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Polymorphonuclear leukocyte myeloperoxidase levels in patients with Behçet's disease

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ABSTRACT

Myeloperoxidase was measured in the plasma and in the supernatants of polymorphonuclear granulocyte cultures from patients with Behçet's disease. High levels were found in both plasma and supernatants from patients with active disease. The addition of pentoxifylline to granulocyte cultures determined a significant decrease of myeloperoxidase levels in active patients only. Hyperactive neutrophils are present during the course of Behçet's disease and may be considered of importance in the pathogenesis of the vascular lesions.

Introduction

Behçet's disease (BD) is a multisystemic inflammatory disease of unknown etiology characterised by involvement of the eyes, skin and mucous membranes. The disease has a chronic course with periodic exacerbations and progressive deterioration. Among major pathophysiologic changes, excessive function of neutrophils, vasculitis with endothelial cell injury and autoimmune responses have been reported. Hyperactive polymorphonuclear neutrophils (PMN) are observed in the biopsy of vascular lesions (1). Many *in vitro* studies on neutrophil function have also been shown to be abnormal. Chemotaxis, phagocytosis and superoxide radical anion generation are increased (2-5) and more recently, high elastase serum levels were found (6).

In a recent study we demonstrated that that the incubation of endothelial cells with neutrophil-derived myeloperoxidase (MPO) is followed by up-regulation of E-selectin expression and that this phenomenon can be reproduced by incubation of endothelial cells with sera from patients with BD that contain high MPO levels (7). Several studies have been published in recent years on the role of neutrophil activation in the course of systemic vasculitides (rev. in ref. 8). In this regard, among emerging factors MPO has been the object of investigation. MPO is primarily located in the azurophil granules and plays a major role as an active component of the PMN phagocytic system (9). After activation MPO is exposed at the membrane level and released (10).

Our major aim in this study was to measure the amount of MPO released *in vitro* by PMN from patients with Behçet's disease and to determine whether plasma MPO levels can be considered an index of disease activity. The effect of pentoxifylline [PTX, a potentially effective drug in the treatment of BD (11)] on MPO release *in vitro* was also assessed.

Materials and methods

Participants

Twenty-one patients with BD and 27 healthy volunteers were studied. Eleven BD patients were males, 10 females. In the control group there were 15 males and 12 females. Duration of disease was 12.5 ± 9.7 years (range 2-40 years). The ages ranged from 14 to 68 years (mean 40 years) in the patient group and from 21 to 47 years (mean 38 years) in the control group. The diagnosis of BD was made according to the criteria of the International Study Group for Behcet's disease (12). Fourteen patients (10 untreated, 4 treated) had at least two of the major manifestations in the active stage at the time of blood withdrawal. Disease activity was determined by physical examination and by laboratory measurements of ESR, CRP and white blood cell count. Clinical parameters and type of treatment are listed in Table I. Blood samples were drawn into tubes containing heparin for the preparation of human peripheral PMN and into tubes with EDTA anticoagulant for the PMN leukocyte count, ESR determination and PMO measurement. An aliquot of blood sample without anticoagulant was also obtained for CRP measurement.

Preparation of human peripheral PMN and culture conditions

Whole blood from patients and healthy donors was drawn into tubes containing heparin. PMN were separated by a standard procedure (13) and the purity was determined by flow cytometric scattergram. Briefly, human neutrophils (> 98% pure) were isolated from fresh heparinized blood by dextran-destrose sedimentation, followed by Ficoll-Hypaque gradient centrifugation. Contaminating red cells were removed by NH_4Cl lysis. Neutrophils were counted using an automatic Coulter counter, re-suspended in

Table I. Clinical parameters in patients with Behçet's disease.

Pts.	Age	Sex	Disease duration	Clinical presentation	Treatment
1	29	M	2	U, OU	UT
2	36	M	2	U, OU	UT
3	62	M	8	OU, GU	UT
4	58	M	12	OU, GU, T	P
5	42	F	16	—	P, A
6	47	F	20	—	Co
7	41	M	5	—	Co, P
8	44	F	6	U, OU, GU	P, Cy
9	47	F	20	U, OU, EN	UT
10	23	M	2	U, OU	UT
11	50	F	5	EN	P
12	37	F	20	EN, OU	UT
13	30	F	15	—	UT
14	68	M	30	U, OU, GU	UT
15	45	F	7	—	UT
16	44	M	11	U, OU	P, Cy
17	44	M	121	U, OU	P, Cy
18	14	M	4	OU, GU, EN	UT
19	22	M	10	—	P, Cy
20	65	F	40	U, OU, EN	UT
21	24	F	15	U, OU, EN	UT

U: uveitis; OU: oral ulcer; GU: genital ulcer, EN: erythema nodosum; T: thrombophlebitis; UT: untreated; P: prednisone; Co: colchicine; A: azathioprine; Cy: cyclosporine.

Hank's balanced salt solution and maintained in this medium for 1 hour at 37°C. After this incubation the medium (that we considered to contain PMO produced by PMN activation during the process of purification) was discharged by centrifugation and the PMN was re-suspended in RPMI medium containing 15% FCS and 100 µg streptomycin at 5 x 10^5 cell/ml concentration. Cells were cultured at 37°C in 5% CO_2 atmosphere under standard conditions or in the presence of various concentrations of TNFα for 1 - 24 hours. Ten ng was the low concentration that produced a significant increase in MPO release. Experiments were also carried out in which 1-10 µg/ml of PTX was added in the medium and the cells were cultured as above. After incubation cultures were centrifuged and supernatants stored at -30° C for MPO.

Measurement of plasma or supernatant MPO

Serum or supernatant MPO was measured by a sandwich enzyme immunoassay that utilizes commercially available monoclonal and polyclonal (Dako, Copenaghen) anti-human MPO antibodies. Wells of microtiter plates (Costar) were absorbed with the monoclonal antibody at a dilution of 1:400 in PBS 0.15 M, pH 7.4 and blocked with PBS containing 2% BSA. Sera diluted 1:10 or supernatants diluted 1:5 were incubated for 1 hr. The wells were then reacted with the polyclonal antibody diluted 1:500 in PBS containing 1% BSA and 0.05% Tween 20, and subsequently with an anti-rabbit IgG conjugated with alkaline-phosphatase. After washing, the specific enzyme substrate (p-nitrophenil-phosphate-disodium 1 mg/ml in 1M diethanolamine HCL buffer pH 9.8) was added and the colour reaction was read in an automated spectrophotometer. The sensitivity was calculated and found to be 40 U/L. The range of the standard curve was 50 - 2,000 U/L. The results were expressed as units per 5 x 10^5 PMN according to a reference standard curve. Inter- and intra-variability was assessed and never exceeded 9% when specimens with low, mid and high levels were used.

Statistics

The statistical analysis of the results was carried out using the Student's t-test for unpaired data. Results in the PTX study were analysed using the Student's t-test for paired data.

Results

Plasma MPO

Plasma MPO levels were 190 ± 120 U/L in the control population. Patients with BD showed levels of 780 ± 680 U/L that were significantly higher than those of controls ($p < 0.001$) (Table II). In particular, plasma MPO levels were 1127 ± 710 U/L in patients with untreated active disease (n=10), 424 ± 323 U/L in treated active patients (n=4) and 549 ± 654 U/L in patients with inactive disease (5 treated, 2 untreated). A higher leukocyte count (12,420 ± 2,800) was found in active patients compared with patients with inactive disease (8,400 ± 1,320; $p < 0.001$). No significant relationship was found, however, between the MPO levels and PMN counts ($r = 0.28$, NS).

Table II. Plasma levels of myeloperoxidase in patients with Behçet's disease and healthy subjects.

Subjects	no.	MPO M ± SD (U/L)
Behcet's disease (BD)		
All	21	780 ± 680[a]
Untreated active BD	10	1127 ± 710[a]
Treated active BD	4	424 ± 323
Inactive BD	7	549 ± 650[b]
Normals	27	190 ± 120

[a] $p < 0.001$ vs normals; [b] $p < 0.01$ vs normals.

MPO levels in supernatants of PMN cultures

MPO was measured in the supernatants of PMN from some of the patients and controls cultured in the presence or absence of TNFα for 1 or 24 hours (Table III). After 1 hour of incubation the concentration of MPO in the supernatants of PMN cultures from patients with active untreated disease (n = 7) was 0.95 ± 0.49 U/5 x 10^5 cells. Levels in the supernatants of PMN from inactive patients (n = 7) and controls (n = 7) were 0.15 ± 0.04 U ($p < 0.01$) and 0.14 ± 0.06 U ($p < 0.01$) respectively. A slight increase was observed after 24 hours of incubation in all patient and control cultures, the difference between the 1-hour and 24-hour cultures being statistically significant only in control group (0.30 ± 0.17 U; $p < 0.05$) and in active patients (1.4 ± 0.24 U; $p < 0.05$). Cells were also exposed to various TNFα combinations. Ten ng of TNFα was the low concentration that produced an increase in PMO release. The addition of TNFα resulted in a significant increase of MPO only in the 1-hour control PMN cultures, however (0.35 ± 0.18 U; $p < 0.05$).

Effect of PTX on MPO release

The effect of PTX was investigated. In preliminary experiments 1-10 µg/ml of PTX were added to all cultures to examine the effect of treatment on *in vitro* MPO release. No effect was obtained with 1 µg/ml of PTX. Five µg/ml was the low concentration that determined significant MPO changes. No further effect was seen when PTX was increased to 10 µg/ml. The addition of the drug was followed by a significant MPO decrease in the 1-hour cultures of PMN from patients with active disease (0.91 ± 0.38 U

Table III. Levels of myeloperoxidase in the supernatant of neutrophil cultures from patients with Behçet's disease in the presence or absence of TNF-α.

Incubation time	1 hour			24 hours		
Presence of TNF-α	Yes	No	p	Yes	No	p
Active disease (n = 7)	1.06 ± 0.40	0.95 ± 0.49[a]	NS	1.40 ± 0.33	1.40 ± 0.24[b]	NS
Inactive disease (n = 7)	0.16 ± 0.04	0.16 ± 0.04	NS	0.29 ± 0.16	0.27 ± 0.13[b]	NS
Healthy subjects (n = 7)	0.35 ± 0.18	0.14 ± 0.067	< 0.05	0.38 ± 0.17	0.30 ± 0.17	NS

[a] $p < 0.01$ vs inactive disease or healthy subjects; [b] $p < 0.05$ 1 hour cultures without TNF-α

vs 0.53 ± 0.30 U, n = 6; p 0.027) (Fig. 1). No effect in MPO secretion was observed in PMN cultures in either controls or patients in the inactive stage (Fig. 1). Similar effects were obtained in the 24-hours cultures (1.44 ± 0.20 U vs 0.73 ± 0.18; n = 6; $p < 0.0001$) (Fig. 1) and in the presence of TNFα (not shown).

Discussion

Neutrophilic polymorphonuclear leukocytes (PMN) are white cells of the myeloid series that are essential for host defence against infection and have functions ideally suited for this role. They are responsive to signals generated in infected or injured tissues which induce them to migrate through tissues to the site of perturbation. Recently it has become apparent, however, that neutrophil mediated processes can also cause inflammatory diseases.

Behçet's disease is a systemic inflammatory disease of unknown etiology. The disease has a chronic course with periodic exacerbations and progressive deterioration. Three major pathophysiologic changes may be operative in the pathogenesis of disease: excessive functions of neutrophils, vasculitis with endothelial injury and autoimmune responses.

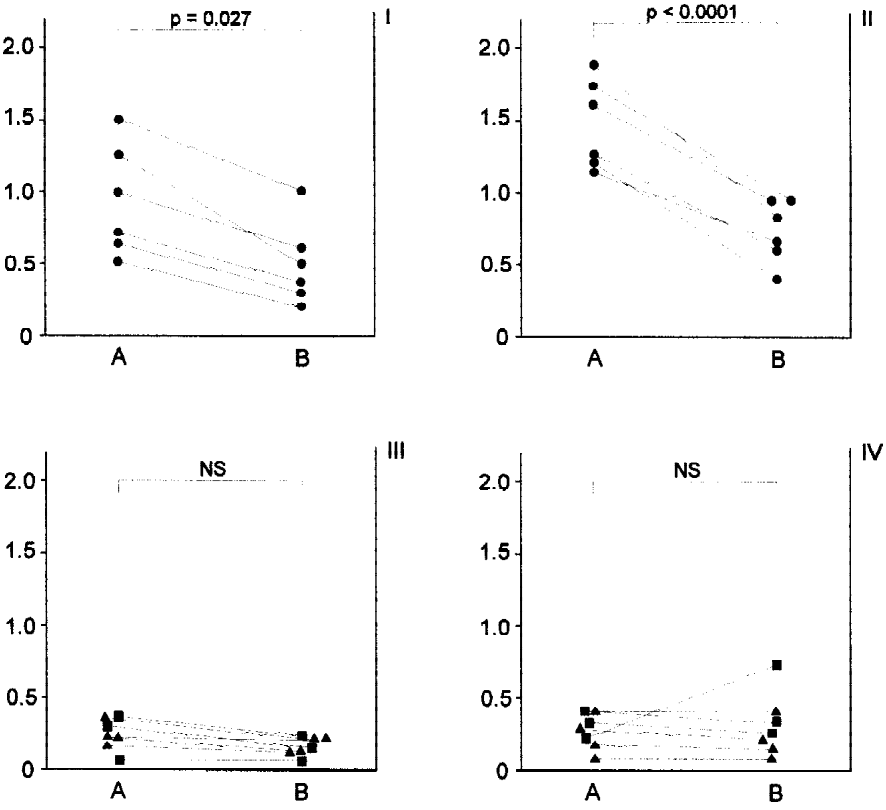

Fig. 1. Effect of pentoxifylline on the levels of MPO in the supernatants of PMN cultures from patients with active Behçet's disease (●), inactive Behçet's disease (▲) and controls (■).
(**A**) untreated PMN; (**B**) PMN treated with μg/ml pentoxifylline. **I** and **III**: PMN cultured for 1 hour; **II** and **IV**: PMN cultured for 24 hours.

In this study we have shown that neutrophils from patients with Behçet's disease in the active stage release *in vitro* increased amounts of myeloperoxidase (MPO) in comparison to those from inactive patients or controls. We consider this MPO release an active process rather than the effect of PMN manipulation during cell isolation. Very low amounts of MPO were observed in the supernatants from control PMN. In order to minimize this possibility cells were also pre-incubated for one hour in the same medium and the supernatants discharged.

To better elucidate the role of neutrophil hyperfunction, we studied the *in vitro* secretion by PMN of MPO in patients with BD and from healthy subjects in the presence or absence of TNFα [interestingly, TNFα has been considered to play a significant role in the pathogenesis of BD either directly or through the induction of other cytokines (14)]. The addition of TNFα to cultures was followed by an increase in MPO secretion only by PMN from controls, suggesting that in the active stage *in vivo* pre-activated PMN could not respond to further stimuli.

Yasui and associates (11) have recently suggested that PTX treatment may improve neutrophil function and clinical symptoms.

In this study we conducted some preliminary experiments in which PMN cultures were treated or not treated with PTX. We found that PTX was able to influence significantly MPO secretion in PMN cultures from patients with active disease.

As shown also in a previous report (7), increased plasma levels were observed in patients with BD. MPO levels however were found to be associated with the disease itself rather than with a particular stage. Indeed, the highest levels were found in the plasma of active patients, although the difference was not statistically significant, suggesting that other variables not evaluated in our study might be operative *in vivo*.

The pathogenetic mechanisms underlying Behçet's disease are multifactorial and still unknown. It is well known, how-

ever, that neutrophil function is enhanced. HLA-B51 molecules themselves may be responsible, at least in part, for the neutrophil hyperfunction (15, 16). In particular, increased serum elastase, and altered chemotaxis, phagocytosis, and superoxide production have been described (2-6) and electronic microscopic studies on biopsy specimens have shown perivascular infiltration with neutrophils and mast cells (17).

Activated neutrophil may release granule constituents, lipid-derived mediators and reactive oxygen intermediates and, as more recently described, secrete cytokines participating in the amplification of inflammation (18, 19).

Many studies have focused on the role of neutrophil activation in the course of vascular damage and it has been demonstrated that MPO is exposed at PMN membrane level upon activation and is then released (10). MPO has been also demonstrated to bind to endothelial cells determining endothelial cell activation and this binding seems to be of importance in the pathogenesis of some ANCA-associated vasculitides (9).

We have recently observed that incubation of endothelial cells with both MPO containing- and AECA containing-plasma from patients with active BD was followed by an increase of E-selectin expression. In the same study a dose-dependent up-regulation of E-selectin expression occurred when endothelial cells were incubated with purified MPO. Other studies have also shown that sera from patients with BD are capable of enhancing the adherence of normal PMN to endothelial cells *in vitro* (20). These observations and our present results may support a putative role of MPO-dependent PMN activation in the pathogenesis of BD vasculitis.

In conclusion, abnormalities of neutrophils, endothelial cells, or both, have been suggested to be responsible for many of the clinical manifestation of BD. Our findings could explain some of the underlying mechanisms of neutrophil function. Hyperactivity of PMN seems to be necessary in the pathogenesis of vasculitis in BD. Indeed, treatment of patients with systemic vasculitis at neutrophil levels seems to reduce the severity of vasculitisc lesions (11, 21) and the *in vitro* release of MPO. The effect of PTX shown in this *in vitro* study and the recent published report on its efficacy in neutrophil chemotaxis and superoxide production (11) indicates that it also would be of interest to learn whether PTX has an *in vivo* effect on MPO secretion.

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