The Role of Apoptotic Proteins in Patients with Systemic Lupus Erythematosis

[f]Faris Q. Alenzi

Department of Med. Lab. Sci., College of Appl. Med. Sci., Al-Kharj University, Al-Kharj, Saudi Arabia

Lymphocytes and granulocytes from healthy donors and SLE patients were used to investigate the role of Fas/FasL system in the pathogenesis of systemic lupus erythematosus (SLE). Determination of lymphocyte subpopulations was carried out by flowcytometry. Fas and FasL expression in lymphocytes and granulocytes were measured by immunofluorescence. Apoptotic cells were measured by TUNEL assay. sFas in the plasma was measured by ELISA. Thirty five normal blood donors and 45 SLE patients were selected for this study. This study was carried out between 2005 and 2007. The number of peripheral leukocytes undergoing apoptosis in SLE patients was greater than those of healthy donors. The degree of DNA damage in lymphocytes and granulocytes of SLE patients was much higher than those of healthy donors (*P*<0.05 & *P*<0.5, respectively). Additionally, a positive relationship was seen between the level of apoptotic lymphocytes and the dosage of prednisolone used for treatment of SLE (r=0.6). The level of Fas and FasL expression on different lymphocyte subpopulations increased depending on the activity of the disease (*P*<0.5 & *P*<0.05, respectively). There was an inverse correlation between the sFas and the stage of disease. Finally, we have demonstrated a relationship between the titre of autoantibodies and the degree of DNA damage in lymphocytes and granulocytes (r=0.7 & r=0.6, respectively). In conclusion, Fas and FasL are likely to play an important role in the pathogenesis of SLE. The real value may be used as a predictor for the activity of disease as well.

Systemic lupus erythematosus (SLE) is a systemic autoimmune disorder which presents with a wide spectrum of clinical and immunological abnormalities. The aetiology of SLE is unknown (Herrmann *et al.*, 1998). SLE is characterized by polyclonal activation of B lymphocytes and the production of a wide range of antibodies to nuclear and cytoplasmic antigens. The persistence of autoreactive B and T lymphocytes is thought to be responsible for hypergamma-globuinaemia and autoantibody production in SLE (Rose *et al.,* 1997, Dean *et al.,* 2002, Fernandez and Perl 2008, Reed *et al.,* 2008a, Reed *et al.,* 2008b).

The Fas pathway is one of the major routes for triggering apoptosis. Cross-linking of Fas by Fas ligand (FasL) triggers apoptotic cell death with the characteristic cytoplasmic and nuclear condensation and DNA fragmentation (Itoh *et al.,* 1991). A possible role of apoptotic cells in the pathogenesis of SLE has been suggested (Mountz *et al.,* 1994, Andrade *et al.,* 2000). Indeed, in SLE patients, there was an increase in the levels of apoptotic lymphocytes (Perniok *et al.,* 1998). Moreover, it has been demonstrated that, during the apoptotic process, autoantigens are exposed at the outer surface of the apoptotic cell. This might allow the development of autoantibodies directed to intracellularly localized antigens (Casciola-Rosen *et al.,* 1994). It was shown that the injection of large numbers of apoptotic cells induced

[f]Corresponding Author:
P.O. Box 422 Al-Kharj 11942, Saudi Arabia.
Fax: (+966 1) 545 4586
E-mail: fqalenzi@ksu.edu.sa

autoimmunity in animal models (Mevorach *et al.,* 1998)

There is a huge dispute on the role of apoptosis on SLE. Lymphocytes from SLE patients display an increase rate of spontaneous apoptosis in vitro compared to healthy donors (Emlen *et al.,* 1994, Lorenz *et al.,* 1997). It has been suggested that this is due to the increase *in vivo* activation of mononuclear cells in SLE resulting in an increased activation-induced cell death. Whether the level of apoptosis in SLE patients is in balance with increased lymphocyte activation is, however, not clear as many factors can influence apoptosis including; soluble Fas (sFas), Bc1-2 and antibodies against FasL have been shown to be elevated in SLE patients (Cheng *et al.,* 1994, Tokano *et al.,* 1996, Rose *et al.,* 1997, Li *et al.,* 2008, Munoz *et al.,* 2008, Martínez *et al.,* 2008, Mackay *et al.,* 2008, Silverman *et al.,* 2008, Telegina *et al.,* 2009).

The aim of this study was to determine the role of Fas/FasL system in the pathogenesis of SLE. This was achieved based on by the assessment of the level of Fas and FasL expression on different leukocytes subpopulations, sFas in serum from SLE and the damage in leukocytic DNA upon disease activity.

Materials and Methods

Characteristic of Patients

The study included 45 with SLE (ages 19-54 years) and the control group consisted of 35 apparently healthy donors (ages 18–35 years) (blood donors). This study was conducted in three governmental hospitals in the eastern region of Saudi Arabia between 2005 and 2007. The diagnosis and stages of SLE were established using the criteria of the American Rheumatism. The study protocol was approved by the university Research Ethics Committee and written informed consents were obtained in all cases.

Isolation of mononuclear and polymorphonuclear leukocytes from human peripheral blood

Mononuclear cells (MNCs) were obtained by density gradient centrifugation over Ficoll-Hypaque (1.077 g/ml) (Nyegaard). After washing with hank's buffered salt solution (HBSS, GibcoBRL), cells were resuspended in HBSS. Granulocytes were prepared from the fraction of cells settled at the bottom of the test tube, through Ficoll centrifugation. Granulocytes were purified from erythrocytes by the standard osmotic shock method in an iced water bath (Paul 1993). To calculate the concentration of nucleated cells, the sample was diluted with 3% acetic acid to lyse red blood cells and an aliquot of cell suspension placed in a hemocytometer counting chamber.

Analysis of Lymphocyte Subpopulations

The analysis of lymphocyte subpopulations was carried out by flowcytometry (Beckman coulter, USA). Suspended lymphocytes were stained with two flourochromes; Fluorescein isothiocyanate (FITC) conjugated with monoclonal antibodies specific to CD25 and CD20 and phycoerythrin (PE) conjugated with monoclonal antibodies specific to CD3, CD4, CD8, and CD19. All conjugated antibodies were obtained from Serotec (NJ, USA).

Estimation of DNA Damage

The DNA structure of human leukocytes was investigated by a direct fluorometric method measuring the rate of unwinding double-stranded DNA in alkali according to the manufacturer's instructions (R&D, MN, USA). Briefly, fluorometric analysis carried out by spectrofluorometer using ethidum bromide as fluorescent. The percentage of double-stranded DNA remained after partial alkali treatment was calculated by the formula: D%= (P-B) / (T-B) x100, where: B= background fluorescence of sample; T= the total fluorescence; P= the fluorescence of DNA after standard alkali lysis. The value of D represents percentage of double-stranded DNA remained after partial alkali treatment. 100%-D% value represents the percentage of double-stranded DNA which underwent unwinding. It can be used as a sensitive measure of strand breaks i.e., percentage of damaged double-stranded DNA.

Determination of Quantity of Apoptotic Leukocytes

The quantity of apoptotic cells was determined both in fresh intact peripheral blood leukocytes and

granulocytes after incubation, in RPMI-1640 culture medium supplemented with 50 IU/ml penicillin , 50 mg/ml of streptomycin, 10% PBS (pH 7.4) and 2mM glutamine, in a CO_2 incubator at 5 % CO2 for 18hr and 36hr. To estimate the degree of apoptosis, cells were pelleted at 200 xg rpm for 5 minutes then resuspended in Dulbecco's MEM (Gibco) at a concentration of $2x10^5$/ml. Aliquots of 100 µl volumes were cytocentrifuged onto cleaned microscope slides at 40 xg for 10 minutes (Shandon Cytospin 2; Shandon, Pittsburgh, PA, USA). Slides were air dried overnight, rehydrated in TBS for 15 minutes at room temperature and then re-dried. Cells were covered by a 5 ml droplet of protein K diluted 1:100 in 10mM Tris (pH 8), incubated 5 minutes at room temperature then dipped three times into TBS and dried. The specimen was covered with 100 µl of supplied equilibration buffer (FragEL™ DNA Fragmentation Detection kit; Calbiochem, Nottingham, UK) and incubated for 30 minutes at room temperature. Excess buffer was poured off and freshly prepared TdT labelling mixture (3 µl TdT enzyme in 57 µl TdT labelling reaction mix (FragEL kit; Calbiochem, Nottingham, UK) was layered onto the cells. Slides were incubated at 37°C in humidified chamber for 1.5 hour, then washed x3 in TBS at room temperature. A cover slip was applied over mounting medium (Frag EL DNA Fragmentation Detection kit) and sealed with nail varnish to prevent evaporation. At least 500 cells from randomly selected fields were scored by fluorescent light microscopy (494 nm). Viable cells stained blue whilst apoptotic cells appeared as small fragmented bodies staining bright green.

Determination Fas/Fas-L in Leukocytes

For Fas and FasL determination, 10^5 cells were cytospun on slides and, anti-Fas FITC and anti-FasL FITC (1:10 diluted) conjugates (Apo-1, Oncor Appligene, UK; Serotec, NJ, USA, respectively) were added, slides incubated at 37°C for 1 hour. Slides were then washed twice with PBS for 10 minutes. Cells on the slides were fixed with 1% paraformaldehyde, mounted with glycerol and checked under an Olympus fluorescence microscope. Matched isotype antibodies were used in parallel as negative controls.

Determination of soluble Fas (sFas) in Plasma by ELISA Method

Soluble Fas in serum samples was detected with a Sandwich ELISA kit (R&D system, MN, USA), according to the manufacturer instructions without modification.

Statistical Analysis

Statistical analysis was carried out using Microsoft Excel spreadsheet and the StatView SE + graphics software. The probability of a significant difference between groups was determined by Mann Whitney test and Wilcoxon Signed rank test. Graphs were plotted using Cricket graph graphics package. All software programs were run on a Macintosh computer.

Results

1. DNA Damage of Peripheral Leukocytes in SLE Patients

-Study the level of DNA damage in leukocytes from SLE patients

The structure of DNA in lymphocytes and granulocytes was investigated from 45 SLE patients. These patients were receiving glucocorticoids and/or cytotoxic therapy. In healthy donors, the proportion of damaged DNA in lymphocytes and granulocytes were 10.1±2.1% and 21.1 ± 1.7%, respectively (Table 1). The proportion of DNA damage in SLE patients was much higher compared to healthy donors (44.5% *vs.* 10.1%). I also found that the highest level of DNA damage was in SLE stage III.

Table 1. Percentage of DNA Damaged Lymphocytes and Granulocytes from Healthy Donors and SLE Patients.

Group investigated	Sample size	% Degree of DNA damage 100% - D% (M±SD)	
		lymphocytes	Granulocytes
Healthy donors (HD)	35	10.1 ± 2.1	21.2 ± 1.7
SLE patients	45	44.5* ± 1.0	44.0±3.0
Stage I of SLE	15	41.0±10.0	25.0±5.5
Stage II of SLE	12	40.0±1.0	30**±6.1
Stage III of SLE	18	70.0*±7.6	42**±7.2

* $P < 0.05$ in comparison with values of HD. ** $P < 0.05$ in comparison with values of Stage I of SLE

-Study of Apoptosis in Lymphocytes and Granulocytes from SLE Patients

A slight increase in the level of apoptosis was found in freshly intact lymphocyte and granulocytes derived from SLE patients (Table 2). Since the lack of significant difference could be due to the fact that apoptotic cells are quickly phagocytosed by macrophages, the commitment to apoptosis in lymphocytes after primary culture for 18 hours was investigated. In contrast to freshly cells, *in vitro* experiments demonstrated that the quantity of apoptotic lymphocytes from SLE patients was higher than those in healthy donors (7 % *vs.* 5.5%). No difference was found in the level of apoptotic granulocytes between normal donors and SLE patients (Table 2). Furthermore, the proportion of apoptotic lymphocytes after *in vitro* experiment was higher in SLE patients and was stage dependent (Table 2). A similar graded pattern of apoptosis in the granulocytes was not observed.

Table 2. Percentage of Apoptotic Cells from Healthy Donors and SLE Patients.

Group investigated	% apoptotic lymphocytes (M±SD)		% apoptotic granulocytes (M±SD)	
	Freshly intact	After 18hr. incubation.	Freshly intact	After 18hr. incubation.
Healthy donors (n=30)	2.0 ± 1	6.0±1.5	3.5±0.8	11.0 ±2.7
SLE (n = 45)	1.5 ± 2	7.0* ± 2.3	4.1 ±1.5	12.0 ± 4.0
Stage I (n=15)	2.5 ± 0.9	7.5 ±1.5	3.6 ± 1.1	15 ±1.4
Stage II (n=12)	1.5 ± 0.5	6.0 ± 1.8	1.8 ± 9.8	12 ±1.3
Stage III (n =18)	1.5 ± 0.3	9.0* ± 2.0	1.1 ±0.5	14±3.2

** $P \leq 0.05$ compared to the appropriate value of level apoptosis freshly intact cells.*

-Study the Correlation Between Autoantibody Titers, Level of DNA Damage, and Apoptosis in Lymphocytes

In SLE patients, a significant correlation between the titers of antibodies to dsDNA and the percentage of DNA damage was found in freshly lymphocytes and granulocytes (r=0.91 and r=0.75, respectively) (Figure 1 A & B). The level of antinuclear antibodies and antibodies to DNA were determined. In 14 patients with active phase of disease (stage III), titers were 1:320, 1:160, respectively. While in stage II (involving 12 patients) and stage I (involving 18 patients) the titers of antinuclear factor were 1:40 and 1:20, respectively.

-Investigation the Level of Apoptosis in Lymphocytes after the Dosage of Prednisolone in SLE Patients

In SLE patients receiving daily prednisolone doses (10 mg-40 mg) the level of lymphocytes apoptosis was analyzed. A positive correlation between the level of lymphocytes apoptosis and the dosage of prednisolone was found (r = 0.71) (Figure-2).

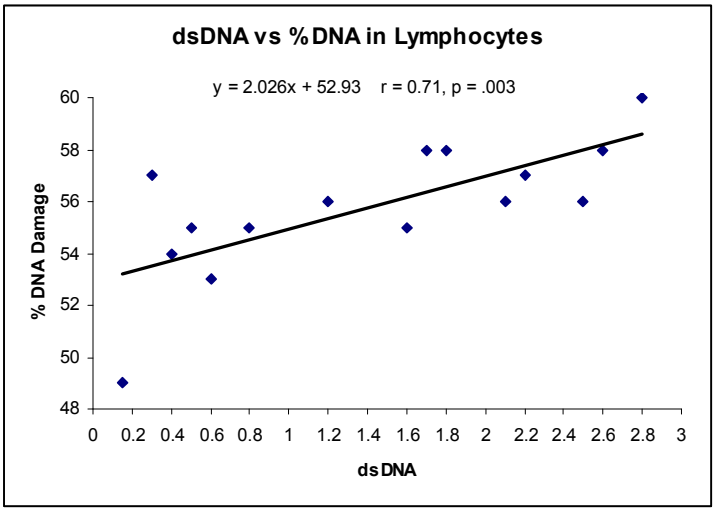

Figure 1A. Correlation between autoantibodies titers to dsDNA and the percentage DNA damages in lymphocytes (r= 0.71). X axis-titers antibodies to dsDNA; Y axis- the percentage damaged DNA.

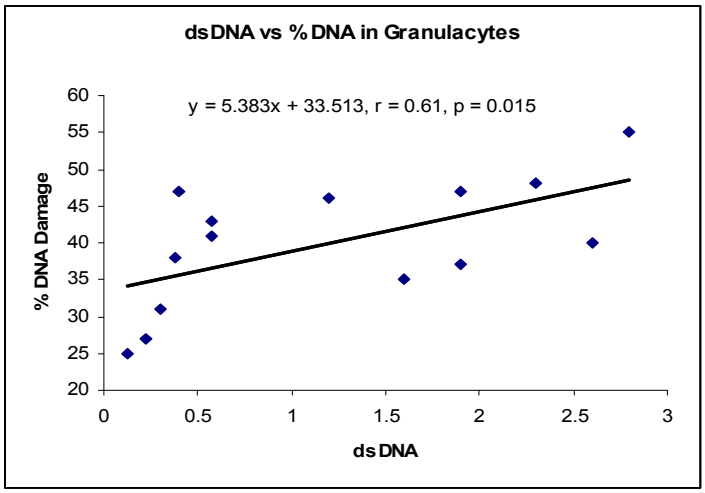

Figure-1B. Correlation between autoantibodies titers to dsDNA and the percentage DNA damages in granulocytes (r= 0.62). X axis-titers antibodies to dsDNA; Y axis- the percentage damaged DNA.

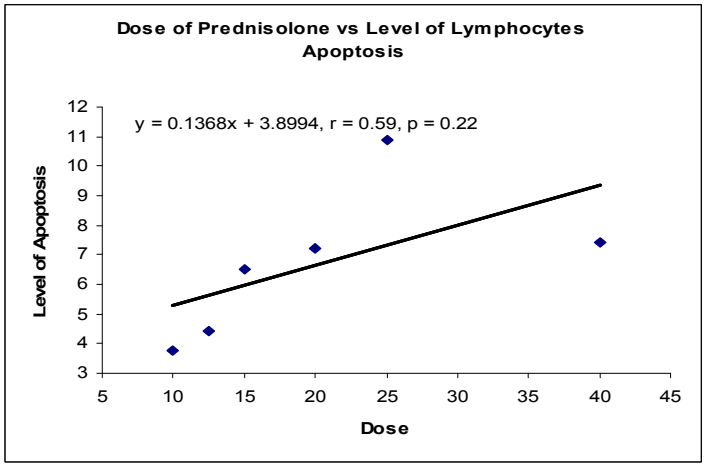

Figure 2. Correlation between the level apoptosis in lymphocytes and the dose of prednisolone (r= 0.6). X axis– dosage prodnisolone (10-40mg); Y axis- percentage apoptotic lymphocytes of SLE patients.

2. Investigation of the Role of the Fas/FasL System in the Regulation of Apoptosis in Patients with SLE

-Estimation the Level of FasL Expression in Leukocytes from Peripheral Blood in Patients with SLE

A significant expression of FasL on the surface of lymphocytes and granulocytes in SLE patients was found compared to the healthy donors (Table 3). Fas expression depended on SLE activity (Table 3). Fas was expressed on 31±5.1 % of lymphocytes, and on 27.1±11.2% of granulocytes of healthy donors.

-Study the Ssoluble Fas in Plasma of Patients with SLE

A slight increase in sFas level in SLE patients was found compared to healthy donors (1.62±0.29 ng/ml *vs*. 1.38±0.06 ng/ml, respectively). The level of sFas decreased in SLE patients who were treated with prednisolone and cyclophosphamide (1.1±0.4 ng/ml) as compared to those treated with prednisolone only (1.9±0.2 ng/ml). A significant inverse relationship (r= -0.3) between the dosage of prednisolone and the level of sFas (Figure 3) was observed.

- Investigation of the Correlation Between the Level of sFas and Titer Antinuclear Antibodies

A positive correlation between the level of sFas and the titer of antinuclear antibodies was found (Data not shown). This association reflects the large number of activated lymphocytes undergoing apoptosis.

Table 3. Level of Fas and FasL expression on lymphocytes and granulocytes from healthy donors and SLE patients.

Group Investigated	Lymphocytes (%) (M±SD)		Granulocytes (%) (M±SD)	
	Fas	Fas-L	Fas	Fas-L
Healthy Donors (n=22)	35.0 ± 3.5	2.5 ± 2.0	27.0 ± 3.0	1.7 ± 2.4
SLE Patients (n= 45)	41.0 ± 2.5	4.4* ± 3.0	31.0 ± 3.2	5.0* ± 2.1
SLE Stage I (n=15)	31.0 ± 5.1	2.3 ± 0.8	27.1 ± 11.2	3.1 ± 0.7
SLE Stage II (n=12)	33.3 ± 9.2	2.1± 0.9	29.2 ± 6.3	4.2 ± 1.5
SLE Stage III (n=18)	39.4 ± 10.7	6.2 ± 1.2	66.2 ± 10.5	9.2 ± 1.2

** P <0.05 in comparison with values for healthy donors.*

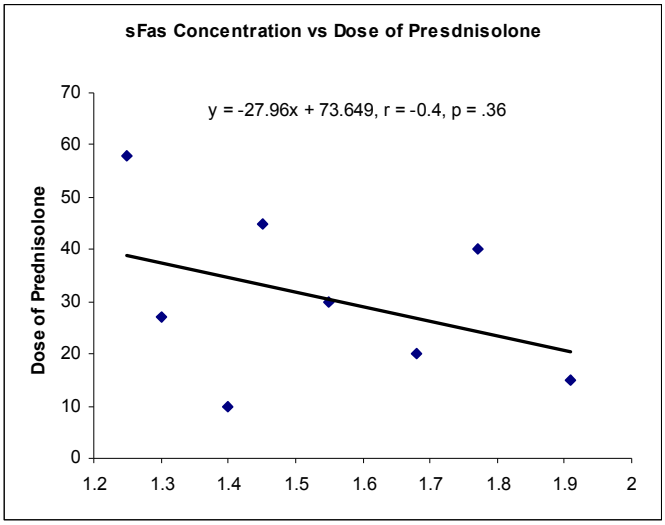

Figure 3. Correlation between sFas concentration and the dose of prednisolone (r= 0.4). X axis-level sFas; Y axis- dosage prodnisolone.

Discussion

Autoimmune diseases are both sufficiently interesting and common to attract major research interest. The production of antibodies against autoantigens is one key feature of autoimmunity which can easily be studied in human. The DNA structure in peripheral blood lymphocytes and granulocytes of SLE patients was clearly compromised. The most significant damage was seen in the lymphocytes of SLE patients. This implies that a genetic mechanism is likely to be involved in the pathogenesis of SLE. A damage of this nature within leukocytic cells may represent a major contributor to the evolving pathogenic processes of this autoimmune disease. Higher levels of DNA damage occurred in lymphocytes and granulocytes of SLE patients as compared to these of healthy controls, while, other studies suggested the absence of endogenous DNA damage of these cells (Boerrigter *et al.,* 1989, Boerrigter *et al.,* 1990).

Apoptosis of lymphocytes represents an important factor in the pathogenesis of SLE whereas in granulocytes do not (Courtney *et al.,* 1998). The increased level of apoptosis in lymphocytes may exceed the capacity of the phagocytic system. If so, it would result in incomplete apoptosis accompanied by release of various intracellular elements (e.g., histones) which would promote the formation of autoantibodies (Huggins *et al.,* 1999)

It is not clear whether a predisposition to lymphocytes apoptosis in SLE patients could be as a consequence of corticosteroids therapy and/or cytotoxic drugs. The majority of SLE patients in the current study, were on prednisolone. Therefore, this prompted the investigation of whether the level of apoptosis in lymphocytes and in granulocytes is linked to corticosteroid therapy. One may assume that, the level of DNA damage in leukocytes might serve as an indicator of the degree of pathogenesis of the disease, and the positive influence of drug treatment of patients. It is known that B-cell hyperactivity in SLE may depend on the cell activation and, in particular, circulating $CD4^+$ cells. Therefore, the decrease in percentage of $CD4^+$ T cell may represent a defense mechanism to protect certain organs from damage that may result from formation and deposition of immune complexes. Moreover, since SLE patients in the current study were under corticosteroid therapies, therefore the reduction in CD4+ cells may suggest that the given subpopulation of cells ($CD4^+$ T cells) is highly responsive to corticosteroids with respect to sensitivity to apoptosis (Seki *et al.,* 1998).

The increased level of FasL on the surface of lymphocytes may promote an apoptogenic signal. The increased level of apoptotic lymphocytes was consistent with lymphopenia. The high level of expression of FasL in lymphocytes and its relationship with the stage of SLE disease may indicate the involvement of lymphocyte activation. Additionally, increased expression of FasL induces apoptosis in Fas-expressing cells, a fact that supports our *in vitro* experimental demonstration of increased apoptosis in lymphocytes (Garrone *et al.,* 1995).

It is known that there are protective mechanisms to prevent the synthesis of autoantibodies (Shinohara *et al.,* 1997). These represent processes of negative and positive selection of lymphocytes in central (e.g., thymus) and peripheral (e.g., lymph nodes) organs of the immune system. The removal of these autoreactive lymphocytes through negative selection occurs via apoptosis. Defects in the mechanism of negative selection of lymphocytes can result in pathogenesis of autoimmune diseases. Soluble Fas blocks apoptosis by its interaction with FasL, thus preventing the removal of activated lymphocytes (Hosaka *et al.,*1998).

Therefore, studying the role of sFas in the regulation of apoptosis in lymphocytes is interesting. It can thus be suggested that Fas expression is increased immediately after the activation of lymphocytes, with the increased level of sFas in SLE patients explaining the proteolytic cleavage of the transmembrane forms of Fas, or via alternative splicing. This represents the most likely mechanism by which the activity of the Fas receptor is regulated (Bijl *et al.,* 1998, Miret *et al.,* 2001, Silvestris *et al.,* 2003).

There was an inverse relationship between sFas and disease activity (in the first stage), but not in SLE second and third stages This decrease in the level of sFas may thus be explained by the use of cyclophosphamide i.e., using immunosuppressive drugs may lower levels of sFas, apparently by inducing apoptosis.

A decrease in the clearance of apoptotic cells might be a contributing factor in systemic autoimmunity (Grodzicky & Elkon 2002; Schulze *et al.,* 2008). Immunologically, a great deal of evidence suggests that during immune repertoire, any lymphocytes that recognize self cells will be deleted by apoptosis (Golstein *et al.,* 1991). Defects in the deletion mechanism of autoreactive T cells might play a critical event in the initiation and maintenance of autoimmune diseases. The MRL/lpr mice (a strain carries a mutation in the Fas gene) encoding a defective Fas gene show resistance in their lymphoid cells to apoptosis (Herron *et al.,* 1993; Kinjoh *et al.,* 1993). Like Fas, FasL-deficient mice develop generalized lymphoproliferative disease (gld) with autoimmunity identical to that in lpr/lpr (Suda *et al.,* 1993). These mice develop lymphoadenopathy rapidly and systemic lupus erythromatosis (SLE)-like autoimmune disease. It has been demonstrated that different strains of lpr mice develop various patterns of auto-antibodies which indicates the involvement of other unknown genetic factors (Cohen *et al.,* 1991). SLE and rheumatoid arthritis are characterized by a common feature which is the imbalance between the production and loss of lymphocytes and synovial cells, respectively. It should be noted that all potent inducers of apoptosis such as steroids and cyclophosphamide are among the identifiable therapies of autoimmune diseases (Botto & Walport 2002; Liu & Pope 2004). It is well established that any defect in the clearance mechanism of lymphoid cells and other cells dying by apoptosis might be crucial in the maintenance of autoimmune diseases. This concept has been developed by findings that unknown factors block phagocytic ingestion of apoptotic cells, so that the dead cells will be engulfed and cleared away whilst intact, but rather leak their contents, which include endonucleases. These circulating nucleosomes were detected in the lupus (Pickering *et al.,* 2001)

In conclusion, data acquired in this study facilitates that Fas/Fas-L system plays an important role in the control of apoptotic death of autoreactive and activated lymphocytes. Further studies are needed to determine whether disturbance of this mechanism may contribute to the pathogenesis of SLE.

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