed noticeable protein citrullination in the samples of SADA isoform purified from SFs of RA patients.

The study of citrullination states of SADA and LADA in the SFs of four studied arthritis

The SADA and LADA isoforms from SFs of patients with ReA, AS, JIA and G were separated and purified by ion-exchange and gel-filtration chromatography also. In the most of these SFs the portion of SADA isoform was on the negligible level. Anyway, in some cases its purification was also possible. The citrullination of the available electrophoretically purified SADA and LADA samples were measured. The analyses did not detect any protein citrullination in the SADA samples obtained from the SFs of the patients with mentioned four arthritis types.

The multiple analyses of LADA samples purified from SFs of all five arthritis types (including RA) did not reveal any protein citrullination.

DISCUSSION

As described earlier^[12], the activity of ADA in SFs from RA patients was statistically higher than in SFs from patients with OA. A rather strong positive correlation between initial ADA activity and SADA/LADA ratio in the RA SFs^[13,14] indicated intracellular SADA release to the extracellular medium in the inflamed tissues. Obviously, the release of intracellular SADA to the extracellular medium due to tissue injuring takes place at the cases of various joint diseases as well. Therefore, the high ADA activity was also registered in some SFs from arthritis types such as ReA, AS, JIA and G^[14].

The present study evidences that the increase of ADA activity is accompanied with the accumulation of SADA in the SFs of RA. The increase of ADA activity in the SFs of patients with four other types of arthritis is accompanied with the accumulation of LADA isoform (Figure 1, Table 1). These data are in concordance with the earlier observation that in the SFs with the initial ADA activities between 54-64 U/L, the SADA/LADA ratio in the cases of ReA, AS, JIA and G patients was lower (between 0.15-0.31), than in the RA SFs (1.12)^[14].

Performed analyses of citrullination states of two ADA isoforms purified from various arthritis types, demonstrated the citrullination of SADA in the RA SFs. In the case of high initial ADA activity the level of SADA citrullination was higher by an order than in the case of moderate initial activity. Usually, the released SADA interacts with DPPIV resulting in accumulation of LADA. Probably, in RA SFs due to citrullination of Arg-142 residue in SADA^[16], the SADA-DPPIV

Table 1 Levels of SADA and LADA in the SFs of RA and ReA.

Arthritis type		ADA initial, U/L	LADA, A630	SADA, A630	SADA/LADA
RA	1	40.23 ± 0.92	0.52 ± 0.13	0.27 ± 0.08	0.52
	2	130.75 ± 25	0.44 ± 0.17	0.7 ± 0.12	1.58
ReA	3	26.8 ± 1.62	0.47 ± 0.58	0.14 ± 0.02	0.29
	4	72.25 ± 4.42	0.88 ± 0.11	0.12 ± 0.03	0.14

Lines 1 and 3 - the subgroups with the low initial activities of ADA; Lines 2 and 4 - the subgroups with high initial activities of ADA; All parameters are presented as mean ± SEM, n = 4 per each subgroup.

interaction is hindered, resulting in accumulation of SADA.

Our observations permit to suggest SADA in SF as a new citrulline containing auto-antigen suitable for differential diagnosis of RA. It is in concordance with the work of Mitsui et al^[20], where on the base of *in vitro* deimination of recombinant ADA, it was suggested as a novel serum ACPA antigen.

Because of insufficiency of available SF samples from AS, JIA and G for the statistical analysis, the significance of differences between ADA activity levels in these arthritis types has not been evaluated. However, the obtained data clearly manifested that in the case of RA and of other arthritis types the different ADA isoforms accumulated.

CONCLUSION

For the first time this study demonstrated the accumulation of citrullinated SADA isoform in SFs of RA, which allows to consider SADA as a new citrulline containing auto-antigen.

The future experiments are urgently needed for confirmation of this suggestion and evaluation of application of citrullinated SADA in the diagnosis and monitoring the dynamics at clinical treatment of RA.

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CONTRIBUTORS: LK, SS, AA and SM designed the study and performed the experiments. SS, AA and SM wrote the manuscript. LK and VV collected the synovial fluids. LK, SS, AA and SM collected and analyzed the data. SM performed the statistical analysis. All authors approved the final version of the manuscript.

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