Sensitivity of B-Cell Chronic Lymphocytic Leukemia to Rituximab and Campath-1H and Correlation with the Expression of Cell Cycle Regulatory Proteins

Mira Grdiša

Division of Molecular Medicine, Ruder Bošković Institute, Zagreb, Croatia

Aim. To assess the effect of monoclonal antibodies anti-CD20 (Rituximab) and anti-CD52 (Campath-1H) on the viability of B cells from patients with B cell chronic lymphocytic leukemia (B-CLL) in comparison with a cytotoxic drug fludarabine (Fluda), and to determine the influence of these agents on the expression of cell cycle regulatory proteins in vitro.

Methods. B-CLL cells were incubated in vitro in the presence of Rituximab, Campath-1H, and Fluda. The viability of the cells was measured by MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Gel electrophoresis and Western blotting were used to determine the effect of these agents on the expression of cell cycle regulatory proteins in vitro.

Results. Both monoclonal antibodies, Rituximab and Campath-1H, were less toxic than Fluda to B-CLL cells. Combination of Campath-1H or Rituximab with Fluda did not have a stronger effect on the cells than Fluda alone. Both antibodies decreased the expression of p27 protein and increased the expression of p23; Fluda had a similar effect. The extent of cyclin D3 and cyclin E expression did not change significantly. The expression of cyclin D2 was slightly increased in the presence of Campath-1H, but in the presence of Rituximab it either decreased slightly or remained the same. Treatment of B-CLL cells with Fluda alone induced significant decrease in cyclin D2 expression.

Conclusion. These results demonstrated that monoclonal antibodies Campath-1H and Rituximab antibodies, as well as a cytotoxic drug fludarabine, had cytotoxic effects on B-CLL cells. They most likely induce apoptosis of B-CLL cells, but their activity is mediated through different pathways.

Key words: antibodies, monoclonal; antigens, CD; antigens, CD20; antineoplastic agents; apoptosis; cell cycle; cyclins; leukemia, lymphocytic, chronic

The treatment of cancer with agents targeting only and specifically tumor cells, while sparing healthy cells of the host, has been one of the main goals of oncology. The development of monoclonal antibodies has raised hopes that tumor-targeted immunotherapy could finally be used in the treatment of cancer. Antibodies can activate the immune system and induce complement-mediated lysis and antibody-dependent cell-mediated cytotoxicity, tumor immunity, apoptosis, and cell cycle arrest, and inhibit cell proliferation, angiogenesis, and metastatic spread.

The pathophysiology of cells in B cell chronic lymphocytic leukemia (B-CLL) is characterized by the inhibition of programmed cell death (apoptosis) and upregulation of the anti-apoptotic protein Bcl-2 (1). Furthermore, B-CLL cells are arrested in G0/G1 phase of the cell cycle (2,3). Dysfunctional apoptosis and cell cycle arrest are the main reasons why CLL cannot yet be cured with conventional chemotherapy. Apoptosis is physiological or programmed cell death, characterized by specific morphological features, including cellular and nuclear pyknosis, cytoplasmic blebbing, and margination of condensed chromatin (4). Cross-linking of various B-cell surface markers has been reported to cause cell cycle arrest and/or apoptosis in mouse and human B cells (5,6). Moreover, cross-linking of CD19 and CD22 with their respective monoclonal antibodies can induce apoptosis in B cells (7). However, the molecular pathways responsible for these characteristics of B-CLL cells are still unknown and require further investigation.

Cell cycle progression is controlled by a series of cyclin-dependent kinases and their activators called cyclins (8). Besides variations in cyclin expression, the activity of cyclin-dependent kinases is regulated by cyclin-dependent kinase inhibitors. The cyclin-dependent kinase inhibitor p27Kip (p27) is a key regulator of the cell cycle, and is responsible for G1/S transition (9). In apoptotic cells, p27 protein is cleaved by the caspases, resulting in p23 protein synthesis (10).

Rituximab, a chimeric antibody directed against CD20, has been recently approved for use against relapsed or refractory follicular or low-grade B-cell
non-Hodgkin’s lymphoma (11,12). It has unique modes of action and can induce killing of CD20+ cells via multiple mechanisms. The direct effects of Rituximab include complement-mediated lysis and antibody-dependent cell-mediated cytotoxicity, whereas indirect ones include structural changes, apoptosis, and sensitization of cancer cells to chemotherapy (13,14).

CD20 antigen is a cell surface non-glycosylated hydrophobic phosphoprotein of 35 kD, expressed during early pre-B-cell development before the plasma cell stage (15). CD20 regulates a step in the cell cycle that is critical for cell-cycle initiation and differentiation (16,17). It is also important for regulatory signals for B cells, although its precise role remains unclear (15-17). The ubiquitous expression of CD20 at high surface densities on malignant human B cells and its lack of internalization after monoclonal antibody binding make it a possible target in the immunotherapy of B-cell lymphomas (14,18). Treatment of resistant B-CLL with Rituximab reported so far has been successful (19,20).

Campath-1H is a genetically reshaped human IgG1 anti-CD52 monoclonal antibody (21), which binds to the cell membrane of more than 95% of all normal human lymphocytes, as well as to most B- and T-lymphoma cells (13,22). CD52 antigen does not change with the binding of antibody, which is important for the therapeutic utility of Campath-1H. This antibody has been used in the treatment of hematopoietic (20,22-24) and non-hematopoietic diseases, such as rheumatoid arthritis (25). It is consistently effective against malignant lymphocytes in the blood, bone marrow, and spleen. B-CLL might be the preferred lymphoproliferative disease for the treatment with Campath-1H (23). The mechanisms by which this antibody lyces cells are still unknown, although both complement-mediated lysis and antibody-dependent cell-mediated cytotoxicity may be involved (26).

Fludarabine (Fluda) is a potent inhibitor of DNA repair and an effective antitumor agent for CLL and low grade non-Hodgkin’s lymphoma (27). In this study, it was chosen as a standard cytotoxic agent against B-CLL cells.

It was shown earlier that cyclin D2 and the p27 were highly expressed in B-CLL (28). Since the expression of p27 has been linked to the defect in apoptosis of B-CLL cells (28), in this study the effect of monoclonal antibodies Rituximab and Campath-1H on the expression of cell cycle regulatory proteins was investigated, as well as their ability to induce apoptosis in B-CLL cells.

The aim of this study as to assess the cytotoxic effect of Rituximab and Campath-1H on B-CLL cells, their ability to induce apoptosis, and changes they caused in the expression of cell cycle regulatory proteins.

**Material and Methods**

**Cell Isolation and Culture**

Anticoagulant agent (heparin) was added to the specimens of peripheral blood from 13 patients with B-CLL, who gave informed consent. The blood specimens were processed within a few hours after blood collection. Criteria for B-CLL cells, besides appropriate clinical and cytopathological features, were based on a characteristic phenotype including CD5 positivity and weak expression of surface immunoglobulins (29). Mononuclear cells (more than 90% B-lymphocytes) were isolated by a density separation technique over Lymphocyte separation medium (Eurobio Biotechnology, Les Ulis, France). After washing, the cells were incubated in a final concentration of 2x10^6 cells/mL, together with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L of glutamine, 50 U/mL of penicillin, and 50 μg/mL of streptomycin in RPMI1640 (Life Technologies Ltd, Paisley, Scotland) at 37 °C in a 5% CO2 humidified atmosphere.

The cells were cultured either in the medium alone or exposed to Rituximab (10 μg/mL; MEDNO Protocol No. GELA LN-98 Group 5), Campath-1H (10 μg/mL; Welcome Research Laboratories, Beckenham, UK), or Fluda (9-β-D-arabinosyl-2-fluoroadenine-monophosphate; 5 μmol/L, Schering AG, Berlin, Germany). The cross-linking antibody, goat-antihuman IgG (50 μg/mL; Sigma, St. Louis, MO, USA), was added to Rituximab and Campath-1H. Viability of cells and analysis of cell lysates by Western blotting were performed in parallel for each sample.

**Cell Viability**

The number of viable B-CLL cells after culturing either in the medium alone or in the presence of Rituximab, Campath-1H, or Fluda was determined by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) at 24, 48, and 72 h (30). The method is based on a reduction of yellow MTT by mitochondrial dehydrogenase from live cells into a purple formazan product, which is then dissolved in dimethyl sulfoxide (DMSO). Absorbance of the product was measured at 570 nm, and the number of living cells was linearly proportional to the amount of reduced MTT. Quadruplicates of each sample of the control and differently treated cells were used in each experiment. The results were expressed as means±SD. To correct for spontaneous apoptosis, the viability of control cells was determined at each time point (0, 48, and 72 h) and used for the calculation of viability of the treated cells.

**Cell Lysates and Western Blotting**

After treatment with monoclonal antibodies, cell lysates were prepared and analyzed for the expression of cell cycle regulatory proteins by Western blotting. The cells (2x10^6) were lysed in a buffer (20 mmol/L TrisHCl pH 7.0, 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L ethylene diaminetetraacetic acid (EDTA), 2 mmol/L Na-orthovanadate, 10 mmol/L NaF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 100 μg/mL phenyl methyl sulphonyl fluoride (PMSF) at 4 °C for 20 minutes. After centrifugation at 20,000 × G for 15 minutes, the supernatants were collected and used either immediately or after freezing at -70 °C. Protein concentration was determined by the Bradford method (http://www. BioRad Laboratories, Richmond, CA, USA).

The effects of both monoclonal antibodies Rituximab and Campath-1H on the expression of the cell cycle regulatory proteins were determined after the separation of the cell lysates by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Briefly, the lysates (50 μg proteins) were separated on a 12%-SDS-PAGE and transferred to Immobiline-P nitrocellulose membranes (Millipore, Bedford, MA, USA). Control of the loading was performed by staining transferred proteins with naphthol blue black (Sigma). After blocking in 5% non-fat milk phosphate-buffered saline (PBS) for 1 h to determine p27 and p23, the membranes were incubated with the antibody specific to p27 (N-20, 0.5 μg/mL, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), or cyclin D2 (2 μg/mL; Pharmingen, San Diego, CA, USA), cyclin D3 (C-16, 0.5 μg/mL; Santa Cruz Biotechnology Inc.), and cyclin E (0.5 μg/mL; Santa Cruz Biotechnology Inc.). Subsequently, the membranes were washed twice in Tris-buffered saline (TBS)-Tween buffer (10 mmol/L Tris-HCl pH 8.0, 200 mmol/L NaCl, and 1% Tween 20) and incubated for 1 h with an appropriate horseradish peroxidase-linked secondary antibody (Dako, Glostrup, Germany). The membranes were then washed three times in TBS-Tween buffer, and the proteins were detected by enhanced chemiluminescence method (ECL™ Western blotting detection reagent, Amersham Pharmacia Biotech, Buckinghamshire, UK). After expo-
sure, the films (Biomax™ film, Kodak, Rochester, NY, USA) were scanned on a densitometer (BioCapt software) and analyzed by Bio-Profil Bio 1-D, Windows Application V97.00 (Vilber-Lourmat, France).

Statistical Analysis
One way ANOVA, followed by Student Newman Keuls method was used for statistical analysis of the data. The level of statistical significance was set at p<0.05. Statistical analysis was performed with Sigmastart software (SigmaQuest, Silicon Valley, CA, USA).

Results
Effect of Rituximab and Campath-1H on Viability of B-CLL Cells
The effect of anti-CD20 (Rituximab) and anti-CD52 (Campath-1H) on the viability of B-CLL was determined on freshly isolated cells treated with these monoclonal antibodies in the presence of cross-linking antibody goat-antihuman IgG and heat-inactivated FBS. The presence of cross-linking antibody was necessary for the activation of both monoclonal antibodies (results not shown). Control cells were incubated under the same conditions without monoclonal antibodies. The viability of the cells was determined at 0, 48, 72, and 120 h, depending on the experiment. Cytotoxic effect of Rituximab on 13 samples, Campath-1H on 9, and Fluda on 13 samples increased with the duration of incubation, especially for Fluda (p=0.022, one-way ANOVA) (Fig. 1). The comparison of the cytotoxic effect of Rituximab and Campath-1H with that of Fluda revealed significant differences in the sensitivity of cells (Figs. 2 and 3). Based on that sensitivity, ie, viability of the cells after 72 h, the samples were divided into three groups. The first group (n=4) was insensitive to Rituximab and significantly more sensitive to Fluda (mean viability, 117±12% vs 42±20%; p=0.025; Fig. 2A). The second group (n=6) was sensitive to Rituximab and Fluda (mean viability, 59±16% vs 22±14%; p<0.001; Fig. 2B), and the third group (n=3) was sensitive to Rituximab but significantly less sensitive to Fluda (mean viability, 52±10% vs 75±8%; p=0.001; Fig. 2C).

The evaluation of the cytotoxic effect of Campath-1H on the samples of 9 patients with B-CLL revealed that all samples were sensitive to Campath-1H (Fig. 3). Treatment of B-CLL in vitro with Campath-1H for 72 h decreased the viability of cells (59±12%) in comparison with control cells (100±22%). The cytotoxic effect of Fluda under the same conditions was significantly stronger (28±24%; p<0.001, F=17.929, df=3,26). The combination of Campath-1H and Fluda did not exert a stronger effect on the cells.
than Fluda alone. The majority of samples (5 out of 9) were equally sensitive to Rituximab and Campath-1H (Fig. 4).

Figure 4. Comparison of cytotoxic effect of Rituximab (closed bars), Campath-1H (gray bars), and Fluda (open bars) among blood samples. Fresh isolated B-cells were treated with monoclonal antibodies (10 \( \mu \text{g/mL} \)) in the presence of goat-antihuman IgG (50 \( \mu \text{g/mL} \)) or Fluda (5 \( \mu \text{mol/L} \)) for 72 h and their viability was determined with MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

**Influence of Rituximab and Campath-1H on Expression of Cell Cycle Regulatory Proteins**

After B-CLL cells were treated with Rituximab and Campath-1H, cell lysates were analyzed by Western blotting and the level of expression of p27, p23, cyc D2, cyc D3, and cyc E was determined in all samples. In vitro treatment of B-CLL cells from 9 analyzed samples with Rituximab and Campath-1H for 72 h caused some changes in the expression of proteins responsible for regulation of the cell cycle (Fig. 5). The level of protein p27 expression decreased, with concomitant increase of protein p23, a truncated form of p27. This effect was consistently seen in all analyzed samples, but at different degree of expression. Fluda also induced similar changes in the expression of protein p27 and the appearance of protein p23. The expression of cyclin D3 was very low in control cells of all examined blood samples and did not change with monoclonal antibodies treatment. The expression of cyclin E did not change significantly with any treatment in comparison with control (untreated) cells, as opposed to the expression of cyclin D2, which increased slightly during the treatment with Campath-1H, and either decreased or remained the same in the presence of Rituximab (variations among the samples). During treatment with Fluda, the expression of cyclin D2 decreased significantly.

**Discussion**

The investigation of cytotoxic effect of monoclonal antibodies on malignant B cells and proteins responsible for regulating the cell cycle is particularly relevant because the reasons for inhibition of apoptosis and cell cycle arrest in B-CLL cells are yet unclear. This study showed that Rituximab and Campath-1H had a similar cytotoxic effect on B-CLL in vitro. The cytotoxic effect of Fluda was greater than that of Rituximab or Campath-1H. The viability of the cells decreased with the duration of incubation. A combination of Rituximab or Campath-1H with Fluda did not have an additive cytotoxic effect. There were some indications that these agents induced apoptosis, perhaps through the activation of different pathways.

Variations in cytotoxic effect were observed among the samples, as well as a difference in the sensitivity to Rituximab and Campath-1H. Most examined samples showed sensitivity to Campath-1H, only to a different extent. Similar effect of Campath-1H was seen in patients with various lymphoproliferative disorders (22-24), including CLL (31-33). In comparison with Fluda, Campath-1H exerted lower cytotoxic effect, whereas the combination of Campath-1H and Fluda in this study did not have stronger effect than Fluda alone, even though the combination of Fluda with other anticancer agents has been proposed (34).

Samples significantly differed also in their sensitivity to Rituximab and Fluda. As shown earlier, the antibodies inhibited proliferation and directly induced apoptosis in some B cell non-Hodgkin’s lymphoma in vitro (35).

Cytotoxic effect of Rituximab depends on the expression of CD20 antigen (receptor) on cells, which was perhaps a reason for different responses of our patients. CD20 antigen is expressed on the surface of most B cell lymphomas and also on normal B cells. Therefore, the Rituximab monoclonal antibody, although providing a targeted treatment, is not entirely tumor-specific (36). The ways in which monoclonal antibodies kill tumor cells are not completely understood, and likely involve several mechanisms. The chimeric monoclonal antibody was engineered to contain human IgG, IgM, or IgG1-constant regions to augment immune-mediated antitumor activity (14). However, both antibodies required cross-linking antibody, Fc-
specific goat-antihuman IgG (37). This suggested that target cell killing is mediated by the binding of monoclonal antibody to the Fc receptor. It seems that both antibody-dependent cell-mediated cytotoxicity and complement-mediated lysis are involved in their cytotoxic mechanisms. Besides activation of immune system, monoclonal antibodies can induce apoptosis, cell cycle arrest, and inhibition of proliferation.

Besides having cytotoxic effects, both antibodies affected the expression of several proteins responsible for regulation of the cell cycle except the expression of cyclins D3 and E. These two cyclins are responsible for a transition from G1 to S phase of the cell cycle. However, B-CLL cells are arrested in G1/G0 phase, which is why their expression of cyclins D3 and E usually does not change. On the other hand, Rituximab caused a decrease in cyclin D2 expression, which was not noticed in the presence of Campath-1H.

Cyclin D2 also plays a regulatory role in the G1/S transition and the p27 protein expression. This observation suggests that the investigated agents kill tumor cells through different mechanisms. Both antibodies caused a decrease in the protein p27 expression and a concomitant increase in the protein p23.

Overexpression of p27 protein leads to a cell cycle arrest. Decreasing expression of p27 protein, caused by different agents, eventually results in apoptosis. This decrease occurs mainly due to proteolytic degradation by caspases, the enzymes activated in the apoptotic pathway of cell death (10). A product of that cleavage is protein p23, which in this study was found after the treatment of B-CLL cells with Rituximab. Thus, apoptosis would be one of the mechanisms of action of this drug. Similar effect was seen with Fluda, an agent that inhibits DNA synthesis (27) and acts as an immunosuppressant (38). Fluda remarkably decreased the expression of p27 protein and cyclin D2, and increased the p23 protein expression in all examined samples.

Although the exact role of the transmembrane CD20 molecule is not known, it can act as a calcium channel either directly or by binding to or activating a calcium channel. Binding of Rituximab initiates a cascade of intracellular signals involved in cell killing, which may also be important in the cell cycle arrest or apoptosis (39). Binding of the antibodies to CD20 may directly inhibit the cell cycle (17). Aggregates of CD20 molecules on the cell surface are believed to create or control a calcium channel, which an important event for the initiation and progression of the cell cycle (40). Activation of the apoptotic pathway by anti-CD20, ie, Rituximab, has been reported earlier (41). Rituximab down-regulated anti-apoptotic protein Bcl-XL and up-regulated the expression of pro-apoptotic proteins Bad and Apaf-1 in Ramos, Raji, and Daudi cell lines (41). Also, in the Burkitt lymphoma cell line Ramos, it induced the release of cytochrome c from mitochondria, and activated caspases 9 and 3 (42).

Altogether, Rituximab, Campath-1H, and Fluda exerted a cytotoxic effect on B-CLL cells in vitro, affecting the cell cycle. Decreasing p27 protein and increasing p23 protein implied that all three agents induced apoptosis, although other mechanisms, as complement-mediated lysis and antibody-dependent cell-mediated cytotoxicity, cannot be excluded.

Acknowledgement

I thank Association Claude Bernarde, Paris, France, which supported my stay in the Laboratoire de cinetique cellulaire, Departement d’Hematologie et d’Oncologie Medicale, Hopital Hotel Dieu, Paris, France where this study was carried out. My sincere thanks go to the staff of this laboratory for all their support and co-operation.

References

16 Tedder TF, Boyd AW, Freedman AS, Nadler LM, Schlossman SF. The B cell surface molecule B1 is func-


