

Increased neutrophil apoptosis during attacks of familial Mediterranean fever

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ABSTRACT Aim

Apoptosis is a programmed form of cell death. Recently much attention has been devoted to the role of apoptosis in rheumatological diseases. We have aimed to analyze apoptosis in the inflammatory pathway of familial Mediterranean fever (FMF).

Methods

26 FMF patients and 12 age and sex matched controls were the subject of the study. Twelve of the patients were analyzed during an FMF attack whereas samples were obtained at least a week after an attack in 14. Four of the patients had renal amyloidosis. Whole blood was treated with ammonium chloride for RBC lysis. Subsequently the cells were stained with propidium iodide and annexin. Neutrophils and lymphocytes were gated separately for analysis by flow cytometry. We have also analyzed cellular Fas and Fas-ligand expression in these cells.

Results

The mean age of the patients was 12.00 ± 3.17 , and was not different than the control subjects. Erythrocyte sedimentation rate and CRP levels were significantly elevated in the attack group as compared to the attack-free group. The mean levels of neutrophil apoptosis in the FMF patients with an attack, attack-free and controls were 12.94 ± 11.78 , 6.60 ± 7.83 and 3.98 ± 4.27 , respectively. Lymphocyte apoptosis in the same groups were 7.84 ± 8.63 , 2.75 ± 2.33 , and 1.22 ± 0.93 , respectively. Neutrophil and monocyte apoptosis was significantly increased during the attack as compared to the controls ($p < 0.05$). However lymphocyte apoptosis was not different between the aforementioned groups. On the other hand, lymphocyte apoptosis was significantly increased in the SLE patients ($p < 0.05$), whereas neutrophil apoptosis was not. Fas staining of neutrophils were not different between the groups ($p > 0.05$). On the other hand the difference between the groups for FasL was significant ($p < 0.05$).

Conclusion

Neutrophil and monocyte but not lymphocyte apoptosis was significantly increased during FMF attacks reminding

us that FMF is an autoinflammation of certain peripheral cells. The increased apoptosis in these patients maybe regarded as a response to clear the unwanted inflammatory cells. On the other hand the increased apoptosis maybe the explanation of the self-limited nature of the FMF attacks. Future studies will enlighten us on the significance of this increased apoptosis in the process of inflammation.

Introduction

Familial Mediterranean fever (FMF) is characterized by self-limited attacks of serosal inflammation. Recent studies have shown that the genetically defective pyrin protein, results in disarrangement in the neutrophil inflammation (1). Why the attacks cease is as intriguing as why the attacks start. The inflammatory attack of FMF almost never results in tissue destruction. This might be due to the nature of the genetic defect *per se*, or due to the transient nature of the stimulus provoking the attack (2). On the other hand this might be due to an increased removal of the inflammatory neutrophils that are responsible for the inflammation. Neutrophil activation is associated with respiratory burst and granule release. The granule contents and respiratory burst may carry the potential to harm the host and initiate more subtle and sustained injury through secondary activation of other cells. Neutrophils are thought to be disposed of through apoptosis. The timing of this process and rate of engulfment of apoptotic cells may be critical in the quantity of inflammatory tissue damage to be produced (3). Thus leukocytes are important in regulating the nature and progression of the inflammatory response. In self-limited diseases, such as FMF, effective apoptosis may be critical in down-regulation of the inflammatory reaction thus preventing chronicity.

Recently much attention has been devoted to the role of apoptosis in the pathogenesis of autoimmune diseases (4). Apoptosis is the programmed form of cell death. Death receptors in the surface of the cell signal 'instructive' apoptosis under certain conditions (5). The best characterized death receptors are

CD95 (also called Fas or Apo1) and tumor necrosis factor receptor (TNFR1). Based on the self-limiting nature of the inflammatory process in FMF, we were interested to analyze apoptosis in FMF to study whether increased apoptotic index of neutrophils plays role in the restriction or down-regulation of inflammation. We also studied lymphocyte and monocyte apoptosis. Furthermore, Fas and FasL expression on neutrophils, and lymphocytes were analyzed as possible effectors of the process.

Patients and methods

The study group consisted of 26 patients diagnosed as FMF in the Department of Pediatric Nephrology and Rheumatology in Hacettepe University, Ankara. All met the criteria for FMF as suggested by the Sheba Medical centers (6). Genetic analysis was performed in 19 of them: in 18 the diagnosis was confirmed genetically by showing mutations of the pyrin gene in two alleles. Only one mutation was defined in the remaining patient.

Twelve age and sex matched children served as controls. Disease controls constituted of 5 patients with systemic lupus erythematosus (SLE). SLE has been chosen because of the well-defined increased apoptosis in the lymphocytes of these patients. Thus we thought SLE would serve as a good 'positive control' for apoptosis. Furthermore we know that the neutrophils are not - at least not the primary- effector cells in SLE whereas the defective protein in FMF is not expressed in lymphocytes. Thus we expected the profile for apoptosis to be different among the cell lineages in FMF and SLE. Ethical consent was obtained for sampling of these children.

Blood samples were obtained at the outpatient clinic. Routine blood samples for acute phase reactants were obtained at the same time. Twelve of the patients were analyzed during an FMF attack. The remaining 14 had no clinical evidence of an FMF attack at the time of sampling. Five of the patients had renal amyloidosis secondary to FMF, only one had developed end-stage renal failure during the study period.

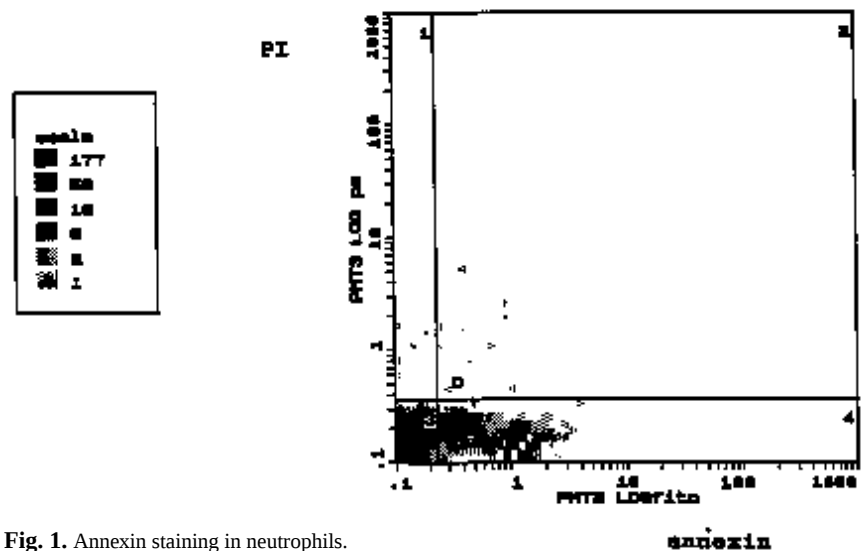


Fig. 1. Annexin staining in neutrophils.

Five of the FMF patients were newly diagnosed and had not received colchicine before. Two of the patients were non-compliant teenagers who had stopped their treatment in the last couple of months. The remaining 19 patients were under colchicine treatment. Four of the seven patients who were not under colchicine treatment were sampled during an attack whereas 3 were not experiencing an attack during sampling.

Flow cytometry (Coulter/Elite) was used for all analyses in this study, including the apoptosis assay by annexin V-binding and for determination of Fas and FasL expression. The analyses were all performed on unstimulated peripheral blood cells.

The phycoerythrin(PE)- or fluorescein isothiocyanate (FITC)-labeled anti-CD95 murine monoclonal antibody, IgG₁ isotype control; purified (unlabeled) anti-CD95L murine monoclonal antibody, FITC-labeled GAM, and isotype control were obtained from Immunotech (Coulter). Annexin-V binding apoptosis kit was also purchased from Coulter.

Whole blood samples were used to avoid the effects of manipulation during isolation of neutrophils. Freshly obtained whole blood samples coagulated by preservative-free heparin were treated with ammonium chloride for RBC lysis. All incubations were performed at room temperature. Briefly, whole blood was subjected to ammonium

chloride for 10 min. In samples with insufficient lysis, a second incubation step for further 5 min was employed. For Fas and FasL expression; following 2 washes in PBS, cells were subjected to 15 min monoclonal antibody incubation. For indirect labeling, as in the case of FasL, stained cells were washed twice in PBS and incubated with GAM for 10 min, then washed twice in PBS and suspended in PBS and fluorescence read immediately.

Apoptosis assay. The lysed samples were washed and cells suspended in binding buffer. Then incubated with 10⁻⁶ l of annexin solution for 10 min and 10⁻⁶ l of propidium iodide (PI) for additional 5 min. The incubations were performed on ice. Following 2 washes in PBS, cells were suspended in binding buffer and analysed immediately by flow cytometry.

For initial set-up control assays were also performed. In selected samples, cells isolated after RBC lysis were cultured in RPMI in 10% FCS; and analysed after 18 hour culture at 37°C, and 5% CO₂. Neutrophils were used as positive controls (Fig. 1) and lymphocytes as negative controls in unstimulated cultures.

Statistical analysis. SPSS software package was used for statistical analysis. Kruskal Wallis and Mann Whitney-u tests were used for non-parametric analysis. Initially Kruskal-Wallis analysis was done and for values > 0.05, two-way comparisons were carried out

Table I. Acute phase reactants in FMF patients*.

	Acute attack (+)	Attack-free
ESR(mm/h)	71.60 ± 40.18**	32.90 ± 16.30
CRP	7.87 ± 2.98**	0.64 ± 1.15
Fibrinogen	562.0 ± 160.6**	319.5 ± 134.78
WBC (mm ³)	14822 ± 5538	8970 ± 2870

* All values mean ± SD

**All p values between the attack and attack-free groups, less than 0.05.

with Mann Whitney-u analysis. If Kruskal Wallis was not significant only p values < 0.008 (= 0.05/6) were considered significant in two way comparisons and in this case confidence intervals (CI) were provided.

Results

The mean age of the patients was 12.00 ± 3.17, and was not different than the control subjects (mean 12.86 ± 3.49). The female:male ratio of the patients and controls were 1.63 and 1.8, respectively. The 12 patients who were sampled during an attack had fever and peritonitis with/without arthritis. Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and fibrinogen levels were significantly elevated in the attack group as compared to the attack-free group (Table I). On the other hand white blood cell counts were not different between the groups of FMF. All these parameters were significantly increased compared to that of controls. The mean levels of neutrophil apoptosis in the FMF patients with an attack, attack-free and controls were 12.94 ± 11.78, 6.60 ± 7.83 and 3.98 ± 4.27, respectively. Neutrophil apoptosis was si-

gnificantly increased during the FMF attack as compared to controls (p = 0.011) (Table II).

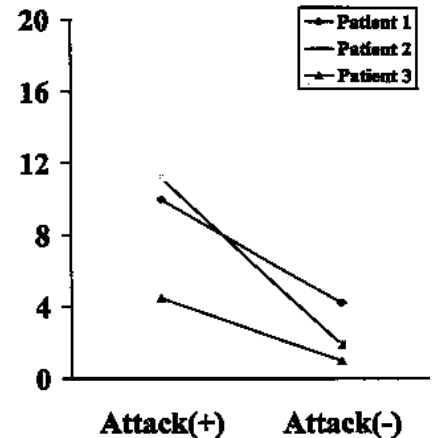
Apoptosis in the monocyte population was also assessed (Table II). Monocyte apoptosis was also significantly increased in FMF patients as compared to controls (p = 0.019). On the other hand lymphocyte apoptosis was not different between the groups (Table II).

Significance was introduced with the lupus patients who had a significantly increased rate of lymphocyte apoptosis (Table II). As disease controls 5 SLE patients were studied with a neutrophil, monocyte and lymphocyte apoptosis of 7.96 ± 9.02, 32.28 ± 28.96 and 11.32 ± 3.26, respectively. Lymphocyte apoptosis was significantly increased as compared to controls (p = 0.008, CI: 1.92 - 18.23) and FMF patients without an attack (p = 0.003, CI: (-)16.14 - (-)0.98). On the other hand neutrophil apoptosis in the SLE patients were not different (p > 0.05).

Apoptosis was not significantly different among the amyloidosis patients, however the number was small to make a judgement.

Fas staining of neutrophils were not different between the groups (Table II) (p > 0.05). On the other hand the difference between the groups for FasL was significantly increased in the FMF patients (p = 0.04) (Table II).

Three patients were sampled during an FMF attack and were followed with samples a week after the cessation of their attacks. Both had marked decrements in their neutrophil apoptosis and neutrophil Fas as well as neutrophil FasL values (Fig. 2).

**Fig. 2.** Decrease of apoptosis, Fas and Fas-ligand expression in peripheral neutrophils in three FMF patients after the cessation of the attack.

Discussion

This study has shown that neutrophil and monocyte apoptosis is increased in FMF patients who were experiencing an attack of the disease. The attacks of FMF are characterized by fever, serositis in the form of peritonitis, synovitis or pleuritis (1, 2). These clinical features are accompanied by an increase in the white blood cell count, ESR, CRP and fibrinogen as acute phase mediators. The attacks are characteristically self-limited. Serositis resolves spontaneously without any sequelae. Thus the local inflammation is somewhat ceased before tissue destruction ensues. This is different than many other rheumatological diseases. The secondary activation of certain molecules and destructive factors such as collagenase may require a more persistent inflammatory trigger to ensue. We thus hypothesized that apoptosis may be important in re-

Table II. Annexin, Fas, Fas-ligand values of the subjects.

		Neutrophil annexin	Lymphocyte annexin	Monosit annexin	Neutrophil Fas	Neutrophil Fas-ligand
FMF Attack(+) (No = 12)	Mean ± SD	12.94 ± 12.78*	7.84 ± 8.63	30.44 ± 25.80*	68.25 ± 28.95	20.33 ± 19.01*
	Median (min-max)	10.6 (1.1-46)	3.9 (0.9-22)	22.95 (4.3-77.1)	75 (16-97)	13.5 (5-63)
FMF Attack(-) (No = 14)	Mean ± SD	6.6 ± 7.8	2.75 ± 2.33*	15.86 ± 18.61	63.31 ± 34.04	11.5 ± 9.26
	Median (min-max)	3.4 (0.3 - 26)	2.6 (0.4 - 6)	8 (1.4 - 60)	61.15 (29 - 99)	11 (2.5 - 21)
SLE (No = 5)	Mean ± SD	7.96 ± 9.02	11.32 ± 3.26*§	32.28 ± 28.99		
	Median (min-max)	2.4 (0.4-18.6)	9.8 (7.8-15.59)	24.1 (8.6-78.2)		
Control (No = 14)	Mean ± SD	3.98 ± 4.27*	1.22 ± 0.93§	9.89 ± 8.21*	57.71 ± 26.15	5.2 ± 5.8*
	Median (min-max)	2.5 (0.8-16)	1.4 (0.2-2.10)	8.3 (0-21)	60 (16-98)	2.4 (2-14)

*, § significant differences, see text for p values and CI.

straining the acute inflammation of FMF. Apoptosis is a physiologic process to dispose of exceeding cells and may be regarded as a normal response as long as the balances are accurate. However, the balance between apoptosis and phagocytosis may indeed be very important in determining the outcome of the inflammation, as has been recently suggested in ANCA(+) vasculitis (7). If there is insufficient clearance or a shift towards necrosis phlogistic events may be overwhelming. Although we have not been able to study the rate of phagocytosis in these patients, the very high rate of apoptosis in FMF, may suggest that this process is an insurance against persistent inflammation.

Although data on neutrophil apoptosis is scarce, mononuclear cells have been well studied in a number of rheumatological diseases (8). Increased apoptosis in the peripheral blood mononuclear cells (PBMC) have been shown in a number of rheumatological diseases including SLE, MCTD, autoimmune vasculitides (8). Lorenz *et al.* (8) have suggested that this might be due to an *in vivo* pre-activation of PBMC and *in vitro* growth factor withdrawal.

In this study the disease control group was SLE patients. We have shown that lymphocyte apoptosis was increased in the SLE patients, whereas it was not different than controls in the FMF patients. On the other hand neutrophil and monocyte apoptosis was significantly increased in FMF patients as compared to controls. This once again confirms that FMF is a disease of neutrophil, and monocyte inflammation. In a recent report, it has been confirmed that pyrin, which is the protein that carries mutations in FMF, is expressed on the monocytes as well (9).

Neutrophil apoptosis was also increased in SLE patients however this did not reach statistical significance. On the other hand Courtney *et al.* (10) have reported a significant increase in the neutrophils of SLE patients. This difference may be due to the varying

disease activity or neutrophil counts of the patients or methodology. In fact Courtney *et al.* (10) have suggested that neutropenia in these patients might be aggravated by the increased apoptosis that they had observed.

It was noteworthy that, in the attack-free FMF patients, the apoptosis of all peripheral blood cells varied in a wide range, especially in the monocyte population. This might be due to the subclinical inflammation that may be present in some FMF patients.

Fas was not significantly different during the attack and this may require further explanation. Apoptosis may be activated by factors other than Fas, since regulation of apoptosis is very complex. The relation between apoptosis and Fas expression is probably influenced by multiple factors (4, 10). The lack of a significant difference between the patients and controls for Fas expression, does not indicate that the patient neutrophils were not susceptible for apoptosis. It may be speculated that, in healthy persons, in the resting state although the Fas expression is present, the apoptosis stimulus (*i.e.* FasL) is absent; or antiapoptotic molecules, Bcl-2 being one, or interactions may be limiting excessive apoptosis. However, in the case of FMF, it may be speculated that the apoptosis key is turned on and the execution takes place, either by apoptosis-inducing molecules (such as FasL as in our study) or down-regulation of anti-apoptotic factors, which we did not study. In fact we have found increased expression of FasL in the patients. Furthermore additional regulatory input may be needed within the Fas pathway itself (4, 10).

A drawback of our study is that we were unable to study another control group such as gout, with again 'limited' inflammatory attacks. It would also be very interesting to study FMF patients who develop more chronic manifestations of the disease such as vasculitis and to analyze the pattern of apoptosis in a cross sectional manner

during an attack. In this preliminary study, neutrophil and monocyte, but not lymphocyte apoptosis was significantly increased during FMF attacks reminding us that FMF is an auto-inflammation affecting certain cells. The increased apoptosis in these patients may be regarded as a secondary response to clear the unwanted inflammatory neutrophils. On the other hand the increased apoptosis may be the explanation of the self-limited nature of the FMF attacks. Future studies will enlighten us on the significance of this increased apoptosis in the process of inflammation.

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