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Autocrine/paracrine involvement of insulin-like growth factor-I and its receptor in chronic lymphocytic leukaemia

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Summary

Chronic lymphocytic leukaemia (CLL) is characterized by the accumulation of long-lived B lymphocytes blocked in $G_{0/1}$ by impaired apoptosis. As insulin-like growth factor-I (IGF-I) is known for its antiapoptotic effects in different cell types, we investigated whether IGF-I and its receptor (IGF-IR) participate in autocrine/paracrine loops affecting the survival of CLL cells. IGF-IR protein and mRNA was present in CLL cells in 44% and 59% of patients respectively. IGF-IR expression in CLL patients was positively correlated with the expression of the antiapoptotic protein Bcl-2 and was involved in CLL cell survival *in vitro*. Serum IGF-I was elevated in CLL patients, but growth hormone (GH) was normal. CLL cells expressed IGF-I mRNA and secreted the growth factor *in vitro*. Therefore, local production of IGF-I can account for the increased levels of serum IGF-I, independently of GH, and may be related to autocrine/paracrine control of lymphocyte survival acting at IGF-IR. This is the first demonstration of IGF-IR expression in a subgroup of CLL patients and of its antiapoptotic effects *in vitro*, highlighting the importance of this growth factor receptor as a possible therapeutic target in CLL.

Keywords: chronic lymphocytic leukaemia (CLL), CLL growth factors, CLL apoptosis, B cells.

Chronic lymphocytic leukaemia (CLL) is a disease characterized by the accumulation of long-lived, usually $CD5^+$, B lymphocytes that are largely blocked in early $G_{0/1}$ of the cell cycle. This neoplastic disorder seems to be caused by the extended survival of a malignant clone rather than by an increase in its proliferative activity. Most CLL cells contain high levels of the antiapoptotic protein Bcl-2 (Hanada *et al*, 1993), which is considered to be central to the evasion of apoptosis by CLL cells (Gottardi *et al*, 1996). As Bcl-2 does not only extend the cell survival, but also renders them resistant to the cytotoxic effects of essentially all currently available anticancer drugs (Reed, 1995), CLL remains incurable.

Growth factors may participate in autocrine or paracrine loops that affect CLL cell growth or survival through the induction of apoptosis-related genes (Bairey *et al*, 2001). There is considerable evidence supporting a role for insulin-like growth factor-I (IGF-I) in the antiapoptotic effects in different cell types (O'Connor *et al*, 1997). IGF-I is a 7·8-kDa peptide, encoded by a member of the insulin gene family (Stewart & Rotwein, 1996), which is mainly produced by hepatocytes in response to growth hormone (GH) stimulation. However, it is also produced locally in many other tissues where it acts in an autocrine/paracrine manner (Grimberg & Cohen, 2000). The transcription product of its gene consists of six exons. The N-terminal part of exon 4 encodes the E peptide, which is present only in the unprocessed hormone. In humans, the E peptide assumes one of two forms, depending on whether exon 4 is spliced to exon 5 (the Eb region) or exon 6 (the Ea region). IGF-I protein translated from Ea mRNA produces an autocrine/paracrine action in non-hepatic tissues, whereas IGF-I translated from Eb mRNA is expressed mainly in the liver after stimulation by GH (Lowe *et al*, 1988). IGF-I produced by bone-marrow stromal cells is involved, as a paracrine factor, in the differentiation of normal pro-B to pre-B lymphocytes, stimulating cytoplasmic μ-heavy chain expression (Landreth *et al*, 1992). IGF-I plays a role in maintaining haematopoietic cells by increasing the proliferation of progenitor cells (Wang *et al*, 1993) and by preventing the apoptosis of interleukin (IL)-3-deprived cells (Rodriguez-Tarduchy *et al*, 1992). Furthermore, the expression of IGF-I receptor (IGF-IR) is undetectable in $CD34^+$ cells but is

positive in committed precursors (Ratajczak *et al*, 1994) and in mature B lymphocytes (Kooijman *et al*, 1992).

It is widely accepted that IGF-I and its receptor are involved in the genesis of cancer. IGF-IR expression is a prerequisite for the development of several tumours because it facilitates transformation by viral and cellular oncogenes (Baserga, 1999). IGF-I synthesis has also been reported in some tumours, and may be an autocrine factor that contributes to malignancy (Werner & LeRoith, 1996). Recent findings have associated high concentrations of circulating IGF-I with an increased risk of breast (Hankinson *et al*, 1998) or prostate cancer (Chan *et al*, 1998), establishing IGF-I as a potential predictor of cancer. Most of these data concern solid tumours. Although CLL is the most common type of adult leukaemia in Western countries (Harris *et al*, 1994), there are no reports on the expression and function of IGF-I and its receptor in this disease. The goal of the present study was to determine IGF-IR expression on CLL cell membranes and its mRNA levels in CLL cells, and to investigate the possible role of IGF-IR on cell survival *in vitro*. We also assessed serum IGF-I and GH levels in healthy controls and patients with CLL, and investigated IGF-I synthesis by CLL cells. These data were correlated with patient staging, and Bcl-2 expression.

Materials and methods

Patients and controls

This study included 27 patients with the clinical, morphological and immunophenotypic criteria ($CD19^+$, $CD5^+$, $CD23^+$ and weak clonally restricted surface immunoglobulins) for CLL (Cheson *et al*, 1996). Peripheral blood samples were collected after obtaining informed consent from patients' who were either untreated or had not been treated for at least 4 months before the time of analysis (range 4–30 months). The research protocol was approved by the local Institutional Ethics Committee. Patients' characteristics are given in Table I. Peripheral blood samples collected from 17 age- and sex-matched healthy donors were used as controls.

Table I. Patient characteristics.

Characteristic	
Number of patients	27
Mean age (years)	68·5 ± 1·4*
Range	55–80
Sex [*n* (%)]	
Male	18 (66·7)
Female	9 (33·3)
Binet stage [*n* (%)]	
A	16 (59·5)
B	6 (22·2)
C	5 (18·3)
Treatment [*n* (%)]	
Not treated	20 (74·1)
Treated	7 (25·9)
Mean haemoglobin level (g/dl)	13·4 ± 0·3
Range	9·0–14·9
Mean WBC (10^9/l)	31·8 ± 8·6
Range	9·2–236·0
Mean platelet count (10^9/l)	177·4 ± 11·3
Range	95–280

*Mean ± standard error

Flow-cytometry analysis

Total leucocytes were obtained from sodium heparin anticoagulated blood by NH_4Cl lysis and were incubated with a panel of antibodies for immunophenotyping [phycoerythrin (PE)-conjugated anti-CD5 and PE-conjugated anti-CD23 were from BD Biosciences (San Diego, CA, USA); PE-cyanine 5 (PECy5)-conjugated anti-CD19 were from Immunotech (Marseille, France); and fluorescein isothiocyanate (FITC)-conjugated anti-kappa and anti-lambda chains were from Dako (Ely, UK)]. IGF-IR staining was performed as described previously (Schillaci *et al*, 1998; Brocardo *et al*, 2001). Briefly, 10^6 leucocytes were incubated with 5 μg/ml α-IR-3 antibody (Oncogene Science Inc., Cambridge, MA, USA), then with an Alexa 488-conjugated $F(ab)_2$ fragment of anti-mouse IgG (Molecular Probes, Eugene, OR, USA), and finally with PE-conjugated anti-CD19. Cells were assessed for fluorescence intensity using a FACScan cytometer (BD Biosciences). A total of 10^4 cells/sample were analysed. Background staining was determined in cells incubated with Alexa 488- or PE-conjugated isotype control antibodies. Data were analysed with Cellquest software (BD Biosciences). Mean fluorescence intensity (MFI) was determined by subtracting the mean fluorescence of the isotype control cells from the mean fluorescence of α-IR-3-treated cells of the CD19-positive cells. MFI is directly proportional to the number of IGF-IR binding sites (Kooijman *et al*, 1992). In all patients, more than 98% of the $CD19^+$ cells were also clonal and $CD5^+$. Triple-colour immunofluorescence was used to detect IGF-IR on normal $CD5^+$ B lymphocytes from six control donors (rheumatoid-factor-negative). After IGF-IR staining for indirect immunofluorescence, as described above, we used PE-conjugated anti-CD5 and PECy5-conjugated anti-CD19 antibodies to analyse 10^6 cells/sample. Triple-colour immunofluorescence was used to examine CD38 surface expression, with FITC-conjugated anti-CD38, PE-conjugated anti-CD5 and PECy5-conjugated anti-CD19 antibodies.

Triple-colour immunofluorescent staining was used to analyse Bcl-2. Cells were incubated with PE-conjugated anti-CD5 and PCy5-conjugated anti-CD-19 antibodies, then fixed using a commercial kit (Fix and Perm; Caltag, Burlingame, CA, USA) and washed with phosphate-buffered saline (PBS) and centrifuged. The pellet was resuspended in permeabilization solution for 5 min and incubated with FITC-conjugated Bcl-2 (Dako) or the isotype-matched negative control.

The cells were washed before flow cytometric analysis, as described above.

Separation and culture of CLL cells and B lymphocytes

The CLL cells and control B lymphocytes were separated by the Rosette-Sep technique (StemCell Technologies, Vancouver, Canada). Heparinized blood was incubated with 50 μl of Rosette Sep human B-cell enrichment cocktail of tetrameric antibodies per millilitre of blood for 20 min at room temperature. The samples were diluted with equal volumes of PBS + 2% fetal calf serum (FCS; Gibco, Grand Island, NY, USA) and separated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). The cells were washed twice with PBS and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) containing 20 mmol/l HEPES, 2 mmol/l L-glutamine (Sigma Chemical Co., St Louis, MO, USA), 5% FCS, and 30 μg/ml gentamicin or in serum-free Dulbecco's medium:Ham F12 (1:1 v/v) medium (Gibco) supplemented with 10 μg/ml human transferrin, 50 μmol/l 2-mercaptoethanol, 2·5 nmol/l sodium selenite, 20 μmol/l ethanolamine, 2 mmol/l L-glutamine, 30 μg/ml gentamicin (all supplements were from Sigma), as described previously (Schillaci *et al*, 1994). The B-cell suspension contained 98·0–99·5% B cells, as determined by flow cytometric analysis using anti-CD19 as a lineage-specific marker.

Measurement of IGF-IR-mediated apoptosis of CLL cells in vitro

The CLL cells positive for IGF-IR membrane expression were purified as described above and cultured in RPMI medium with 5% FCS at 37°C in a 5% carbon dioxide atmosphere in the presence of anti-α-IR-3 antibody (5 μg/ml, GR11L, sodium azide-free; Oncogene) to block IGF-IR function (Rohlik *et al*, 1987) or an irrelevant antibody as the control (IgG1*k*, MOPC-21; Sigma). The percentage of apoptotic cells was estimated after 48 h by light-scattering properties: reduction in forward scatter and increase in side scatter parameters were used to gate the apoptotic population, as described by Pepper *et al* (1999). The concentration of IGF-I in FCS was 145 ± 15 ng/ml, measured by radioimmunoassay (RIA, see below).

Determination of IGF-IR and IGF-I mRNAs by reverse transcription polymerase chain reaction

Total RNA was prepared from 10^7 purified CLL cells or control B lymphocytes using Trizol reagent (Gibco), and reverse transcription (RT) was performed with 1 μg RNA in a 25-μl reaction volume, according to the method of Quinn *et al* (1996). The transcribed cDNA fragments were amplified in a 50 μl reaction volume with 100 μmol/l each dNTP, 0·4 μmol/l each primer, and 1 U *Taq* DNA polymerase (Promega Corp., Madison, WI, USA). Amplification was performed in an Eppendorf Master Cycler thermal cycler (Eppendorf, Hamburg, Germany). Primers for human IGF-IR were as follows: (F) 5′-ATTGAGGAGGTCACAGAGAAC-3′ and (R) 5′-TTCATATCCTGTTTTGGCCTG-3′ (Quinn *et al*, 1996). Primers for Ea IGF-I were as follows: (F) 5′-GTGGATGCTCTTCAGTTCGTGTGTG-3′ and (R) 5′-TGGCATGTCACTCTTCACTCC-3′ (Swolin *et al*, 1996). Primers for glycerol-3-phosphate dehydrogenase (G3PDH) were as follows: (F) 5′-TGAAGGTCGGAGTCAACGGATTTG-3′ and (R) 5′-CACCACCTGGAGTACCGGGTGTAC-3′ (Zhou *et al*, 1994). Amplification conditions for IGF-IR, Ea IGF-I and G3PDH cDNAs were: two cycles of denaturation at 94°C for 2 min, annealing at 62°C for 2 min and extension at 72°C for 5 min, followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min, as described previously (Brocardo *et al*, 2001; Segretin *et al*, 2003). The polymerase chain reaction (PCR) products were separated by electrophoresis on a 1·8% agarose gel, and visualized by ethidium bromide staining under ultraviolet transillumination.

Levels of mRNA were evaluated by densitometric analysis of the PCR products using ImageQuant software (Molecular Dynamics, Amersham, Little Chalfont, UK). A 100-bp ladder (Gibco) was used to verify the sizes of the PCR products. The identity of the PCR fragments was confirmed by Pst1 cleavage.

IGF-I determination in conditioned medium and serum samples

Purified CLL and control B lymphocytes were cultured for 48 h at 2×10^6 cell/ml in serum-free Dulbecco's medium:Ham F12 (1:1) medium supplemented as described above. The conditioned media were collected, centrifuged and stored at −70°C. Peripheral venous blood samples were collected in tubes for serum isolation, and after centrifugation, sera were stored at −70°C. For IGF-I RIA, serum samples, conditioned media and IGF-I standards were subjected to the acid–ethanol cryoprecipitation technique, as previously described by Breier *et al* (1991). IGF-I was determined using antibody (UB2-495) provided by Drs L. Underwood and J. J. Van Wyk, and distributed by the Hormone Distribution Program of the National Institute of Diabetes and Digestive, and Kidney Diseases. Recombinant human IGF-I (produced by bacterial fermentation; Sigma) was used as the radioligand and unlabelled ligand. IGF-I was radiolabelled by the chloramine-T method (Lacau-Mengido *et al*, 2000). Assay sensitivity was 6 pg/tube. Intra- and interassay coefficients of variation (CV) were 7·2% and 12·8% respectively. The amount of IGF-I in Dulbecco's medium:Ham F12 (1:1) supplemented medium not exposed to cells was 0·06 ± 0·01 ng/ml and was subtracted from the IGF-I concentrations calculated for the conditioned media.

Growth hormone determination in serum samples

Sera were collected as described previously for IGF-I determination. A commercially available kit, a two-site fluorometric

GH assay based on two monoclonal antibodies, was used (Delfia Human GH, Euro/DPC, Gwynedd, UK). Fluorescence was analysed with the Immulite Analyzer (DPC, Los Angeles, CA, USA). The detection limit was 0·1 ng/ml and the intra- and interassay CVs were 5·8 % and 8·4% respectively.

Statistical analysis

Results are expressed as mean ± standard errors (SE). Comparisons between groups were made with paired or unpaired Student's *t*-test, or one-way ANOVA. Pearson's correlation was used for statistical comparisons of Bcl-2 and IGF-IR expression. Fisher test was used in IGF-IR and CD38 coexpression analysis. $P < 0{\cdot}05$ were considered significant.

Results

IGF-IR expression on CLL cells and control B lymphocytes

In order to assess IGF-IR expression in plasma membranes on control B lymphocytes and CLL cells, we performed an immunofluorescence assay and analysed the results by flow cytometry. Figure 1 shows a representative set of histograms for IGF-IR expression on B lymphocytes from a control donor (Fig 1A) and from two CLL patients. The leukaemic cells of one of these patients expressed IGF-IR (Fig 1B), whereas those of the other had no detectable IGF-IR (Fig 1C). IGF-IR expression was homogeneous in all samples. Fig 1D shows that IGF-IR, expressed as MFI, was detected in all controls: 3·6 ± 0·4 (range 1·3–8·1). Meanwhile 12 of 27 CLL cells were positive for IGF-IR expression (MFI > 1) and their MFI was 3·3 ± 0·7 (range 1·3–8·4). When IGF-IR expression and Binet stages were represented as a biparametric dot-plot (Fig 1E), CLL IGF-IR$^+$ cells were unevenly distributed in all stages of the disease (56% in A, 17% in B and 40% in C stages).

Regarding that CLL cells are CD5$^+$ and only a subpopulation of control B lymphocytes, called B1a cells (Lydyard *et al*, 1999) express the CD5$^+$ marker, we studied IGF-IR expression on this subset. IGF-IR expression was similar on CD5$^+$ B lymphocytes (MFI 4·1 ± 0·4) and on their CD5-$^-$ counterparts (MFI 3·8 ± 0·3, $n = 6$).

As IGF-IR was expressed in a subgroup of CLL patients, we explored the possible coexpression with CD38, a strong prognosis determinant of a poor response to conventional treatment (Del Poeta *et al*, 2001). Figure 1F shows that of 12 patients with IGF-IR-positive CLL cells, five were also CD38$^+$ (>30% positive cells), whereas the cells of the other seven expressed only IGF-IR. Of 15 patients without IGF-IR expression on CLL cells, only two expressed CD38. There was a significant association between CD38 and IGF-IR expression ($P < 0{\cdot}05$). In control B lymphocytes CD38 expression was detected in 63·3 ± 5·6 % of the population (MFI = 12·4 ± 1·6, $n = 6$).

Then, we investigated the presence of IGF-IR mRNA in the cytoplasm of purified B lymphocytes (>98% purity) of leukaemic patients and healthy controls by RT–PCR (Fig 2). All CLL cells that expressed IGF-IR on the plasma membrane and the cells of five additional patients who were IGF-IR-negative, expressed detectable levels of IGF-IR mRNA. All control B lymphocytes tested expressed IGF-IR mRNA (Fig 2).

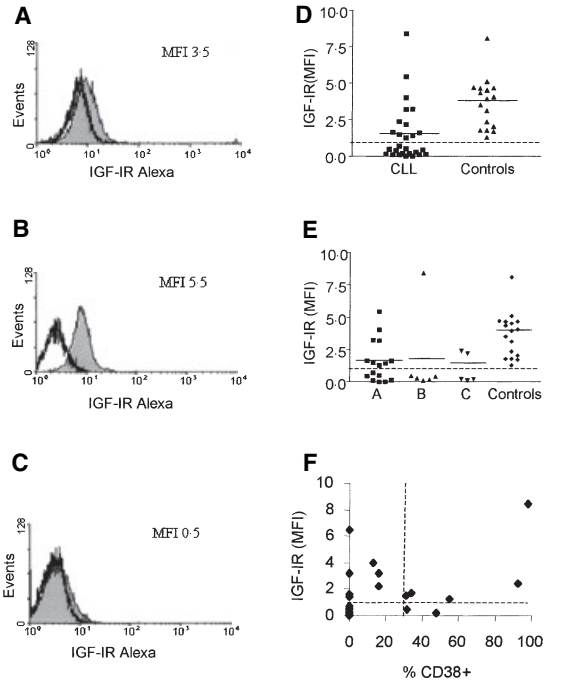

Fig 1. IGF-IR expression in CLL cells in different Binet stages and in control B-lymphocytes. Relationship between IGF-IR and CD38 expression. IGF-IR expression was assessed by indirect immunofluorescence using α–IR3 antibody on CD19$^+$ cells and analysed by flow cytometric analysis. (A) Representative histogram of IGF-IR expression in control B-lymphocytes. (B) and (C) Representative histograms of IGF-R expression in CLL cells. In all panels, grey areas correspond to cells incubated with α–IR3 antibody superimposed over histograms of cells stained with an irrelevant isotype-matched antibody (white area). Data are expressed as mean fluorescence intensity (MFI), obtained by subtracting the mean fluorescence of the isotype control cells from the mean fluorescence of α–IR3-treated cells of the CD19-positive cells. (D) IGF-IR expression in CLL cells of 27 patients and 17 controls (3·6 ± 0·4 MFI). (E) IGF-IR expression in CLL classified by Binet staging and in controls. Horizontal solid line indicates mean level. Horizontal dotted lines define IGF-IR positive cases. Data are expressed in each group as mean ± standard error. (F) IGF-IR expression (as MFI) was plotted against CD38 expression (as % of positive cells) in CLL cells. This analysis includes the 27 CLL patients. The horizontal broken line defines IGF-IR$^+$ cells and the vertical broken line defines CD38$^+$ cells. There was a positive association between CD38 and IGF-IR expression (Fisher's test $P < 0{\cdot}05$).

IGF-IR expression and its relationship to CLL cell survival

One of the principal antiapoptotic proteins overexpressed in CLL is Bcl-2. As IGF-I can enhance the expression and function of Bcl-2 in other cells types (Minshall *et al*, 1997), we investigated the expression of both molecules in CLL cells. In IGF-IR negative patients the MFI of Bcl-2 expression was

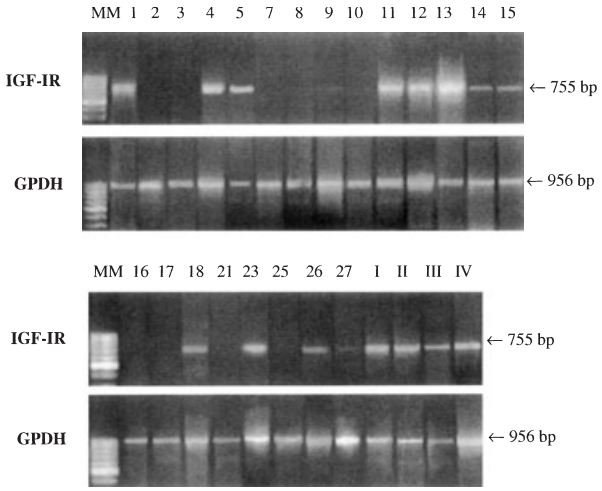

Fig 2. IGF-IR mRNA expression in CLL cells and control B lymphocytes. B lymphocytes from CLL patients and controls were purified (>98%) and IGF-IR and GDPH mRNAs were detected by RT-PCR analysis. Lanes 1–27, patients; lanes I–IV, four representative control B lymphocytes. MM, molecular weight marker.

34·5 ± 4·0 and in IGF-IR positive patients it was 30·9 ± 5·3. Although there was no difference between Bcl-2 expression between these groups we found a highly significant direct correlation between Bcl-2 and IGF-IR expression only in the positive group (Fig 3, $R^2 = 0{\cdot}54$, $P < 0{\cdot}02$; $n = 10$).

To prove that IGF-IR functions on CLL cells, we cultured the purified CLL IGF-IR$^+$ cells of nine patients and six control B lymphocytes for 48 h in RPMI medium supplemented with 5% FCS in the presence of a blocking anti-IGF-IR antibody or an irrelevant antibody as control. In this way, we impeded the interaction between the IGF-I present in the FCS or in cells, and its receptor. There was a significant increase in apoptotic CLL cells after culture with anti-IGF-IR antibody (Table II, $P < 0{\cdot}0005$) while there were no effects on control B lymphocytes.

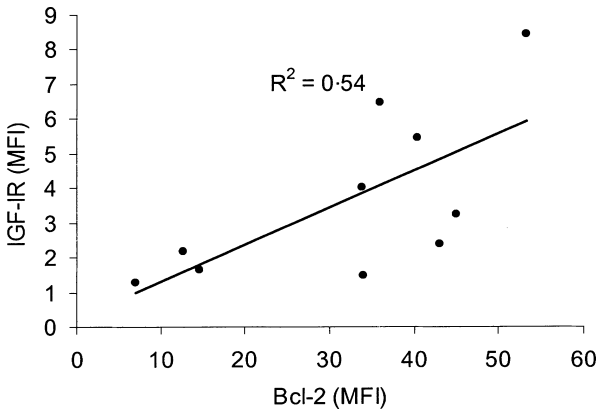

Fig 3. Relationship between IGF-IR expression and the antiapoptotic protein Bcl-2 in CLL cells. Only CLL cells positive for IGF-IR expression were used in this analysis. IGF-IR expression was plotted against Bcl-2 expression in CLL cells. Data are expressed in MFI. R, Pearson correlation coefficient; $P < 0{\cdot}02$; $n = 10$ patients.

IGF-I serum levels

Our next objective was to assess the presence of the IGF-IR ligand, IGF-I. We first determined IGF-I levels in the sera of patients and healthy controls. Figure 4A shows that the mean IGF-I serum levels of CLL patients was 265 ± 20 ng/ml. These levels were significantly higher than the mean serum concentration detected in controls (170 ± 12 ng/ml, $P < 0{\cdot}0019$), which were similar to previously reported control levels (Zapf *et al*, 1981). When we analysed these data, grouping patients according to Binet stages (Fig 4B), we observed increased IGF-I levels in all stages: A stage 294 ± 31 ng/ml, B stage 238 ± 27 ng/ml, C stage 251 ± 34 ng/ml ($P < 0{\cdot}0004$, $P < 0{\cdot}01$, $P < 0{\cdot}01$, relative to the control group).

Growth hormone serum levels

Serum IGF-I is mainly produced by hepatocytes in response to GH. Therefore, we examined GH levels in patients and controls. Figure 4C shows that serum GH levels were similar in controls and CLL patients (1·5 ± 0·3 ng/ml vs. 1·5 ± 0·5 ng/ml, respectively). Similar results were obtained when data were analysed according to Binet staging (Fig 4D).

IGF-I expression and synthesis by CLL cells

Circulating IGF-I is principally secreted by the liver, and also by other tissues (Yakar *et al*, 1999). To determine whether IGF-I can act in an autocrine manner, we used RT–PCR to assess whether CLL cells express IGF-I mRNA. Figure 5 shows that IGF-I mRNA was present in 19/22 samples. In all cases, when high IGF-I serum levels were detected, mRNA was present in CLL cells.

As CLL cells express IGF-I mRNA, we sought to determine whether they are able to secrete this factor. We cultured purified CLL cells from nine IGF-I mRNA positive patients or control B lymphocytes in serum-free medium for 48 h and then measured the IGF-I in the conditioned medium. IGF-I levels in conditioned medium of CLL cells were significantly higher than in controls: 0·42 ± 0·06 ng/ml and 0·19 ± 0·07 ng/ml respectively ($P < 0{\cdot}05$).

Discussion

The fundamental role of IGF-IR in malignant transformation is well established for solid tumours. The transforming activity of IGF-IR depends, to a large extent, on its potent antiapoptotic activity, in addition to its mitogenic effects. The ability of IGF-IR to protect cells from apoptosis has been demonstrated in fibroblasts, haematopoietic cells and other cell types (O'Connor *et al*, 1997). CLL is a disease characterized by a quite variable clinical course, reflecting its heterogeneity. Our

Table II. Effect of IGF-IR blockage on CLL cell survival *in vitro*.

Patient number	IGF-IR (MFI)	% apoptosis	% apoptosis with anti IGF-IR Ab	Control number	IGF-IR (MFI)	% apoptosis	% apoptosis with anti IGF-IR Ab
4	8·4	36	43	1	1·5	18	18
5	5·4	25	37	2	1·3	13	14
9	4·0	17	31	3	1·5	13	9
11	1·6	24	30	4	2·3	19	20
13	2·2	63	82	5	3·1	20	20
20	1·5	35	38	6	2·7	29	28
22	1·6	15	17				
23	3·2	28	38				
27	3·2	39	43				
Mean ± SE		31 ± 5	41 ± 6*			19 ± 2	20 ± 1

Purified CLL cells and control B lymphocytes were cultured for 48 h in RPMI with 5% FCS in presence of anti IGF-IR antibody or an irrelevant antibody. Apoptosis was determined by side and forward scatter properties of the cells. The *P*-value was calculated by using Student's *t*-test for paired samples.

MFI, mean fluorescence intensity.

*$P < 0{\cdot}0005$.

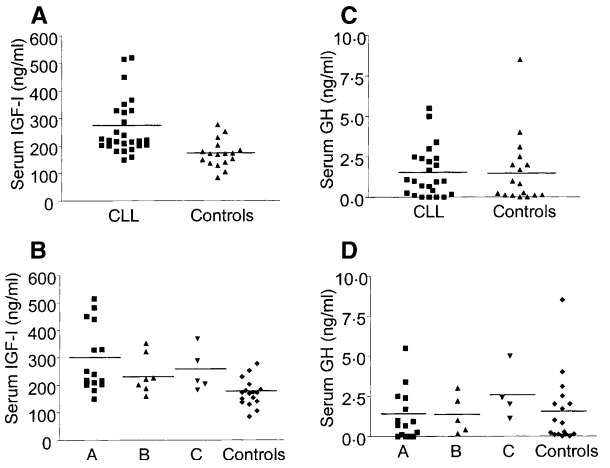

Fig 4. Serum IGF-I and GH levels in CLL patients and in controls. The IGF-I determinations were performed by RIA analysis and GH by Delfia analysis. (A) Serum IGF-I levels in 27 CLL patients (265 ± 20 ng/ml) and 17 controls (170 ± 11 ng/ml) $P < 0{\cdot}0019$. (B) Serum IGF-I levels in CLL patients classified by Binet staging and controls. Binet A stage (294 ± 31 ng/ml), B stage (238 ± 27 ng/ml), C stage (251 ± 34 ng/ml) *versus* controls (170 ± 11 ng/ml) $P < 0{\cdot}0004$, $P < 0{\cdot}01$, $P < 0{\cdot}01$ respectively. (C) Serum GH levels in 24 CLL patients and 17 controls. (D) Serum GH levels in CLL patients classified by Binet staging and controls. Horizontal solid lines indicate mean level. Data are expressed in each group as mean ± standard error.

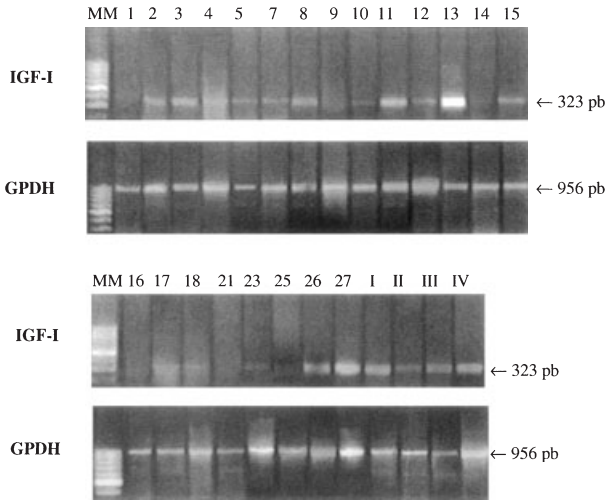

Fig 5. IGF-I mRNA expression in CLL cells and control B lymphocytes. B lymphocytes from CLL patients and controls were purified (>98%) and IGF-I mRNA and GDPH was detected by RT–PCR analysis. Lanes 1–27, patients; lanes I–IV, four representative control B lymphocytes; MM, molecular weight marker.

results show that IGF-IR is expressed in 44% of CLL patients and possibly defines a subgroup of patients within this disease. Although we have not been able to find a correlation between IGF-IR expression and Binet stages, we have demonstrated its association with CD38 expression, a marker associated with cells with poor response to treatment and shorter patient survival. All CLL cells that were IGF-IR$^+$ had detectable levels of IGF-IR mRNA, as did the cells of five additional patients with CLL who were IGF-IR-negative by flow cytometric analysis. We have also demonstrated that blocking IGF-IR with a neutralizing antibody induces apoptosis of CLL cells and not in control cells *in vitro*, and that IGF-IR expression correlates directly with levels of the antiapoptotic protein Bcl-2. These results showed that abolition of IGF-IR function on CLL cells had a biological response. Therefore, IGF-IR expression could be useful for a new tailored therapeutic approach that would involve blocking its expression or function on this specific subgroup of CLL patients whose leukaemic cells are IGF-IR$^+$. This can be achieved through neutralizing antibody, antagonistic peptides, selective kinase

inhibitor (Mitsiades *et al*, 2004) or through antisense oligodeoxynucleotide (ODN)-based strategies, dominant-negative mutants, or interference mRNA directed against IGF-IR (Baserga *et al*, 1997). In line with these strategies, we have recently demonstrated that breast cancer growth can be inhibited by direct *in vivo* administration of IGF-IR antisense ODN in Balb/c mice. Tumours from IGF-IR antisense ODN-treated mice showed a significant decrease in the degree of insulin receptor substrate-1 tyrosine phosphorylation, phosphatidyl inositol 3-kinase/Akt and p42/p44 mitogen-activated protein kinase activation in comparison with sense ODN treated mice (Salatino *et al*, 2004). In humans, there has been one report of a phase I clinical trial using antisense IGF-IR mRNA to treat malignant astrocytoma (Andrews *et al*, 2001). This type of therapy is now in use among patients with CLL, using Oblimersen, an antisense ODN designed against Bcl-2 mRNA (Klasa *et al*, 2002).

There are several reports correlating IGF-I levels and increased risk of breast (Hankinson *et al*, 1998) or prostate cancer (Chan *et al*, 1998). The present study has demonstrated a significant increase in IGF-I serum concentrations in patients with CLL regardless of the Binet stage of the disease. IGF-I mediates the anabolic action of GH in skeletal tissues in an endocrine manner. However, Yakar *et al* (1999) reported normal growth and development in the absence of hepatic IGF-I, demonstrating an autocrine/paracrine role for local IGF-I that is independent of GH. Therefore, localized expression of IGF-I in different tissues is an important growth-regulating factor that acts in an autocrine/paracrine way, as previously suggested by Jansen *et al* (1991). About 30% of the IGF-I content of serum may be of tissue origin (Yakar *et al*, 1999). We found that CLL cells express the Ea form of IGF-I mRNA, and secrete *in vitro* higher IGF-I levels than control lymphocytes that may therefore act in a paracrine/autocrine way (Lowe *et al*, 1988). The fact that serum GH levels were normal in these CLL patients suggests that the increase in IGF-I serum levels is independent of GH, and IGF-I may be secreted by the CLL cells themselves. Taken together, we suggest, on the one hand, that CLL cells are able to synthesize and secrete IGF-I in an autocrine/paracrine manner and could account for the downregulation of IGF-IR observed in several patients. On the other hand, in a subpopulation of CLL patients, where IGF-IR expression is conserved and it has *in vitro* antiapoptotic activity, IGF-IR targeting could be a useful therapeutic approach.

We detected IGF-IR in B lymphocyte plasma membranes in all control subjects, similar to the results of Kooijman *et al* (1992). However, there has been no report to date of IGF-IR expression in a subpopulation of B lymphocytes called B1a cells that express the CD5 marker. Our results show that there is no difference in IGF-IR expression between $CD5^+$ or $CD5^-$ B lymphocytes, suggesting that there is no correlation between IGF-IR and CD5 expression. We also investigated IGF-I production in control B lymphocytes. A previous report demonstrated an IGF-I production of 2·2 ng/ml in 3×10^4 Epstein–Barr-transformed B lymphocytes during 1 week of culture, but it detected no IGF-I secretion in normal B lymphocytes (Merimee *et al*, 1989). In the present work, we detected IGF-I secretion in control B lymphocytes (0·19 ± 0·07 ng/ml per 2×10^6 cells during 48 h). This difference may be the result of the presence of more cells in our culture or because of the high IGF-I background levels in the study of Merimee *et al* (1989), probably the result of albumin in the culture medium.

In conclusion, we report for the first time, IGF-IR expression in a subgroup of patients with CLL and its antiapoptotic effect *in vitro*. The present results highlight the importance of this growth-factor receptor as a possible therapeutic target in CLL disease. Furthermore, we demonstrated that CLL cells secrete IGF-I *in vitro* and that this autocrine/paracrine production may account for the observed increased levels of serum IGF-I, which are independent of the action of GH.

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