Apoptosis and autoantibodies in systemic lupus erythematosus
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Chapter V

Effects of smoking on activation markers, Fas expression and apoptosis of peripheral blood lymphocytes

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Abstract

Smoking influences numbers and function of peripheral blood lymphocytes (PBL) by a process that is badly understood. We conducted this study to evaluate whether the immune impairment of smoking might be related to changes in the expression or functionality of Fas, a cell surface molecule that plays a central role in immune homeostasis and cytotoxic activity.

PBL from 10 smoking and 10 non-smoking healthy volunteers were isolated. Flowcytometry was performed to measure the state of activation, Fas expression and apoptosis of PBL. Functionality of Fas was tested by assessing apoptosis after incubation of isolated lymphocytes with agonistic anti-Fas antibodies in 4 smoking and 4 non-smoking individuals.

Smoking was associated with an increase in the percentage of Fas expressing CD4+ T- and B-lymphocytes. A decrease in the percentage of activated (CD38+) B cells was observed. In vitro Fas-induced apoptosis did not appear different between smokers and non-smokers. No differences in the percentages of circulating apoptotic lymphocytes could be demonstrated between smoking and non-smoking individuals. Smoking is associated with increased Fas expression on PBL in general, and on B cells in particular. This might render these cells more susceptible for apoptosis. As Fas is functionally intact this may also explain the reduced percentage of activated (CD38+) B cells found in smoking individuals. The latter may contribute to the reduced humoral immune response observed in smokers.

Introduction

Chronic cigarette smoking results in an impaired immune response. T cell responsiveness is affected and T cell dependent antibody responses are decreased [1;2]. Numbers of peripheral blood leukocytes cell counts change. For example, smoking results in decreased numbers of natural killer (NK) cells [3]. Data concerning the influence of smoking on lymphocyte subsets are conflicting. Tollerud et al. found a significant increase in the percentage of CD4+ T-lymphocytes in smokers, without changes in the percentage of B-lymphocytes [4]. Hockertz et al., however, demonstrated a decrease in the percentage of T- as well as B-lymphocytes in smokers [5]. The mechanisms underlying these changes in immune function and peripheral blood lymphocyte (PBL) distribution are badly understood.

Fas, (APO-1, CD95) is a membrane receptor that plays a central role in immune homeostasis and mediates essential signals for apoptosis in activated mature lymphocytes [6]. In order to get more insight in possible mechanisms underlying selective losses and impairment of immune function of lymphocytes we hypothesized that chronic cigarette smoking is associated with increased Fas expression on PBL rendering these cells more
Fas expression in smokers

susceptible for apoptosis. Fas expression and the state of activation of PBL was analyzed in smoking and non-smoking individuals. In addition, we assessed the occurrence of apoptosis of PBL \textit{in vivo} as well as the functionality of the Fas-mediated apoptotic pathway \textit{in vitro}.

\textbf{Materials and Methods}

Twenty healthy individuals were studied. All denied the presence of clinical symptoms or signs, in particular wheezing, fever or cough, and physical examination was unremarkable. Ten of them were cigarette smokers (age 42.9 ± 6.9 years, mean ± SD), smoking 18.0 ± 2.8 cigarettes per day, with a number of 22.6 ± 8.6. pack-years. Ten were non-smokers (age 30.9 ± 8.3 years).

\textit{FACS analysis and apoptosis assay}

Peripheral blood mononuclear cells were isolated by lymphoprep density gradient centrifugation within one hour after collection from heparinised blood. For surface staining the following phycoerythrin (PE) or fluorescein isothiocyanate (FITC) and allophycocyanine (APC) conjugated monoclonal antibodies (mAb) were used: CD4 (APC), CD19 (APC), CD25 (PE), CD38 (FITC), CD95 (PE) and HLA-DR (PE) (Becton Dickinson, Mountain View, CA); CD8 (APC) (Pharmingen, Hamburg, Germany). After washing cells with phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) staining of $10^6$ cells was done by adding labeled mAb with subsequent incubation for 45 minutes on ice in the dark. Samples were washed with PBS/BSA. After the addition of 222.5 µl binding buffer (10 mM hepes pH=7.4, 140 mM NaCl, 5 mM CaCl$_2$), 25 µl propidiumiodide (10 µg/ml, Molecular Probes, Leiden, The Netherlands) and 2.5 µl FITC-labeled annexin V (Nexins Research BV, Hoeven, The Netherlands) diluted 1:50 were added. Immediately after incubation for 10 minutes on ice in the dark, three-color immunofluorescence analysis was performed on a Coulter Epics-Elite equipped with a gated amplifier (Coulter Electronics, Mijdrecht, The Netherlands). Cells were gated for lymphocyte characteristics using both forward and sideward scatter. Percentages of cells staining positive for a particular marker within the population analyzed were compared between smokers and non-smokers. In addition, we compared mean fluorescence intensity (MFI) of staining between both groups. MFI represents the intensity of the signal on those cells positively staining for the respective marker. All analyses were performed consistently by the same person.

\textit{Apoptosis induction}

After isolation (t0), cells were resuspended in medium (RPMI 1640 + 2 mM glutamin + 60 µg/ml gentamycin + 5 % human poolserum) with and without the addition of 5 µg/ml anti-Fas (CLB-CD95/15), kindly provided by dr. L. Aarden (CLB, Amsterdam, The
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Netherlands). Subsequently, cells were cultured for 24 hours (t1). Both immediately after isolation and after 24 hours of culture cells were analysed for apoptosis assessment.

Statistics
Differences in parameters between smokers and non-smokers were evaluated by the Mann-Whitney U test (2-tailed). Results of apoptosis induction were analysed within groups using the Wilcoxon signed rank test. A p value ≤ 0.05 was considered significant.

Results
Smoking individuals had increased numbers of neutrophils and total lymphocyte counts in their peripheral blood compared to non-smokers (table 1a). Absolute numbers and percentages of circulating peripheral blood CD4⁺, CD8⁺ and CD19⁺ lymphocytes in smokers were not significantly different compared to non-smoking individuals (table 1). Interestingly, the percentages of Fas (CD95) expressing B-lymphocytes and CD4⁺ T-lymphocytes were increased in smokers compared to non-smokers (table 1b).

Table 1a:

<table>
<thead>
<tr>
<th></th>
<th>non-smokers (n = 10)</th>
<th>smokers (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>6.33 ± 2.02</td>
<td>8.42 ± 1.64⁺</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.80 ± 0.61</td>
<td>2.48 ± 0.41⁺</td>
</tr>
<tr>
<td>CD19⁺ lymphocytes</td>
<td>0.16 ± 0.08</td>
<td>0.24 ± 0.09</td>
</tr>
<tr>
<td>CD4⁺ lymphocytes</td>
<td>0.78 ± 0.32</td>
<td>1.11 ± 0.32</td>
</tr>
<tr>
<td>CD8⁺ lymphocytes</td>
<td>0.58 ± 0.24</td>
<td>0.79 ± 0.15</td>
</tr>
</tbody>
</table>

Mean ± SD values; *p<0.05, p values were calculated using the Mann-Whitney U test (2-tailed).

As Fas is upregulated upon cell activation we analysed the relation between the expression of Fas and the expression of activation markers on the various lymphocyte subsets. Percentages of activated B-lymphocytes (CD38⁺), CD4⁺ T-lymphocytes (CD25⁺) and CD8⁺ T-lymphocytes (HLA-DR⁺) are shown in table 1b. Percentages of activated B cells were significantly decreased in smoking individuals. The difference in Fas expression on B cells between smokers and non-smokers could be accounted for by an increased percentage of Fas expressing, non-activated (CD38⁻) B-lymphocytes in smokers (table 2).

Besides an increase in the percentage of Fas expressing CD4⁺ T-lymphocytes also mean fluorescence intensity (MFI) of Fas on these cells was increased in smokers compared to
Fas expression in smokers

non-smokers (median value of 187 units and 129 units, respectively, p<0.05). MFI of Fas on CD19+ cells and CD8+ cells and MFI-values of activation markers on the respective subpopulations of lymphocytes were comparable between both groups.

Table 1b:

Percentages of lymphocyte subsets and expression of activation markers and Fas (CD95) within these subsets in smokers and non-smokers

<table>
<thead>
<tr>
<th></th>
<th>non-smokers (n = 10)</th>
<th>smokers (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD19+</td>
<td>7.4 ± 2.2</td>
<td>8.4 ± 3.1</td>
</tr>
<tr>
<td>% CD38+</td>
<td>73.4 ± 6.1</td>
<td>54.0 ± 14.7***</td>
</tr>
<tr>
<td>% CD95+</td>
<td>18.8 ± 6.5</td>
<td>36.3 ± 10.4***</td>
</tr>
<tr>
<td>% CD4+</td>
<td>40.9 ± 4.9</td>
<td>44.6 ± 6.2</td>
</tr>
<tr>
<td>% CD25+</td>
<td>4.0 ± 1.3</td>
<td>4.9 ± 1.5</td>
</tr>
<tr>
<td>% CD95+</td>
<td>43.5 ± 9.1</td>
<td>57.7 ± 8.4**</td>
</tr>
<tr>
<td>% CD8+</td>
<td>32.4 ± 6.1</td>
<td>30.2 ± 6.7</td>
</tr>
<tr>
<td>% HLA-DR+</td>
<td>5.6 ± 2.5</td>
<td>4.2 ± 2.4</td>
</tr>
<tr>
<td>% CD95+</td>
<td>55.8 ± 11.2</td>
<td>62.5 ± 10.6</td>
</tr>
</tbody>
</table>

Mean ± SD values; percentages CD19+, CD4+, and CD8+ are expressed as percentages of total lymphocytes. Activation markers and Fas expression are expressed as percentage cells positive within the CD19+, CD4+, and CD8+ subpopulations; **p<0.01, ***p<0.001, p values were calculated using the Mann-Whitney U test (2-tailed).

To analyse whether the Fas-mediated pathway of apoptosis induction was functionally intact in smokers, lymphocytes from 4 non-smokers and 4 smokers were incubated with an agonistic anti-Fas mAb. Results of anti-Fas induced apoptosis of CD19+ cells are shown in figure 1. The percentage Fas expressing lymphocytes increased in medium after 24 hours; increase of Fas expression occurred also after incubation with anti-Fas, though less pronounced (data not shown). Percentages of apoptotic B cells were low directly after isolation, increased after 24 hours incubation in medium alone, and, compared to the latter value, reached significantly higher percentages after incubation with anti-Fas, demonstrating the functionality of the Fas-induced apoptotic pathway (figure 1).

In smokers the percentage Fas expressing B cells after isolation was higher. Despite this difference, after apoptosis induction in B cells percentages of apoptosis did not differ between smokers and non-smokers (figure 1). Also, percentages of apoptosis reached in T cells after apoptosis induction were comparable between smokers and non-smokers (data not shown).
Table 2:

Co-expression of activation markers and Fas in CD19+ B-lymphocytes from non-smokers and smokers

<table>
<thead>
<tr>
<th></th>
<th>non-smokers</th>
<th>smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>CD95+</td>
<td>10.0 ± 4.7</td>
<td>12.4 ± 4.7</td>
</tr>
<tr>
<td>CD95+</td>
<td>7.5 ± 3.8</td>
<td>22.7 ± 9.8***</td>
</tr>
<tr>
<td>CD95−</td>
<td>69.3 ± 11.3</td>
<td>42.5 ± 17.8***</td>
</tr>
<tr>
<td>CD95−</td>
<td>13.3 ± 7.4</td>
<td>22.6 ± 10.4*</td>
</tr>
</tbody>
</table>

Mean ± SD values; percentages are expressed as percentage cells positive for the respective activation markers within the subpopulations; *p<0.05, ***p<0.001; p values were calculated using the Mann-Whitney U test (2-tailed).

As we found Fas-induced apoptosis similar in smokers and non-smokers in vitro, we wondered whether differences in percentages of apoptotic lymphocytes could be demonstrated between smoking and non-smoking individuals in vivo. Percentages of circulating apoptotic lymphocytes were assessed by annexin V staining. Although percentages of apoptotic CD19+ cells, CD4+ cells and CD8+ T cells were higher in smokers versus non-smokers, differences were not statistically significant (table 3).

Table 3:

Percentages of total lymphocytes and lymphocyte subsets staining with annexin V in smokers and non-smokers

<table>
<thead>
<tr>
<th></th>
<th>non-smokers</th>
<th>smokers</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>% lymphocytes annexin V+</td>
<td>4.0 ± 3.4</td>
<td>5.6 ± 8.9</td>
<td>0.82</td>
</tr>
<tr>
<td>% CD4+ annexin V+</td>
<td>2.0 ± 0.8</td>
<td>3.2 ± 4.3</td>
<td>0.97</td>
</tr>
<tr>
<td>% CD8+ annexin V+</td>
<td>2.0 ± 4.3</td>
<td>4.3 ± 5.2</td>
<td>0.28</td>
</tr>
<tr>
<td>% CD19+ annexin V+</td>
<td>7.9 ± 3.2</td>
<td>9.4 ± 13.4</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Mean ± SD values; p values were calculated using the Mann-Whitney U test (2-tailed).

Discussion

The influence of smoking on numbers and function of PBL has been demonstrated in several studies. T cell dependent and independent antibody responses are decreased. The mechanisms responsible for these effects, however, are not well understood [2;3]. We show that smoking is associated with increased expression of Fas on PBL, on B-lymphocytes in particular.
As can be observed from the proportions of peripheral blood lymphocyte subsets which express Fas, relatively larger effects of smoking were found on B cells. In smokers, the percentage Fas expressing B cells was nearly doubled compared to non-smokers whereas in the CD4+ subpopulation the proportion Fas positive cells only rose from 43.4 up to 57.7 percent. Thus, differential effects of smoking on lymphocytes subpopulations cannot be excluded. Differential effects of smoking on B- and T-lymphocytes might be explained through fundamental differences between these cells. Apoptosis of B and T-lymphocytes seems to be initiated via different pathways [7]. B cells need stimulation via the B cell receptor in conjunction with a costimulatory signal for proliferation and differentiation [8]. Inadequate signaling results in apoptosis that ensures a specific immune response because bystander B cells will be eliminated. For example, stimulation of resting B cells via CD40 ligand only leads to upregulation of Fas and increased susceptibility to Fas-mediated cell death [9]. It is tempting to speculate that smoking serves as an inadequate activating signal for lymphocytes, resulting in increased Fas expression. A higher percentage of resting B cells will become Fas positive and will, upon further activation, disappear by apoptosis. This hypothesis is supported by our in vitro results showing comparable Fas-mediated apoptosis between smokers and non-smokers, although the numbers of individuals studied were small. The decreased percentage of CD38+ B cells that we found in smokers might be explained by elimination of CD38+, Fas expressing cells via apoptosis. Seemingly in contradiction with this hypothesis is the lack of evidence that we found for increased apoptosis of lymphocytes in smoking individuals, as percentages of annexin V staining lymphocytes did not differ between smokers and non-smokers. However, as apoptosis is an ongoing event and cigarette smoking in all participants in this study could be considered a chronic stimulus, smoking might result in slightly elevated levels of apoptotic lymphocytes only (table 3), due to continuous clearance of apoptotic cells from the circulation. Only in cases when decreased clearance of apoptotic cells has been suggested, such as in systemic lupus erythematosus [10], a significant increase of circulating apoptotic cells will be observed. Nevertheless, the absolute as well as relative numbers of circulating B cells were not decreased in smokers although we are not informed about possible differences in the total (intra- and extravascular) numbers of (B-) lymphocytes between smokers and non-smokers. Fas expression on PBL of smoking individuals has been reported to be unaltered in one other study [11]. However, this study measured Fas expression on total lymphocytes only, and no analyses of subpopulations were performed. As B cells constitute a minority of PBL, changes in Fas expression on these cells may have been missed. It is noteworthy that in the same study it is demonstrated that Fas ligand is constitutively expressed on the majority of T cells of individuals with chronic cigarette smoking. This supports our concept that smoking stimulates lymphocytes since FasL is expressed on T cells after activation only [12].
In conclusion, our data demonstrate that smoking is associated with elevated percentages of Fas expressing CD4⁺ and CD19⁺ PBL in healthy individuals. Probably, as the Fas pathway did not seem to differ between smokers and non-smokers, activated CD19⁺ PBL are eliminated at a higher rate. The increase in Fas expression on these lymphocyte subsets therefore might have consequences for the immune response and might contribute to the decreased (humoral) responsiveness of smoking individuals.

References
