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EFFECTS OF α-INTERFERON AND STEROIDS ON CD23 EXPRESSION AND RELEASE IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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ABSTRACT

Background. **Since high CD23 expression and release have been reported in B-chronic lymphocytic leukemia (B-CLL), we investigated whether α-interferon or corticosteroids were able to modulate the expression and/or the release of this factor.**

Methods. **CD23 expression was determined with FITC-labelled anti-CD23 monoclonal antibody, and sCD23 release with a sandwich enzyme immunoassay. Twenty-one patients affected by B-CLL (stage A or B) were studied before and after three different treatment regimens (α-interferon, corticosteroids, α-interferon + corticosteroids).**

Results. **CD23 was highly expressed in the B-cells of all patients, and expression was not modified by any of the therapies. sCD23 release from leukemic cells was significantly greater ($p<0.00001$) in untreated subjects than controls, and *in vitro* treatment with phorbol myristate acetate (PMA) led to a 10-fold increase ($p<0.0001$) in sCD23 secretion. On the contrary, PMA did not increase sCD23 release in normal B cells. Treatment with corticosteroids (either alone or associated with α-interferon) reduced sCD23 secretion from leukemic cells, whereas α-interferon alone was not able to modify sCD23 release.**

Conclusions. **Our data support the hypothesis that CD23 plays a role in the maintenance and progression of B-CLL and that the pharmacological modulation of this receptor/lymphokine could be useful in the therapy of B-CLL.**

Key words: CD23, chronic lymphocytic leukemia, α-interferon

B-cell-derived chronic lymphocytic leukemia (B-CLL) is characterized by monoclonal proliferation of B lymphocytes arrested at an intermediate stage of differentiation.[1,2] The disease is accompanied by several poorly understood immunological alterations including elevated serum levels of the soluble interleukin 2 receptor (sIL-2R),[3,4] interleukin-6,[5] and tumor necrosis factor-α.[6,7]

The presence of an autocrine pathway supporting the growth of B-cells has been suggested.[8] Recently, the presence of low-affinity IgE receptors has been observed in normal B cells,[9] B-CLL cells,[10] and on a small fraction of T-cells,[11,12] macrophages,[13] eosinophils[14] and platelets.[15] Moreover, a substance in the supernatant cultures of IgE-receptor-bearing cells has been reported to react with antibodies against IgE receptors.[16] This substance was recently identified as CD23 and its soluble form (sCD23).[17] CD23 is rapidly upregulated on the B-cell surface after treatment with phorbol esters or after transfection by Epstein-Barr virus (EBV).[18]

Furthermore, EBV-infected cells release a soluble product (sCD23) that demonstrates autocrine growth-promoting activity.[19] Recent studies indicate that sCD23 is a potential differ-

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entiation factor for B-cells.[20] These data suggest that CD23 is involved in general B-cell regulatory pathways. Since previous observations reported high CD23 expression[21] and high sCD23 serum levels[22] in B-CLL, the purpose of the present study was to determine whether corticosteroids and/or α-interferon modify CD23 expression or release in B-CLL.

Materials and Methods

Criteria for eligibility

Twenty-one patients affected by B-cell CLL were included in the study. Diagnosis of CLL was based on a peripheral lymphocyte count greater than 10.0×10^9/L and bone marrow lymphocyte infiltration (defined as more than 30% lymphocytes in the aspirate). In subjects with an absolute lymphocyte count less than 15.0×10^9/L the monoclonality of peripheral lymphocytes was evaluated by FACS determination of surface light-chain distribution.[23] The summation curves of the histograms were analyzed with Kolmogorov-Smirnov statistics, and a D value higher than 10.0 was considered significant for monoclonality.[24] A bone marrow biopsy was also obtained from all patients. These biopsies were placed in B5 solution and two hours later in ethanol 70%. All samples were examined by the same experienced pathologist following standard criteria.[25] Subjects were classified according to the staging proposed by Binet et al.[26] Since IFN therapy seems to be useful only in stages A and B,[27,28] patients in stage C were excluded from this study. Those with associated neoplasia, positive Coombs' test, prolymphocytic features, over 80 years old or with a prevalence of T-lymphocytes were also excluded. Participants were either untreated or had not received any chemotherapeutic agent for six months prior to enrolling in the study.

Treatment schedules

After informed consent was obtained, the patients were randomly assigned to one of three therapeutic regimens: (A) recombinant α-2a interferon (α-IFN; Roferon-A, Hoffman-La-Roche) 3,000,000 U sc daily (7 subjects); (B) intermittent (three times a week) administration of α-IFN 3,000,000 U sc associated with 50 mg oral prednisone divided into two doses (after breakfast and after lunch)(8 patients); (C) prednisone (0.25 mg/Kg) daily (6 patients).

The long-term effects of these different therapeutical regimens on blood lymphocyte count and disease progression will be discussed elsewhere. CD23 expression and release were determined before and after two months of treatment. Ten normal volunteers, recruited from the medical staff of our laboratory, were used as controls.

Phenotyping

Mononuclear cells were separated from blood samples and marrow aspirates on Ficoll density gradients. Cells were stained with specific monoclonal antibodies and, after incubation and washing, immunofluorescence was measured by FACScan flow cytometry (Becton Dickinson, USA). Monoclonal antibodies against CD5 and CD10 (from Dako, USA) and CD3, CD4, CD8, CD19, CD20, CD21 and HLA-DR (from Becton & Dickinson, USA) were used.

Lymphocyte separation

Heparinized peripheral blood was diluted with phosphate buffered saline (PBS) and layered on Ficoll. After centrifugation, mononuclear cells were collected, washed, and monocyte-depleted by plastic adherence for 45 min at 37°C. B-cell purification was accomplished by immunomagnetic separation. Briefly, lymphocytes were incubated for 30 min at 4°C with anti-CD4, anti-CD8, anti-CD16 and anti-CD14 (Immunotech) monoclonal antibodies; after three washings, cells were incubated with goat anti-mouse immunoglobulin-coated magnetic beads (Dynabeads, Unipath) for 30 min at 4°C with continuous mixing. At the end of the incubation, the suspension was placed in a magnetic field to separate free B lymphocytes from the other subsets bound to the magnetic beads. The entire procedure was repeated twice. Purity, as determined by flow cytometric measurement of CD19 reactivity, was between 96 and 98%.

Culture conditions

All media and supplements were purchased from Gibco (UK). Purified B cells from controls, untreated and treated B-CLL patients were cultured in 24-well culture plates at 37°C in an atmosphere of 95% O_2 and 5% CO_2, at a density of 1×10^6 cells/mL. The culture medium was composed of RPMI 1640, 10% fetal calf serum, 100 g/mL streptomycin and 100 IU penicillin. Lymphocytes were cultured in the absence or presence of pokeweed mitogen (PWM) (final concentration 1:400) or phorbol myristate acetate (PMA) (final concentration 1 ng/mL). Culture supernatants were collected after five, seven and ten days, centrifuged and stored for cytokine determinations.

CD23 expression and sCD23 release

CD23 expression was determined in leukocyte-enriched buffy coats from cultures or fresh blood (0.5×10^6/mL) by incubating an appropriate dilution (2 μg/sample) of FITC-labelled anti-CD23 monoclonal antibody. Data were obtanied by examining 5000 cells per sample, and results were expressed as percentage of CD23-bearing cells after subtraction of background fluorescence, obtained using the lysis program. Mean fluorescence intensity (MFI), as determined with the lysis program, was expressed in arbitrary units. sCD23 was measured by a sandwich enzyme immunoassay (sCD23 EIA, Bioline). Briefly, samples and reference standards were applied to the wells coated with anti-sCD23 monoclonal antibodies. After incubation, the wells were washed and sheep anti-human sCD23 added; after a second incubation, an anti-sheep IgG conjugated with horseradish peroxidase was added. The bound conjugate was visualized using O-phenylenediamine and hydrogen peroxide. The results were expressed as μg/L.

Cell viability

The viability of cultured and fresh cells was determined by dye exclusion using nigrosine (naphthalene black) or trypan blue 0.4% in PBS. One drop of cell suspension was added to one drop of stain, and the percentage of unstained viable cells was microscopically evaluated. Recovery of viable cells after one-week culture was greater than 85%.

Cell cycle analysis

Peripheral blood lymphocytes were washed in PBS. After centrifugation the cell pellet was washed again with GM-EDTA buffer, resuspended at 1×10^6 cells/mL, immediately stained with propidium iodide (50 μg/mL) in Na citrate 0.1%, Nonidet P40 1%, RNAse 0.5%, and filtered through a 50 μ membrane to avoid cell clumps. Fluorescence light emission was determined after passing a 620 LP filter; the laser was set at 488 nm and 290 mW. Data were collected in list mode with a computer, and cell cycle analysis (percentage of cells in G1, S or G2M phases) was carried out with a consort 30 DNA-polynomial model program.

Cytoplasmic immunoglobulins

Cells were harvested from the cultures, washed in PBS, suspended at a density of 4.0×10^6 cells/mL and applied to glass slides by cytocentrifugation. The preparations were fixed in a 5% acetic acid/95% ethanol solution for 30 min at –20°C and washed twice in PBS. The slides were stained with fluorescein-labelled goat or rabbit anti-human immunoglobulins (IgG, IgM, k and λ light chain) for 30 min at room temperature. The slides were then washed twice with PBS. A drop of fixing solution was placed on the slide and a cover slip applied. They were read under immersion oil with a fluorescence microscope.

Statistical analysis

Data were expressed as mean±SD. Statistical analysis was performed using the statistical package SPSS. Analysis of variance between two groups was calculated (one-way). For categorical variables, cross tabulation with a Pearson X^2 was used to test whether the row and column variables were independent.

Results

Patients

The main clinical features of the patients are reported in Table 1. The three treatment groups

were equal in terms of age, male/female ratio, stage of disease and hematological parameters. The monoclonality of peripheral lymphocytes was confirmed in all patients by FACS determination of surface light-chain distribution. Surface immunoglobulins were at low density in all subjects.

Cell phenotype

Surface membrane immunophenotype was common in 19 (90.5%) patients as indicated by the positivity of CD5, CD19 and HLA-DR monoclonal antibodies. Two patients (9.5%) were CD5 negative but CD19 and HLA-DR positive.

CD23 expression

Lymphocytes obtained from normal subjects showed a low number of CD23-positive cells (2.82±0.9%), whereas a large fraction of lymphocytes from B-CLL patients was found to be CD23 positive (62.7±14.2%, p<0.0001) (Figure 1). The number of CD23-positive cells was closely correlated (r = 0.89) with CD5-positive cells. In 5 patients all CD23-positive cells were also found to be CD5-positive by the double fluorescence method, as recently reported in normal human B lymphocytes. After PMA the percentage of CD23-positive cells increased significantly (p <0.001) in both controls (10.9±82) and patients (76.0±10.7), while no changes were observed after PWM. Determination of MFI (in arbitrary units) yielded a value of 60.7 ±10.4 in normal subjects, which was significantly lower (p <0.0001) than that for B-CLL patients (472.6±50.2), indicating that a large number of CD23 molecules were expressed on the cell surface. After PMA stimulation MFI rose to 106.2±22.8 (p<0.001 vs basal condition) in normal subjects, whereas lymphocytes derived from B-CLL patients showed a minor increase (489.3±40.1; <0.05). The number of CD23-positive cells and the MFI did not change for either patients or controls after culture in medium alone. Similar CD23 expression was found before and after treatment, and no difference was found among the three therapeutic regimens.

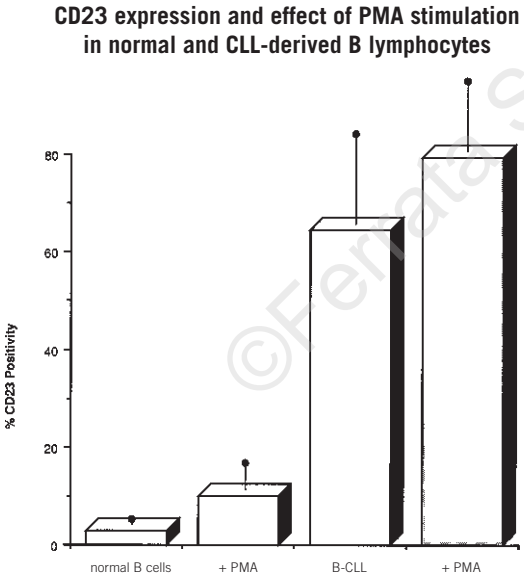

Figure 1. The results are expressed as percentage of CD23 positive cells. Normal subjects showed a lower number of positive cells (p<.0001) than B-CLL patients. Final concentration of PMA: 1 ng/mL. PMA determined a significant (p<.001) increase of CD23 expression in both normal subjects and patients.

sCD23 release

Normal B lymphocytes released very small amounts of sCD23 (7.1±9.9 μg/L). B lymphocytes from untreated B-CLL patients released a significantly higher (p<0.00001) amount of sCD23 in basal conditions (125.8±57.5 μg/L). PWM did not increase sCD23 release in either normal or malignant lymphocytes. After PMA stimulation the level of sCD23 release from normal lymphocytes did not change significantly (10.7±11.6 μg/L, p: NS), whereas it increased more than ten times (1,706±190.7 μg/L, p<0.0001) in lymphocytes derived from untreated B-CLL patients (Figure 2). The time course of sCD23 release from normal and neoplastic lymphocytes did not show significant differences after 5, 7 and 10 days of culture (Table 2). After two months of therapy the three groups of patients showed similar spontaneous sCD23 release after 10 days of culture in the presence of the medium alone (131.1± 42.2

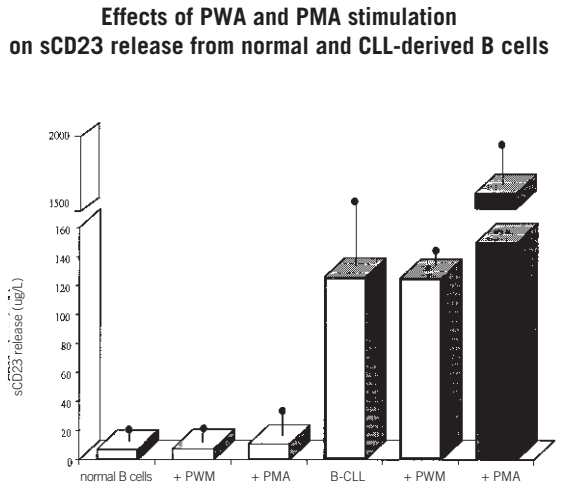

Figure 2. The results are expressed as µg/L. Final concentration of PMA was 1 ng/mL and of PWM, 1:400. In basal conditions sCD23 release was significantly (p<.00001) higher from neoplastic lymphocytes than from controls. PWM did not affect sCD23 release. PMA induced significantly higher (p<.0001) sCD23 release from CLL patients, whereas no changes were observed in control lymphocytes.

µg/L). After PMA stimulation, the lymphocytes collected from α-IFN treated patients produced sCD23 at a rate comparable to that before therapy (1,670.0±230.7 µg/L, p: NS); conversely, sCD23 release was significantly reduced (160.7 ±54.2 µg/L, p<.05) in corticosteroid-treated patients. sCD23 release in the group receiving combined therapy (α-IFN+corticosteroids), was comparable to that of patients under corticosteroid therapy (182.7±77.8 µg/L, p: NS vs corticosteroid treatment and p<.05 vs untreated patients) (Figure 3).

Cell cycle analysis

A similar pattern was found in both normal and CLL patients with a low number of cells in S (2%) or G2M (1%) phase. The number of normal lymphocytes in S phase rose significantly in controls (13.5±2.4% p <.05) after PMA stimulation; a slight increase of cells in G2M phase (2.0±0.2%) was also observed. Similarly, in CLL patients PMA stimulation led to an increased number of cells in S (18.6±4.9%, p <.05) and G2M phase (3.5±1.3%).

Cytoplasmic immunoglobulins

A marginal fraction of lymphocytes (2%) in all normal subjects showed cytoplasmic immunoglobulins (CyIg). Only two patients (9.5%) showed a small fraction (4%) of lymphocytes with CyIg. After PMA treatment the percentage of lymphocytes with CyIg increased in normal subjects (5.7% p<0.05). In contrast, no modification was observed in lymphocytes collected from B-CLL patients. Due to the small number of positive cases in each group of patients, no statistical analysis was performed among the different therapy groups.

Discussion

Knowledge about CD23 expression and func-

Table 1. Clinical and histological parameters of the patients.

treatment	*A*	*B*	*C*
# of patients	7	8	6
Age	61±7	62±6	59±10
Male/female ratio	1.2	1.0	1.5
Stage A/B	5/2	7/1	3/3
Lymphocyte count (×10⁹/L)	32.525±5.297	28.704±6.835	31.993±4.971
Hb level (g/dL)	12.2±0.9	12.7±1.1	13.0±2.0
Platelet count (×10⁹/L)	172±23.5	123±29.5	144±41.5
Performance status 0/1/2	4/3/0	4/4/0	3/2/1

Data are expressed as mean±standard deviation. No significant difference was found among the three groups for each parameter. A: IFN alone; B: IFN+steroids; C: steroids alone.

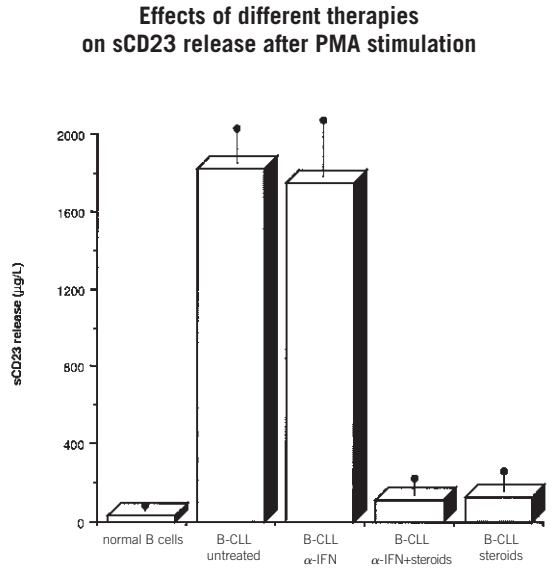

Figure 3. The results are expressed as μg/L. sCD23 release in untreated and α-IFN treated patients did not differ; on the contrary, a significant difference ($p<.05$) was found between subjects receiving corticosteroids (alone or in combination) and untreated or IFN-treated patients.

tion is at present rather limited. Kikutani[29] and Waldschmidt[30] demonstrated in both humans and mice that CD23 was expressed on more than 90% of η or δ B normal lymphocytes, whereas no such expression was seen on cells that had switched to IgG or IgA. Since CD23 is normally expressed at this intermediate stage of B-cell differentiation (before isotype switching), it is not surprising that CLL malignant B-cells present CD23 on their surface. Since sCD23 is a breakdown product of surface CD23, and CLL B-cell release more sCD23 than normal cells, it is likely that CD23 is upregulated in B-CLL. Our results support this hypothesis since a high percentage of CD23-bearing cells and a high number of CD23 molecules were found on the cell surface in CLL patients.

Multiple mechanisms can induce CD23 upregulation, the best example being transfection of lymphoid cell lines by EBV.[33] The high (more than 10^5 receptors/cell) CD23 expression is due to an EBV antigen (EBNa-2). Transfection of lymphoid cells with cDNA for this component produces high CD23 expression and sCD23 secretion in culture media.[32] The role of viruses, such as EBV, cytomegalovirus, HTLV-I or -II in the pathogenesis of B-CLL is currently unknown. Several years ago a preliminary report described the isolation of retrovirus-like particles and reverse transcriptase activity in cultured B-CLL lymphocytes,[33] but that finding remains unconfirmed. Direct involvement of EBV seems unlikely; B-CLL cells can be transfected by the virus, but this does not lead to cell immortalization as occurs in normal B cells.

CD23 upregulation can be induced by IL-4,[34] which increases the CD23 synthesis rate as well as IgE production.[35] IL-4 was once considered the main physiological mechanism for CD23 upregulation, but recently IL-2[36] was also shown to be capable of increasing sCD23 release and CD23 expression. High levels of several cytokines, i.e. TNF-α, IL-6, and IL-2, have been found in B-CLL. Accordingly, CD23 upregulation may reflect high serum levels of these cytokines, although we did not find any correlation between TNF-α, IL-2, sIL-2R serum levels and CD23 expression and release in our patients (unpublished data).

The difference between normal and malignant B cells is accentuated by PMA stimulation: secretion of sCD23 from B-CLL lymphocytes was 100-fold higher than from normal B cells, and CD23 expression was also increased. PMA does not need specific membrane receptors, such as plant lectins[37] but directly activates protein kinase C,[39] which is the second messenger for cell activation. Malignant B cells seem to be unable to increase CD23 expression significantly after PMA stimulation, probably because CD23 expression is maximal and cannot be artificially augmented.

Since no significant difference was found between normal and neoplastic lymphocytes regarding the number of cells in S and G2M phases after PMA stimulation, the increased sCD23 secretion demonstrated by neoplastic lymphocytes does not seem to be related to the proliferation process, but rather to some pecu-

Table 2. Time course of sCD23 release from normal and leukemic B lymphocytes. The data are expressed as μg/L.

	5th day	*7th day*	*10th day*
normal lymphocytes	6.8±5.9	7.1±9.9	7.1±8.9
normal lymphocytes+PWM	6.9±6.3	6.9±9.1	7.2±7.8
normal lymphocytes+PMA	9.1±7.4	10.7±11.6	10.5±8.6
B-CLL lymphocytes	122.3±62.1	125.8±57.5	131.2±64.5
B-CLL lymphocytes+PWM	128.4±55.8	131.2±63.5	124.7±72.8
B-CLL lymphocytes+PMA	1694.3±211.5	1810.0±100.6	1706.0±190.7

liar behavior of leukemic cells.

Several studies have focused on the function of sCD23 as an autocrine B-cell growth factor, although this area remains quite controversial.[39,40] Based on our data, corticosteroids seem to interrupt this possible autocrine pathway, as indicated by the significant reduction in sCD23 secretion from the cells of patients treated with corticosteroids or combined therapy. Corticosteroids bind to intracellular receptors[41] and suppress the signal transduction cascade.[42] Since the synthesis and release of several cytokines (e.g. IL-1, IL-2, IL-6 and γ-IFN) is regulated via this pathway,[43-45] CD23 can also be controlled by a similar mechanism.

Recently, in an *in vitro* study prednisolone was able to suppress CD23 expression and sCD23 release from normal B cells.[46] Although extrapolation from *in vitro* to *in vivo* observations must be made cautiously, these findings are in line with our data. Whether corticosteroid therapy modifies serum levels of other cytokines in B-CLL is now under investigation. B lymphocytes derived from α-IFN-treated patients showed sCD23 release similar to that found in untreated patients. Since α-IFN produces an anti-proliferative effect in early-stage B-CLL, its action does not seem to be mediated by CD23 modulation: expression and release of this substance do not differ from what is observed in untreated subjects.

Only patients in stages A and B were considered, and therefore a correlation between CD23 expression and/or release and disease prognosis was not possible. In fact, CD23 expression seems to be low only in advanced disease (Stage C).[21]

In conclusion, our data support the hypothesis that CD23 may play a role in B-CLL, although further studies are needed to determine the utility of pharmacological modulation of this receptor/lymphokine in the therapy of the disease.

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