Flowcytometric Study of Expression of Perforin and CD134 in Patients with Systemic Lupus Erythematosus

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Perforin is a membrane-disrupting protein that allows the entry of granzymes into a target cell inducing degradation of target substances in the cytoplasm and nucleus thus leading to programmed cell death or apoptosis. CD134 was originally described as an activation antigen found on activated T cells. In this work, Flowcytometry was used to evaluate the expression of perforin and CD134 in patients with Systemic Lupus Erythematosus (SLE) to elucidate their role in the pathogenesis of SLE and disease severity. The study was conducted on 15 patients with SLE, 6 patients out of the 15 patients were suffering from lupus nephritis, 10 healthy subjects were included as controls. The results revealed that absolute number of circulating CD3+ lymphocytes in the patients was significantly lower than the controls (P=0.013). The percentage of CD8+ CD3+ T cells was significantly increased in the SLE group when compared to that of CD4+ CD3+ T cells in same group (P=0.001) Perforin expression on both CD4+ and CD8+ cells was significantly increased in patients compared to controls. (P=0.002 & P=0.001, respectively). In addition, a significant increase was observed in the percent of pf+CD8+CD3+ in the patient group compared to that of pf+CD4+CD3+ in the same group (P=0.001). There was a significant increase in the expression of CD134 on CD4+ and CD8+ cells (P=0.001 & P=0.001 respectively). Also, in the same group of patients a significant increase was detected in the frequency of CD134+CD4+T cells compared to that of CD134+CD8+CD3+ T cells (P=0.032). A significant positive correlation was detected in the patient group between CD134 and perforin expression on both CD4+ and CD8+ T cells (r=0.523). Moreover, CD134+CD4+CD3+ was also correlated positively with urinary proteinuria (P=0.023, r=0.524). Our data suggest the role of Perforin + cytotoxic T lymphocytes and CD134+ cells in the pathogenesis of autoimmunity of SLE. Thus, inhibition of perforin could be beneficial for SLE patients. Targeting pf and CD134 could be a new therapeutic approach in patients with SLE.

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with multiorgan involvement characterized by an immune response against nuclear components (Ruiz et al., 2001). SLE patients experience a waxing and waning disease course and a wide array of clinical manifestations reflecting the systemic nature of the disease. The skin, kidneys, joints, and central nervous system may become the target of SLE-induced inflammation at its onset or during the course of the disease (Wakeland et al., 2001).

Importantly, T cells from lupus patients promiscuously kill autologous monocytes/macrophages, which may contribute to disease pathogenesis by both increasing the amount of potentially antigenic apoptotic material as well as decreasing its clearance (Kaplan et al., 2002). The killing is completely inhibited by antibodies (Abs) to class II MHC determinants and partially inhibited by Abs to class I, suggesting that CD4+ T cells are primarily responsible for the killing, but are assisted by CD8+ T cells (Richardson et al., 1992). Fas ligand, and tumor necrosis factor (TNF) like weak inducer of apoptosis play a role in this response, but inhibition of these molecules does not completely inhibit the killing, thus, additional cytotoxic molecules, such as perforin, may contribute (Kaplan et al., 2002).

Perforin (pf) is expressed mainly in activated cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, although some reports suggest its expression in microglia as
well (Gasque et al., 1998). In CTLs, pf is stored in cytoplasmic granules and is a major effector of cytolysis by these cells. On pf release, it inserts itself into the plasma membrane of target cells and polymerizes into pore forming aggregates. Pores of pf lead to osmotic lysis of target cells and induce apoptosis by allowing granzymes to enter the target cells (Malipiero et al., 1997). Perforin-deficient mice have confirmed its function as an effector molecule and in the immune response to viruses and tumors as well as in other aspects of immune regulation such as activation-induced cell death (AICD), antibody production and spontaneous autoimmunity (Peng et al., 1998). Expression of perforin in peripheral blood lymphocytes and psoriatic lesions of exacerbated psoriasis was detected in previous studies (Massari et al., 2007). The role of pf in rheumatoid arthritis is not clear, although some observations suggest a role in disease pathogenesis, because pf expressing CTLs has been demonstrated in the rheumatoid synovium (Bauer et al., 2005).

T cell activation requires not only the interaction between the T cell receptor and MHC/antigen complexes, but also co-stimulation is provided by molecules expressed on antigen presenting cells (APCs). In addition to the well-characterized T cell co-stimulatory molecules CD28 and CD152 which bind to CD80 and CD86 on APCs, several members of the TNF superfamily, especially CD134 (OX40), CD137 (4–1BB) and CD154, have been shown to induce co-stimulatory signals upon binding to their cognate receptors (Finney et al., 2004). OX40 was originally described as an activation antigen found on rat activated T cells, which is formally called CD134 (Paterson et al., 1987). Then the gene was cloned and shown to be a member of tumor necrosis factor receptor (TNF-R) gene family, which includes CD40, Fas, CD30, and 4-1BB (Weinberg, 1998). A common function of TNF-R family members is the regulation of cell mediated activation and or apoptosis (Li et al., 2007). The expression of CD134 ligand (CD134L) has been shown to be upregulated in proliferative lupus nephritis, suggesting a role for the CD134–CD134L pathway in its pathogenesis. Biopsies from lupus associated skin lesions revealed the expression of CD134L; in addition, leukocyte infiltrates contained moderate numbers of CD134 cells (Aten et al., 2000).

The aim of this study was to evaluate the expression of perforin and CD134, as a costimulatory molecule on T cells, in patients with SLE in an attempt to elucidate their role in pathogenesis of SLE and disease activity.

Patients and Methods

The study was conducted on 15 consenting patients with SLE admitted to the Department of Dermatology and Venereology, Tanta University and Urology and Nephrology Center, Mansoura University. The patients were 14 females and one male. Their ages ranged from 14-37 years with a mean of 18.36±3.54 years. Diagnosis of SLE was done according to the American Rheumatism Association (Tam et al., 1982).

The mean duration of the disease was 5.68±2.71 years. Six patients out of the 15 patients were suffering from renal involvement (lupus nephritis). All patients were advised to stop immunosuppressive drugs 3 weeks before commencement of the study. Ten healthy subjects were also included as controls.

All patients were subjected to the following:

-Complete history taking.
-Thorough general and dermatological examination.
-Laboratory investigations including:
-Complete blood picture, complete urine analysis, 24-hours urine protein, blood urea and serum creatinine.
-Erythrocyte sedimentation rate (ESR), LE cells, Rheumatoid factor, antinuclear antibody titre (ANA), antidouble stranded DNA (anti ds. DNA).
-Two ml venous blood were taken from both patients and controls under minimal tourniquet pressure for flowcytometric study.

Flowcytometry

Monoclonal antibodies used were anti-CD3- FITC, anti CD8- PE, anti CD134- PE, anti perforin-PE and anti-
CD4-PE (BD PharMingen, San Diego, CA). Antibody concentrations used in cell staining were based on data supplied by the manufacturers. 100 µl of blood were placed together with 10 µl of appropriate fluorescent conjugated monoclonal antibody or the matched isotype control. This mixture was incubated in the dark on ice for 30 minutes. After incubation, the blood was lysed by adding 450 ml of 1x FACS lysis buffer (BD) and incubation in the dark for 20 minutes. Then, the sample was washed twice in PBS. After last wash, the pellet was dissolved in 300 ml of 2% paraformaldehyde solution. The percentage of the target cells was determined with a fluorescence activated cell sorter (FACS Calibur system; Becton Dickinson) using three colour staining. 5000 cells were routinely acquired and analysis was done using CellQuest Pro software. Gate was made on lymphoid populations. Gating and instrument settings were controlled in every experiment and were maintained throughout the whole study in all control and patient samples analyzed. Dot blot was set as FL1 and FL2 markers (HuanZhong et al., 2004).

Statistical Analysis

The collected data were organized, tabulated and statistically analysed using SPSS software, statistical computer package version 12. For quantitative data, the mean and standard deviation were calculated. The difference between means was statistically analysed using t test and chi square. Pearson correlation coefficient (r) was calculated to test the association between two variables. P<0.05 was considered significant.

Results

Lymphocyte subset distribution in the peripheral blood of SLE patients and healthy controls are shown in table (1). The absolute number of circulating CD3+ lymphocytes (u/l) in the patients was significantly lower than that in the controls (898±234 versus 1257±219, P= 0.013).

Moreover, in the control group the percentage of CD4+ CD3+ T cells in the PBMC was significantly higher than the percentage of CD8+ CD3+ T cells (26.62±6.12 versus 8.95±0.82, P= 0.001), meanwhile, the percentage of CD4+ CD3+ T cells (figure 3C) was significantly decreased in the SLE group when compared to that of CD8+CD3+ T cells (Figure3 A) in same group (28.59±5.18 versus 35.36±7.90, P= 0.032).

The percent expression of perforin is shown in table (2). Perforin expression on both CD4+ and CD8+ cells was significantly increased in patients compared to the controls (3.01±1.11 versus 0.73±0.40, P=0.002 & 25.06±5.65 versus 6.07±1.49, P= 0.001, respectively). In addition, a statistically significant increase was observed in the percent expression of pf+CD8+CD3+ in the patient group (Figure3 B) compared to the expression of pf+CD4+CD3+ in the same group (25.06±5.65 versus 3.01±1.11, P= 0.001).

Table 1. Lymphocyte subsets in peripheral blood of patients and controls.

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<tr>
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<th>Mean ± SD</th>
<th>*P value</th>
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<tr>
<td>Patient CD3+ (cells/µl)</td>
<td>898 ± 234</td>
<td>0.013</td>
</tr>
<tr>
<td>Control CD3+ (cells/µl)</td>
<td>1257 ± 219</td>
<td></td>
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<tr>
<td>Patient %CD4+CD3+</td>
<td>28.59 ± 5.18</td>
<td>0.032</td>
</tr>
<tr>
<td>Patient %CD8+CD3+</td>
<td>35.36 ± 7.90</td>
<td></td>
</tr>
<tr>
<td>Control %CD4+CD3+</td>
<td>26.62 ± 6.12</td>
<td></td>
</tr>
<tr>
<td>Control %CD8+CD3+</td>
<td>8.95 ± 0.82</td>
<td>0.001</td>
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* P<0.05 is significant
Table 2. Comparison between percent perforin expressions on CD8+ and CD4+ cells of the SLE patients and control groups

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<th></th>
<th>Mean ± SD</th>
<th>*P value</th>
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<tr>
<td>Patient % pf+CD4+CD3+</td>
<td>3.01 ± 1.11</td>
<td>0.002</td>
</tr>
<tr>
<td>Control % pf+CD4+CD3+</td>
<td>0.73 ± 0.40</td>
<td></td>
</tr>
<tr>
<td>Patient % pf+CD8+CD3+</td>
<td>25.06 ± 5.65</td>
<td>0.001</td>
</tr>
<tr>
<td>Control % pf+CD8+CD3+</td>
<td>6.07 ± 1.49</td>
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Table 3. Comparison between percent expression of CD134 on CD4+ and CD8+ cells of the SLE patients and control groups.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>*P value</th>
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<tr>
<td>Patient % CD134+CD4+CD3+</td>
<td>18.44 ± 1.44</td>
<td>0.001</td>
</tr>
<tr>
<td>Control % CD134+CD4+CD3+</td>
<td>9.13 ± 1.33</td>
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<tr>
<td>Patient % CD134+CD8+CD3+</td>
<td>4.11 ± 0.79</td>
<td>0.001</td>
</tr>
<tr>
<td>Control % CD134+CD8+CD3+</td>
<td>1.02 ± 0.44</td>
<td></td>
</tr>
<tr>
<td>Patient % CD134+CD4+CD3+</td>
<td>18.44 ± 1.44</td>
<td>0.032</td>
</tr>
<tr>
<td>Patient % CD134+CD8+CD3+</td>
<td>4.11 ± 0.79</td>
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There was also a significant increase in the urinary proteinuria (mg/dl) in the patient group compared to that of the control group (594.7±483.2 versus 56.10±37.49, *P* = 0.008).

A significant positive correlation was detected in the patient group between CD134 and perforin expression on both CD4+ and CD8+ T cells (*P* = 0.045, *r* = 0.523) (Figure 1), and between CD134+CD4+CD3+ and urinary proteinuria in the same group of patients (*P* = 0.023, *r* = 0.524) (Figure 2).
Figure 2. Correlation between percent CD134+CD4+CD3+ and urinary proteinuria of patient group.

(A) Flow cytometry dot blot shows the percentage of CD3+CD8+ cells in patient with SLE. Gate was done on the lymphoid populations

(B) Flow cytometry dot blot shows the percentage of CD3+CD8+ perforin+ cells in patient with SLE. Gate was done on the CD3+CD8+ populations in (A)

(C) Flow cytometry dot blot shows the percentage of CD3+CD4+ cells in patient with SLE. Gate was done on the lymphoid populations

(D) Flow cytometry dot blot of CD134+CD4+CD3+ cells in patient with SLE. Gate was done in CD3+CD4+ populations in (C)

Figure 3. Flow cytometric dot blot in a case of SLE patient group.

$r = 0.524$

$P = 0.023^*$
Discussion

The present study reveals a significant quantitative increase in CD8+ cytotoxic T lymphocytes accompanied with a decrease in CD4+ T lymphocytes in the SLE patients. These results were in accordance with that of Blanco et al., (2005) who demonstrated that CD8+ T lymphocytes were both qualitatively and quantitatively increased and highly correlated with SLE disease activity. They proved that CD8+ T lymphocytes freshly isolated from the peripheral blood of SLE patients with active disease had the intrinsic capacity to generate nontolerized granzyme B autoantigen fragments without any inhibition of the caspase pathway, suggesting a peculiar status for these cells in patients with active SLE. This implies a previously ignored role of CD8+ T lymphocytes in the generation of high amounts of nuclear autoantigens which, as a consequence, may overwhelm the physiologic clearance pathway.

In the present study, the expression of perforin in the SLE group was higher than that of the control group. The percentages of perforin+ CD8+ T cells in the patient group were significantly higher than that in the control group. Moreover, perforin + CD4+ T cells in SLE group of patients were significantly lower than perforin + CD8+ T cells in the same group. Our results were consistent with that of Li et al., (2007); they suggested that the hemolysis activities of perforin were increased in SLE active patients, and this might be caused by increase of its expression. Perforin has been demonstrated to play an important role in the pathology of SLE (Peng et al., 1998). It is generally considered to be a marker of lymphocytes having active cytotoxic function (Denyer et al., 2006).

The results of the present study were also in accordance with those obtained by Lu et al., (2003). They proved that overexpression of perforin in both CD4 and CD8 T cells in SLE patients was due to demethylation of a conserved region located between the promoter and upstream enhancer and identified perforin as a transcript that reproducibly increased 2-fold in the hypomethylated cells. In addition, Blanco et al., (2005) demonstrated that patients with SLE disease flares were characterized by higher proportions of perforin- and or granzyme B–positive lymphocytes.

Cell death causes inflammation that can induce apoptosis and cell death in turn. It is conceivable that the pf/granzyme pathway could contribute to the pathology of rheumatoid arthritis in at least two ways: promotion of autoimmunity by blocking peripheral tolerance and activation induced cell death (AICD) or destruction of target tissues (Sower et al., 1996).

The expression of perforin in subpopulations of peripheral blood lymphocytes in lichen planus disease was investigated by Massari et al., (2007). A significant increase of pf expression in cytotoxic T lymphocytes (CD3+ perforin+ cells) in the exacerbation phase of disease which was mostly located in the CD8+ subpopulation (CD8+ perforin+) was observed. They suggested an important role for perforin in this autoimmune disease. So, owing to its important role in SLE, perforin might be a potential target of its clinical treatment (Trapani & Smyth, 2002).

CD134 has been shown to play an important role in many CD4 responses in vivo, including regulating memory T cell development. Signaling through CD134 via antibody stimulation or membrane-bound CD134 ligand (CD134L) causes T cell proliferation when coupled with T cell receptor stimulation (Godfrey et al., 1994).

This study showed that the expression of CD134 on peripheral CD4+ and CD8+ T cells was increased in SLE group compared with control group. The percentage of
CD134+CD4+ T cells was significantly higher than that of CD134+CD8+ T cells in the SLE group of patients. In addition, CD134 percent expression was positively correlated with perforin percent expression on both CD4+ and CD8+ T cells in SLE group. Consistently, Aten et al., (2000) detected the expression of CD134+ T lymphocytes in Peripheral blood mononuclear cells (PBMC) from SLE patients. They found that among the CD4+ T lymphocytes, CD134 was expressed only on the subset expressing CD45RO, but they could not detect CD134 expression on resting CD8+ T lymphocytes. Furthermore, all lymphocytes expressed CD134 also coexpressed CD3.

A significant positive correlation was detected between CD134+ CD4+ and proteinuria in SLE patients. This suggests that these CD134 cell populations play a pivotal role in the pathogenesis of this disease. Patschan et al., (2006) indicated that the expression of CD80, CD86 and CD134 on peripheral CD4+ T cells was associated with nephritis and increased disease activity in patients with SLE.

There are two hypothetical ways in which CD134+ CD4+ T cells could mediate lupus nephritis. Firstly, these cells could provide help to B cells producing anti-dsDNA antibodies and also to other antibodies that may contribute to the kidney lesions (Zhao et al., 2005). Secondly, it is also possible that the CD134+ T cell populations infiltrate glomerular endothelial cells after ligation with CD134L and cause direct damage (Lathrop et al., 2004).

The role of CD134 for effector functions has been shown by the observation that treatment with a stimulatory anti-CD134 antibody enhances T cell expansion and differentiation to effector cells in mice. This evidently promoted the secretion of IFN gamma and the upregulation of various interleukin (IL) receptors with subsequent cytokine-mediated kidney cell damage (Patschan et al., 2006).

Anti-CD134 antibody has been used to strengthen the effects of CD134+CD4+ or CD134+CD8+ cells, which could be used to treat tumor or infection. Immunologic toxins have been also conjugated to be anti-CD134 antibodies to clear autoreactive T cells that could be used to treat autoimmune disorders (Weinberg, 2002).

It was demonstrated that CD134 monoclonal antibody could decrease expression and hemolysis activities of perforin in PBMCs in the SLE active group (Li et al., 2007). CD134 Appears to be a beneficial target for autoimmune therapy because the T cells that express CD134 are highly enriched for the cells that react with autoantigens at the site of inflammation and peripheral T cells do not express CD134L in normal or diseased individuals. Therefore, antibodies against CD134 might delete the pathogenic T cells specifically (Weinberg, 1998).

In conclusion, our data indicate that perforin + cytotoxic T lymphocytes and CD134+ cells may have a role in the pathogenesis of SLE. Targeting CD134 and perforin could be a new therapeutic approach in patients with SLE. This issue, however, needs to be further investigated with a larger scale of patients.

References


in patients with active systemic lupus erythematosus. Arthritis Rheum. 35:647-54


