Mechanisms of disease

A loss-of-function polymorphic mutation in the cytolytic P2X7 receptor gene and chronic lymphocytic leukaemia: a molecular study

Summary

Background Chronic lymphocytic leukaemia (CLL) has a familial incidence nearly three times higher than expected for the general population and one predisposing factor might be an inherited failure of mechanisms involved in apoptosis of lymphocytes. Our aim was to ascertain whether or not a defect in a proapoptotic pathway, caused by a single nucleotide polymorphism that results in loss-of-function of P2X7 in healthy individuals, was present in leukemic B lymphocytes of patients with CLL.

Methods We extracted genomic DNA from the peripheral blood leucocytes of 36 unrelated individuals with CLL, four individuals with familial CLL, and 46 age-matched controls. We sequenced a PCR product to detect mutations in exon 13 of P2X7. In most patients with CLL, we measured expression and function of the P2X7 receptor by flow cytometry in B lymphocytes and T lymphocytes.

Findings The prevalence of the polymorphic mutation and the frequency of the mutant allele were three-fold greater in individuals with CLL than in white, elderly controls. Individuals homozygous for the polymorphic allele had no P2X7 receptor function and heterozygotes had half the mean function of that seen in individuals homozygous for the wildtype allele; amounts of ATP-induced apoptosis varied accordingly. In two families, in which we studied a father-son pair and a sister-sister pair with CLL, loss of P2X7 function arose because of inheritance of one or two 1513A→C alleles for P2X7.

Interpretation Activation of the P2X7 receptor leads to apoptosis of lymphocytes in individuals with CLL, and reduced function of this receptor has an anti-apoptotic effect, resulting in an increase in B-cell numbers. Thus, inheritance of a loss-of-function polymorphic mutation at position 1513 in the P2X7 gene could contribute to the pathogenesis of CLL.

Introduction Chronic lymphocytic leukaemia (CLL) is the most frequent type of leukaemia in the more-developed world, and results in the progressive accumulation of mature CD5+ B lymphocytes in blood and bone marrow of the affected person.12 Anaemia and thrombocytopenia are features of advanced disease, but recurrent infections and splenomegaly, with or without lymphadenopathy, arise at all stages of CLL. Leukemic CD5+ B lymphocytes have reduced ability to undergo apoptosis in vivo, a feature generally attributed to the anti-apoptotic effects of BCL2 overexpression,13 although a defect in a proapoptotic pathway might also contribute to the prolonged survival of B lymphocytes in individuals with CLL.

Since the first report of identical twins with CLL,4 there have been many other reports of clustering of the disease in small families. Results of studies in more than 1500 families indicate that the familial incidence of CLL is nearly three times higher than that expected for the general population,14 though linkage analyses5 have excluded an effect of genes within the MHC region. Since CLL has a familial component, an inherited germline mutation could be a predisposing factor. However, neither cytogenetic nor molecular studies have identified deficiencies in recognised B lymphocyte apoptotic pathways other than that of BCL2 overexpression.

Purinergic P2X7 CYTOLYTIC RECEPTORS (a type of P2 PURINORECEPTOR) are ligand-gated cation channels that are expressed on, and mediate ATP-induced apoptosis of, cells of the immune system.1 Purinergic P2X7 is also expressed on the CD5+ B lymphocytes that accumulate in people with CLL. The P2X receptor family have two transmembrane domains with intracellular amino and carboxyl termini and a trimeric structure in the plasma membrane.5 P2X7 differs from other family members because it has a long carboxyl terminus of 240 amino acids that extends from the inner membrane face, and which is necessary for the permeability properties of the receptor (figure 1).10 When P2X7 receptors are activated by extracellular ATP, cation-selective channels open, resulting in potassium efflux from the cell and subsequent stimulation of intracellular CASPASES, leading to apoptosis.11 ATP initiates apoptosis of macrophages and the destruction of any engulfed intracellular mycobacteria, hence implicating the P2X7 receptor in the control of tuberculous infection.12,13 Our aim was to ascertain whether or not this receptor plays a part in apoptosis of B lymphocytes and hence the development of B-cell malignancies.
Methods

Patients
We enrolled unrelated individuals with CLL who attended Nepean Hospital between June, 1999, and Dec, 2001. We diagnosed CLL if patients had sustained lymphocytosis (lymphocyte concentration >4·0×10⁹/L) and a monoclonal B-lymphocyte population typed as CD5, CD19, CD20, and CD23 positive. We included only patients who had not received chemotherapy within a month of the start of the study, and, wherever possible, we assessed individuals before they were treated. If possible, we enrolled partners of patients as age-matched controls, though we also recruited controls from staff at Nepean Hospital. We included in analyses only patients and controls who were white and had no previous history of haematological or immunological disorders or chronic infections. We also studied members of three families in which CLL was present. The local ethics committee approved the study, and all participants provided written informed consent.

Study protocol
We obtained samples of peripheral venous blood from patients and controls. We measured the degree of expression of P2X7 on the surface of different mononuclear cell subsets, derived from samples, by staining cells with a fluorescein-conjugated antibody against CD19 or CD3. We identified specific antibody against P2X7 (courtesy of I Chessell, GlaxoSmithKline, Harlow, UK) or with an isotype control monoclonal antibody against CD19, CD3, or CD14, and incubated lymphocytes from patients with CLL on gated CD19+ cells as described.¹⁶ We incubated lymphocytes (1×10⁹/mL) with or without 200 μmol/L benzoyl benzoyl ATP, a potent agonist of the P2X7 receptor,¹⁷ in Hapes-buffered sodium chloride medium at 37°C for 20 min, washed them once, and resuspended in}

Figure 1: Membrane topology of the P2X7 receptor
The P2X7 receptor has two transmembrane domains with intracellular amino and carboxyl termini. ATP binds to a pocket that contains lysine residues in the extracellular domain to activate the receptor. The carboxyl terminal domain contains a loss-of-function single nucleotide polymorphism 1513A→C, which converts glutamic acid to alanine at aminoacid 496. This action might impair the assembly of the P2X7 channel within the membrane complex. The carboxyl terminal domain also contains a binding motif, which is important in trafficking and signal transduction.

Figure 2: Pedigree of three families two of which show a familial incidence of chronic lymphocytic leukaemia (CLL)
Black symbols=individuals with CLL. Genotype at position 1513 of P2X7 also shown.

GLOSSARY

CYTOLYTIC RECEPTOR
A receptor that, upon binding with its specific ligand, activates a cascade of signalling events that result in cell death.

CASPASE
An intracellular proteolytic enzyme that converts its substrate from an inactive to an active form. Caspase activation results in apoptosis (programmed cell death).

METALLOPROTEASE
An enzyme that requires a metal ion—eg, zinc, iron—to act as a cofactor for it to function.

P2 PURINOCEPTOR
A receptor that is activated by extracellular purine nucleotides—eg, ATP—or pyrimidine nucleotides—eg, UTP. P2 purinoceptors are subdivided into P2X receptors, which form ligand-gated ion channels, and P2Y receptors, which are G protein coupled receptors.
RPMI-1640 medium with 10% fetal calf serum. We incubated the lymphocytes for 24 h and then labelled the cells with the viability dye 7-amino-actinomycin D together with fluorescein-conjugated monoclonal antibody against CD19. We then calculated the proportion of apoptotic or dead cells within the appropriate quadrants.

Statistical analysis
Comparison of the prevalence and allele frequency of the 1513A→C mutation was calculated by the Fisher’s exact test. The Wilcoxon sum-rank test was used to compare differences in P2X7 receptor function between different groups.

Role of the funding source
The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results
We analysed results from 40 individuals with CLL and 46 age-matched controls. Of these, four individuals had familial CLL (figure 2). We also studied the healthy children of one of the non-familial CLL patients. 15 of the 36 unrelated patients with CLL had a heterozygous nucleotide substitution at position 1513 of P2X7, and one patient had a homozygous substitution. By contrast, only five controls were heterozygous and one homozygous for the substitution at this position. The prevalence of this single nucleotide polymorphism was three-fold greater in patients with CLL than in controls (p=0.0023; table 1). The frequency of the mutant allele was also significantly higher in patients than in controls (p=0.0066; table 1).

The patient who was homozygous at 1513 (C/C) had complete loss of receptor function in leukaemic B lymphocytes and in non-leukaemic T lymphocytes (figure 3), whereas heterozygosity conferred P2X7 function about half that of cells with wildtype sequence. This result was substantiated by measurement of P2X7 function in B lymphocytes from 33 unrelated patients with CLL, in which receptor function in heterozygous individuals was half that of those carrying two wildtype alleles (figure 4; p=0.0216). A similar and negative effect of the polymorphic gene dosage on P2X7 function has been described in leucocytes of healthy individuals.16 It is noteworthy that there was some variation in the functional response of the P2X7 receptor, and several patients who were either wildtype or heterozygous in genotype had an extremely low P2X7 channel function.

We studied inheritance of the polymorphic 1513A→C allele by genotyping the children of a patient diagnosed at age 65 years with CLL, who was homozygous

<table>
<thead>
<tr>
<th>Controls (n=46)</th>
<th>CLL (n=36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean, SD) (years)*</td>
<td>62·7 (12·5)</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>40</td>
</tr>
<tr>
<td>A/C</td>
<td>5</td>
</tr>
<tr>
<td>C/C</td>
<td>1</td>
</tr>
<tr>
<td>Proportion of Individuals with A/C or C/C genotype</td>
<td>13·0</td>
</tr>
<tr>
<td>Allele frequency</td>
<td>0·08</td>
</tr>
</tbody>
</table>

*At presentation of disease for patients with CLL.

Table 1: Frequency of the 1513A→C polymorphic allele for the P2X7 receptor in unrelated patients with chronic lymphocytic leukaemia (CLL) and in aged-matched unrelated controls

![Figure 3: ATP-induced ethidium uptake curves in B lymphocytes and T lymphocytes from five patients with chronic lymphocytic leukaemia according to P2X7 1513A→C polymorphism](image)

Ethidium uptake into B lymphocytes and T lymphocytes measured in the same mononuclear preparation.

![Figure 4: P2X7 function in CD19 lymphocytes from 33 unrelated patients with chronic lymphocytic leukaemia, who are wildtype, heterozygous, or homozygous for the loss-of-function 1513A→C allele](image)
for 1513A→C alleles (family 1, figure 2). All five children were heterozygous for this allele although none had CLL. We grew fibroblasts from a skin biopsy of this patient with CLL and analysed DNA from the cells. Sequence analysis showed that the fibroblasts contained the same homozygous mutation at nucleotide 1513 as was present in the peripheral blood. We also studied two pedigrees with familial CLL. In one family, both father and son had CLL diagnosed at age 74 and 53 years, respectively (family 2; figure 2). The father was diagnosed after routine blood tests, and his son was diagnosed when he presented with herpes zoster of the face, and he was found to have lymphadenopathy, splenomegaly, and lymphocytosis. At the start of our study, both men had recently received chlorambucil and the son was still on this treatment. Table 2 shows that the 1513A→C allele was inherited in the father-son pair, whereas P2X7 function was nearly absent in both patients. The mother, who was heterozygous for this allele was phenotypically normal and had good P2X7 function. In the other family, two sisters were diagnosed with CLL (mild lymphocytosis but no lymphadenopathy or splenomegaly) at ages 52 and 53 years; their mother (now deceased) had CLL, which was diagnosed at 74 years of age. Both sisters were heterozygous for the 1513A→C allele and their lymphocytes had very few functioning P2X7 receptors (table 2). Table 2 shows that the four familial CLL cases had significantly reduced P2X7 function in their non-leukaemic T cells (p=0.009) as well as in their monocytes (data not shown).

We compared P2X7-mediated cytotoxicity in the leukemic B lymphocytes of three patients who were wildtype, heterozygous, or homozygous for the 1513A→C polymorphic allele. The polymorphic P2X7 allele reduced P2X7-induced apoptosis from 39% in wildtype cells, to 9% of heterozygous cells, and 6% of homozygous cells (figure 5). T-lymphocytes showed a similar but less pronounced rank order of benzoyl benzoyl ATP-induced apoptosis.

---

Table 2: Expression and function of P2X7 receptor in T lymphocytes from members of two families in figure 2 with chronic lymphocytic leukaemia (CLL)

<table>
<thead>
<tr>
<th>Family 2</th>
<th>Phenotype</th>
<th>Genotype at 1513</th>
<th>Relative P2X7 expression (mean channels of fluorescence intensity)</th>
<th>P2X7 function (arbitrary units of area under ATP-induced ethidium uptake curve at 5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>CLL</td>
<td>A/C</td>
<td>4-2</td>
<td>125</td>
</tr>
<tr>
<td>Mother</td>
<td>Healthy</td>
<td>A/C</td>
<td>4-6</td>
<td>811</td>
</tr>
<tr>
<td>Son</td>
<td>CLL</td>
<td>C/C</td>
<td>3-7</td>
<td>235</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Family 3</th>
<th>Phenotype</th>
<th>Genotype at 1513</th>
<th>Relative P2X7 expression (mean channels of fluorescence intensity)</th>
<th>P2X7 function (arbitrary units of area under ATP-induced ethidium uptake curve at 5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sister</td>
<td>CLL</td>
<td>A/C</td>
<td>3-0</td>
<td>0</td>
</tr>
<tr>
<td>Sister</td>
<td>CLL</td>
<td>A/C</td>
<td>3-2</td>
<td>210</td>
</tr>
<tr>
<td>Controls</td>
<td>Healthy</td>
<td>A/A</td>
<td>7-2 (1-3†)</td>
<td>4008 (842‡)</td>
</tr>
</tbody>
</table>

*Data are mean (SEM); †n=10; ‡n=11.

---

Figure 5: P2X7-induced cytotoxicity of lymphocytes from three patients with chronic lymphocytic leukaemia (CLL), who were wildtype, heterozygous, or homozygous for the loss-of-function 1513A→C allele

Viable cells are in the lower region with non-viable cells (boxed) in the upper left region.
Discussion

Activation of the P2X7 receptor is an important mechanism of apoptosis in cells of the immune system, including thymocytes,24 B lymphocytes,25 macrophages,26 and dendritic cells.27 Cell-to-cell contacts are thought to generate sufficient extracellular ATP to activate P2X7 and trigger downstream signalling pathways, including membrane METALLOPROTEASES28 and caspases,29 resulting in apoptosis of the target cell. A single nucleotide polymorphism (1513A→C) in the P2X7 receptor changes glutamic acid to alanine at aminoacid 496 and abolishes its function, without affecting expression of the receptor on the surface of lymphocytes. Our results show that the prevalence of the polymorphic 1513A→C allele for P2X7, in either heterozygous or homozygous dosage, is threefold greater in patients with CLL than in controls.

We measured the function of the P2X7 receptor with flow cytometry, which provides a sensitive measure of the initial rate of ethidium uptake, via the P2X7 channel, into cells. Two-colour flow cytometry also permits the analysis, through gating, of ethidium uptake into particular lymphocyte subsets within the population, facilitating accurate measurement of P2X7 expression and function in leukemic B lymphocytes in peripheral blood, which we correlated with their P2X7 genotype. The presence of one inactive allele reduces the function of the P2X7 receptor by half, and two inactive alleles abolishes P2X7 function. Assays of ATP-induced apoptosis confirmed that the presence of one or two inactive alleles for the P2X7 receptor reduced the susceptibility of B lymphocytes to undergo ATP-induced apoptosis, possibly accounting for prolonged survival of these cells in vivo.

Few, if any, genetic polymorphisms have been described in which one allele encodes a non-functional channel. Thus the most likely explanation for our data is that the mutant monomeric protein exerts a dominant-negative effect when it assembles with functional monomers to form a homo-oligomer, which is necessary for P2X7 channel function. Other examples of channelopathies include generalised epilepsy with febrile seizures in a large pedigree, which is caused by a defect in a sensitive potassium channel or its associated sulfonylurea receptor abolish function of this channel in the pancreatic β-cell and lead to persistent hypersecretion of insulin.30 However, these inherited channelopathies are rare and contrast with the far greater prevalence, of around 10%, in the general population of a poorly functioning or inactive P2X7 receptor channel on cells of the immune system.

There is little data on the genes implicated in familial CLL, despite extensive study of the mutations that result in development and progression of CLL. Prime candidates for these mutations are inactivating mutations of the p5331 and the ataxia telangiectasia genes,32 and there is cytogenetic evidence that deletion of DNA at 17p and 11q (at the site of these genes), respectively, is associated with aggressive disease and poor prognosis.33 Deletions of chromosome 12q24, which contains the P2X7 gene, are rare, and trisomy of chromosome 12 is the more usual finding in CLL. Molecular studies have also shown mutations in the ataxia telangiectasia gene in around 20% of patients with CLL and, in two individuals, these mutations were germline.34 Several of our results indicate that the 1513A→C allele is present in germline DNA and is not a somatic mutation that arises in B cells during their transformation to cancerous cells. We noted, for example, that healthy T lymphocytes and cancerous B lymphocytes had defective P2X7 receptor function in patients who were either heterozygous or homozygous for the 1513A→C allele. Furthermore, fibroblasts from a skin biopsy of one patient with homozygous 1513A→C in B lymphocytes were also homozygous for this allele. Finally, our data from the study of two separate families with CLL show heritability of the 1513A→C allele within these pedigrees along with pronounced loss of P2X7 function. Thus, loss-of-function mutations in the P2X7 receptor could explain the molecular basis of familial CLL. Additionally, our data exclude the possibility that the 1513A→C mutation is somatically acquired in the B cell lineage. Whether homozygous dosage of this polymorphic allele further increases the risk of CLL remains uncertain, since only two patients in our series fell into this category. However, both homozygotes were relatively young for this diagnosis (53 and 65 years), and a young age of onset has been proposed as a feature of familial CLL.35 Three other patients with CLL in our series showed absent or nearly absent P2X7 function despite a wildtype genotype in leukaemic B lymphocytes in peripheral blood. This further supports the hypothesis that other loss-of-function mutations might arise in the genes for P2X7 or in its several protein partners in the membrane complex.36 We assessed loss of function for the P2X7 receptor by the failure of ATP to induce cellular uptake of ethidium, a large organic cation. However, the P2X7 channel is selective for calcium and, to a lesser extent, to potassium, and sodium whose fluxes we did not measure in all patients. Nevertheless, our previous data12,13 suggests that loss of function mutations will abolish the permeability of the P2X7 channel equally to both large and small cation permeants.

Data support the notion that some genes expressed at half normal values cannot fully suppress development of leukaemia. Thus, inheritance of one mutated copy of the AML1 gene predisposes individuals to the development of acute myeloid leukaemia.37 Results of another study30 show that heterozygous loss of the PML gene contributes to development of leukaemia by enhancing the survival of immature myeloid cells and thus increasing incidence of the disease in PML-RARα transgenic mice. The increased prevalence of a heterozygous non-functional P2X7 allele in CLL would place P2X7 within this new class of tumour suppressor gene, in which genetic haploinsufficiency of P2X7 is an early contributor to the pathogenesis of CLL.

Contributors

J Wiley designed and coordinated the study, recruited most patients, and wrote the report; P Dao-Ung did cytotoxicity assays, helped measure P2X7 expression and function, and did DNA work; B Cru did most of the P2X expression and function measurements and analysed data; R Suyler did some P2X, measurements, analysed data, and did DNA work; A Shemon did DNA work; C Li did some DNA analyses; J Taper provided clinical detail and access to eight patients; J Gallo and A Manoharan also contributed clinical detail, and provided access to patients of the two pedigrees with CLL.

Conflict of interest statement

None declared.

Acknowledgments

We thank Julian Barden, Maria Ban, Peter Masson, and Prof Graeme Stewart for help and advice, Susan MacCallum for referral of a patient, the Lee family for their generous assistance, and Shelley Spicer for typing the report.

The work was funded by the Leo and Jenny Foundation, the Community Health and Anti-Tuberculous Association, the National Health and Medical Research Council of Australia, and the Cecilia Kilkeary Foundation.
References


