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LETTER TO THE EDITOR

Apoptotic induction by anti-CD20 antibodies in chronic lymphocytic leukemia: comparison of rituximab and obinutuzumab

Lina Reslan[1,2,3,4], Stéphane Dalle[1,2,3,4,5], Stéphanie Herveau[1,2,3,4], Emeline Perrial[1,2,3,4] & Charles Dumontet[1,2,3,4,5]

[1]*Université Lyon, Lyon, France,* [2]*Université Lyon 1, Lyon, France,* [3]*Inserm U1052 and* [4]*CNRS UMR5286, Centre de Recherche en Cancérologie de Lyon, Lyon, France and* [5]*Hospices Civils de Lyon, Lyon, France*

Monoclonal antibodies (MAbs) are becoming an essential component of the treatment of patients with chronic lymphocytic leukemia (CLL). CLL is a heterogeneous disease with a variable clinical course. The use of rituximab, an anti-CD20 MAb, has changed the therapeutic landscape of B-cell malignancies, particularly in patients with non-Hodgkin lymphoma (NHL), with more recent indications in the setting of CLL [1]. CD20 is commonly expressed by CLL cells, albeit at a lower level than in NHL cells, which might partly explain the lesser efficacy observed in patients with CLL treated with rituximab [2].

Obinutuzumab is a novel type II glycoengineered immunoglobulin G1 (IgG1) anti-CD20 MAb with enhanced antibody-dependent cell-mediated cytotoxicity, less complement-dependent cytotoxicity and superior cell death induction in comparison with rituximab. It is currently in phase II/III clinical trials for the treatment of NHL and CLL [3]. The aim of this study was to compare the mechanism of induction of apoptosis of the two anti-CD20 MAbs, rituximab and obinutuzumab, in fresh CLL samples. We studied the effects of these two MAbs on the intrinsic pathway of apoptosis, by evaluating the change of mitochondrial membrane potential and the expression levels of apoptosis-related proteins.

Because our study is based on 32 clinical samples (Table SI in Supplemental data available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.788175) and specifically on freshly isolated CLL cells known to have a spontaneous apoptotic effect, and taking into consideration the high heterogeneity observed among patients, we show results for only one representative patient in some assays. Similar results were obtained in three different patients.

Freshly isolated CLL cells were incubated up to 24 h with obinutuzumab or rituximab at a final concentration of 10 μg/mL. Most of the samples of CLL that were available for this study presented a high degree of spontaneous apoptosis (greater than 30%), which led us to use 30% as the threshold. The percentage of apoptotic cells assessed by Annexin V binding was higher in samples exposed to obinutuzumab than that in controls after 24 h. Data from nine representative patients are presented in Figure S1 (Supplemental data available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.788175).

Obinutuzumab exhibited significant induction of apoptosis after 24 h in a caspase-dependent manner [Figure 1(a)], which was not the case for rituximab, a type I antibody. Moreover, exposure of CLL cells to obinutuzumab was associated with significant dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$) after DiOC6(3) (3,3'-dihexyloxacarbocyanine iodide) staining, 3 h and 6 h after exposure to antibodies, in comparison to untreated cells [Figure 1(b)]. The effect of obinutuzumab was similar to that observed with carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), a potent uncoupler of oxidative phosphorylation that induces dissipation of $\Delta\psi_m$. The loss of $\Delta\psi_m$ induced by obinutuzumab was almost totally abolished by Z-VAD.fmk, a pan-caspase inhibitor, suggesting that the loss of $\Delta\psi_m$ is at least partially caspase-dependent [Figure 1(c)]. These results suggest that mitochondria may be among the earliest targets of caspase activation during apoptosis induced by obinutuzumab. A possible explanation for the different cellular responses elicited by obinutuzumab and rituximab is that obinutuzumab binds CD20 in a completely different orientation compared with that of rituximab, with an elbow angle almost 30° wider than that of type I antibodies, potentially resulting in different spatial arrangements of two CD20 molecules bound to a single obinutuzumab or rituximab molecule [4]. The reduction of $\Delta\psi_m$ was associated with the production of reactive oxygen species (ROS) in obinutuzumab-exposed cells (Figure S2 in Supplemental data available online at http://informahealthcare.com/doi/abs/10.3109/10428194. 2013.788175). However, this increase was not statistically significant in our fresh CLL cells, and antioxidants were unable to prevent the loss of $\Delta\psi_m$ or to circumvent obinutuzumab-induced apoptosis, suggesting that increased ROS content was not the *primum movens* of obinutuzumab-induced cell death. These data are in contradiction with a recent study showing that ROS are critical for programmed cell death evoked by a range of MAbs, including type II anti-CD20 MAbs (tositumomab and obinutuzumab),

Correspondence: Pr. Charles Dumontet, MD, PhD, INSERM 590, Faculté Rockefeller, 8 avenue Rockefeller, 69008 Lyon, France. Tel: 33-4-78-77-72-36. Fax: 33-4-78-77-70-88. E-mail: charles.dumontet@chu-lyon.fr

There is an accompanying commentary that discusses this paper. Please refer to the issue Table of Contents.

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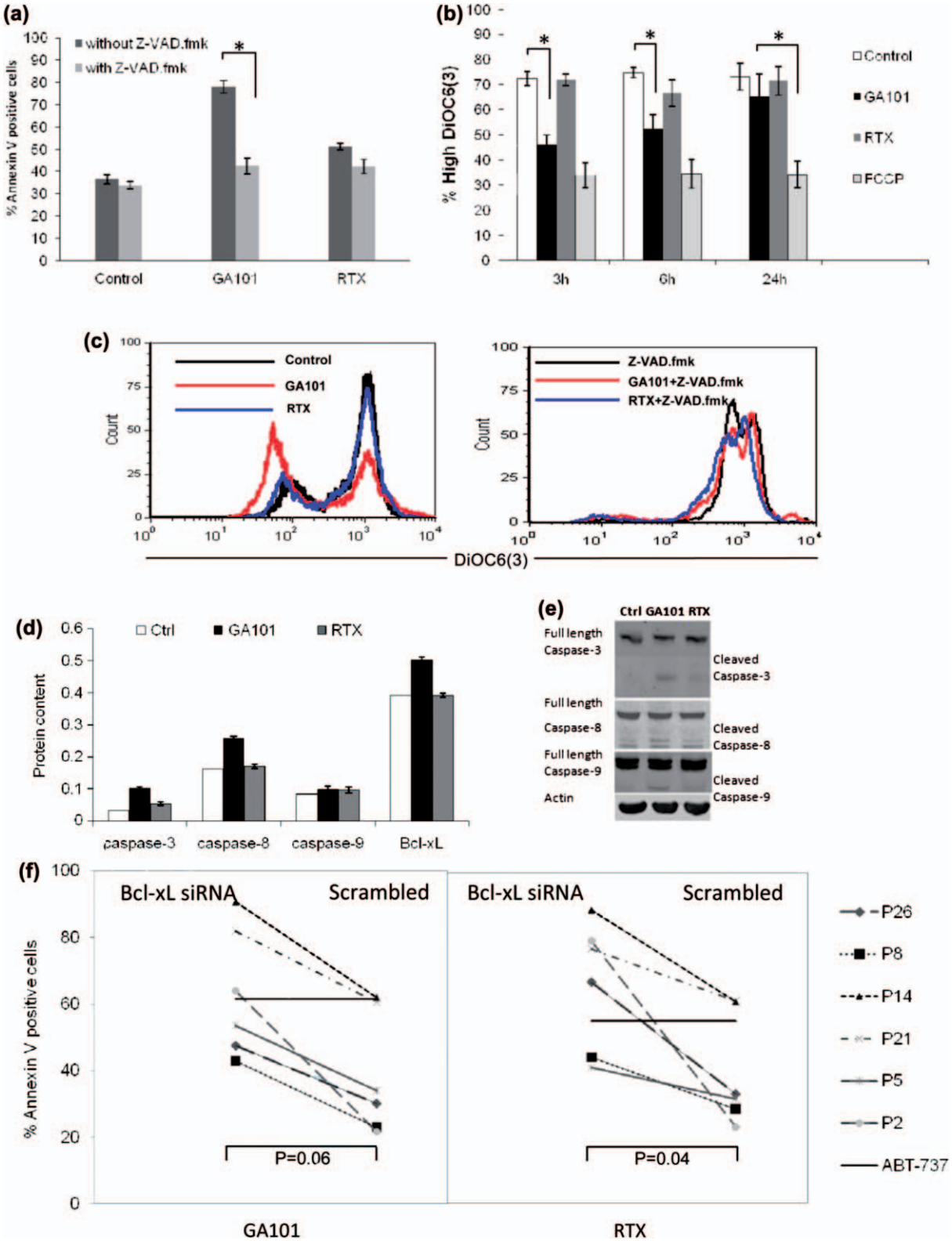

Figure 1. Evidence of caspase dependence and involvement of Bcl-xL in apoptosis induced by anti-CD20 antibodies. (a) Differences in Annexin V positive cells after exposure to GA101 or rituximab (RTX) at 10 μg/mL for 24 h with or without pre-incubation with 50 μM Z-VAD.fmk in a representative CLL patient sample (P26). (b) Alterations in mitochondrial membrane potential evaluated by DiOC6(3) labeling in nine CLL samples exposed to anti-CD20 antibodies (10 μg/mL) for up to 24 h. FCCP was used as a positive control. (c) DiOC6(3) labeling in a representative CLL patient sample (P20) incubated with 50 μM of Z-VAD.fmk for 1 h prior to exposure to antibodies. (d) Effect of anti-CD20 antibodies on expression levels of caspase protein and Bcl-xL content in fresh CLL cells. Diagram showing relative average increases of Bcl-xL (six patients) and caspases (three patients) normalized against expression levels of β-actin is presented. (e) Immunoblot analysis of representative patient CLL cells treated with GA101 or rituximab (RTX) (10 μg/mL) for 24 h in comparison to control (Ctrl) and analyzed for expression and activation of caspase-3, caspase-8 and caspase-9. Cells were lysed then immunoblotted using specific antibodies. (f) Percentages of Annexin V positive cells in fresh CLL cells from six patients, after transfection with anti-Bcl-xL or control siRNA by sonoporation followed by exposure to GA101 or rituximab (RTX) for 24 h. ABT-737 (1 nM) was used as a positive control for silencing of Bcl-xL. Values of ABT-737 represent the average for six patients. Error bars represent standard deviation (SD). Statistical significance of differences between treatments was determined by paired Student's *t*-test. $^{*}p < 0.05$.

in human B-lymphoma cell lines and primary B-CLL cells [5]. This contradiction could be due to the use of different types of cell lines and primary B-CLL cells, while we used freshly isolated CLL cells in our experiments.

The sensitivity of CLL cells to therapy has been reported to be influenced by the relative ratios of pro- and anti-apoptotic proteins. Kitada *et al*. reported that patients whose cells had a high Bcl-2:Bax ratio had a lower response rate to fludarabine [6]. In this study, we found that exposure to obinutuzumab caused conformational activation and mitochondrial translocation of Bax as well as increased content of Bak (Figure S3 in Supplementaldata available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.788175). The conformational changes of these proteins have been suggested to modify the protein–protein interactions that are required for the integration of damage signals and commitment of the cell to apoptotic death [7].

The mitochondrial pathway is commonly activated by cytotoxic agents active in CLL cells, such as fludarabine and other cytotoxic anticancer drugs [8]. Many studies have shown that rituximab-induced caspase-3 and -9 activation is concurrent with the induction of apoptosis [9,10]. Our study confirmed that obinutuzumab may induce apoptosis by activation of the mitochondrial pathway involving the subsequent cleavage of caspase-3 and -9, in five out of nine patient samples studied [Figures 1(d) and 1(e)]. Caspase-8 was also found to be processed in some patient samples examined. The involvement of caspase-8 in the intrinsic mitochondrial pathway observed in our study could result from activation of other caspases, independent of Fas [11].

Notwithstanding the heterogeneity observed among patient samples, the content of Bcl-xL was found to be increased consistently after exposure to obinutuzumab and rituximab in six samples (Figure S4 in Supplemental data available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.788175). Bcl-xL has been suggested to be a major actor in preclinical models of resistance to rituximab [12]. We found that Bcl-xL was not present in most CLL samples at baseline, but increased significantly after exposure to rituximab or obinutuzumab [Figure 1(d)]. Furthermore, decreasing Bcl-xL content by transfection of a specific siRNA sensitized CLL cells to the cytotoxic effects of rituximab or GA101 [Figure 1(f), Figure S5 in Supplemental data available online at http://informahealthcare.com/doi/abs/10.3109/10428194.2013.788175]. The degree of sensitization was similar to that observed with ABT-737, a small molecule inhibitor of Bcl-xL. Our data strongly support the results obtained by Herting *et al*. when combining obinutuzumab with Bcl-2 family inhibitors such as ABT-737 and ABT-263 [13]. Thus, Bcl-xL might constitute an interesting molecular target to potentiate the antitumor effect of therapeutic MAbs.

In conclusion, our results suggest that apoptotic signaling pathways differ between rituximab and obinutuzumab, with a greater involvement of the mitochondrial pathway in cells exposed to obinutuzumab. Inhibition of Bcl-xL could constitute a means to sensitize CLL cells to the apoptotic effects of anti-CD20 antibodies. Insofar as these antibodies have little activity against CLL *per se* but sensitize cells to chemotherapy, it is important to identify the pathways influenced by these antibodies in CLL cells. Further experiments should determine the role of relocalization of target antigens to rafts in the cytotoxic mechanisms of MAb-induced cell death and apoptotic signaling. As the family of MAbs targeting CD20 and other lymphoid antigens is steadily growing, a better understanding of the mechanisms of toxicity is required to improve the use of these antibodies and possibly to determine which patients are most susceptible to benefit from a given therapeutic MAb.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

Supplementary material available online

Table giving patient characteristics and figures showing further results.

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