
The level of soluble Granzyme A is elevated in the plasma and in the V γ 9/V δ 2 T cell culture supernatants of patients with active Behçet's disease

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ABSTRACT

Objective. Granzyme A (GrA) is a serine proteinase with trypsin-like activity that is released extracellularly during the degranulation of cytotoxic cells. Among the cytotoxic cells, $\gamma\delta$ T cells participate in the early phases of the immune response and are known to express perforin and granzymes constitutively in agreement with their cytolytic potential.

Methods. GrA activity was detected using the synthetic substrate N- α -benzyloxycarbonyl-L-lysine thiobenzyl ester in the plasma and supernatants of peripheral blood mononuclear cell cultures in the presence of Dimethylallyl pyrophosphate to obtain V γ 9/V δ 2 T cell expansion.

Results. Significantly high levels of GrA were found in the serum and supernatants of lymphocytes from patients with active Behçet's disease cultured in the presence of DMAPP. Levels were found to be significantly lower after remission. A positive correlation was observed between GrA levels in the supernatants and the V γ 9/V δ 2 T cell expansion factor.

Conclusion. These results strongly suggest that V γ 9/V δ 2 T cells are active participants in the pathogenesis of the disease through their degranulation and granzyme release.

Introduction

Granzyme A (GrA) is a serine proteinase with trypsin-like activity that is released extracellularly during the degranulation of cytotoxic cells (1). Multiple functions have been proposed for granzymes, including the degradation of extracellular matrix (2) and myelin basic proteins in myelin membranes (3) and the activation of pro-urokinase (4). Compelling evidence also supports a role for

granzymes in granule-mediated target cell apoptosis (5).

Circulating NK cells, and $\gamma\delta$ T cells in particular, participate in the early phases of the immune response and are known to constitutively express perforin and granzymes in agreement with their cytolytic potential (6, 7). In contrast, unstimulated CD3⁺CD8⁺ CTL express only marginal levels of GrA mRNA, but synthesis is rapidly induced after IL-2 stimulation (8). *In vitro* studies have shown that granzymes may be released extracellularly during cytotoxic cell degranulation (1).

The immunopathogenesis of Behçet's disease (BD) is believed to be T cell-mediated. Cytotoxic T cells are considered to play a role in the development of disease (9). Our recent data also point to a role of activated V γ 9/V δ 2 T lymphocytes in the progression and probably in the pathogenesis of the disease (10).

Circulating levels of GrA have not been previously investigated in BD. In the present study, we measured the amount of GrA in the serum of patients with active and inactive BD. To extend studies on the role of V γ 9/V δ 2 T lymphocytes in the disease, we also investigated GrA levels in the supernatants of lymphocytes from BD patients, cultured in the presence of Dimethylallyl pyrophosphate (DMAPP) to obtain V γ 9/V δ 2 T cell expansion (10).

Materials and methods

Patients

31 patients with Behçet's disease (17 males, 14 females, mean age 42 ± 24 years), classified according to the International Study Group for Behçet's disease (11), were studied. At time of sampling 18 patients were in the active and 13 were in the inactive stage. Patients

were considered to have active disease when at least two of the following criteria were present: recurrent aphthous ulceration, erythema nodosum, thrombophlebitis, ocular and CNS involvement. Inactive patients included in this study were in complete remission (absence of all signs and symptoms of disease). Pathergy test positivity was present only in 27.7% of the patients in the active stage. The HLA-B51 allotype was present in 16 of the patients. None of the case patients or control subjects had evidence of viral (EBV, CMV, HIV or HSV) or bacterial infection. In 6 of the active patients blood for serum and lymphocyte studies was obtained after the induction of remission. Remission in these patients was achieved by anti-TNF (Infliximab) therapy in 4 and high dose prednisone and cyclosporine in 2. All patients were using colchicine, an immunosuppressive agent such as cyclosporin (n=8), azathioprine (n=2), and/or low dose corticosteroids (n= 16). Twenty-one healthy volunteers (age range 21-54 years, mean 38 years) were enrolled as controls. In addition, plasma from 5 patients with rheumatoid arthritis, 8 patients with osteoarthritis and 6 patients with active tuberculosis were also investigated. Human studies committee approval and individual informed consent from each patient were obtained.

Esterase assay for GrA

GrA activity was tested using the synthetic substrate N-benzyloxycarbonyl-L-lysine thiobenzyl ester (z-lys-SBzl; Sigma, St Louis, USA). The esterolytic activity against z-lys-SBzl was assayed as previously described (12) with minor modification. Briefly, 20 µl of supernatants (obtained at the 10th day of culture) or diluted (1:100 in PBS) sera were co-incubated with 35 µl 1 mM z-lys-SBzl and 35 µl 1 mM 5,5 dithio-bis-(2 nitrobenzoic acid) (Sigma). After incubation at 37°C for 2 hours and 30 min. respectively, the absorbance at 405 nm was determined. Esterolytic activity of GrA was reported as mOD units. Because z-lys-SBzl can be hydrolyzed by granzyme K (GrK) as well as GrA, esterolytic activity in the plasma or culture superna-

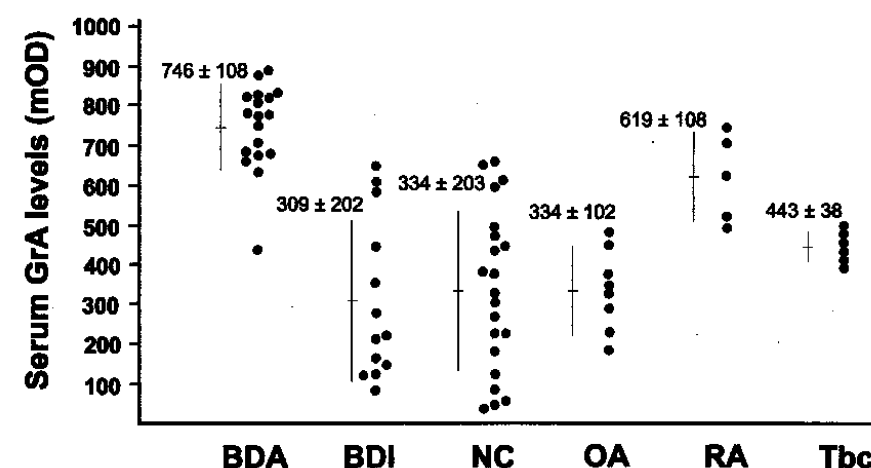


Fig. 1. Distribution of Granzyme A (GrA) serum levels in patients with active (BDA) and inactive (BDI) Behçet's disease, in patients with rheumatoid arthritis (RA), osteoarthritis (OA) and active tuberculosis (Tbc) and in normal controls (NC). Results are expressed as mOD units at 405 nm. BDAs vs BDI, $p < 0.001$; BDAs vs NC, $p < 0.001$; BDAs vs RA, NS; BDAs vs OA, $p < 0.001$; BDAs vs Tbc, $p < 0.01$; BDI vs OA vs Tbc, NS.

tants may be either the result of GrA or GrK or both. The tryptase-like activity identified in the granules is, however, primarily attributable to GrA and both GrA and GrK have been proposed to play a role in target cell death (13).

Monoclonal antibodies and flow cytometry

Monoclonal antibodies (mAbs) specific for human surface anti-TCR V 2 FITC (PharMingen, San Diego, Ca) were used as followed: PBMC (10^6 in 100 ml PBS with 1% heat-inactivated foetal calf serum and 0.02% Na-azide) were incubated at 4°C for 30 min. with anti-TCRV 2 FITC conjugated mAb. After washing, the cells were suspended in PBS with 1% FCS and analysed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) by using forward scatter/side scatter gating to select the lymphocyte population for analysis.

Cell separation and expansion in vitro of Vγ9/Vδ2 T lymphocytes.

Peripheral blood mononuclear cells (PBMC) were obtained from each individual by separating heparinized venous blood on Ficoll (Euroclone, Wetherby, Yorkshire, UK). The cells were washed in RPMI-1640 medium (Euroclone), and cultured in 24-well plates (Costar, Cambridge MA, USA) at a concentration of 5×10^5 cells/ml in

RPMI 1640 supplemented with 10% fetal calf serum (FCS, Euroclone), heparin 20 mM (Euroclone), 2 mM l-glutamine (Euroclone), penicillin/streptomycin 100 U/ml (Sigma) at 37°C, 0.5% CO₂. For the expansion of V 9/V 2 T cells (10), PBMC were cultured for 10 days in medium alone, or in the presence of Dimethylallyl pyrophosphate (DMAPP; 0.5 mM, final concentration; Sigma). After 72 hours, cultures were supplemented with a 0.5 ml medium containing 20 U/ml recombinant human interleukin (IL-2; Genzyme, Cambridge MA, USA). Every 72 hours 0.5 ml medium was replaced with a 0.5 ml fresh medium containing 20 U/ml of IL-2. Results were expressed as expansion factor (EF) (10).

Statistical analysis

Serum and supernatant GrA levels from each group were compared using the Mann-Whitney U test. Levels for each patient before and after remission were compared using the student t test for paired data. The correlation between GrA levels in supernatants and V 9/V 2 T cells expansion was analysed by the Spearman's rank correlation test. A p value < 0.05 was considered to be significant.

Results

Serum levels of GrA

Serum GrA levels were significantly

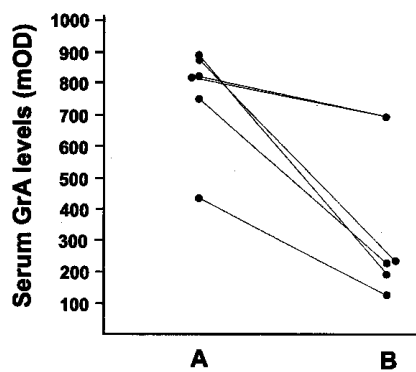


Fig. 2. Serum level distribution of Granzyme A (GrA) in patients with Behçet's disease before (A) and after (B) the induction of clinical remission. Results are expressed as mOD units at 405 nm. Avs B $p < 0.01$.

higher in the active (746 ± 108 mOD units; median 777; range 435-888) than in the inactive patients (309 ± 202 ; median, 233; range, 85-692; $p < 0.001$) or healthy controls (334 ± 203 ; median, 326; range, 47-659; $p < 0.001$) (Fig. 1). GrA was also determined in a group of patients ($n=6$) before (765 ± 169 ; median, 819; range, 435-888) and after remission (361 ± 260 ; median 230; range, 125-692; $p < 0.01$) (Fig. 2). Serum levels in patients with active rheumatoid arthritis were similar (619 ± 108 ; median, 625; range 498-741) to those obtained in active BD. Low levels were found in the sera from patients with osteoarthritis (334 ± 102 ; median, 325; range, 185-481). Levels in patients with

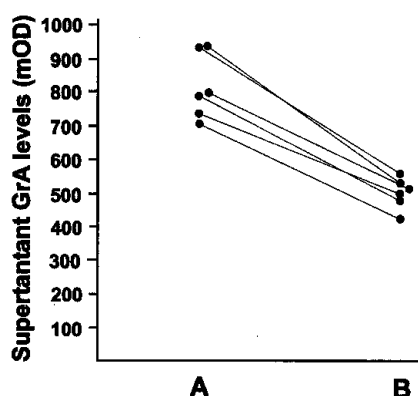


Fig. 4. Level distribution of Granzyme A (GrA) in the supernatants of lymphocytes from patients with Behçet's disease before (A) and after (B) the induction of clinical remission cultured in the presence of dimethylallyl pyrophosphate (DMAPP) to obtain the V 9/V 2 T cell expansion. Results are expressed as mOD units at 405 nm. Avs B, $p < 0.001$.

tuberculosis infection were 443 ± 37 (median 429; range, 396-497).

Supernatant levels of Gr A and V γ 9/V δ 2 T cell expansion

GrA levels were determined in the supernatants of PBMC cultured in the presence of DMAPP to investigate the relationship between its release and the V 9/V 2 T cell expansion ability.

GrA levels were 818 ± 97 mOD units in the supernatants obtained from cultures of active patients ($n=6$) (Fig. 3). In inactive patient cultures ($n=5$) GrA levels were 589 ± 21 , similar to those obtained in control cultures ($n=5$; 550 ± 30). The difference between levels in patients with active disease and controls or patients with inactive disease was statistically significant ($p < 0.001$) (Fig. 3). In the group of patients studied before and after remission, levels were 818 ± 97 and 502 ± 47 ($p < 0.001$; Fig. 4) respectively. The V 9/V 2 expansion factor was 80 ± 19 in active patients. In inactive patients and controls the expansion factor was 22 ± 12 and 6 ± 5 , respectively. Supernatant GrA levels in BD were significantly correlated with the V 9/V 2 expansion factor obtained in the same cultures ($n=11$, $r=0.82$, $p < 0.01$) (Fig. 5).

Discussion

Granzyme A (GrA) is a serine proteinase with trypsin-like activity that is released extracellularly during the degranulation of cytotoxic cells (14, 15). Cytotoxic T cells have been shown to secrete a significant portion of their synthesized granzymes following activation (15); in particular, GrA is secreted by T lymphocytes in response to antigen or stimuli that mimic antigen (16). Analysis of highly purified lymphocyte population subsets suggests that GrA is endogenously expressed in NK and T cells, whereas T cells do not express high GrA levels (7). The constitutive expression GrA in T cells may be of significance for their potential cytotoxic activity.

Behçet's disease is particularly frequent in countries along the Silk Road from the Mediterranean area to Japan, and it is strongly associated with HLA-B51 and MIC-A genes (17,18). The eti-

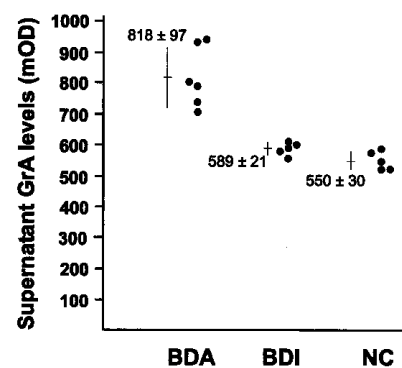


Fig. 3. Level distribution of Granzyme A (GrA) in the supernatants of lymphocytes from patients with active (BDA) and inactive (BDI) Behçet's disease or normal controls (NC) cultured in the presence of dimethylallyl pyrophosphate (DMAPP) to obtain the V 9/V 2 T cell expansion. Results are expressed as mOD units at 405 nm. BDA vs BDI, $p < 0.001$; BDA vs NC, $p < 0.001$; BDI vs NC, NS.

ology and pathogenesis of BD remain unknown. Various micro-organisms such as streptococci and herpes simplex virus have been implicated as causative agents of BD in genetically susceptible individuals (19-21). There is also evidence of immunological dysregulation, including neutrophil hyperfunction, autoimmune manifestations and several phenotypic and functional lymphocyte abnormalities, possibly resulting from complex interactions of genetic and environmental factors (22-26). Most of the immunological studies suggest a central role for T cells in the pathogenesis of BD. Moreover, significant

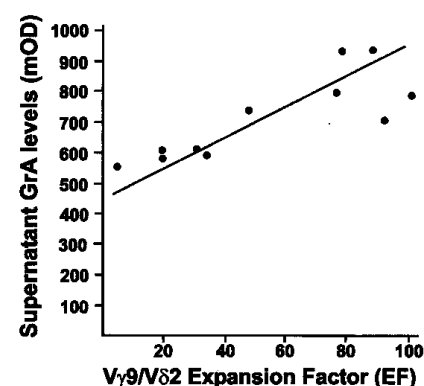


Fig. 5. Correlation between the levels of Granzyme A (GrA) in the supernatants of lymphocytes from patients with Behçet's disease, cultured in the presence of dimethylallyl pyrophosphate (DMAPP) to obtain the V 9/V 2 T cell expansion, and the V 9/V 2 T cell expansion factor (EF); ($n=11$, $r=0.82$, $p < 0.01$).

proliferative responses of T cells to peptides derived from both mycobacterial and human 65-kD heat shock proteins has been demonstrated (27).

We and others have been recently postulated a role for T cells in the immunopathogenesis of BD (10, 28).

In the present study we analysed the levels of GrA in serum samples and in supernatants of PBMC from patients with active or inactive BD cultured in the presence of DMAPP, to obtain purified T cell populations (10). We utilized the BLT esterase assay to detect granzyme A activity. Other methods have been recently described (western blotting and Enzyme immunoassays), but these detect the protein concentration and not enzymatic activity. Significantly high levels of GrA were found in the serum of patients with active disease, while levels in inactive patients and healthy controls were similar. V 9/V 2 cells from patients with active disease, but not from inactive patients or control individuals responded to DMAPP *in vitro* with expansion. Increased GrA levels were found in the culture supernatants of patients with active disease but not in inactive patients. A positive correlation was also found between GrA levels and the V 9/V 2 expansion factor.

V 9/V 2 T lymphocytes from BD patients are activated (10). They may be responsible for the development of inflammatory processes through cytokine production and the subsequent induction of adhesion molecules, which permit the accumulation of reactive T lymphocytes at the site of inflammation. Involvement of T cells in the local injury process has been also demonstrated by their presence in the infiltrate of mucosal ulcerations (29). We postulate the GrA secretion as a possible effector function of V 9/V 2 T cells in BD.

GrA secretion is induced by target cells during the cytotoxic cell response. Cytolytic cells have been reported to constitutively secrete the GrA following activation (15). T cells constitutively express GrA (6,7). GrA has been reported to degrade extracellular matrix proteins *in vitro* (2) and myelin basic proteins in myelin membranes (3). In ad-

dition, GrA induces the production of IL-6 and IL-8 in fibroblasts and epithelial cells (30) and stimulates phagocytic activity and IL-6, IL-8 and TNF-production in monocytes (31). Our results do not indicate that increased GrA levels are specific for BD, high levels being obtained in other inflammatory conditions (RA), but do suggest a potential role for the GrA in the tissue damage of BD lesions.

GrA levels were observed in the serum of active patients. GrA in the serum might be the consequence of degranulation of T cells, but also of NK or CTL. Impaired clearance of granzymes during a cytotoxic response may contribute, however, to an increase in extracellular granzymes. Detectable levels of extracellular GrA were also present in the plasma of inactive patients and healthy donors. Since normal peripheral blood contains a small percentage of activated granzyme-positive lymphocytes, the levels in normal subjects may reflect the constitutive release of the proteases from these activated cells (32). In active patients, however, circulating GrA should not only be the result of the degranulation of CTL. CTL, in fact, are known to synthesize new proteins, some of which acquire the granule targeting signal and re-fill granules, and some of which are secreted via a non-granule pathway (15). In particular, it has been observed that up to one-third of the GrA secreted after TCR cross-linking to release the lytic granules is due to the newly synthesized proteins secreted via the constitutive secretory pathway; this protein is enzymatically active since it can be detected by cleavage of a synthetic substrate (15). The potential extracellular functions of the granzymes in disease have not been extensively evaluated. Taken together, the elevated levels of GrA in serum and, in particular, in the V 9/V 2 T cell supernatants of patients with active Behçet's disease strongly suggest that cytotoxic cells are active participants in the pathogenesis of the disease.

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