Increased mononuclear cell membrane fluidity and increased B lymphocyte HLA class I expression in IgA nephropathy

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Abstract

Background. The functions of membrane bound proteins are regulated by the physical properties of the cell membrane. Lymphocyte dysfunction in IgA nephropathy may therefore be related to abnormal cell membrane fluidity. In this study we have examined peripheral blood mononuclear cell membrane fluidity and the expression of HLA antigens on the surface of lymphocytes in IgA nephropathy subjects compared to normal controls and disease controls.

Methods. Twenty-two IgA nephropathy subjects with normal or mildly elevated serum creatinine (serum creatinine < 130 µmol/l), 21 normal control subjects, and 16 disease control subjects were studied. Fluorescence anisotropy was determined using diphenylhexatriene, which partitions deep into the cell membrane, and trimethylammonium-DPH, which partitions more superficially. The lymphocyte expression of HLA antigens was measured by flow cytometry.

Results. Fluorescence anisotropy for diphenylhexatriene of mononuclear cells from IgA nephropathy subjects was significantly lower compared to mononuclear cells from normal control subjects and disease control subjects, indicating higher membrane fluidity (median values, IgA nephropathy 0.161; normal control 0.175; disease control 0.175; P<0.001 and P<0.001). Fluorescence anisotropy for trimethylammonium-DPH was lower in the IgA nephropathy group compared to the normal control group (median values, IgA nephropathy 0.268; normal control 0.274; P<0.001), but not significantly different compared to the disease control group (0.272). HLA class I expression on the surface of B lymphocytes was significantly higher in the IgA nephropathy group compared to the normal control and disease control groups (median values, IgA nephropathy 30364; normal control 15495; disease control 16907; P=0.0001 and P=0.002 respectively).

Conclusion. This study provides evidence of abnormal cell membrane architecture and increased HLA class I expression in IgA nephropathy.

Key words: fluorescence anisotropy; HLA expression; IgA nephropathy; lymphocyte; membrane fluidity

Introduction

The precise immune defect responsible for IgA nephropathy remains unknown. The close temporal association between upper respiratory tract infections and exacerbations of haematuria in a proportion of subjects with IgA nephropathy indicates that there may be defects in mucosal immune defence mechanisms. The immunoglobulin produced is polyclonal and is generally reported to be of the IgA1 isotype in Caucasians [1]. It is therefore likely to originate from systemic sources rather than from mucosally associated lymphoid tissue. It is well recognized that B lymphocytes from patients with IgA nephropathy, when cultured, respond inappropriately to a mitogen such as pokeweed (PWM) by producing more immunoglobulins, particularly of the IgA class [2,3]. Abnormalities of T lymphocytes and lymphocyte cytokine networks have also been identified [4].

Such responses to a mitogen are mediated by cellsurface receptor coupling leading to intracellular events. The behaviour of receptors and other proteins are influenced by the physical properties of the lipid bilayer in which they are positioned. Thus the composition of the cell membrane and its organization have regulatory effects on cellular function. Altered cell membrane composition is associated with changes in the function of membrane bound proteins such as the Na⁺–H⁺ exchanger [5] and the insulin receptor [6]. In addition, alteration of cell membrane fluidity by dietary supplementation has also been shown to affect the expression of major histocompatibility antigens (MHC) in mice splenocytes [7], the function of natural killer cells [8], and to attenuate T lymphocyte proliferation in response to a mitogen [9].

It has been reported that abnormal erythrocyte sodium–lithium countertransport (SLC) activity iden-
tifies those patients with IgA nephropathy who are at most risk of progression of their renal disease [10]. In other conditions, such as hyperlipidaemia and insulin-dependent diabetes mellitus, abnormalities in SLC are associated with altered erythrocyte membrane fluidity [11,12]. Deranged SLC in IgA nephropathy may reflect abnormal erythrocyte membrane architecture which may also be present in other cell types and cause disruption of other aspects of cell function. Lymphocyte dysfunction in IgA nephropathy may therefore be related to abnormal cell membrane structure. This may in turn provide a rationale for the use of membrane modifying agents for the treatment of the condition. Trials of dietary highly unsaturated fatty acids (HUFA) supplements have recently shown a significant benefit in terms of disease progression [13].

In the present study we have examined peripheral blood mononuclear cell (PBMC) membrane fluidity in IgA nephropathy patients compared to normal and disease control subjects using 1,6-diphenyl-1,3,5-hexatriene (DPH) and 4-trimethylammonium-DPH (TMA) fluorescent probes. The expression of lymphocyte surface molecules has been quantified by flow cytometry in order to examine any relationship between cell membrane fluidity and the function of membrane bound proteins.

Subjects and methods

Subjects

Twenty-two patients with biopsy-proven IgA nephropathy with either normal or mildly elevated serum creatinine (serum creatinine < 130 umol/l) were selected from outpatient clinics at the Freeman Hospital, Newcastle upon Tyne. Subjects taking medication other than antihypertensives or diuretics were considered to be hypertensive. Twenty millilitres of venous blood was then collected. Blood samples for isolation of mononuclear cells were collected into lithium heparin tubes and samples for measurement of serum creatinine, total serum cholesterol and serum triglyceride collected into plain tubes.

Methods

Subjects attended outpatient clinics at Freeman Hospital, Newcastle upon Tyne or were visited at home. After 5 min recumbency, blood pressure was measured three times using a standard sphygmomanometer and the mean of the second and third readings recorded. Systolic and diastolic readings were taken at phases I and V respectively. Expressed values are the mean arterial pressure (diastolic plus one-third of the pulse pressure). Patients with a systolic blood pressure above 160 mmHg or a diastolic blood pressure above 90 mmHg and those taking antihypertensive medication were considered to be hypertensive. Twenty millilitres of venous blood was then collected. Blood samples for isolation of mononuclear cells were collected into lithium heparin tubes and samples for measurement of serum creatinine, total serum cholesterol and serum triglyceride collected into plain tubes.

Mononuclear cell preparation

Whole blood was diluted 1:1 with phosphate-buffered saline (PBS), layered onto Ficoll–Hypaque (d = 1.077) and centrifuged at 400 g for 25 min. The mononuclear cell layer was then washed to remove platelets (centrifugation at 250 g for 15 min) and resuspended in PBS. A cell count of the suspension was then determined using a Coulter counter (Model S+ STKR) and the lymphocyte concentration adjusted as required for fluorescence anisotropy and flow cytometry described below.

Cell membrane fluidity

Mononuclear cell suspensions were prepared with a cell concentration of 5 x 10^6 per ml in 3 ml of PBS with 5 mmol/l glucose. The fluorescent probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 4-trimethylammonium-DPH (TMA), dissolved in acetone and dimethylformamide respectively (to 2 x 10^{-3} M), were added to separate suspensions to a final concentration of 2 x 10^{-6} M. Fluorescence anisotropy readings were made immediately for TMA and after 30 min incubation for DPH using a Perkin Elmer LS 50B fluorimeter with polarizing filters. The mean of four readings was taken at 37°C with an excitation wavelength of 360 nm and an emission wavelength of 430 nm. Fluorescence anisotropy (r) is defined by the equation below where lvv and lvh are the intensities measured in directions parallel and perpendicular to the vector of the vertically polarized exciting light, lhv and lhv are the intensities measured in directions parallel and perpendicular to the vector of the horizontally polarized exciting light, and G is the grating correction factor [14]. Fluidity is inversely related to fluorescence anisotropy.

\[ r = \frac{lvv - (lhv \times G)}{lvv + 2(lhv \times G)}, \quad G = \frac{lhv}{lhv} \]

Data are expressed as median and range.

<table>
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<th></th>
<th>n</th>
<th>Age (years)</th>
<th>Serum creatinine (umol/l)</th>
<th>Serum triglyceride (mmol/l)</th>
<th>Serum cholesterol (mmol/l)</th>
<th>Mean arterial pressure (mmHg)</th>
<th>Urinary protein (n%) with &gt; 1 g/24 h</th>
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<tbody>
<tr>
<td>NC</td>
<td>21</td>
<td>31 (20–56)</td>
<td>79.5 (58–106)</td>
<td>0.8 (0.5–2.2)</td>
<td>5.3 (3.5–6.6)</td>
<td>89.3 (81–100)</td>
<td>0 (0)</td>
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<tr>
<td>IGAN</td>
<td>22</td>
<td>40 (18–73)</td>
<td>103 (71–130)</td>
<td>1.6 (0.4–5.2)</td>
<td>5.6 (3.8–8.6)</td>
<td>97.5 (78.3–116.7)</td>
<td>8 (36)</td>
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<tr>
<td>DC</td>
<td>16</td>
<td>44 (25–75)</td>
<td>105 (63–221)</td>
<td>2.5 (0.8–5)</td>
<td>7.6 (3.8–13.7)</td>
<td>104 (83.7–123.3)</td>
<td>6 (38)</td>
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Table 1. Characteristics of normal control (NC), IgA nephropathy (IGAN) and disease control subjects (DC).
Flow cytometry

Ten-microlitre aliquots of the mononuclear cell suspensions (10^5 cells) were labelled with either monoclonal antibody to CD19 (to identify B lymphocytes) or to CD3 (to identify T lymphocytes). Both antibodies were conjugated with fluorescein–isothiocyanate (FITC). Saturating concentrations of monoclonal antibody specific for HLA class I (Serotec Ltd-W6/32) or HLA class II (Serotec Ltd-WR18) were used to quantify the surface expression of HLA class I and II antigens on the lymphocytes (antibodies were conjugated with phycocerythrin-PE). Each suspension of mononuclear cells was labelled with the conjugates for 30 min at 4°C and then washed to remove unbound antibody. Two-colour flow cytometry was performed using a Becton Dickinson FACSCAN. Standardized latex beads were used to calibrate the channel number into fluorescence molecule equivalents (PE Quantum beads, Flow Cytometry Standard Corporation).

Other biochemical analyses

Serum triglycerides, total cholesterol, and serum creatinine were measured using the Olympus AU560 and AU5000 respectively. Subjects whose serum contained chylomicrons were excluded from the study.

Statistical analysis

Unpaired t tests were used to compare variables that were normally distributed. Plasma triglyceride, plasma creatinine, DPH anisotropy, TMA anisotropy, and HLA expression were not normally distributed and were tested using Mann–Whitney U test. Pearson or Spearman correlation analyses were used to examine any relationship between test variables that had normal or non-parametric distributions respectively.

Results

Ten of 22 of the IgA nephropathy subjects were hypertensive (9 were taking antihypertensive medication; 4 β-blockers, 3 calcium antagonists, 1 β-blocker and calcium antagonist, 1 thiazide and β-blocker) and five of 16 of the disease control group were hypertensive (4 were taking antihypertensive medication; 1 β-blocker, 3 β-blocker and diuretic). Eight of the 22 IgA nephropathy subjects and 10 of the disease control subjects had serum creatinine values above the upper limit of normal (females, 90 μmol/l; males, 110 μmol/l). Serum creatinine values were higher in the disease control group than in the IgA nephropathy group. Serum triglyceride values were significantly higher in IgA nephropathy subjects compared to the normal control group, but IgA nephropathy and disease control groups had similar values. Total plasma cholesterol values were not significantly different between groups. There was no significant difference between the mean ages of the groups. Eight of the IgA nephropathy subjects and six of the disease control subjects had a urinary protein excretion greater than 1 g per 24 h.

The fluorescence anisotropy for DPH was lower in the mononuclear cells from IgA nephropathy subjects compared to mononuclear cells from normal controls and disease controls (median (range): IgA nephropathy 0.161 (0.147–0.174), normal control 0.175 (0.159–0.183), disease control 0.175 (0.162–0.186) P<0.001 and P<0.001, Mann-Whitney U test, Figure 1A). The fluorescence anisotropy of TMA was lower in the mononuclear cells from IgA nephropathy subjects than in the mononuclear cells from normal controls (median (range): IgA nephropathy 0.268 (0.256–0.294), normal control 0.274 (0.269–0.291), P<0.001, Mann–Whitney U test, Figure 1B) but was not significantly lower compared to mononuclear cells from the disease control subjects (median (range): 0.272 (0.258–0.284), P = 0.18). Comparison of normotensive and hypertensive IgA nephropathy subjects showed no difference in...
mononuclear cell anisotropy. Neither measure of anisotropy was related to age, serum creatinine, total serum cholesterol, serum triglyceride, or presence of proteinuria. Assessment of intrasubject variability of fluorescence anisotropy measurements, made by repeating the measurements for five normal control subjects and five subjects with IgA nephropathy, showed consistently lower values for the IgA nephropathy group compared to the normal control group. The interval between measurements ranged from 4 months to 12 months (Figure 2).

The expression of HLA class I of B lymphocytes from IgA nephropathy subjects was significantly higher than of B lymphocytes from normal controls and disease controls (Table 2, Figure 3). There was no significant correlation between HLA class I expression and age, total serum cholesterol, serum triglyceride, serum creatinine, DPH anisotropy or TMA anisotropy. There was no difference between the groups in the measures of expression of HLA class I on T lymphocytes and HLA class II on B lymphocytes and T lymphocytes (Table 2). Neither the percentage of B lymphocytes nor the percentage of monocytes were different between the IgA nephropathy and normal control groups (mean (SEM) B lymphocytes: IgA nephropathy 2.99 (0.41), normal controls 3.24 (0.42%), Monocytes: IgA nephropathy 9.27 (1.24), normal controls 9.45 (1.03)).

**Discussion**

The lower anisotropy values for DPH imply that membrane fluidity is significantly higher in mononuclear cells from IgA nephropathy subjects compared to mononuclear cells from normal controls and disease controls. This phenomenon cannot be attributed to alterations in the proportion of B lymphocytes and monocytes in the cell suspension as these remained similar between the groups studied. There was a wide range of overlap between anisotropy values with DPH for all groups. IgA nephropathy is often characterized by relapses and remissions and it may be that only subjects with active disease have abnormal mononuclear cell membrane fluidity. However the subjects of this study had no overt evidence of an active phase

<table>
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<th>Table 2. HLA class I and II expression of B and T lymphocytes for normal control (NC), IgA nephropathy (IGAN) and disease control groups</th>
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<td>NC</td>
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Flow cytometry data expressed as fluorescence molecule equivalents (median (range). Mann–Whitney U test: \( P = 0.0001 \) compared to NC, \( P = 0.002 \) compared to DC.
Lymphocyte membrane fluidity and HLA expression in IgA nephropathy

in their disease. Cell membrane fluidity is dependent upon many factors.

The range of anisotropy values for each of the three groups was similar, which does not suggest the presence of a subgroup with lower anisotropy, but is consistent with a general lowering of anisotropy values in the IgA nephropathy group. Assessment of fluorescence anisotropy measurements on two separate occasions in this study demonstrated a degree of variability in both DPH and TMA anisotropies, but showed a consistent lowering of the values for the IgA subjects compared to the normal controls. Mononuclear cell membrane fluorescence anisotropy for DPH has been shown to increase significantly with age [15], but there was no significant difference in age between the three groups in this study. The observed differences in TMA anisotropy may be a non-specific feature of renal impairment as the values for the IgA nephropathy group were not significantly lower than the disease control group.

Surface expression of HLA class I of the B lymphocytes of IgA nephropathy subjects was significantly higher compared to the B lymphocytes of normal control and disease control subjects. As for cell membrane fluidity there was a wide overlap between values for the groups. This may be related to a spectrum of disease activity represented in the IgA nephropathy group. No significant relationship between HLA class I expression and either measure of membrane fluorescence anisotropy was demonstrated.

It is arguable that increased lymphocyte membrane fluidity in this study simply represents a normal phenomenon associated with activation of components of the mononuclear cell population. In vitro stimulation of T lymphocytes has been shown to lead to significant changes in the fatty acid composition of both neutral lipids and phospholipids of the cell membrane and an increase in fluidity measured using nitroxide spin-label e.s.r. and DPH anisotropy [16]. However, the expression of HLA class II antigens was not increased for B lymphocytes or T lymphocytes in the subjects with IgA nephropathy. This indicates that these cells were not activated.

It has been shown that alteration of cell membrane composition by enrichment either with highly unsaturated fatty acids (HUFA) [9] or cholesterol [17] markedly suppresses activation of lymphocytes to blast cells in response to mitogens in vitro. The effect of HUFA has been attributed to altered eicosanoid production resulting from the substitution of omega-3 polyunsaturated fatty acids for arachidonic acid as substrates for cyclo-oxygenase and lipoxygenase [18]. An additional explanation for this phenomenon may be the optimal fluidity hypothesis, proposed by Shinitzky [19], that perturbation of membrane fluidity in either direction from its optimum results in suboptimal membrane protein function. It is possible that a defect of cell membrane architecture, leading to a decreased fluorescence anisotropy for DPH is responsible for at least some of the observed lymphocyte abnormalities in IgA nephropathy by altering the function of membrane-associated proteins such as receptor molecules and surface antigens.

Increased HLA class I expression in IgA nephropathy may reflect abnormal presentation of viral or endogenous proteins at the cell surface. Surface expression of HLA class I may therefore reflect disease activity. Failure to show a correlation between anisotropy and HLA class I expression in this cross-sectional study may be due to other factors known to affect HLA class I expression. Abnormal membrane fluidity may not directly influence HLA expression, but may alter the response to activating stimuli. The significance of this observation could be further examined in a longitudinal study.

This study clearly demonstrates that both membrane fluidity and B lymphocyte HLA class I expression are increased in IgA nephropathy. An alteration in membrane fluidity may be of significance in the pathogenesis of the condition, or may be an associated phenomenon that affects disease progression. Manipulation of cell membrane fluidity in vitro has been shown to alter the function of membrane associated proteins [5, 6]. It remains to be shown whether this principle can be applied in vivo to correct the functional abnormalities of the cell membrane, but the possibility of correcting cell membrane dysfunction and thereby modifying the risk of end-organ damage is a worthwhile prospect. We feel that further studies are justified to elucidate the nature of the relationship between these parameters and the relationship to disease activity.

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References


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