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Alemtuzumab induces enhanced apoptosis in vitro in B-cells from patients with chronic lymphocytic leukemia by antibody-dependent cellular cytotoxicity

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Abstract

Alemtuzumab, a monoclonal anti-CD52 antibody has been shown to be highly effective in B-cell chronic lymphocytic leukemia, even in fludarabine-refractory disease. The mechanism of action is currently unknown, but may rely on complement-mediated cell lysis and antibody-dependent cellular cytotoxicity. The aim of this study was to assess the proapoptotic activity of alemtuzumab in chronic lymphocytic leukemia and to describe pathways potentially underlying this effect. Peripheral blood mononuclear cells from 21 chronic lymphocytic leukemia patients were treated in vitro in the absence of complement with fludarabine alone, alemtuzumab alone, or with the additional presence of a cross-linking anti-Fc-antibody. Apoptosis was quantified after 24 h by flow cytometry analysis. Expression of several pro- and anti-apoptotic proteins was determined at different time points. Apoptosis of peripheral blood mononuclear cells treated with alemtuzumab alone was significantly enhanced compared to untreated cells suggesting a minor potentially cytotoxic mechanism by direct signaling independent from antibody-dependent cellular cytotoxicity. The presence of a cross-linking anti-Fc-antibody induced the formation of cell clusters and enhanced apoptosis significantly suggesting a potential role of antibody-dependent cellular cytotoxicity in alemtuzumab induced apoptosis. Alemtuzumab activated a CD52-dependent signaling pathway which induced a significant increase in caspase 3 and 8 expression. Alemtuzumab significantly enhances apoptosis in chronic lymphocytic leukemia cells in vitro, especially in combination with a cross-linking anti-Fc-antibody, this effect being mediated by a caspase-dependent pathway.

Keywords: Chronic lymphocytic leukemia; Alemtuzumab; Apoptosis

1. Introduction

B-cell chronic lymphocytic leukemia is the most common type of leukemia in the Western world. It is characterized by the accumulation of long-lived, functionally inactive, mature-appearing neoplastic B-lymphocytes (Rozman and Montserrat, 1995). The clonal excess of B-cells is caused mainly by a suppression of cell death rather than by increased cell proliferation (Reed, 1998). In normal tissues the balance between proliferation and apoptosis, which is controlled by a large variety of different genes regulating this process, maintains homeostasis. Overexpression of Bcl-2 has been reported for chronic lymphocytic leukemia cells suggesting that suppression of apoptosis may contribute to the pathophysiology of chronic lymphocytic leukemia (Schena et al., 1992). Moreover, a reduced expression of Bax, a pro-apoptotic member of the Bcl-2 protein family, is associated with a poor prognosis in patients with CLL (Schimmer et al., 2003). On the other hand, the activation of caspases by different stimuli plays a

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key role in apoptosis of chronic lymphocytic leukemia cells (Decker et al., 2004; Chapman-Shimshoni et al., 2003).

Although no curative treatment is currently available for patients with chronic lymphocytic leukemia, several drugs including purine analoges, glucocorticoids, alkylating agents, the anti-CD20 antibody rituximab, and the anti-CD52 antibody alemtuzumab have shown a high activity against the disease (Byrd et al., 2000; Lin et al., 2003; Faderl et al., 2003; Lozanski et al., 2004).

Alemtuzumab (Campath-1H®) is a humanized monoclonal antibody directed against the cell surface antigen CD52. CD52 is a small glycosyl-phosphatidylinositol linked protein of unknown function that is expressed on both B and T lymphocytes (Waldmann, 2002; Rowan et al., 1998). Alemtuzumab has been shown to be highly effective in chronic lymphocytic leukemia, even in fludarabine refractory patients (Rai et al., 2002; Andritsos et al., 2002; Keating et al., 2002; Kennedy et al., 2002; Osterborg et al., 1997). The precise mechanism of action of alemtuzumab in chronic lymphocytic leukemia is currently unknown, but it is assumed to rely on complement-mediated cell lysis and antibody-dependent cellular cytotoxicity (Bindon et al., 1988; Villamor et al., 2003; Greenwood et al., 1993). An additional mechanism by which alemtuzumab could mediate lymphocyte depletion is by inducing programmed cell death (apoptosis) through direct activation of distinct signaling pathways.

In the present study we investigated potential pro-apoptotic mechanisms initiated by alemtuzumab in chronic lymphocytic leukemia cells. Moreover, we examined the expression of apoptosis-related proteins in cells following incubation with alemtuzumab.

2. Materials and methods

2.1. Patients

The study included samples from 21 patients with chronic lymphocytic leukemia (15 men and 6 women) with a median age of 60 years. For each patient morphologic diagnosis of chronic lymphocytic leukemia was confirmed by flow cytometry (Durig et al., 2001, 2002, 2003) which revealed a typical CD19+, CD20+, CD5+, CD23+, Ig light chain- (κ or λ light chain) restricted immunophenotype. CD38 expression was confirmed by flow cytomery as described (Durig et al., 2002, 2003). The percentage of CD5+CD20+ cells in the mononuclear fraction was over 90% and was represented almost exclusively by B-cells. Peripheral blood was usually obtained during routine follow-up visits to our institution with all patients giving informed consent according to institutional guidelines. None of the patients were on corticosteroids or any anti-inflammatory treatment, which may interfere with the study conditions.

2.2. Reagents and antibodies

The biologically active form of fludarabine, 5-fludarabine-monophosphate, and alemtuzumab (Campath-1H®) were obtained from Medac-Schering (Munich, Germany).

The following antibodies were used: Rabbit polyclonal anti-Bcl-2 antibody (Santa Cruz Biotechnology, CA, USA), rabbit polyclonal anti-Bax antibody (Santa Cruz Biotechnology, CA, USA), rabbit polyclonal anti-caspase 3 antibody (BD-Pharmingen, Germany), rabbit polyclonal anti-caspase 8 antibody (Upstate cell signaling solutions, Lake Placid, NY, USA), rabbit polyclonal anti-caspase 9 antibody (Santa Cruz Biotechnology, CA, USA), mouse monoclonal anti-actin antibody (Chemicon International, CA, USA) and goat anti-human IgG Fc fragment specific antibody (Serotec, Germany).

2.3. Isolation and culture of cells

Peripheral blood mononuclear cells were isolated from whole blood by centrifugation on a Ficoll/Hypaque gradient and cryopreserved in liquid nitrogen. Freezing/thawing did not influence the examined cell responses as confirmed in samples from 3 patients (data not shown). Peripheral blood mononuclear cells were cultured at a cell concentration of 2.5×10^6/ml in Iscove's modified Dulbecco's Medium (GIBCO™, Germany) supplemented with 15 % heat-inactivated fetal calf serum, 1% penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were incubated for 24 h with either 10 μg/ml fludarabine or 10 μg/ml alemtuzumab alone, or with 10 μg/ml alemtuzumab in combination with a goat anti-human IgG Fc fragment specific antibody at a concentration of 10 μg/ml. Alternatively, the cells were exposed to 10 μg/ml fludarabine in combination with 10 μg/ml alemtuzumab and a goat anti-human IgG Fc fragment-specific antibody at a concentration of 10 μg/ml. As a control, cells were incubated in medium without any drug or with a goat anti-human IgG Fc fragment specific antibody alone at a concentration of 10 μg/ml. Doses for fludarabine and anti-Fc antibody were assigned as published before (Schimmer et al., 2003; Bellosillo et al., 2002). Physiological in-vitro doses for alemtuzumab were recommended from Medac-Schering.

2.4. Analysis of cell viability by annexin V binding

For morphological studies, cytospin preparations were stained with May-Grünwald-Giemsa. To quantitate the extent of apoptosis, cells were harvested, washed twice in phosphate-buffered saline (PBS), gently resuspended into 0.4 ml binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl), and incubated with 0.5 μg/ml annexin V-fluorescein isothiocyanate and 10 μl propidium iodide for 15 min in the dark (Apoptosis Detection Kit I, BD-Pharmingen, Germany) according to

the manufacturer's instructions. Annexin V binds the membrane phospholipid phosphatidylserine, which is externalized from the inner to the outer leaflet of the plasma membrane in the early stage of apoptosis. When membrane integrity is lost, as seen in the later stage of cell death resulting from apoptotic process, propidium iodide staining becomes positive.

Ten thousand events were acquired on a FACSort flow cytometer and analyzed using the CellQuest software. Apoptotic cells were defined as cells expressing annexin-V (propidium iodide positive and propidium iodide-negative). Necrotic cells were excluded by gating on forward and side scatter. Representative flow cytometry analysis of cells from a patient with chronic lymphocytic leukemia is shown in Fig. 1. Consistent with previous reports (Koopman et al., 1994; Ormerod, 1998; Darzynkiewicz et al., 1997), three cell populations could be clearly distinguished: viable cells (annexin V-FITC$^-$ propidium iodide$^-$), cells in the early phase of apoptosis (annexin V-FITC$^+$ propidium iodide$^-$), and cells in the late phase of apoptosis (annexin V-FITC$^+$ propidium iodide$^+$). The percentages of apoptotic cells (annexin V-FITC$^+$ propidium iodide$^-$ and annexin V-FITC$^+$ propidium iodide$^+$) of all cells were determined. To obtain the net effect of drugs on apoptosis in vitro, drug-induced apoptosis was corrected for spontaneous apoptosis in untreated control samples.

2.5. Western blot analysis

Cells were washed once in ice-cold PBS and subsequently lysed in ice-cold lysis buffer (0.1 M dithiothreitol [DTT], 0.3 M benzamidine, 1 mg/ml trypsin inhibitor, 0.5 M EDTA, 1 M Tris pH 7.5, 150 mM NaCl, 1% NP-40) for 30 min. Debris was sedimented by centrifugation for 20 min at 13,000 × g. Protein was determined according to Bradford (1976). After mixing with 3 × Laemmli's and denaturation for 1 min at 95 °C, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with the use of equal amounts of the supernatant whole cell lysate (100 μg), followed by transfer to nitrocellulose filters. After blocking the reaction with 5% milk and incubation with the primary antibodies, the nitrocellulose sheet was further incubated with a rabbit or mouse peroxidase-conjugated secondary antibody (Sigma, Germany), and developed using an enhanced chemiluminescence system according to the manufacturer's instructions (Western Lightning, PerkinElmer life sciences). To detect different proteins using the same blot, blots were stripped twice for 30 min. in strip buffer (0.2 M Glycine, 0.1% SDS, 10 ml Tween 20 in 1000 ml aqua dest.) at room temperature. Thereafter, blots were washed three times, and subsequently blocked and probed with primary antibody as described above. Films were scanned and densitometry was used to quantify the immunoblot signal (NIH Image, Scion, Frederick, MD). To compare protein expression between the different time points, the average intensity of the signal was multiplied by the number of pixels in that area.

2.6. Statistical analysis

Differences in drug-induced apoptosis and protein expression were analyzed by the Wilcoxon nonparametric test with the use of SPSS for Windows® 11.0 (SPSS,

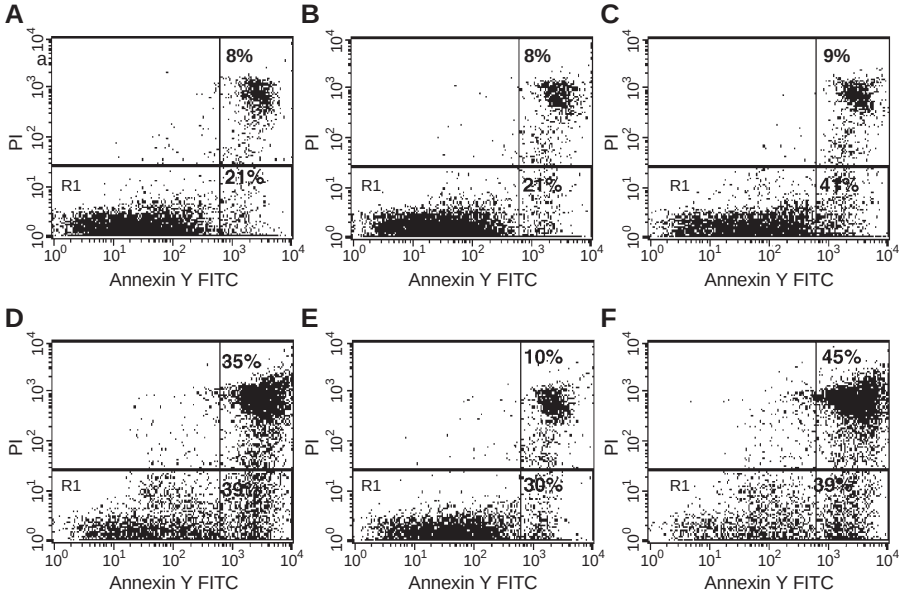

Fig. 1. Flow cytometric analysis of drug-induced apoptosis in chronic lymphocytic leukemia cells. Shown are representative results from a patient with chronic lymphocytic leukemia. Cells were incubated (A) in medium only, (B) with a goat anti-human IgG Fc fragment specific antibody alone, (C) with alemtuzumab alone, (D) with alemtuzumab plus goat anti-human IgG Fc fragment specific antibody, (E) with fludarabine alone, (F) with alemtuzumab, goat anti-human IgG Fc fragment specific antibody and fludarabine. The percentage of Annexin V-positive cells is indicated in each panel.

Chicago, IL, USA). Differences were regarded significant at $p < 0.05$.

3. Results

3.1. Alemtuzumab- and fludarabine-induced apoptosis

We studied the effects of alemtuzumab and fludarabine in peripheral blood mononuclear cells from 21 patients with chronic lymphocytic leukemia, of whom 17 were untreated and 4 were refractory to or had relapsed after fludarabine. None of the patients had received alemtuzumab. For these investigations we used one apoptosis assay by double staining with annexin V-FITC and propidium iodide.

Incubation of peripheral blood mononuclear cells with a cross-linking anti-Fc antibody alone did not increase apoptosis over that observed in medium alone (Fig. 2). Alemtuzumab alone significantly increased apoptosis compared to control, and the combination of alemtuzumab with a cross-linking anti-Fc antibody significantly increased this process. Moreover, we could confirm increased apoptosis rates induced by fludarabine alone ranging between 0 and 20% above controls. The combination of alemtuzumab, a cross-linking anti-Fc antibody and fludarabine was the most effective combination with apoptosis rates, corrected for spontaneous apoptosis, ranging from 33% to 75% (Fig. 2).

We also determined the potential influence of chemotherapeutic pre-treatment of patients ($n = 4$) on alemtuzumab-induced apoptosis. Chronic lymphocytic leukemia cells of treated and untreated patients did not differ in their sensitivities towards alemtuzumab in the presence or absence of a cross-linking anti-Fc antibody or the combination of alemtuzumab, a cross-linking anti-Fc antibody, and fludarabine (data not shown).

Since CD38 status has been described as a predictive marker for disease progression, we analyzed the impact of the CD38 status on apoptosis rate of chronic lymphocytic leukemia cells in vitro. There was no significant difference in total apoptosis rate in the controls. However, comparing annexin/propidium iodide positive and annexin/propidium iodide negative cells, CD38 positive cells showed significantly more propidium iodide positive cells and less propidium iodide negative cells than CD38 negative cells ($p = 0.036$, data not shown). There were no differences in apoptosis rates induced by alemtuzumab or fludarabine related to CD38 status (data not shown).

3.2. Morphological changes evoked by alemtuzumab

Cytospin slides were prepared from 2×10^5 cells and stained with May-Grünwald-Giemsa. Alemtuzumab in combination with a cross-linking anti-Fc antibody caused formation of small cell clusters after 6–8 h which were initially tightly adherent and subsequently did not disintegrate over the next 7–10 days (Fig. 3). Alemtuzumab alone did not induce such cell clusters. However, cell clusters seem to be more frequently present in cell cultures treated with the combination of alemtuzumab, a cross-linking anti-Fc antibody, and fludarabine.

3.3. Drug-induced expression of apoptosis-related proteins

In order to better understand the mechanisms potentially underlying alemtuzumab-induced apoptosis we performed Western blot analysis to determine changes in the expression of pro- and anti-apoptotic proteins in cells from chronic lymphocytic leukemia patients. Representative results are summarized in Figs. 4 and 5. Following incubation of cells in medium alone, expression levels of Bax, Bcl-2, caspase 3

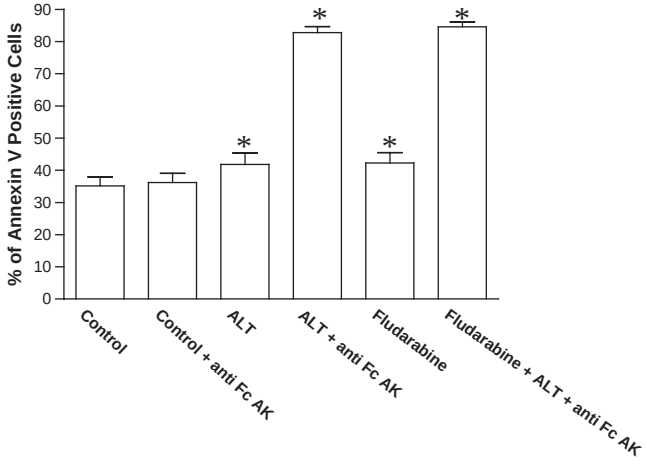

Fig. 2. Apoptosis rates as measured by flow cytometry in 21 patients. Anti-CD52-induced apoptosis demonstrated by FITC-labeled Annexin V staining. Apoptosis rates between control with or without goat anti IgG Fc antibody did not differ significantly. Drug-induced apoptosis rates are significantly higher than in control and control plus goat anti-human IgG Fc fragment specific antibody, respectively. Anti Fc AK: goat anti-human IgG Fc fragment specific antibody; ALT: alemtuzumab; *: significantly different from control and from control plus goat anti-human IgG Fc fragment specific antibody. Columns represent means ± S.E.M.

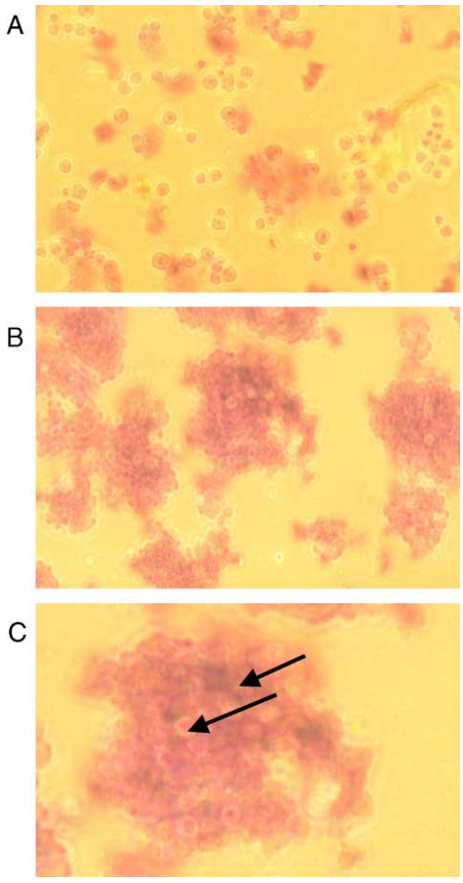

Fig. 3. Effect of alemtuzumab on isolated chronic lymphocytic leukemia cells. 2.5×10^6/ml isolated chronic lymphocytic leukemia cells were cultured for 24 h in the absence (panel A) or presence (panel B) of alemtuzumab and a goat anti-human IgG Fcγ fragment specific antibody. Cytospin slides were prepared from 2×10^5 cells and stained with May-Grünwald-Giemsa alemtuzumab in combination with a cross-linking anti-Fc antibody caused formation of small cell clusters after 6–8 h that were initially tightly adherent but subsequently did not disintegrate over the next 7–10 days. Original magnification × 100. Panel C shows an enlarged section of panel B. Apoptotic cells are indicated by arrows.

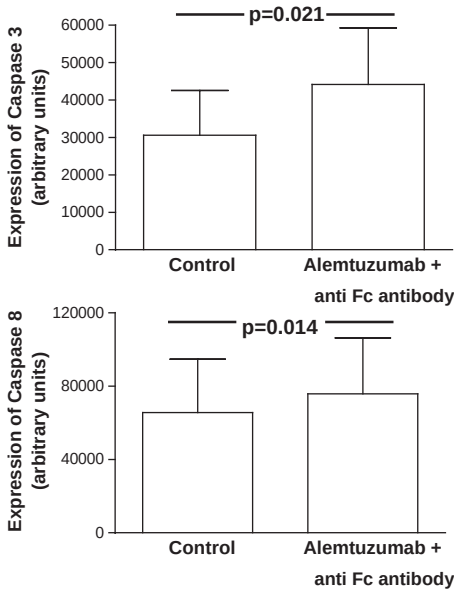

Fig. 5. Expression of caspase 3 and caspase 8 at different time points. Nitrocellulose blots were exposed to X-ray film. Films were scanned and densitometry was used to quantify the immunoblot signal (NIH Image, Scion, Frederick, MD). To compare protein expression at different time points the average intensity of the signal was multiplied by the number of pixels within the respective area. Results are represented from 3 independent experiments. Expression of both caspases 3 and 8 was significantly increased in cells treated with alemtuzumab plus anti Fc antibody compared to control after 24 h. Columns represent means ± S.E.M. anti Fc antibody: goat anti-human IgG Fc fragment specific antibody.

and caspase 9 remained largely constant at 4 and 24 h, while we observed a significant increase in the expression of caspase 8 after 24 h (31% increase, $p = 0.026$) compatible with a certain degree of spontaneous apoptosis under these conditions (cf. Fig. 2). Following incubation with alemtuzumab the expression of Bcl-2, Bax and caspase 9 remained largely constant compared to controls, whereas the expression of the pro-apoptotic caspase 8 (6% increase; $p = 0.036$)

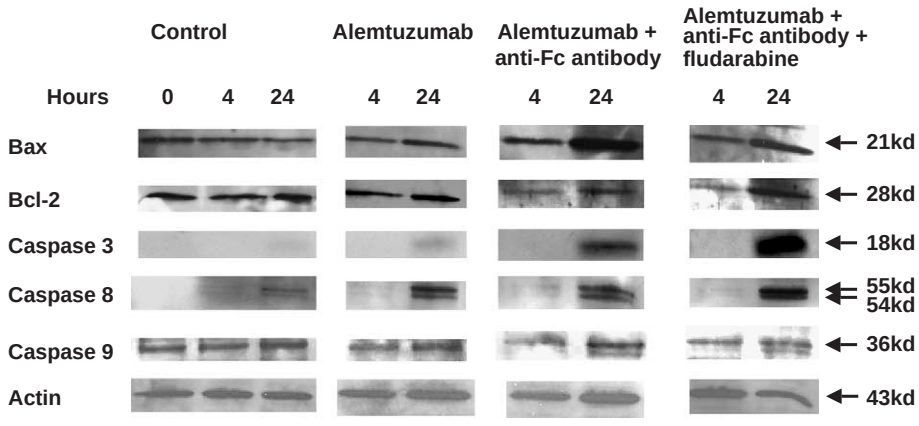

Fig. 4. Expression of apoptosis-related proteins during drug-induced cell death. Whole-cell lysates were obtained from representative patients with chronic lymphocytic leukemia incubated with complete medium (control), alemtuzumab in the absence or presence of anti-human IgG Fc fragment specific antibody and with a combination consisting of alemtuzumab, goat anti-human IgG Fc fragment specific antibody and fludarabine. Using Western blot analysis the samples were analyzed for the expression of Bax, Bcl-2, caspases 3, 8 and 9. Results are represented from 3 independent experiments. Similar expression of actin confirmed equal loading of protein for all samples.

and caspase 3 (10% increase, $p=0.021$) increased slightly but significantly after 24 h, respectively. These effects were strongly enhanced on incubation of cells with both alemtuzumab and a cross-linking anti-Fc antibody. Under these conditions we also observed an increased expression of caspase 3 (31% increase, $p=0.013$) and caspase 8 (9% increase, $p=0.033$) in comparison to alemtuzumab alone. The combination of alemtuzumab, a cross-linking anti-Fc antibody plus fludarabine caused strongest expression of caspase 3 (3.2 fold higher expression, $p=0.011$) and caspase 8 (19% increase, $p=0.026$) in comparison to baseline values (Fig. 5). In addition the expression of caspase 3 and caspase 8 after 24 h was significantly increased in each group compared to the 4 h value.

Together, these findings are compatible with the action of external death stimuli while the expression of caspase 9, which is typically induced through intrinsic stimuli, remained largely unaffected in our experiments.

4. Discussion

Anti-CD52 antibody-based immunotherapy of chronic lymphocytic leukemia has been shown to be effective even in fludarabine refractory patients (Keating et al., 2002). The mode of action of alemtuzumab in chronic lymphocytic leukemia has remained obscure. Possible mechanisms include complement-mediated cell-lysis and antibody-dependent cellular cytotoxicity.

Recently, Zent et al. (2004) described a rapidly cytotoxicity induced by alemtuzumab plus complement, but they did not see any apoptosis induced by alemtuzumab alone in a serum free medium.

Using cells from 21 patients with chronic lymphocytic leukemia we demonstrate that alemtuzumab in combination with a cross-linking anti-Fc antibody induces enhanced apoptosis of human chronic lymphocytic leukemia cells in vitro in a complement-free system suggesting a potential role of antibody-dependent cellular cytotoxicity in alemtuzumab induced apoptosis. As many Fc-positive cells also express cytolytic activity resulting in antibody-dependent cellular cytotoxicity, cross-linking the antibody was required while in vivo Fc-positive cells may mediate these effects.

We show here that treatment with alemtuzumab alone or in combination with a cross-linking anti-Fc antibody for 24 h increases staining in 4% and 44% of the cells, respectively, over baseline using the annexin V-FITC/propidium iodide dual staining with flow cytometry analysis. Apoptosis induced by alemtuzumab alone was in fact significantly increased compared to baseline. However, the effect was small suggesting a minor, potentially cytotoxic mechanism by direct signaling independent of antibody-dependent cellular cytotoxicity. These results are in contrast to those of Zent et al. (2004) who did not see any effect of alemtuzumab on apoptosis in chronic lymphocytic leukemia cells maintained in a serum-free medium. Stanglmaier et al. (2004) demonstrated in only 1 out of 8 patients with chronic lymphocytic leukemia an effect of alemtuzumab on apoptosis. However, these authors also showed a slightly increased apoptosis rate in Ramos cells by alemtuzumab alone compared to control (17% versus 13% annexin V^+ cells, respectively). The addition of a cross-linking anti-Fc antibody, however, evoked significantly higher apoptosis rates (median 44% over baseline) suggesting a major effect of antibody-dependent cellular cytotoxicity in alemtuzumab-induced apoptosis. Moreover, we show slightly, but not significantly increased apoptosis ($p=0.092$) following the additional administration of fludarabine (apoptosis rate 48%) which may provide a rational for the combination of fludarabine and alemtuzumab in the treatment of chronic lymphocytic leukemia in vivo. Elter et al. (2004) showed in a phase-II-trial the safety and efficacy of the combined modality treatment with fludarabine and alemtuzumab. Moreover, others also demonstrated an increased apoptosis rate induced by a goat anti-human antibody which is in line with our results (Stanglmaier et al., 2004).

Chronic lymphocytic leukemia cells of previously treated or treatment-naive patients did not differ in their sensitivities to alemtuzumab both in the presence or absence of a cross-linking anti-Fc antibody or the combination of alemtuzumab, a cross-linking anti-Fc antibody and fludarabine. All treated patients had previously received fludarabine. Thus, our in vitro observations are in line with the clinical experience showing a good responsiveness towards alemtuzumab in patients previously treated with fludarabine.

The exact mechanism by which alemtuzumab induces apoptosis in chronic lymphocytic leukemia cells has not been investigated before. We, therefore, determined expression of the Bcl-2 and Bax proteins as well as the expression of pro-apoptotic caspases after incubation with alemtuzumab. Bcl-2 is overexpressed in chronic lymphocytic leukemia and an increased Bcl-2/Bax ratio is predictive of an unfavorable clinical outcome (Schena et al., 1992; Schimmer et al., 2003). In our study, Bax and Bcl-2 protein expression were not changed after 24 h incubation with alemtuzumab suggesting a minor role of these two proteins in alemtuzumab-induced apoptosis. Caspase 9 protein expression remained unchanged over time which is in line with the notion that caspase 9 is predominantly activated through intrinsic death stimuli (Chapman-Shimshoni et al., 2003; Decker et al., 2004). However, we demonstrate that alemtuzumab, especially in combination with a cross-linking anti-Fc antibody, obviously activates a CD52-dependent signaling pathway which results in apoptosis and which may depend on caspase 3 and 8 activation. Caspase 8 initiates cell death in response to extracellular apoptosis-inducing ligands and is activated in a complex associated with the receptors' cytoplasmic death domains (Jones et al., 2001). Caspase 3 appears to amplify caspase 8 signals into full-fledged commitment to disassembly. Taken together, alemtuzumab induces apoptosis which appears

through a caspase-dependent pathway in a manner compatible with that observed for other extrinsic death stimuli.

Recently, Stanglmaier et al. (2004) described a non-classic, caspase-independent apoptotic pathway in freshly isolated chronic lymphocytic leukemia cells. Moreover, these authors proposed that the mitochondrial pathway may not be involved in alemtuzumab-induced apoptosis. This would be in line with our results which showed unchanged expression of bcl-2, bax protein, and caspase 9. However, in their report Stanglmaier et al. (2004) measured the cleavage of poly-ADP-ribose polymerase (PARP), an indirect way to assess caspase-3 activity. In contrast, we determined activation of the caspase pathway more directly by means of Western blot analysis and our data suggest activation of a caspase-dependent pathway. In line with our findings, Byrd et al. (2002) observed caspase-3 activation in three patients with chronic lymphocytic leukemia by rituximab, an anti-CD20 monoclonal antibody, in vivo. This latter finding and caspase activation by alemtuzumab described here suggest that one general mechanism of action of monoclonal antibodies involves pro-apoptotic signals mediate by caspase activation.

In conclusion we demonstrate here that alemtuzumab significantly enhances apoptosis in chronic lymphocytic leukemia, especially in combination with fludarabine and a cross-linking anti-Fc antibody. This effect was observed even in cells from patients whose disease was refractory to or had relapsed after fludarabine treatment. Alemtuzumab upregulates the caspase 8 and caspase 3 proteins while expression of the Bcl-2 protein, Bax protein and caspase 9 was not influenced. Our work and the paper of Zent et al. (2004) and Stanglmaier et al. (2004) demonstrate that antibody-dependent cellular cytotoxicity and the complement-mediated cell lysis are the two major mechanisms by which alemtuzumab induces apoptosis in chronic lymphocytic leukemia cells in vitro.

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