Expression of CD10 by B-chronic lymphocytic leukemia cells undergoing apoptosis in vivo and in vitro

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CD10 is a 100 kDa, type II integral membrane protein, characterized by a single hydrophobic sequence that functions as a signal peptide and a transmembrane region. CD10 has neutral endopeptidase (NEP) activity, the function of which on the surface of lymphoid cells has yet to be elucidated.\(^1,2\) CD10 was initially discovered on the surface of acute lymphoblastic leukemia (ALL) cells and has long been considered a tumor-specific marker.\(^3\) Subsequent studies demonstrated that CD10 is expressed on a variety of cells from different tissues.\(^4\) Among the cells of hematopoietic lineage, CD10 is found on immature and mature T- and B-lymphocytes and on granulocytes.\(^7,8\) The reason why CD10 is expressed only at particular stages in the maturation of cells of a given lineage is presently unknown, although recent evidence has demonstrated a correlation between apoptosis and CD10 expression.\(^9,10\) CD10 is also found on the cells of a number of lymphoid malignancies, and in some cases there is evidence for a correlation between its expression and the propensity of the cells to undergo apoptosis.\(^11\)

B-cell chronic lymphocytic leukemia (B-CLL) is an accumulating disease of slowly proliferating cells.\(^12\) The malignant cells are characterized by a CD5-CD23- and CD19+ surface phenotype and by low expression of surface Ig.\(^15,16\) The presence of CD10 on the surface of the cells often raises questions regarding the diagnosis of B-CLL, although cases of unquestionable diagnosis of B-CLL have been reported in which the malignant cells stained brightly for CD10.\(^16,17\)

The finding of CD10 on the surface of B-CLL cells raises several issues concerning its meaning. In principle, CD10 expression could define a minor B-CLL subset, with special features; alternatively it could indicate a special propensity of the cells to apoptosis as has been observed in the case of other malignancies. This work was intended to clarify these issues.

Design and Methods

Patients and peripheral blood specimens

Peripheral blood was obtained from 31 untreated patients diagnosed with B-CLL as confirmed by clinical, pathologic and flow cytometry criteria.\(^15,16\) Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation (Biocell Separating Solution, Seromed, Biochrom KG, Berlin, Germany). In the suspensions containing less than 96% malignant B cells, the contaminating monocytes and T cells were removed by anti-
CD3 and CD14 MoAbs (Becton Dickinson & Co., Sunnyvale, CA, USA) sensitization followed by magnetic beads (Dynabeads Goat anti mouse IgG, Dynal Biotech ASA, Oslo, Norway). The cells were resuspended at a concentration of 5–10×10^6/mL in RPMI 1640 medium (Seromed, Biochrom KG) supplemented with 10% heat-inactivated fetal calf serum (FCS, Seromed Biochrom KG), 100 IU/mL penicillin and 100 µg/mL streptomycin (Seromed, Biochrom KG), 2 mmol/L L-glutamine (Seromed, Biochrom KG) and 1% sodium pyruvate (Seromed Biochrom, KG) and cultured with the same medium in a humidified atmosphere containing 5% CO₂ at 37°C. All patients gave their informed consent and the study was approved by the Institutional Review Board.

**Tonsillar B cells**

B cells were purified from tonsils obtained at surgery as previously reported. The cells were fractionated on Percoll-density gradients and only the cells in the high density fractions (50–60% Percoll) were collected. These were devoid of germinal center cells and plasma cells.

**Immunofluorescence and cell separation**

The following MoAb were used for direct immunofluorescent staining: CD19 FITC, CD3 FITC (Becton Dickinson & Co.), anti-IgM FITC, anti-IgD FITC (DAKOPATTS, Glostrup, Denmark), CD5 PE, CD23 PE, CD38 PE (Becton Dickinson & Co.), CD10 (J5) PE (Coulter Corp., Hielah, FL, USA), and CD5 Cy-Chrome, CD8 Cy-Chrome (BD Biosciences Pharmigen, San Diego, CA, USA). Triple staining was performed using the following combination of reagents: CD19-PerCP (Becton Dickinson & Co), CD10-Pe (J5) (Coulter Corp), and annexin-V conjugated with FITC (1 µg/mL) (ApoTox Detection Kit; MBL Co., Ltd., Japan). The cells were analyzed using a FACScan flow cytometer (Becton Dickinson & Co). CD10-positive and -negative cells were physically separated by a MiniMACS System (Miltenyi Biotec Inc., Bergisch, Gladbach, Germany) as previously described. Briefly the cells were incubated with anti CD10 MoAb for 30 min at 4°C, followed by goat anti-mouse Ig microbeads (Miltenyi Biotec) for 10 min at 4°C.

**Apoptosis assays**

Apoptosis was measured by staining with annexin-V conjugated with FITC (1 µg/mL) (ApoTox Detection Kit; MBL Co.). Alternatively, to detect the hypodiploid cells by flow cytometry, cells were permeabilized by treatment with hypotonic solution and then stained with propidium iodide (PI). In selected experiments, morphologic analysis of apoptotic cells was carried out following Giemsa staining.

Cells were induced into apoptosis by exposure to either goat μ chain antibodies (CD38-positive cases only) (Gaδ-Ab, 10 µg/mL) (Southern Biotechnology Associates, Birmingham, AL, USA), etoposide (50 µmol/L) (VEPESID; Bristol-Myers Squibb, Rome, Italy) or fludarabine monophosphate (1 µg/mL) (FAMP, Shering, Italy). Inhibition of protein synthesis during apoptosis was achieved by incubating the cells for 2 hours with cycloheximide (50 µg/mL) (Sigma Aldrich). Inhibition of apoptosis in the cells exposed to apoptotic stimuli was obtained by incubating the cells with VAD–FMK (10 µmol/L; final concentration, ICE family protease inhibitor; MBL Co.) and, in the CD38-positive B-CLL cases, with goat anti δ chain antibodies (Gaδ-Ab, 10 µg/mL) (Sigma Aldrich).

The inhibition of apoptosis was observed by flow cytometry using annexin-V, FITC-conjugation, and PI staining.

**Demonstration of CD10 expression by the malignant cells of fludarabine-treated patients**

Peripheral blood samples were taken from three patients who underwent fludarabine treatment (25mg/m²/die for five consecutive days). Blood was always taken 1 hour after the fludarabine infusion. Cells were purified and analyzed as above.

**Statistical analysis**

All calculations were performed using the SAS/STAT software package, release 6.06 of SAS Institute Inc., 1993. The non-parametric Wilcoxon rank sum test was employed to test the distribution of two related variables.

**Results**

**Increased proportion of CD10-positive cells in B-CLL cell suspensions undergoing spontaneous apoptosis in vitro**

Highly purified malignant cells from 31 B-CLL cases were cultured for 12, 24 and 48 hrs and analyzed for CD10 expression and the presence of apoptotic cells. In all but one of the cases (case 21), there was a progressive increase in the percentage of apoptotic cells (as determined by annexin-V and PI staining to detect the hypodiploid DNA following cell permeabilization). Although substantial variations were observed, in every case there was a close correlation between the increase in the proportion of apoptotic cells and that of CD10-positive cells which were generated in culture. This is evident in Figure 1A, which summarizes the data observed at 48 hrs of culture, when the maximum increases in the proportion of CD10-positive and apoptotic cells were observed. For the sake of clarity, the B-CLL cases are subdivided into three groups: those with high, medium, or low levels of apoptosis (and corresponding high, medium, or low expression of CD10). The cor-

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relation between CD10 expression and apoptosis determined by Spearman’s test (p) was 0.830 and 0.774 depending on whether annexin-V or PI staining, respectively, was employed. Figure 1B illustrates the flow cytometry profiles observed in two cases (cases 20 and 21) that are representative of the two extremes observed.

**Expression of CD10 by B-CLL cells treated with etoposide or fludarabine in vitro**

Treatment with drugs such as etoposide or fludarabine causes apoptosis of B-CLL cells in vitro. Here, we investigated whether treatment with these drugs could induce CD10 expression concomitantly with apoptosis. As shown in Figure 2, in which the results of four different cases are presented, B-CLL cells treated with etoposide were consistently induced into apoptosis to a greater extent than those cultured in medium alone. Similar results were obtained when the same cases were treated with fludarabine in vitro (data not shown). The results confirm a correlation between the extent of apoptosis induced and the concomitant CD10 expression.

**Inhibition of CD10 expression by B-CLL cells following treatment with VAD-fmk**

If CD10 expression is directly correlated to apoptosis, then the inhibition of apoptosis would be expected to result in inhibition of CD10 expression. B-CLL cells were treated with etoposide for 48 hrs in the presence or absence of VAD-fmk, which
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Exposure of CD10 by B-CLL cells following exposure to Gaμ-Ab but not to Gaδ-Ab. B-CLL cells from patient 22 were cultured with the indicated stimuli for 48 hrs, harvested and stained for annexin-V and CD10: flow cytometry profiles without selective gating (first vertical column on the left) and apoptotic cells electronically separated from non-apoptotic cells (gates R1 and R2, respectively) (second column). These cells were analyzed separately for annexin-V and CD10 expression (third vertical column). The experiment is representative of 9 CD38-positive B-CLL cases tested.

Expression of CD10 by B-CLL cells exposed to Gaμ-Ab or Gaδ-Ab in vitro

Exposure of the cells of CD38-positive B-CLL cases to Gaμ-Ab induced more apoptosis than medium alone.22,30 Thus, if a correlation between apoptosis and CD10 expression exists, then exposure of the cells to Gaμ-Ab would induce both CD10 expression and apoptosis. Initial experiments were carried out on 18 B-CLL cases, 9 of which were CD38-positive cases known to respond to Gaμ-Ab stimulation in vitro.22,30 Exposure of these cases to Gaμ-Ab caused a significant increase in the expression of CD10 (33±15%) concomitant to the appearance of apoptotic cells (52±20). In contrast, when the same cases were treated with Gaδ-Ab, a significant block of the expression of CD10 (14±7%) and annexin-V (25±9) was observed. In the remaining nine CD38− cases there was very little spontaneous apoptosis and this minimal apoptosis was not increased by Gaμ-Ab treatment. In these cases there was very little if any CD10 expression. Figure 4 shows one representative experiment on a CD38+ B-CLL case in which treatment with Gaμ-Ab resulted in the appearance of a much larger number of cells co-expressing CD10 and annexin-V than that observed in cells cultured...
in medium alone. In contrast, no or very few CD10/annexin-V double positive cells were seen in the cultures exposed to Gaδ-Ab. The suspensions cultured in medium or Gaμ-Ab revealed two different cell populations when analyzed for FSC and SSC features, whereas Gaδ-Ab-treated cells showed a single cell population similar to that observed in the suspension prior to culture. The two cell populations seen in the untreated or Gaμ-Ab-treated cells were gated separately as shown in Figure 4 and analyzed for CD10 and annexin-V expression. As expected, both markers were detected predominantly on cells with the physical characteristics of apoptotic cells (gated in R1, Figure 4). The majority of cells with the physical characteristics of viable cells (gated in R2), did not express either CD10 or annexin-V. A minority of the cells gated in R2 expressed annexin-V and not CD10, particularly in the suspensions treated with Gaδ-Ab, suggesting that phosphatidylserine externalization detected by annexin-V staining preceded the expression of CD10.

**Identification of CD10-positive cells as apoptotic cells**

In these experiments, we investigated whether the cells induced into apoptosis were the very same cells capable of expressing CD10 in vitro. To this end, purified CD38-negative B-CLL cells were induced into apoptosis by etoposide treatment for 48 hrs, harvested and stained for CD10 and CD19 (Figure 5). The CD10-positive cells were subsequently separated by magnetic beads and the purified cells analyzed for their morphology and for PI and annexin-V staining. As apparent in the figure, the cells that expressed CD10 were apoptotic as assessed by annexin-V expression and PI staining. Moreover, a large fraction of the cells displayed fragmented nuclei, whereas the remaining cells showed vacuole and chromatin condensation. In contrast, negligible apoptosis was observed in CD10− cells and the cell morphology also failed to indicate apoptosis. However, these cells did undergo apoptosis and became CD10-positive after a long time in culture.

**Expression of CD10 by B-CLL cells undergoing apoptosis in vivo**

In these experiments, we investigated whether treatment of B-CLL patients with fludarabine resulted in the appearance of CD10-positive cells in the blood. The tests were carried out on three different patients (I, II and III) treated with the same protocol. The results of the tests on patients I and II are reported in Figure 6A. Patient I had very few circulating CD10-positive cells prior to treatment. On day 1 of treatment, a few CD10-positive cells appeared in the periphery and their proportion increased on day 2 and again on day 3. Tests on day 12 showed that the CD10-positive cell levels had virtually returned to those observed prior to treatment. In connection with this, note that this patient had considerably lower, but still detectable CD19−CD5+ malignant cells in circulation following this cycle of therapy. The cells obtained from patient I on day 3 of therapy (Figure 6B) were stained for CD10 and CD19. The CD10−positive cells were separated by magnetic beads. The CD10+ cells were apoptotic as assessed by both annexin-V and PI staining following permeabilization to detect

Figure 5. Analysis of apoptosis in purified CD10-positive B-CLL cells. B-CLL cells from case 28 were cultured with etoposide for 24 hrs. The cells were stained for CD19 and CD10 (A) and separated into CD10-positive and negative B cells by magnetic beads (B). The CD10-positive and CD10-negative cells (top and bottom, respectively) were analyzed for apoptosis or morphology (Giemsa staining). Apoptosis was measured by both annexin-V and PI-staining. This experiment is representative of three carried out on different B-CLL cases.
Expression of CD10 by B-CLL

Expression of CD10 by B-CLL cells following fludarabine treatment. (A) B-CLL cells from two patients treated with fludarabine were purified and analyzed for CD10 expression on the indicated days of treatment. On days 1-3, staining was carried out 1 hour after fludarabine infusion. Patient I proved to be a responder and patient II to be refractory to fludarabine treatment. (B) Cells from patient I were purified on day 3 of fludarabine treatment and stained for CD19 and CD10. The CD10 positive cells were separated from the CD10 negative cells by magnetic beads and analyzed for apoptosis by annexin-V and PI staining.

A

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B

in vivo (day 3)

Expression of CD10 by normal B cells undergoing apoptosis in vitro

In these experiments, we investigated whether normal B cells could express CD10 upon apoptosis induction in vitro. Low density tonsillar B cells were cultured in the presence or in the absence of etoposide for 48 hrs. As shown in Figure 7A, the proportion of double positive CD10–annexin V cells was low among the cells cultured with medium alone, but increased considerably in the suspensions treated with etoposide. Because it is generally accepted that CD5+ cells represent the normal counterpart of B-CLL cells, we investigated whether the CD5+ cells were able of expressing CD10 in vitro. Cell fractionation procedures using CD5 MoAb were avoided because stimulation via surface CD5 can influence the B-cell apoptotic capacity.

Notably, the expression of CD10 in vivo never reached the intensity of that observed on apoptotic cells in vitro. The explanation of this phenomenon is not readily apparent. However, it is tempting to speculate that the cells at more advanced stages of apoptosis were cleared from the circulation, a hypothesis supported by the finding that the expression of annexin-V was also never very bright.
four different populations could be identified. These four cell subsets where electronically gated and reanalyzed for CD10 and annexin-V expression. As apparent in Figure 7B, both CD5+ and CD5− cells were capable of expressing CD10 upon apoptosis induction in vitro.

**Discussion**

CD10 is a surface marker rarely expressed by B-CLL cells, although cases have been reported of patients in whom a substantial fraction of cells with otherwise typical phenotypic features of B-CLL expressed CD10.16,17 The present study shows that CD10 can be a B-CLL marker in particular circumstances, namely when the malignant cells undergo apoptosis in vivo or in vitro.

The notion of a close correlation between CD10 expression and apoptosis was supported by the observation that the proportion of CD10+ cells increased in parallel with that of apoptotic cells in all of the experimental conditions used. In addition, it was possible to isolate the CD10+ B-CLL cells that progressively appeared in culture and ascertain their apoptotic status. CD10 expression was observed in B-CLL cells undergoing apoptosis following etoposide or fludarabine treatment. In those cases that expressed CD38, apoptosis (and CD10 expression) could also be induced by Gaμ-Ab (and not with Gaδ-Ab) treatment in vitro. Thus, it appears that CD10 expression is observed on apoptotic cells irrespective of the stimuli used to induce apoptosis and, unlike other markers such as CD38 or ZAP-70,15,30,32,33 the capacity to express CD10 may not be a marker for subsets of B-CLL. CD10 expression was also observed in normal CD5+ B cells induced into apoptosis in vitro. Therefore, CD10 expression is not only a feature of the malignant cells, but also of their normal counterpart. This concept is also in line with the observation that many normal lymphoid cells of T and B lineage can become CD10+ upon apoptotic induction.9-11,19

CD10 expression was also observed in vivo, when the cells were induced into apoptosis by fludarabine treatment. In the one patient, who proved to be refractory to fludarabine treatment, very little CD10 expression was observed in vivo. Although these observations need to be confirmed in a larger number of patients, CD10 expression, which can be determined with simple and relatively inexpensive flow-cytometry techniques, appears to represent a practical tool for assessing the response of B-CLL cells to a variety of treatments, including certain monoclonal antibodies such as anti CD20 or Campath34,35 which appear to induce apoptosis in B-CLL cells.36,37

Recent data have indicated that B-CLL cells (or at least certain subsets thereof) are apoptosis-prone in vitro.22,38-40 Such a propensity to undergo apoptosis appears to be counterbalanced in vivo by the action of certain stromal or other accessory cells which activate a variety of anti-apoptotic
mechanisms within the malignant cells themselves. It has also been suggested that rescue from apoptosis by these mechanisms could take place in particular areas such as pseudo-follicles observed in bone marrow. Thus, these mechanisms seem to be instrumental in the in vivo expansion of the malignant cells. The availability of a marker such as CD10 that can be detected in tissue sections, may help to elucidate the issue further by tracing the sites where apoptosis of malignant cells occurs more frequently.

The present observations raise the question of whether the physiologic function of CD10 during apoptosis stems from its particular enzyme activity (NEP), or of B cells (this study; data not shown).

There are several hypotheses for the potential role of CD10/NEP on the surface of cells undergoing apoptosis. For example, CD10/NEP could prevent the cells’ possible rescue from apoptosis by degrading those cytokines capable of delivering anti-apoptotic signals. From this perspective, CD10 expression could be seen as a safety device intended to drive the cell to its final destiny once the decision to undergo apoptosis has been made. An alternative and not mutually exclusive hypothesis is that lymphoid cells, which are physiologically capable of releasing a number of cytokines, continue to do so even after the apoptotic pathway has been activated. In this case, the role of CD10 /NEP would be that of degrading these peptide signals released by the cells during apoptosis, one of preapoptotic T cells or of B cells (this study; data not shown).

In lymphoid cells, cytokine release, cell proliferation and apoptosis are closely connected events. Thus, the expression of CD10/NEP and perhaps of other similar enzymes may serve the function of preventing unwanted inflammatory reactions in the sites surrounding the proliferation of lymphoid cells. Since these interactions are likely to be maintained following malignant transformation, it is possible that the expression of CD10/NEP to some extent prevents the onset of inflammatory reactions in the surroundings of leukemia cells undergoing apoptosis and thereby contributes to protecting these cells from potential attacks by the immune system.

References

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Pre-publication Report & Outcomes of Peer Review

Contributions
FM: coordinator of clinical studies; MM: laboratory investigator; RD, ZS, OB-M, FA-M, SM, RE, SC, CV: collection, study and treatment of patients; CG: supervisor of the laboratory studies; FM: supervisor of the clinical and laboratory data. MM and FM contributed equally to this study.

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Redundant publications: no substantial overlapping with previous papers.

Manuscript processing
This manuscript was peer-reviewed by two external referees and by Dr. Estella Matutes, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Matutes and the Editors. Manuscript received April 28, 2003; accepted June 2, 2003.

In the following paragraphs, Dr. Matutes summarizes the peer-review process and its outcomes.

What is already known on this topic
CLL cells are usually CD10 negative. It is known that the apoptotic pathway is impaired in CLL and some drugs act by inducing apoptosis. Recent studies have shown a correlation between apoptosis and CD10 expression.

What this study adds
This study demonstrated that CLL cells induced to apoptosis by \textit{in vitro} culture express \textit{in vitro} studies in 3 patients treated with fludarabine showed transient upregulation of CD10. Although CD10 expression may be useful to evaluate efficacy of therapy, as the authors suggest, the \textit{in vivo} data are very preliminary and drugs may act by a variety of mechanisms which do not involve apoptosis.