Graves' ophthalmopathy: in search of better markers and better treatment
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Chapter 4

Smoking and disease severity are independent determinants of serum adhesion molecule levels in Graves' ophthalmopathy


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Adhesion molecules play a key role in autoimmune disorders, and serum concentrations of soluble adhesion molecules are increased in Graves' ophthalmopathy (GO). Whether this is due to the strong association with smoking is unknown. It is also not known if the severity or activity of GO determine the serum levels of adhesion molecules. We measured serum concentrations of sICAM-1, sVCAM-1 and sELAM-1 in 62 euthyroid Graves' patients with untreated GO, in 62 healthy controls, matched for sex, age and smoking habits, and in 26 euthyroid Graves' patients without GO. GO severity was assessed by the Total Eye Score and the activity by the Clinical Activity Score. Adhesion molecules were measured by highly sensitive ELISAs. GO patients had higher levels than controls (median values in ng/ml with range): sICAM-1 300 [171–575] vs. 244 [119–674], P<0.001; sVCAM-1 457 [317–1060] vs. 410 [238–562], P<0.001; and sELAM-1 61 [19–174] vs. 53 [23–118], P=0.021. Euthyroid Graves' disease patients without GO had levels similar to controls: sICAM-1 273 [138–453], sVCAM-1 386 [260–1041] and sELAM-1 46 [22–118]. Smoking had an independent effect and was associated with higher levels of sICAM-1 and lower levels of sVCAM-1 in both GO patients and controls; sELAM-1 levels were comparable. In the 62 GO patients, sICAM-1 correlated significantly with severity of eye disease: r =0.40, P=0.002. No correlation was found with the duration of GO, the Clinical Activity Score or TBI levels. Multivariate analysis of all 150 subjects showed that the presence of GO and smoking are independent determinants of sICAM-1 and sVCAM-1 concentrations. In GO patients the Total Eye Score was a stronger determinant than smoking.

It is concluded that i) smoking is associated with increased sICAM-1 and decreased sVCAM-1 levels; ii) independent from smoking, euthyroid GO patients have higher levels of sICAM-1, sVCAM-1 and sELAM-1 than patients with euthyroid Graves' disease or healthy controls; iii) the major determinant of sICAM-1 in GO patients is the severity of their eye disease.

Introduction

Adhesion molecules are derived from three families of proteins: the immunoglobulin (IG) superfamily, the integrin family and the selectin family. They play an important role in trafficking of lymphocytes and their interaction with connective tissue cells and the extracellular matrix.

The aetiology of Graves' ophthalmopathy (GO) is unknown, but adhesion molecules are probably involved in this autoimmune attack. Heufelder et al. showed that Intracellular Adhesion Molecule-1 (ICAM-1) is expressed on cultured orbital fibroblasts upon incubation with various cytokines or with purified serum IgG's from Graves' disease patients. Immunohistochemistry of orbital connective tissue samples from GO patients showed an increased expression of ICAM-1, Vascular Cell Adhesion Molecule-1 (VCAM-1) and Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1) compared with tissues from controls. Moreover, Pappa et al. found increased expression of ICAM-1, VCAM-1 and ELAM-1 associated with early and thus, probably active eye disease.

Soluble forms of these adhesion molecules can be measured in serum, and several studies report that patients with GO have higher levels of sICAM-1 and sELAM-1 in serum than
patients with Graves’ disease without GO or healthy controls.\textsuperscript{7-9} This has attracted our attention, because a serum marker for GO would be very helpful. First, if levels would correlate with the severity of the eye disease, they might be useful as an objective parameter. Second, it is difficult to distinguish clinically the active, inflammatory stage of the disease from the inactive fibrotic endstage.\textsuperscript{10-12} If serum adhesion molecules would correlate with disease activity, they might serve as an activity marker, which could be helpful in selecting patients who might benefit from immunosuppressive therapy. The studies performed so far did not correlate serum adhesion molecule levels with the severity or the activity of the eye disease.

Table 1 : Characteristics of 62 healthy controls, 62 patients with Graves’ ophthalmopathy (GO) and 26 patients with Graves’ thyroid disease without GO (GTD).

<table>
<thead>
<tr>
<th></th>
<th>Controls n=62</th>
<th>GO n=62</th>
<th>GTD n=26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in yrs mean±SD</td>
<td>52.5±10</td>
<td>52.6±10</td>
<td>37±12*</td>
</tr>
<tr>
<td>Female/Male</td>
<td>44/18</td>
<td>44/18</td>
<td>22/4</td>
</tr>
<tr>
<td>Smoking yes/no</td>
<td>32/30</td>
<td>32/30</td>
<td>10/16</td>
</tr>
<tr>
<td>TSH (mU/l) mean±SD</td>
<td>2.0±1.3</td>
<td>1.8±2.2</td>
<td>1.55±2.0</td>
</tr>
<tr>
<td>fT\textsubscript{4} (pmol/l) mean±SD</td>
<td>14.7±3.4</td>
<td>15.5±3.9</td>
<td>14.6±2.7</td>
</tr>
<tr>
<td>TBII (U/l) median(range)</td>
<td>-</td>
<td>16 (3–405)</td>
<td>3 (3–22) **</td>
</tr>
<tr>
<td>duration of GTD in months median(range)</td>
<td>-</td>
<td>18 (0–276)</td>
<td>12 (6–60)</td>
</tr>
<tr>
<td>duration of GO in months median(range)</td>
<td>-</td>
<td>13 (1–156)</td>
<td>-</td>
</tr>
</tbody>
</table>

* Students t test: P<0.001 vs. controls and GO patients **Mann-Whitney U-test: P=0.006 vs. GO patients

Smoking is a well established risk factor for GO.\textsuperscript{13-15} The proportion of smokers among GO patients is much higher than in patients with Graves’ disease without GO or in the general population. There have been studies in patients without thyroid disease, mainly in patients with atherosclerosis, showing that smoking is associated with increased serum levels of sICAM-1 and sVCAM-1.\textsuperscript{16,17} The effect of smoking was not taken into account in the earlier studies in Graves’ disease patients.

We therefore decided to measure serum levels of sICAM-1, sVCAM-1 and sELAM-1 in 62 euthyroid Graves’ patients with GO, 62 healthy controls matched for sex, age and smoking habits, and 26 patients with euthyroid Graves’ disease without GO. The aims of
this study were: i) to establish whether smoking has an effect on serum adhesion molecules; ii) to evaluate whether GO patients have higher serum levels of adhesion molecules than controls and Graves' patients without GO; iii) to analyse whether serum levels of adhesion molecules are correlated to the activity and/or severity of the eye disease.

**Patients and Methods**

**Patients** We studied 62 patients with untreated GO who had been rendered euthyroid mostly by antithyroid drugs. The ophthalmopathy varied from mild to very severe and its severity was graded using the Total Eye Score. The Total Eye Score is calculated as the sum of each NOSPECS class present times the grade in that class (for that purpose we substituted 1, 2 and 3 respectively, for grades a, b, and c). The activity of the ophthalmopathy was scored using the Clinical Activity Score based upon classic inflammation parameters: rubor, dolor, tumour and functio laesa. The Clinical Activity Score consists of 10 items; for each item present 1 point is given, and the maximal score is 10. We also noted the duration of both the eye disease and the thyroid disease in months since the first signs and symptoms. Controls were recruited from the general population by advertisement, and participated in a study to determine reference values. They were matched with GO patients for age, sex and smoking habits. Smokers were defined as individuals who currently smoked cigarettes and non-smokers as those who did not. We further studied 26 patients with Graves' disease without any eye symptoms. They were all treated with antithyroid drugs, and had been euthyroid clinically and biochemically for at least 2 months.

**Methods** Serum samples were kept stored at -20°C, until use. All samples were measured in duplicate. Highly sensitive, commercially available ELISA kits (Quantikine, R&D Systems, Minneapolis, MN) were used to measure serum concentrations of sICAM-1 (detection limit 0.35 ng/ml), sVCAM-1 (detection limit 2.00 ng/ml), sELAM-1 (detection limit 0.1 ng/ml). Free $T_3$ ($FT_3$) was determined with either a coated tube $^{125}$I radioimmunoassay (SPAC, Byk-Sangtec Diagnostica, Dietzenbach 2, Germany), or a solid phase time-resolved fluoroimmunoassay (Delfia, Wallac Oy, Turku, Finland). Thyrotropin receptor antibodies (TBII) were measured by TRAK assay (BRAHMS Diagnostica, Berlin, Germany). TSH was measured in a chemiluminescent enzyme immunoassay (Immulite Third Generation TSH kit, DPC, Los Angeles, CA).

**Statistical analysis** To analyse differences between the three groups, we used two tailed t-tests or in cases of abnormal distribution, the Mann-Whitney U-test. Correlations were calculated with two-tailed Pearson correlation coefficients or Spearman correlations. Multivariate analysis was used to assess whether GO and smoking were independent factors for adhesion molecule levels, and whether these levels were influenced by characteristics of thyroid disease or eye disease. In short, each variable that correlated (P<0.05) with one of the adhesion molecule levels in a univariate analysis, was entered in a multivariate model using multiple linear regression analysis. The SPSS 10.0.7 software was used.

**Results**

The general characteristics of the three groups are given in Table 1. Thyroid function was similar in all three groups. Graves' patients without GO were younger than the GO patients and the healthy controls. TBII levels were higher in the GO patients than in the Graves' disease patients.

In the 88 Graves' patients (62 patients with GO and 26 without GO) there was no cor-
relation with the duration of the thyroid disease or the TBII titer in the 58 patients (49 patients with GO and 9 Graves' patients without GO) in whom TBII was measured. GO patients had higher levels of sICAM-1, sVCAM-1 and sELAM-1 compared with healthy controls and Graves’ patients without GO (Figure 1). In the 62 GO patients, no correlation was found with the Clinical Activity Score or the duration of the eye disease. However, there was a correlation between sICAM-1 levels and disease severity as assessed with the Total Eye Score (r =0.40, P=0.002; Figure 2).

Univariate analysis in all subjects showed that levels of sICAM-1, sVCAM-1 and sELAM-1 not only correlated with presence of GO but that sICAM-1 and sVCAM-1 were also influenced by smoking habits. The 74 smokers had higher levels of sICAM-1 than the 76 non-smokers (291[172–674] ng/ml vs. 253[119–438] P=0.001) whereas sVCAM-1 levels were lower in smokers than in non-smokers (413[238–603] ng/ml vs. 449[260–1060] P=0.001). Smoking did not influence sELAM-1 levels. (Table 2).

Multivariate analysis in all 150 subjects showed that the presence of GO (P=0.001) and smoking (P<0.001) are independent determinants of sICAM-1 levels. Using multivariate analysis within the group of 62 GO patients, disease severity (Total Eye Score) was a stronger determinant of sICAM-1 (P=0.006) than smoking (P=0.09). sVCAM-1 levels were related to the presence of GO (P<0.001) and smoking (P=0.001), but not to the Total Eye Score.

Discussion

In this study we demonstrated that smokers have higher sICAM-1 levels and lower sVCAM-1 levels than non-smokers. Since Graves’ patients often smoke, this finding
Table 2: Effect of smoking on serum levels of sICAM-1, sVCAM-1 and sELAM-1 in 62 healthy controls, 62 patients with Graves’ ophthalmopathy (GO) and 26 patients with Graves’ thyroid disease without GO (GTD).

<table>
<thead>
<tr>
<th></th>
<th>smokers</th>
<th></th>
<th>non-smokers</th>
<th></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td></td>
<td>n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sICAM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>controls</td>
<td>32</td>
<td>253 (172–674)</td>
<td>30</td>
<td>229 (119–322)</td>
<td>0.013</td>
</tr>
<tr>
<td>GO</td>
<td>32</td>
<td>319 (232–575)*</td>
<td>30</td>
<td>290 (171–438)*</td>
<td>0.062</td>
</tr>
<tr>
<td>GTD</td>
<td>10</td>
<td>280 (220–453)</td>
<td>16</td>
<td>257 (138–377)</td>
<td>ns</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>controls</td>
<td>32</td>
<td>400 (238–517)</td>
<td>30</td>
<td>434 (336–562)</td>
<td>0.011</td>
</tr>
<tr>
<td>GO</td>
<td>32</td>
<td>432 (317–603)*</td>
<td>30</td>
<td>504 (334–1060)*</td>
<td>0.003</td>
</tr>
<tr>
<td>GTD</td>
<td>10</td>
<td>377 (278–579)</td>
<td>16</td>
<td>396 (260–1041)</td>
<td>ns</td>
</tr>
<tr>
<td>sELAM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>controls</td>
<td>32</td>
<td>57 (24–98)</td>
<td>30</td>
<td>50 (23–118)</td>
<td>ns</td>
</tr>
<tr>
<td>GO</td>
<td>32</td>
<td>65 (19–85)</td>
<td>30</td>
<td>59 (27–174)**</td>
<td>ns</td>
</tr>
<tr>
<td>GTD</td>
<td>10</td>
<td>56 (22–118)</td>
<td>16</td>
<td>44 (27–79)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values given as median and range in ng/ml. Statistical analysis was performed with the Mann-Whitney U-test. * = P<0.01 between GO patients and controls; **=P<0.05 between GO patients and controls

constitutes a confounding factor in the earlier studies showing higher sICAM-1 levels in ophthalmopathy patients. However, our study clearly showed that ophthalmopathy, apart from smoking, is also associated with increased sICAM-1, sVCAM-1 and sELAM-1 levels because (i) we compared our GO patients with a healthy control group matched for smoking habits, (ii) we compared the GO patients with a group of Graves’ patients without GO and (iii) using multiple linear regression analysis, the presence of ophthalmopathy was an independent determinant of sICAM-1 levels. There was no relationship between serum adhesion molecule levels and the activity of the eye disease (duration of GO, Clinical Activity Score). However, sICAM-1 levels did correlate with the severity of the eye disease as assessed by the Total Eye Score.

It thus appears that in patients with Graves’ disease, sICAM-1 levels are higher in the presence of orbital disease. This would be in agreement with De Bellis et al., who followed a group of 103 newly diagnosed Graves’ hyperthyroidism patients for development of GO, and found an increase in sICAM-1 concentrations in 12/14 patients just before onset of GO. We could not confirm a correlation with disease activity, disqualifying serum adhesion molecules as an activity parameter. We have no good explanation why adhesion molecules in serum do correlate with severity, but not with activity. However, it should be noted that there is no feasible gold standard for activity of the eye disease and the Clinical Activity Score is at best a weak surrogate marker. It might still be that serum adhesion molecule levels reflect histologically assessed active inflammation in orbital tissues.

Nevertheless, GO patients did have higher levels of sICAM-1, sVCAM-1 and sELAM-1 than healthy controls. However, there is considerable overlap in adhesion molecule levels between controls and Graves’ patients without and with GO (Figure 1), precluding the use of sICAM-1 as a serum parameter for
ophthalmopathy. This overlap was not found in earlier studies. In the case of sICAM-1 this might have been caused by selection of patients and controls, because less overlap may be expected if controls are predominantly non-smokers as opposed to smoking patients who, because they smoke, may also have more severe GO. In the group of Graves' thyroid disease patients without GO, the difference between smokers and non-smokers was less marked, probably because of a small sample size.

It is intriguing to speculate how smoking affects the expression of adhesion molecules. Bergmann et al. found that healthy smoking women had higher levels of sICAM-1 than non-smoking women as well as a higher leukocyte count and, subsequently, more monocytes. These data suggest that smoking leads to leukocyte recruitment, increased adhesion molecule expression and thus, more cell adhesion to vessel walls. This phenomenon might lead to atherosclerotic plaque formation and trafficking of lymphocytes. We could not find any reports of studies on smoking and sICAM-1 serum levels in other autoimmune diseases.

The relative risk of smoking is 7.7 for GO, but only 1.9 for Graves' hyperthyroidism without eye disease. This strong association with GO puzzles many researchers. There might be a role for hypoxemia, as was shown in an in vitro study by Metcalfe et al. in which hypoxic culture conditions induced orbital fibroblasts from healthy individuals to produce more glycosaminoglycans and to synthesize more DNA. Recently, it was found that in cultured orbital fibroblasts HLA-DR expression could be upregulated by a combination of nicotine or tar with interferon gamma. The combined data may indicate that smoking induces a general increase in tonus of the immune system, in line with our previous report that smoking tended to increase the serum levels of sIL-1RA and IL-6R, and the titre of Heat Shock Protein 72 antibodies. Thus, smoking will result in a more severe autoimmune attack in subjects at risk for Graves' disease.

In conclusion, smoking is associated with higher sICAM-1 and lower sVCAM-1 levels. Patients with GO have higher levels of sICAM-1, sVCAM-1 and sELAM-1 than controls and than Graves' patients without GO. sICAM-1 levels are positively correlated with the severity of GO.

References

with Graves' disease with or without ophthalmopathy and in patients with toxic adenoma. J Clin Endocrinol Metab 1995; 80 (7) 2118–21.


