Distinctive effects of G-CSF, GM-CSF and TNFα on neutrophil apoptosis in systemic lupus erythematosus

D.J. Armstrong[1], E.M. Whitehead[1], A.D. Crockard[2], A.L. Bell[3]

[1]United Hospitals Trust, Antrim, Co Antrim; [2]Regional Immunology Laboratory, Royal Victoria Hospital, Belfast; [3]Queen's University of Belfast, Belfast, UK.

Abstract

Objective

To investigate the influence of culture with G-CSF, GM-CSF and TNFα on neutrophil apoptosis, comparing neutrophils from SLE patients with rheumatoid arthritis (RA) patients and healthy control subjects.

Methods

Neutrophils were isolated from SLE (n=10), RA (n=10) and healthy control subjects (n=10), and cultured with two different concentrations of G-CSF, GM-CSF and TNFα. Proportion of apoptotic neutrophils at T=0, T=2hrs and T=24hrs was measured using FITC-labelled annexinV and flow cytometry.

Results

Significantly more neutrophils were apoptotic at T=0 in the SLE subjects than in the other groups (median, range - Control 3.5% (0.3-7.9) SLE 9.5% (2.9-29.1) RA 3.0% (0.4-23.0) p<0.05). Following culture for 24 hours with 1ng/ml G-CSF, the proportion of apoptotic neutrophils from SLE subjects was significantly increased (median, range = 51.6% (27.0-84.0) without G-CSF v 66.8% (31.8-89.2) with G-CSF, p<0.05). This was not observed with RA or control subjects, in whom the trend was towards inhibition of apoptosis. Similar trends were seen with GM-CSF. There was significant induction of apoptosis in SLE neutrophils after 2 hr culture with 1ng/ml TNFα (median, range = 2.3% (0.1-8.0) without TNFα v 5.2% (1.0-22.4) with TNFα). No significant change was seen in the other groups. There was an inverse correlation between total neutrophil count and the degree of induction of apoptosis by G-CSF and GM-CSF, determined at a range of time-points and cytokine concentrations

Conclusions

Neutrophils from SLE patients display resistance to the apoptosis-inhibiting effects of G-CSF and possibly GM-CSF, and appear more susceptible to the apoptosis-inducing action of TNFα, the greatest resistance being observed in the more neutropenic patients.

Key words

SLE, neutrophils, apoptosis, G-CSF, GM-CSF, TNFα, rheumatoid arthritis.

David J. Armstrong MD, MRCP, Clinical Research Fellow; Esme M. Whitehead MD, BSc, FRCP, DCH, Consultant Rheumatologist; Alistair D. Crockard PhD, FRCPath, Consultant Clinical Scientist; Aubrey L. Bell MD, FRCP, Consultant Rheumatologist and Senior Lecturer.

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Please address correspondence and reprint requests to: Dr. David J. Armstrong, Specialist Registrar, Department of Rheumatology, Musgrave Park Hospital, Stockman's Lane, Belfast BT9 7JB, UK. E-mail oswald17727@hotmail.com

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Introduction

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease characterised by circulating autoantibodies, the majority of which are directed against components of the cell nucleus (1, 2). Apoptosis is a tightly controlled process that effects cell death without the release of inflammatory mediators (3). However there is increasing evidence pointing to apoptotic cells as a source of autoantigens in SLE, leading to a T-cell regulated humoral autoantibody response (4-7), and establishing disturbance of apoptosis as central to the pathogenesis of SLE.

Our group has demonstrated increased circulating apoptotic neutrophils in SLE, correlating to levels of anti-ds-DNA antibody and clinical measures of disease activity (8). An increase in the number of apoptotic neutrophils in the circulation could arise as a result of increased rates of apoptosis, and/or decreased clearance of apoptotic cells (9). There is evidence suggesting abnormal clearance mechanisms for apoptotic cells in SLE (9-11). Neutrophils from SLE patients have also been shown to demonstrate increased nuclear DNA damage (12), in line with an increased rate of apoptosis. Altered sensitivity of lupus neutrophils to cytokines and growth factors would provide one possible explanation for altered susceptibility to apoptosis.

Granulocyte Colony Stimulating Factor (G-CSF) and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) are key survival factors for granulocytes (13). G-CSF and GM-CSF, at several time intervals and at a range of concentrations, have been shown to inhibit neutrophil apoptosis in healthy subjects *in vitro* (14). We have observed significantly raised levels of serum G-CSF in SLE patients with neutropenia (8), raising the possibility of a relative insensitivity to the effects of G-CSF as an explanation for the increased circulating apoptotic neutrophils in SLE.

Tumour Necrosis Factor Alpha (TNFα) is a cytokine responsible for a large and functionally diverse range of signalling molecules within cells, including control of apoptosis (15, 16). It can have both pro- or anti-apoptotic effects depending on time and concentration (17, 18).

In this study we have sought to further characterise neutrophil apoptosis in SLE by examining the influence of relevant cytokine factors G-CSF, GM-CSF and TNFα on apoptosis in cultured cells from lupus patients and controls subjects.

Materials and methods

Patients and controls

SLE patients (n = 10, all female, mean age = 46.5 years, range = 28-63) who fulfilled at least four of the Revised American Rheumatology Association (ARA) criteria for SLE (19) were recruited from rheumatology outpatient clinics and inpatient wards. RA patients (n = 10, all female, mean age = 60.3 years, range = 50-83) were recruited as disease controls from the same sources, and normal healthy subjects (n = 10, 9 female, 1 male, mean age = 41.0 years, range = 25-69) were recruited from hospital staff. The same subjects were used for both the G-CSF and GM-CSF culture experiments. A different group of SLE patients, including some of those involved in the G-CSF and GM-CSF studies, was used for the TNFα experiments (n = 10, all female, mean age = 43.5 years, range = 28-62).

Written informed consent was obtained from all subjects. Ethical approval was granted by the Queen's University Belfast medical ethical committee.

Disease activity was measured for the SLE patients using the Systemic Lupus Activity Measure (SLAM) score and British Isles Lupus Assessment Group (BILAG) score (20), and for the RA patients using the modified Disease Activity Score (DAS28) (21). Full Blood Count, erythrocyte sedimentation rate (ESR), C-Reactive Protein (CRP) and autoantibody titres were also measured for each of the patients, and their current drug therapy recorded.

For the neutrophils cultured with G-CSF or GM-CSF, 5 out of 10 in each of the SLE and RA groups were on immunosuppressive drugs excluding hydroxychloroquine (SLE patients – azathioprine 3, methotrexate 1, cyclophos-

phamide 1; RA patients – methotrexate 3, myocrisin 1, cyclosporin 1).
Five of the SLE subjects and 5 of the RA subjects were taking prescribed oral corticosteroids (mean daily dose 5.3 mg and 6.7 mg respectively).

Neutrophil isolation
Neutrophils were isolated from 20 ml samples of heparinised peripheral blood using techniques similar to those already described by our group (9). Briefly, the samples were sedimented through a 6% dextran solution (DextranT500 Amersham Pharmacia Biotech) for 15 minutes to remove erythrocytes, and then washed with phosphate buffered saline (PBS). The leucocyte pellet was resuspended and laid on a discontinuous Percoll (Amersham Pharmacia Biotech) density gradient. After centrifugation, the neutrophil layer was aspirated and washed in PBS.

Culture experiments
Recombinant G-CSF and GM-CSF (R&D Systems) and TNFα (BD Biosciences) were reconstituted and diluted to the desired concentration with sterile de-ionised water. Neutrophils were counted and resuspended at a concentration of $1x10^6$ in RPMI medium supplemented with 100U/ml penicillin (Sigma) 100μg/ml streptomycin (Sigma) and 2mM L-glutamine (Life Technologies).
A pilot study was performed (data not shown) to establish the range of concentrations over which detectable changes in percentage of apoptotic neutrophils could be determined. G-CSF, GM-CSF or TNFα was added to the supplemented RPMI at concentrations from 0.01 μg/ml to 100 μg/ml, and the neutrophils were incubated for 2 or 24 hours at 37°C in a 5% CO_2 atmosphere before determination of annexin V positivity. For the main study, concentrations of 0.1 μg/ml and 1 μg/ml of each of the three cytokines was chosen. This was within the range of concentrations at which G-CSF and TNFα are known to be present in human serum. For this study, neutrophils were again cultured for 2 or 24 hours at 37°C in a 5% CO_2 atmosphere, before annexin V labelling and flow cytometry.

Annexin V staining
Annexin staining was performed using Annexin V from R&D systems. $1x10^6$ neutrophils were suspended in a solution of 10 μl of binding buffer and 79 μl sterile distilled water, and incubated for 15 mins with 1μl annexin V-FITC (25μg/ml) and 10 μl propidium iodide (all R&D) at room temperature in the dark, according to manufacturer's instructions. Cells were analysed immediately using a Coulter EPICS ELITE flow cytometer which was standardised for inter-run variability by calibration with Immunocheck Fluorospheres (Coulter Corporation). Neutrophils were gated appropriately after analysis of side and forward scatter signals, and the proportion of apoptotic neutrophils determined (22, 23).

Statistical analyses
The Wilcoxon signed rank test and Spearman rank correlation were used as appropriate.

Results
The same group of subjects (SLE, RA and controls) was used for the G-CSF and GM-CSF experiments, and a different group of SLE patients for the TNFα work. The mean values for the clinical and laboratory parameters in the SLE subjects are shown below (Table I).
Our study populations were drawn from patients in whom musculoskeletal disease features prominently: all 10 lupus patients fulfilled the arthritis criterion of the ARA Diagnostic Criteria for SLE (20), 6 fulfilled at least one of the skin criteria and 5 the haematological criterion. By contrast, only one patient in the study had biopsy-proven SLE nephritis.
The mean DAS score for the RA patients in the CSF experiment was 6.21 (5.38–7.56), and almost identical for the TNFα experiment subjects.

Neutrophil apoptosis – Basal levels
The percentage of circulating apoptotic neutrophils (positive for Annexin V) was measured at T = 0 to establish basal levels. The number of apoptotic neutrophils in the SLE patients was significantly greater ($p < 0.05$) than either the RA patients or control subjects (Table II).
There was no significant difference between the percentage of apoptotic neutrophils in any of the groups at T = 2 or at T = 24. There was however a fall from the T = 0 levels in the percentage of apoptotic neutrophils in the gated region at T= 2 in the SLE and RA groups. We do not believe that this represents

Table I. Clinical and laboratory data for SLE patients in G-CSF and GM-CSF study, and TNFα study.

	G-CSF and GM-CSF study		TNFα study	
	Median	Range	Median	Range
SLAM	8.0	3-27	11.0	4-27
BILAG	11.0	6-21	11.0	8-21
SLICC	1.0	0-9	1.0	0-9
ESR (mm/hr)	24.0	5-140	28.0	8-140
CRP (mg/l)	5.7	0-115	8.7	0-49
Total WCC (x 10^6/ml)	6.7	3.6-13.2	5.4	3.6-13.4
Neutrophils (x 10^6/ml)	4.0	2.6-8.6	3.7	2.1-12.2
Lymphocytes (x 10^6/ml)	1.2	0.6-4.4	0.9	0.6-2.0

Table II. Spontaneous neutrophil apoptosis in culture (percent of Annexin V positive cells).

	Controls (median, range)	SLE (median, range)	RA (median, range)
T=0	3.5 (0.3-7.9)	9.5 (2.9-29.1)*	3.0 (0.4-23.0)
T=2	3.6 (1.0-12.6)	2.6 (0.2-4.9)	1.6 (0.2-3.8)
T=24	65.0 (43.3-83.3)	52.2 (27-78.5)	57.3 (27.9-81.0)

* SLE v control or RA subjects ($p < 0.05$) at T=0.

Table III. Percentage of apoptotic neutrophils from SLE, RA and healthy control subjects after culture for 2 hr and 24 hr with two different concentrations of G-CSF.

	T=2	T=2 0.01 ng/ml G-CSF	T=2 1 ng/ml G-CSF
SLE (median, range)	1.9 (0.2-8.0)	4.7 (1.1-6.7)	2.5 (1.0-4.6)
RA (median, range)	1.4 (0.2-3.8)	2.3 (0.3-5.5)	1.5 (0.2-3.7)
Control (median, range)	2.3 (1.0-12.6)	3.2 (1.5-16)	2.6 (0.8-5.6)
	T=24	T=24 0.01 ng/ml G-CSF	T=24 1 ng/ml G-CSF
SLE (median, range)	51.6 (27.0-84.0)	65.8 (24.2-87.8)	66.8 (31.8-89.2)*
RA (median, range)	60.7 (27.9-81.0)	60.7 (26.9-74.8)	55.5 (26.6-73.4)
Control (median, range)	65.1 (43.3-83.3)	65.1 (44.8-80.2)	69.8 (35.4-86.6)

$p<0.05$, SLE neutrophils cultured for 24 hr with 1 ng/ml G-CSF v SLE neutrophils cultured for 24 hr with no cytokine.

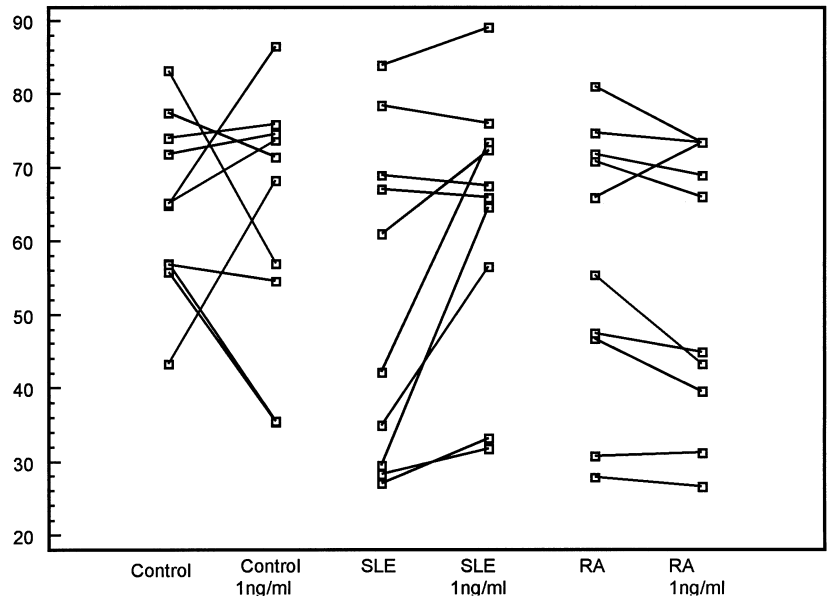

Fig. 1. Dot and Line diagram, showing change in percentage of apoptotic neutrophils after culture for 24 hours with and without 1ng/ml G-CSF in subjects from three patient groups.
SLE neutrophils cultured for 24 hours v SLE neutrophils cultured for 24 hours with 1 ng/ml G-CSF, $p < 0.05$.

Table IV. Percentage of apoptotic neutrophils from SLE, RA and healthy control subjects after culture for 2 hr and 24 hr with two different concentrations of GM-CSF.

	T=2	T=2 0.01 ng/ml GM-CSF	T=2 1 ng/ml GM-CSF
SLE (median, range)	1.9 (0.2-8.0)	2.5 (0.2-4.4)	2.0 (0.3-5.0)
RA (median, range)	1.4 (0.2-3.8)	2.1 (0.1-3.3)	1.3 (0.3-3.4)
Control (median, range)	2.3 (1.0-12.6)	2.4 (1.7-6.7)	2.1 (1.2-10.6)
	T=24	T=24 0.01 ng/ml GM-CSF	T=24 1 ng/ml GM-CSF
SLE (median, range)	51.6 (27.0-84.0)	61.2 (28.6-87.2)	60.9 (29.7-84.5)
RA (median, range)	60.7 (27.9-81.0)	57.7 (22.2-72.4)	62.2 (24.2-71.1)
Control (median, range)	65.1 (43.3-83.3)	66.9 (42.1-85.3)	62.3 (30.2-81.0)

revival of apoptotic cells, as once apoptosis has proceeded to the degree that phosphatidylserine is expressed on the outer membrane of cells (and cells are therefore annexin V 'positive' and are designated as apoptotic in this study) there is no evidence to suggest that they can become viable again.

Instead we believe that many of the neutrophils which were apoptotic at time T = 0 have become necrotic and disappeared from the neutrophil 'gate' on flow cytometry at T = 2, while many of those neutrophils labelled as 'apoptotic' at T = 2 may have been viable at T = 0, and entered apoptosis during the first 2 hours of culture.

Neutrophil apoptosis – Following culture with G-CSF, GM-CSF and TNFα

G-CSF. There was a statistically significant increase in the number of apoptotic neutrophils in the SLE population ($p < 0.05$) when cultured with 1ng/ml of G-CSF for 24 hours, as compared with the culture with no cytokine. This was not seen in the other two groups, in which the trend was towards inhibition of apoptosis (Table III, Fig. 1).

GM-CSF. There was no statistically significant change in any group, although in the SLE population the trend was towards induction of apoptosis after 24 hours and in the other two groups the trend was towards inhibition (Table IV).

TNFα. There was a statistically significant increase in the percentage of apoptotic neutrophils after 2 hour culture with 1ng TNFα in the SLE not seen in either the control subjects or RA groups. 24 hour culture with 0.01ng/ml TNFα caused statistically significant inhibition of apoptosis in the RA group, but not in the control or SLE subjects. 1 ng/ml TNFα caused significant inhibition of apoptosis in the SLE cells ($p < 0.05$) and in the Control and RA groups ($p < 0.01$) (Table V).

Correlations with clinical and laboratory data

For each subject, the response to G-CSF, GM-CSF and TNFα at each time interval and each concentration was expressed as a positive or negative percentage of the proportion of apoptotic

Table V. Percentage of apoptotic neutrophils from SLE, RA and healthy control subjects after culture for 2 hr and 24 hr with two different concentrations of TNFα.

	T = 2	T = 2 0.01 ng/ml TNFα	T = 2 1 ng/ml TNFα
SLE (median, range)	2.3 (0.1-8.0)	3.7 (0.9-30.6)	5.2 (1.0-22.4)*
RA (median, range)	1.6 (0.2-5.5)	1.6 (0.2-5.4)	2.6 (0.8-5.3)
Control (median, range)	2.3 (1.0-5.4)	2.9 (1.3-18.5)	5.7 (1.8-11.6)
	T = 24	T = 24 0.01 ng/ml TNFα	T = 24 1 ng/ml TNFα
SLE (median, range)	51.6 (27.0-87.3)	60.8 (33.5-80.3)	39.7 (20.1-68.4)**
RA (median, range)	48.5 (27.9-71.9)	47.8 (14.7-69.6)	13.6 (3.4-50.4)#
Control (median, range)	68.5 (43.3-90.6)	70.7 (35.5-88.7)	47.7 (7.7-86.2)##

*$p < 0.05$, SLE neutrophils cultured for 2 hrs without TNFα v SLE neutrophils cultured with 1 ng/ml TNFα.
**$p < 0.05$, SLE neutrophils cultured for 24 hrs without TNFα v SLE neutrophils cultured for 2 hrs with 1 ng/ml TNFα.
#$p < 0.01$, RA neutrophils cultured for 24 hrs without TNFα v RA neutrophils cultured for 24 hrs with 1 ng/ml TNFα.
##$p < 0.01$, Control neutrophils cultured for 24 hrs without TNFα v Control neutrophils cultured for 24 hrs with 1 ng/ml TNFα.

Table VI. Correlation between response to 2 hr culture with G-CSF or GM-CSF and total neutrophil count or CRP (r value, p value).

	Neutrophil count	CRP
G-CSF 0.01ng/ml	r= -0.69, $p < 0.05$	r= -0.88, $p < 0.05$
G-CSF 1ng/ml	r= -0.66, $p < 0.05$	r= -0.88, $p < 0.05$
GM-CSF 0.01ng/ml	r= -0.55, $p = 0.09$	r= -0.75, $p < 0.05$
GM-CSF 1ng/ml	r= -0.67, $p < 0.05$	r= -0.76, $p < 0.05$

neutrophils in the sample without cytokine at the same time interval.

There was a negative correlation between the SLAM score and the response to GM-CSF 0.01 μg/ml at 24 hours ($r = -0.83$, $p < 0.05$) and 0.01 μg/ml G-CSF at 2 hours ($r = -0.7$, $p < 0.05$). There was however no clinically relevant relationship between response to G-CSF, GM-CSF or TNFα and SLAM or BILAG scores, or with steroid or immunosuppressive therapy.

There was a statistically significant negative correlation between neutrophil count and apoptotic response to G-CSF at 2 hours for both 0.01 μg/ml and 1 μg/ml (Table VI). Patients with the lowest neutrophil counts were those in whom neutrophil apoptosis was induced most easily by G-CSF.

There was a similar statistically significant negative correlation between response of neutrophils in SLE patients after 2 hour culture with 1μg/ml GM-CSF and overall neutrophil count (Table VI).

Those SLE patients in whom there was the greatest induction of apoptosis by G-CSF and GM-CSF had the lowest levels of CRP (Table VI).

There was no correlation between response to any of the three cytokines and the DAS score in the RA patients.

Discussion

Our results show that neutrophils from SLE patients respond less well to the apoptosis-inhibiting action of 1μg/ml G-CSF after 24 hours compared with neutrophils from healthy individuals and rheumatoid arthritis patients, and raise the possibility of a similar response to 1 μg/ml TNFα. The same trend was identified for both cytokines at the lower 0.01 μg/l concentration. We have also demonstrated a pro-apoptotic effect of 1μg/ml TNFα in 2 hour culture not seen in healthy and RA neutrophils, and a similar trend at the 0.01 μg/ ml concentration. There was a trend towards the same response at both concentrations of GM-CSF as that seen in G-CSF, but statistical significance was not achieved. There is a significant negative correlation between neutrophil count and the proportional increase in apoptotic neutrophils after culture with both concentrations of G-CSF and the higher concentration of GM-CSF, and a strong trend towards correlation with GM-CSF after 24 hour culture. There was no relationship between the response to cytokines and either steroid use or immunosuppressive drug use.

Our work suggests that lupus neutrophils may resist the apoptosis-inhibiting action of G-CSF and TNFα after 24 hour culture, and may be more sensitive to the apoptosis-inducing action of TNFα in early culture. Neutropenic SLE patients appeared to be the most resistant to the apoptosis inhibiting effects of G-CSF and GM-CSF.

Resistance to apoptosis inhibition may contribute to the increased number of circulating apoptotic neutrophils in SLE. It would provide a possible explanation for the increased levels of G-CSF we have described in neutropenic lupus patients (8).

Induction of SLE has been described in RA patients who have received TNFα blocking drugs (24). Blocking of the effects of TNFα in patients who are already resistant to the cytokine could lead to a dramatic increase in the number of apoptotic neutrophils in the circulation, providing a source of autoantigens for the humoral autoantibody response of SLE. Similarly, induction of a flare of disease activity in SLE patients receiving recombinant G-CSF has been described (25); increased neutrophil apoptosis in response to G-CSF might also contribute to increased SLE activity.

There are a number possible explanations for resistance to apoptosis control in SLE neutrophils.

Cell population shift

Neutrophils in the circulation of SLE patients might be less mature than those in the healthy and RA subjects. It

has been suggested that SLE patients do indeed exhibit immature neutrophils in the circulation and display up-regulation of granulopoesis-related genes, especially those related to interferon, which is assuming a central role in the pathogenesis of SLE (26). Other studies have described abnormal response to G-CSF and GM-CSF in immature neutrophils (27), in particular resistance to normal induction of apoptosis.

Other influences on apoptosis, e.g. autoantibodies

Induction of apoptosis by anti-dsDNA autoantibodies in neutrophils from healthy control subjects has been described (28). It is possible that anti-dsDNA antibody in the sera of the SLE patients might directly influence rates of neutrophil apoptosis, and indeed we have previously shown that the number of apoptotic neutrophils in SLE is proportional to serum levels of anti-dsDNA antibody (9).

Signaling transduction defects

G-CSF acts via interaction with its cell surface receptor G-CSFR, activating Janus Kinases (JAK) 1 and 2, and transcription factors including STAT1, STAT3 and STAT5. Suppressors of cytokine signalling (SOCS) regulate signal transduction by binding to and inhibiting JAK activity, and SOCS3 production has recently been shown to be induced by G-CSF (29). The Bcl family of proteins are also important in apoptosis control, and evidence exists suggesting that the protein Mcl-1 may be responsible for resistance to apoptosis in a variety of tumours, and vital in the control of apoptosis in normal neutrophils (30).

Abnormalities at post-translational, transcriptional and even genomic levels of protein kinases and G-proteins have been shown to have an important clinical influence in apoptosis resistance in other conditions (31), and, together with SOCS and Mcl-1, would be prime targets for further investigation in SLE.

Other growth factor or cytokine effects not studied

A range of other cytokines has been implicated in the control of apoptosis, and may be relevant in neutrophils in SLE. For example interleukin-10 (IL-10), which activates B-lymphocytes and inhibits T-lymphocyte apoptosis, is found in elevated levels in SLE. (31). It is also known to modulate neutrophil function (33), although its effects on neutrophils in SLE have not been studied. Other interleukins may be involved; for example, IL-3, IL-6 and IL-15 are known to mediate apoptosis in neutrophils (34).

Experimental caveats and drug effects

We have studied a relatively small group of patients, who had in some cases quite different disease profiles, although all suffered from lupus arthritis. They were also being treated with a variety of different drugs including immunosuppressives and steroids. Nevertheless, SLE is a heterogeneous disease, and the sample reflected the type of patient encountered in a standard rheumatology outpatient department rather than specialised subgroups studied elsewhere. Steroid treatment is known to inhibit apoptosis, but the fact that the response to the CSFs was opposite in the SLE and RA subjects, who were taking an equivalent amount of prednisolone, makes it unlikely that use of steroids significantly influenced the results.

Experiments measuring G-CSF, GM-CSF and TNFα receptor expression would clarify further the role of cytokine resistance in increased neutrophil apoptosis, and comparison with serum levels of G-CSF, GM-CSF and TNFα might also be important, although the relevance of the serum value with that in the microenvironment of the neutrophil is open to debate.

In conclusion, this study provides evidence that neutrophils from SLE patients may resist the anti-apoptotic effects of G-CSF and GM-CSF, and may be more susceptible to the pro-apoptotic effects of TNFα, than either healthy controls or rheumatoid arthritis patients. The effect is particularly seen in those with low neutrophil counts, and does not appear related to overall disease activity or therapy. This may have implications for the pathogenesis of SLE, and the treatment of neutropenia in SLE patients with recombinant G-CSF.

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