Serum levels of granzyme B decrease in patients with rheumatoid arthritis responding to abatacept

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Abstract

Objective

A possible role of granzyme B (GZMB) in the pathogenesis of joint erosions in rheumatoid arthritis (RA) has been suggested. Since CD28neg T-cells may be an important source of GZMB, and we have previously shown that co-stimulation blockade by abatacept can prevent the generation of the CD28neg T-cell populations, we evaluated the effect of abatacept therapy on GZMB serum levels in patients with RA.

Methods

The serum levels of GZMB were evaluated by an indirect solid-phase enzyme immunoassay before the start of treatment with abatacept (T0) in 53 patients with RA and after 6 months of therapy (T6) in 25 patients.

Results

At T0, GZMB serum levels were correlated with disease activity measured by DAS28-CRP (p=0.0022) and percentages of circulating CD4+CD28neg and CD8+CD28neg T-cells (p=0.007; p=0.031). The levels of GZMB in 18 patients with a moderate or good EULAR clinical response to ABA significantly decreased from T0 to T6 (p=0.023), whereas no variation was observed in 7 non responders. The variation of GZMB levels was directly correlated with that of DAS28-PCR (p=0.040), but not with those of circulating CD28-neg T-cell subsets.

Conclusion

Costimulation blockade by ABA can decrease the serum levels of GZMB in RA patients responding to the treatment, suggesting that this might be one of the mechanism by which abatacept can prevent radiographic erosions. However, the lack of correlation of such decrease with the numbers of circulating CD28-neg T cells suggests that these cells probably are not the main source of serum GZMB.

Key words

abatacept, granzyme, rheumatoid arthritis, T cells

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Introduction

Rheumatoid arthritis (RA) is characterised by several changes of the T-cell compartment which include, in most patients, an increased number of T cells lacking the CD28 costimulatory molecule (1). These populations, which display some functional characteristic of cytotoxic memory T cells, may be pathologically relevant in RA development. Accordingly, their increase is associated with worse prognosis and extra-articular manifestations (2).

Granzymes are a family of serine proteases which play key roles in the induction of target cell death by cytotoxic T-lymphocytes and natural killer (NK) cells. In particular, granzyme B (GZMB) is expressed by activated CD4+ and CD8+ T-cells, while it is less abundant in NK cells (3). Soluble granzymes, including GZMB are found extracellularly in normal sera and are elevated in a number of diseases, ranging from infections to autoimmune diseases, and, in particular, in RA (4). The possible pathogenic role of granzymes in RA was suggested by *in vitro* data demonstrating their enzymatic activity for the cleavage of cartilage aggrecan proteoglycans (5). The interest in the role of GZMB in the pathogenesis of joint damage was reinforced by the associations of increased serum levels of GZMB with early development of radiographic erosions (6), and of a genetic variant of *GZMB* with progression of joint destruction in RA (7).

Abatacept (ABA) is a fusion protein (CTLA4-Ig) approved for the treatment of RA; its CTLA4 portion binds CD80 and CD86, the CD28 ligands, on antigen-presenting cells, and competing with the engagement of CD28 on T cells, it influences the subsequent T-cell activation (8). In patients treated with ABA, we have observed a reduction of the number of circulating CD28neg T cells, correlated with the improvement of RA disease activity, suggesting that the co-stimulation blockade by ABA can prevent the generation of CD28neg T-cell populations (9, 10).

Therefore, since CD28neg T-cells may be an important source of granzyme, in this study we evaluated the effect of ABA therapy on GZMB serum levels in patients with RA.

Materials and methods

Patients

Fifty-three consecutive RA patients, treated for at least 3 consecutive months with ABA were enrolled in the study. Their main clinical and demographic characteristics are shown in Table I. The study was approved by the Institution Ethics Committee and patients' written consent, according to the Declaration of Helsinki, was obtained. The clinical disease activity and the response to the treatment were evaluated respectively with the DAS28 (based on CRP) and the EULAR criteria of response to the treatment (11).

Flow cytometry

T cell counts were determined by flow cytometry (Cytomics FC-500, Beckman Coulter Inc., Fullerton, CA, USA), as described (9). Briefly, 100 µl of fresh whole blood were stained for 20 min at 4°C with a combinations of monoclonal antibodies (CD3, CD4, CD8, CD28) from Beckman Coulter. Absolute cell count was determined by single-platform analysis using Flow-Count beads (Beckman Coulter).

Serum GZMB levels analysis

Serum samples were collected and stored at -80°C immediately before the first administration of ABA (T0) and then after 6 months (T6). GZMB levels were measured by an indirect solid-phase enzyme immunoassay, with a sensitivity limit of 20 pg/ml (CellScience Inc., Canton, MA, USA). Intra-assay CV% was 10.4. The analysis was carried out simultaneously on all serum samples at the end of the study.

Statistical analysis

Data are expressed as the median (10^{th}-90^{th} percentile). Wilcoxon-signed rank test was applied to assess variation within paired quantitative variables. The correlations between variables were evaluated with the linear simple regression.

Results

The percentages and the absolute numbers of circulating $CD4^+CD28neg$ and $CD8^+CD28neg$ T-cells decreased after 6 months of treatment with ABA (Table II).

Table I. Main clinical and demographic features of RA patients.

	Entire cohort of patients at baseline (n=53)	Patients followed for 6 months (n=25)
Sex (male/female)	12/41	4/21
Age (years)	52 (39–71)	53 (39–66)
Disease duration (years)	6 (1–16)	6.5 (1–16)
Smokers (%)	19 (36%)	12 (48%)
Rheumatoid Factor positive (%)	44/51 (86%)	19/24 (79%)
Anti-CCP antibodies positive (%)	41/47 (87%)	19/24 (79%)
Number of previous DMARDs	3 2–5)	3 (1–5.5)
Number of previous biological agents:	2 (0–2.8)	2 (0–2)
TNF alpha blocking agents	38	19
Rituximab	7	5
Tocilizumab	4	2
Anakinra	6	5
ABA as first line biological treatment	5	5
Median dosage of methotrexate at baseline (mg/week)	12.5 (5.62–15)	10 (0–15)
DAS28-CRP at baseline	5.1 (4.0–6.3)	5.1 (4.4–6.3)

Data are expressed as the median (10^{th}-90^{th} percentile), if not otherwise indicated.
CCP: cyclic citrullinated peptide; DMARDs: disease-modifying anti-rheumatic drugs: ABA: abatacept; TNF: tumour necrosis factor; DAS: disease activity score; CRP: C-reactive protein.

Table II. Variations of T-cell subpopulations after 6 months of therapy with abatacept.

	T0	T6	*p*-value
$CD4^+CD28^-$ (% of $CD4^+$ T cells)	5.3 [0.9–16.1]	3.7 [0.9–11.3]	0.018
$CD4^+CD28^-$ (cells/μL)	33 [7–143]	24 [5–97]	0.018
$CD8^+CD28^-$ (% of $CD8^+$ T cells)	40.7 [22.5–67.8]	32.9 [14.3–58.4]	0.005
$CD8^+CD28^-$ (cells/μL)	118 [42–323]	88 [24–227]	0.008

Data are expressed as the median (10^{th}-90^{th} percentile).

Before starting treatment with ABA (T0), soluble GZMB was measurable in 51 out of 53 sera from RA patients evaluated. Median level was 50.3 pg/ml (24.3–103.3). As shown in Figure 1, GZMB serum levels were correlated with disease activity, evaluated by DAS28-CRP (p=0.0022), and with the percentages of circulating $CD4^+CD28neg$ and $CD8^+CD28neg$ T-cells (p=0.007 and p=0.031), but not with disease duration (data not shown). GZMB levels did not differ between rheumatoid factor- or anti-CCP antibodies-positive and negative patients.

In 25 patients GZMB serum levels were evaluated also after 6 months of ABA treatment (T6). Considering this whole population, no variation of GZMB levels was observed (from 64.4 pg/ml (46.8–106.3) to 58.3 (46.1–92.6) pg/ml). However, in 18 patients with a good or moderate clinical response to ABA, GZMB levels significantly decreased (p=0.023; Fig. 2), whereas no variations was observed in 7 non responders. Indeed, there was a direct correlation between the variation of GZMB levels and that of DAS28-CRP (p=0.040; Fig. 2). No significant correlation was observed between the variation of GZMB serum levels and those of circulating CD28neg T-cell subsets (p=0.89, and p=0.56, as far as $CD4^+$ and $CD8^+$ T-cells, respectively). The variations of GZMB levels were not different between rheumatoid factor- or anti-CCP antibodies-positive and negative patients (p=1).

Discussion

Granzymes are serine proteinases which are stored in the granules of activated cytotoxic T cells and NK cells. Previous studies have shown that the levels of GZMB in the plasma or serum of patients with RA are increased as compared with healthy controls, or patients with osteoarthritis or reactive arthritis (4, 6).

In this study, we observed that soluble GZMB levels are correlated with RA activity, as measured with the composite index DAS28-CRP, whereas Goldbach-Mansky *et al*. have found a significant correlation with rheumatoid factor titers, but not with some individual component of DAS28-CRP evaluated by them (namely, swollen and tender joint counts, and CRP levels) (6). It is not known whether this discrepancy might be explained by different characteristics of the disease in the two case series (early RA in the study of Goldbach-Mansky *et al.;* established disease in the present study). Alternatively, the composite index may be more sensitive to detect a correlation than individual items, or other components of the index not evaluated by Goldbach-Mansky *et al*. may be relevant.

Moreover, we first observed a significant correlation of soluble GZMB levels with the percentages of circulating $CD4^+$ and $CD8^+$ CD28neg T lymphocytes, a potential source of GZMB.

For this reason we planned to evaluate the variations of soluble GZMB in patients treated for 6 months with ABA, an agent known to reduce the percentages of these T-cell populations (9, 10). We observed a significant reduction of GZMB serum levels in patients with clinical response to ABA, but not in non-responders. Not much information is available on the effect of other therapies on GZMB levels. In 28 Japanese patients with RA treated with the TNF-blocking agent etanercept for 6 months, no significant variation of GZMB serum levels was observed, but a separated analysis of responders versus non responders was not performed in that study (12). It is not clear therefore whether the result of this study really indicates, as suggested by the authors, that GZMB production in RA is not dependent by TNF.

Interestingly, we found a correlation between the variation of disease activity and that of soluble GZMB. Data from our longitudinal study therefore confirm the association found in the cross-sectional study. It is well known that ABA not only can reduce disease activity in patients with RA, but also inhibits progression of structural damage (13). Although other mechanisms, including a direct effect of ABA on osteoclast precursors differentiation may play a role in the prevention of

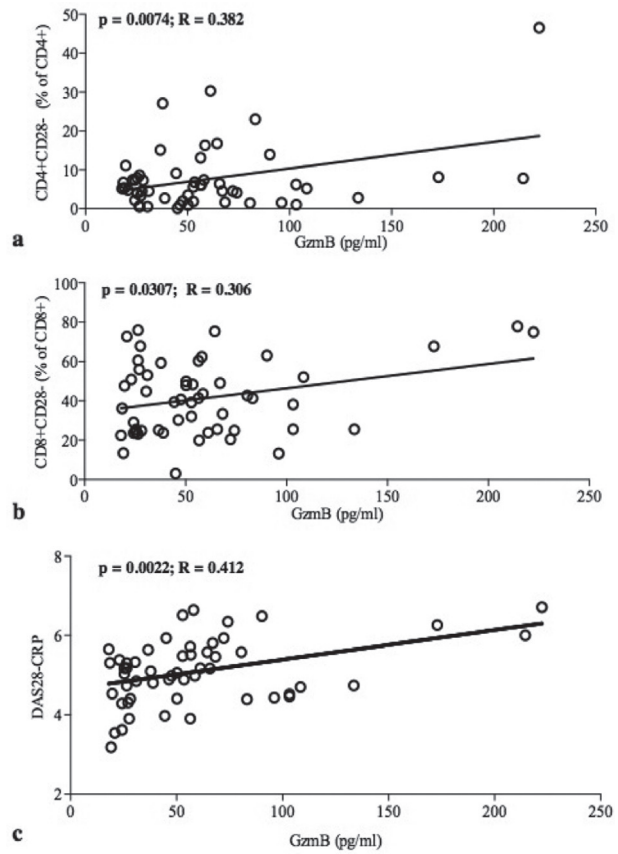

Fig. 1. Correlations of serum levels of Granzyme B (GzmB) with:
a) the % of $CD4^+CD28^-$ b) the % of $CD8^+CD28^-$ c) DAS28-CRP.

DAS: disease activity score
CRP: C-reactive protein.

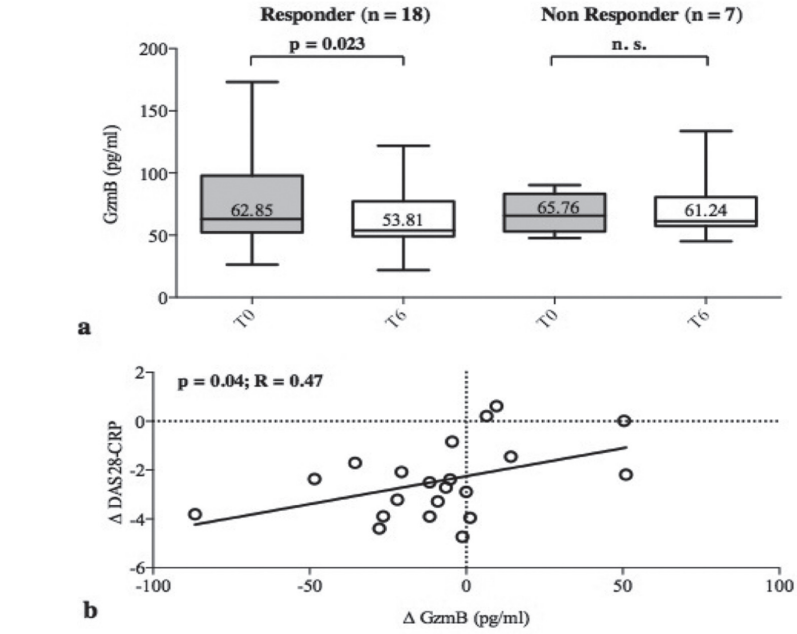

Fig. 2 a. Variations of granzyme B (GzmB) serum levels in patients treated with abatacept for 6 months. **b**. Correlation of variations of granzyme B serum levels and DAS28-CRP in patients treated with abatacept for 6 months. n.s.: not significant.

radiographic erosions (8), our observation suggests that reduction of GZMB might be one of the mechanism by which ABA displays this protective activity. In fact, it has been shown that GZMB can cleave cartilage aggrecan proteoglycans, an early event in the course of destructive arthritis (5).

On the other hand, we have not found a correlation between the variation of soluble GZMB levels and those of circulating $CD4^+$ and $CD8^+$ CD28neg T lymphocytes. Although we should acknowledge that the relatively small number of patients evaluated may limit the statistical power to detect such a correlation, these results suggest that these cells probably are not the main source of serum GZMB. This is not fully known, but since the synovial tissue from RA is enriched of GZMB expressing cells (14), and levels of GZMB are much higher in the synovial fluid than in the serum of these patients (4), it has been suggested that the rise in serum GZMB may originate from extracellular release in the inflamed joint (4, 6). It should be considered that although granzymes are constitutively expressed by cytotoxic T lymphocytes, only small percentages of the granzyme positive cells in the synovium are $CD8^+$ and $CD4^+$ CD28neg T lymphocytes, whereas most are NK cells (15). However, we are not aware of data on the effect of ABA on synovial tissue granzyme positive NK cell infiltration, and the hypothesis that an effect of ABA on this population is responsible of the decrease of soluble GZMB levels deserves therefore further studies.

In conclusion, the data presented here show that soluble GZMB levels are correlated with disease activity and decrease in patients responding to ABA, suggesting that this might be one of the mechanisms by which ABA reduces the progression of joint erosions.

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