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Understanding rituximab function and resistance: implications for tailored therapy

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1. ABSTRACT

The addition of anti-CD20 monoclonal antibody (rituximab) to chemotherapy has significantly improved survival in B-cell lymphoma. However, a substantial number of patients relapse after treatment with rituximab. Understanding of anti-CD20 antibody molecular function may facilitate the development of pharmacologic strategies to overcome resistance. Cell death have been demonstrated to be caused by rituximab binding to CD20 throughout direct and indirect mechanisms. The direct mechanism comprises growth inhibition, induction of apoptosis and sensitization of cells to chemotherapy. While, the indirect mechanisms to Rituximab include complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). However, these mechanisms are still poorly understood. To shed light on this issue, we have analyzed the most significant results showing the role of Rituximab as a signal-inducing antibody and as a chemosensitizing agent through negative regulation of major survival pathways. Mechanisms of resistance to Rituximab are also discussed. Additionally, studies here reported show that, cellular targets are modified after treatment with Rituximab and may become useful for novel therapeutic strategies in the treatment of patients resistant to standard therapy.

2. NON HODGKIN'S LYMPHOMAS

Non-Hodgkin lymphomas (NHL) represent 2-4% of all malignancies. The incidence increases with age, with a peak at 80 years, and is higher in males and in the Caucasian population. In the last 40 years the most cases occurred in America, Europe and Australia, while Asia had the lowest incidence (1-2). However, a heterogeneous geographic distribution can be observed for the different variants of NHL. The aging of population in the industrialized countries, HIV infection and the professional exposure to carcinogenic substances are the major responsible of NHL increased incidence. Nevertheless, in most cases the aetiology of NHL in unknown. Hereditary immunodeficiencies, such as SCID and hypogammablobulinemia, increase the risk of NHL, as well as severe autoimmune disoreders, such as Hashimoto's thyroiditis. EBV plays a specific role in increasing the risk of Burkitt lymphoma, nasal T cell lymphoma and post-transplant lymphoproliferative disorders. Human lymphotropic virus type 1 (HLV-1) is responsible of T cell leukemia in adults, with the highest incidence in Japan, South America and Africa. Helicobacter Pylori has been recognized to be involved in the genesis of gastric lymphomas (3). Other professional, environmental and dietetic associations have been observed, although no

confirming data, at the moment, have been obtained. Protooncogene mutations and oncosoppressor deletions have also been implicated in tumoral transformation.

A causative association between HCV and NHL has recently been postulated. On the basis of epidemiological data, emerging biological investigations and clinical observations, HCV appears to be involved in the pathogenesis of at least a proportion of patients with NHL, as well as of non malignant B cell proliferative disorders, including type II mixed cryoglobulinemia. Some HCV-associated NHL appears to be highly responsive to antiviral therapy. The understanding of the pathophysiological process leading from HCV infection to B-cell clonal expansion has improved significantly. Data support an antigen-driven indirect stimulation of clonal expansion model, leading from oligoclonal to monoclonal expansion and in some instances to frank malignancy (4-6).

The variability in the clinical behaviour of NHL depends on the different maturation stages of the cells from which the tumour originates. In general, the tumors coming from mature cells display an indolent behaviour. On the contrary, tumors of lymphoid precursors, actively proliferating, show a very aggressive behaviour.

According to the WHO classification, NHL are divided into T and B cell malignancies: both can be further distinguished into immature precursors and mature cell neoplasms. From a clinical point of view NHL present indolent forms with low malignity grade and aggressive forms with high malignity grade. Such clinical entities show particular morphological features that can be recognized by an histopathological examination, though a definite confirmation often requires additional immunophenotipc analysis and molecular genetics studies.

Management of NHL depends in large part on the classification (indolent, aggressive, highly aggressive) and specific diagnosis and/or subtype. In indolent NHL, chemotherapeutic strategies include both single agents (e.g., chlorambucil, cyclophosphamide, cladribine, fludarbine, and pentostatin) and combination therapy (e.g., cyclophosphamide, vincristine, and prednisone [CVP] and fludarbine with mitoxantrone and dexamethasone regimens). The anti-CD20 monoclonal antibody Rituximab has been used alone or in combination (e.g. R-CVP, or R-CHOP, cyclophoshamide-doxorubicin-vincristine-prednisolone) and has demonstrated considerable efficacy in multiple clinical studies. Maintenance therapy with Rituximab provides improved outcomes in relapsed and refractory cases. Diffuse large B-cell NHL is treated initially with 6 to 8 cycles of R-CHOP given every 3 weeks. Highly aggressive lymphoma is mainly treated with short courses of multiagent chemotherapy regimens (7-8). Rituximab has also been used in the salvage setting treatment of diffuse large B-cell lymphoma, as monotherapy, in combination with chemotherapy, or in the context of autologous stem cell transplantation (9).

In this review we have explored the most significant studies on Rituximab, its structure and mechanisms of action; in particular, the alteration of the major survival pathways in B-NHL cell lines, caused by Rituximab, has been examined, along with the studies concerning the mechanisms of acquired resistance to Rituximab. A clear understanding of these mechanisms is crucial for detecting the weak points that may be susceptible of new therapeutic intervention.

3. RITUXIMAB AND CD20: STRUCTURE AND FUNCTION

Rituximab, anti-CD20 monoclonal antibody, was at first used in the treatment of follicular lymphoma, being eventually extended to all $CD20^+$ malignancies (10). Nowadays, however, several other tumoral as well as non tumoral conditions seem to take advantage from Rituximab therapy, like HCV-associated B cell proliferations, type II mixed cryoglobulinemia (4), cryoglobulinaemic vasculitis type III (11), refractory kidney transplant rejection (12), rheumatoid arthritis (13) and other autoimmune diseases, including systemic lupus erythematosus, Sjögren's syndrome, vasculitides, autoimmune cytopenias, and neurologic and dermatologic autoimmune diseases (14-15). Interestingly, the administration of Rituximab in HCV-associated B-cell proliferations has been shown to prevent further evolution of these conditions towards a lymphoma (4).

Rituximab is a genetically engineered chimeric murine-human anti-CD20 monoclonal antibody, where variable light- and heavy-chain regions originated from murine anti-CD20 antibody are linked to a human IgG-*k* constant region. The resulting protein is for 95% of human origin (16). This is particularly important as the human portion of Rituximab allows it to trigger all antibody-dependent cytotoxicity mechanisms, such as ADCC (antibody-dependent cell-mediated cytotoxicity) and CDC (complement-dependent cytotoxicity). At the same time, such a chimeric antibody does not display the unwanted development of anti-mouse antibodies (HAMA) that is a common event when using a mouse antibody. The development of anti-chimeric antibodies (HACA) has been to show to be present only in a small percentage of patients treated with Rituximab (around 1%) (17).

The success of Rituximab is basically due the ideal properties of its target, CD20. The CD20 receptor is expressed in a lineage-specific and developmentally regulated manner (18). It is exclusively expressed on B cells and appears during the pre-B-cell stage, but is absent during the earlier or later stages of B-cell differentiation such as pro-B cells and plasma cells. However, it is not expressed on other cell types. The human CD20 gene is located on chromosome 11, close to the site of the translocation [(11;14) (q13;q32)], found in a subset of B-lineage malignancies; this might explain the observed alterations in the CD20 expression occurring after t(11;14) translocation (17-19). CD20 belongs to the four-transmembrane protein family, which includes also the beta chain of the high-affinity receptor for IgE (FcεRIβ), the myeloid and lymphoid specific protein HTm4 and the testis specific non-hematopoietic human gene, TETM4 (20). The

exact function of CD20 is not completely understood. Earlier studies by Deans *et al* (21) have demonstrated that cross-linking CD20 resulted in increases in intracellular calcium and show that CD20 is associated with the Src family tyrosine kinases, suggesting the involvement of CD20 in transmembrane signaling. Some evidences seem to indicate that the cell signaling resulting from the cross-linking of CD20 by Abs can depend on a re-distribution of CD20 molecules to specialized microdomains at the plasma membrane, known as lipid rafts (22). This seems to result in a decrease of total protein kinase activity (23) and, in turn, to inhibition of proliferation or even apoptosis induction (24). The relation between phosphorylation and cell proliferation is documented by evidences reporting that CD20 isolated from proliferating or malignant B cells or B-cell lines is highly phosphorylated, whereas CD20 detected in non-proliferating B cells is non-phosphorylated. Crosslinking cell surface CD20 by Abs or by phorbol esters results in enhanced phosphorylation (25). A role of CD20 as a Ca^{2+} channel emerged from studies showing that transfection of CD20 cDNA in different lymphoid (human T, and mouse pre-B lymphoblastoid) and non-lymphoid (human K562 erythroleukemia and mouse NIH-3T3 fibroblasts) cell lines increases transmembrane Ca^{2+} conductance (26).

As humanized, chimeric antibody, Rituximab shows a longer half-life than the one showed by other equivalent mouse or rat antibodies, due to the lack of HAMA or HARA development (17). This positively affects the efficacy of Rituximab.

3.1 Rituximab mechanisms of action

The advantage given by such a humanized chimeric antibody is also due to its ability to interact with the host cellular and humoral mechanisms of immune responses. These mechanisms consist of Antibody-dependent cell-mediated cytotoxicity (ADCC) and Complement-dependent cytotoxicity (CDC). However, Rituximab is known to exhibit also an intrinsic antitumor activity, by the induction of apoptosis.

3.1.1. ADCC

The constant region of the heavy chain of Rituximab, which has a human origin, allows it to interact with the Fc receptors for IgG (FCγRIIIa) of the host cells (Natural Killer cells, macrophages, neutrophils). Three kinds of Fc receptors have been studied in their ability to modulate Rituximab mediated ADCC induction: one, FcγRIIb, has hinibitory function and abrogates effector cell activity; two others, FcγRIIa and FcγRIIIA, have activatory function, allowing effector cell activation in proximity of the target cell. Once effector cells are activated, they are able to mediate a cytotoxic effect towards the target, the so-called antibody-dependent cell-mediated citotoxicity (ADCC). ADCC is a recognized major anti-tumor mechanism, and the efficiency of the interaction between Rituximab and FCγRIIIa is of crucial importance. Some authors have shown that the responses to Rituximab are related to FCγRIIIa receptor polimprphisms; indeed, the position 158 of the protein seems to determine the efficiency of ADCC. It has been shown that only the homozygosity of the FcγRIIIa-158V aplotype was the parameter associated with the clinical and molecular responses (27), although more recent works seem to accept that even the heterozigosity for position 158 V/F should be able to elicit efficient ADCC and Rituximab response if appropriate expression of FCγRIIIa (CD16) is present (28). Similar results came out from another group who investigated on the FcγRIIa polimorphisms, where the homozygosity for histidine at position 31 was found to be associated with the response rate to Rituximab and progression-free survival (29). However, most of the molecular mechanisms of resistance to ADCC remain still unknown. Another work by Inagaki *et al* focused on ADCC displayed by NK cells, which are the most efficient cells in mediating anti-tumor activity. According to the authors, the response to Rituximab could be in part dependent on the expression levels of some of the NKG2D ligands, especially UL16-binding proteins (ULBPs), on the tumor cells (30).

3.1.2. CDC

Rituximab is also capable of binding to C1q, eliciting the activation of the complement cascade via the classical pathway (31). The C1q binding sites of the antibody IgG1 molecule correspond to the residues D270, K322, K326, P329, P331 and E333 (32-33). An important synergism between CDC and ADCC has been demonstrated, related to the complement ability to promote inflammation and enhance the activation status of innate effectors (34). Different sensitivity to Rituximab-induced CDC has been observed among lymphoma cells, FL cells being the most sensitive, and SLL the most resistant (35). Such different sensitivity to CDC has been explained with different expression levels of membrane complement regulatory proteins (mCRP): in particular, a negative correlation to CDC has been recognized for CD46 (membrane cofactor protein), CD55 (decay accelerating factor, DAF) and CD59 (membrane inhibitor of the reactive lysis). mCRPs are expressed in the most of cancer cells. The expression levels may be regulated by several stimuli, such as the stage of differentiation (36-37), host factors depending on neighboring tumoral or stromal cells (38), and the stressing condition created by the complement attack, which can also act as a selective pressure stimulus (39). Several authors have shown how the use of neutralizing antibodies abrogating the function of CD46, CD55, and CD59 markedly enhanced the antitumor activity of Rituximab *in vitro* and *in vivo* (40-42).

Overcoming tumoral cell resistance to CDC represents a major goal among the efforts to increase the efficacy of Rituximab. Several studies have been made in this direction; besides the use of mCRP-blocking antibodies, other molecules have been studied, capable of inhibiting mCRPs, such as fludarabine, downregulating the expression of CD55 (43), or the *Streptococcus intermedius* toxin, Intermedilysin, able to abrogate the function of human CD59 (44); alternative strategies focused on the possibility to either increase CD20 expression by the use of Bryostatin-1 (45), or Synthetic CpG oligodeoxynucleotides (46), or enhance the complement function by the use of

antibodies conjugated to complement activators like the C3b, or the cobra venom factor (CVF) (47).

A recent work by Racila and coworkers has shown that the clinical response and the response duration to Rituximab therapy of follicular lymphoma can be influenced by C1qA polymorphism (276A/G). In this study, the authors demonstrated that patients who were homozygous for the alanine residue at position 276 achieved complete response at a higher rate compared to the heterozygous or homozygous for glycine patients (48). It is worth to remind that some authors have pointed out the possibility that complement activation can be involved in some of the side-effects of Rituximab treatment (49).

3.1.3. Induction of apoptosis

Besides the ability to trigger host cellular and humoral immune responses against tumor cells, Rituximab is also able to induce apoptosis on target cells, and to exert a synergic effect with different chemotherapeutic agents (43, 50-53). Such immuno-chemosensitization has been shown to be independent from Rituximab Fc functions (54).

The major pro-apoptotic pathways triggered by Rituximab involve caspase-dependent mechanisms, although still unclear caspase-independent pathways have been postulated (38, 55-56). Rituximb-mediated caspase-dependent apoptosis has been shown to occur by three main pathways: the activation of Src family tyrosine kinases (Lyn, Fyn, and Lck), the activation of Fas apoptotic signalling, and the inhibition of the major survival pathways: p38 MAPK, ERK1/2, NFkB and Akt, which are constitutively activated in lymphoma cells.

3.1.3.1. Activation of Src family tyrosine kinases

The studies of Deans and coworkers from 1993 to 2005 demonstrated that CD20 is associated to membrane microdomains known as lipid rafts, enriched in Src-family tyrosine kinases and other signalling effectors, suggesting a role of CD20 in signal transduction (21-22, 57-59). Parallel to these studies, other authors have elucidated how the activation state of Src-tyrosine kinases is controlled by an adaptor protein, PAG (phosphoprotein associated with glycosphingolipid-enriched membrane microdomains), localized exclusively into lipid rafts. PAG, also known as Csk-binding protein, can bind to the Src kynases, thus maintaining resident Src family tyrosine kinases Lyn, Fyn, and Lck in an inactive state. After Rituximab binds to CD20, a redistribution of lipid rafts occurs, subsequently transactivating Src tyrosine kinases, and initiating downstream signaling pathways resulting in apoptosis (60-61).

3.1.3.2. Activation of Fas apoptotic pathway

Bonavida and coworkers showed that treatment of the Fas-resistant NHL cell lines, 2F7, Ramos and Raji, with Rituximab sensitized the cells to apoptosis induced by CH-11 (FasL agonist monoclonal antibody), with a synergic effect. Such effect has been shown to be dependent from an up-regulation of Fas, occurring within 6 hours from treatment, due to the inhibition of the expression and activity of the transcription repressor Yin-Yang 1 (YY1) that negatively regulates Fas transcription. According to the authors, the downregulation of YY1 expression is the result of Rituximab-induced inhibition of both the p38 mitogen-activated protein kinase (MAPK) signaling pathway and the constitutive activity of nuclear factor kappa B (NF-kappaB) in the cells (62).

Moreover, Rituximab binding to CD20 has been shown to induce apoptosis through Fas dependent activation of caspase-8 pathway, in Ramos B cells. However, this activation has been shown not to involve a direct death receptor-ligand interaction, as blocking the death receptor ligands, Fas-Ligand or TRAIL, using neutralizing Abs, did not inhibit apoptosis. Caspase-8 activation has been shown to be achieved following membrane clustering of Fas molecules leading to formation of the death inducing signaling complex (DISC); upon CD20 cross-linking, Fas-associated death domain protein (FADD) and caspase-8 were recruited into the DISC. The clustering of Fas molecules seems to be dependent from Fas translocation to lipid rafts, together with CD20, induced by Rituximab ligation (63).

However, previous studies have shown that drug-induced caspase-8 activation in B-lymphoma cells can occur independently from Fas or FADD expression, and can, instead, be mediated by postmitochondrial caspase-3 activation (64).

3.1.4 .Inhibition of survival and antiapoptotic signaling pathways

Initial studies with monomeric Rituximab on several B-NHL cell lines were shown to result in the inhibition of cell proliferation and sensitization of drug-resistant NHL cell lines to chemotherapy-induced killing with various drugs (65). These findings suggested that Rituximab signals cells to modulate the intracellular pathways that regulate proliferation and resistance, and thereby potentiates the cytotoxicity of drugs. Studies revealed that Rituximab treatment preferentially inhibited the expression of the antiapoptotic gene products Bcl-2/Bcl-xL. Investigating the Rituximab induced modifications of the molecular signaling pathways that regulate these gene products, it has been demonstrated that Rituximab mediates the inhibition of p38 mitogen-activated protein kinase (MAPK), nuclear factor (NF)-kB, extracellular signal-regulated kinase-1/2 (ERK-1/2), and Akt survival pathways. The inhibition of these antiapoptotic pathways sensitizes B-NHL cells to chemotherapy and to undergo apoptosis (24, 66).

3.1.4.1. Inhibition of the p38 MAPK/STAT3/NF-kB/SP1/Bcl-2 pathway

In some studies, it has been shown that the secretion of certain cytokines by tumor cells renders them resistant to the cytotoxic effect of chemotherapeutic drugs (67-68). Some authors hypothesized that the chemosensitization of NHL cell lines might be due to Rituximab-mediated inhibition of these tumor-derived protective factors. This hypothesis was tested in the B-NHL cell line 2F7 (69), which produces cytokines such as

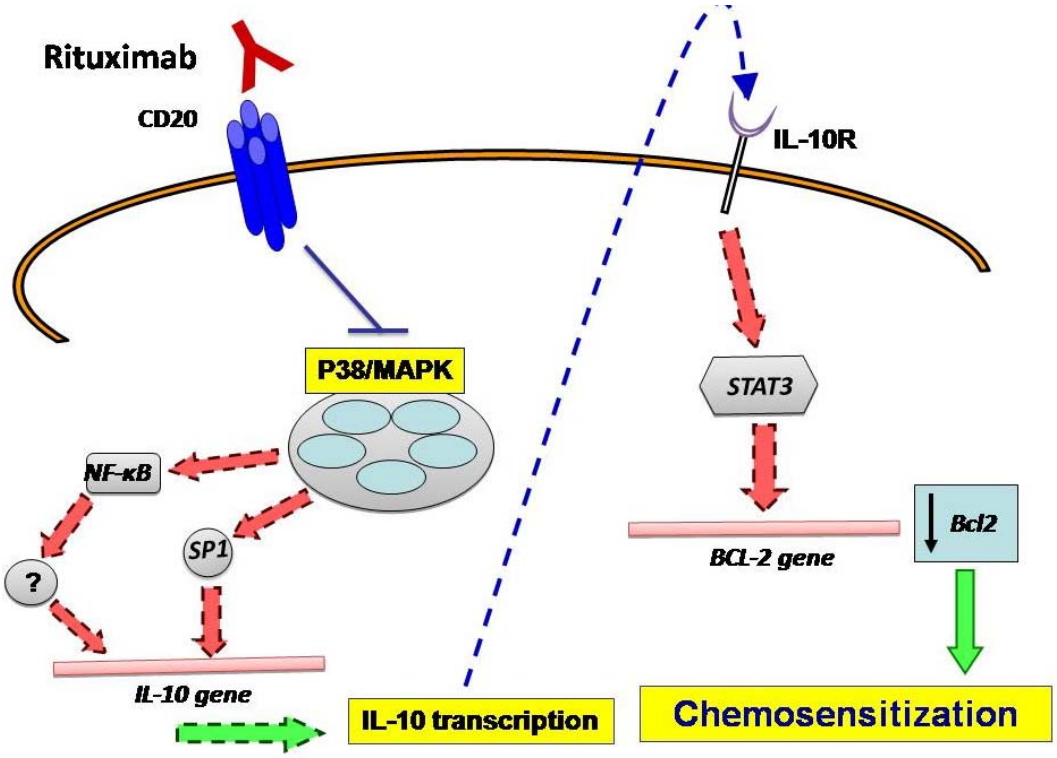

Figure 1. Rituximab mediated inhibition of the P38 MAPK signaling pathway. Inhibition of p38MAPK pathway by Rituximab results in NK-kB and SP-1 downregulation and consequent reduction of IL-10 synthesis. The reduction of IL-10 leads to downregulation of STAT3 and Bcl-2, thus sensitizing NHL cells to drug induced aopoptosis.

TNFalpha and IL-10. The study demonstrated that Rituximab significantly inhibited IL-10 synthesis and secretion, and the neutralization of IL-10 by anti-IL-10 monoclonal antibody inhibited cell-proliferation similar to the findings obtained with Rituximab and selectively inhibited expression of Bcl-2 (50). These findings suggest that IL-10 behaves as a protective factor and may control antiapoptotic regulatory gene products.

It was shown that Rituximab-mediated inhibition of IL-10 secretion resulted in downregulation of the constitutive activity of signal transducer and activator of transcription 3 (STAT3) seen in those cells (through IL-10–IL-10R interaction), and STAT3 inhibition resulted in inhibition of Bcl-2 transcription and expression (70). It has been reported that IL-10 induction is accompanied by an enhanced phosphorylation of p38/SAPK2 in the Burkitt's Lymphoma cell line BL-2 (71). Other studies have shown that activation of the mitogen-activated protein kinase (MAPK) signaling pathway regulates activation of the transcription factor Sp-1, which in turn regulates the transcription of the IL-10 gene (72). Based on these findings, it was hypothesized that Rituximab-mediated inhibition of IL-10 production may be due to inhibition of the MAPK pathway. In addition to the demonstration of Rituximab-mediated inhibition of p38/MAPK activity and inhibition of IL-10 transcription, Rituximab also inhibited constitutive NF-kB activity in 2F7 cells (73). The inhibition of NF-kB was shown to be downstream of p38 MAPK activity. The direct role of NF-kB in the transcriptional regulation of IL-10 is controversial and may occur via an indirect mechanism (74) (Figure 1).

3.1.4.2. Inhibition of the Src/Raf 1/MEK1/2/ERK1/2/AP-1/Bcl-xL pathway

Treatment of Ramos and Daudi cell lines with Rituximab resulted in inhibition of cell proliferation and sensitization of drug-resistant cells to chemotherapy-induced apoptosis with various drugs; Rituximab treatment also resulted in the selective downregulation of Bcl-xL expression, with minimal effect on Bcl-2 expression in Daudi and Ramos cells, the latter remaining Bcl-2 negative (75).

In previous reports, the analysis of the bcl-x gene promoter region, revealed the presence of NF-kB and AP-1-bindings sites, leading to the conclusion that and NF-kB and AP-1, at least in part, regulate Bcl-xL gene expression (76-82). As it was well known that AP-1 activation is regulated by the ERK1/2 pathway (83-84), these findings suggested that bcl-x is regulated by NF-κB and AP-1, and that Rituximab negatively affects one or both these pathways, resulting in inhibition of Bcl-xL expression. This hypothesis was tested directly, and Rituximab was indeed found to inhibit both ERK1/2 and NF-κB signaling pathways in B-NHL cell lines (85-87) (Figure 2).

3.1.4.3. Inhibition of the NF-kB/Bcl-xL pathway

Constitutive NF-kB activation has been observed in various malignancies including NHL, either via the

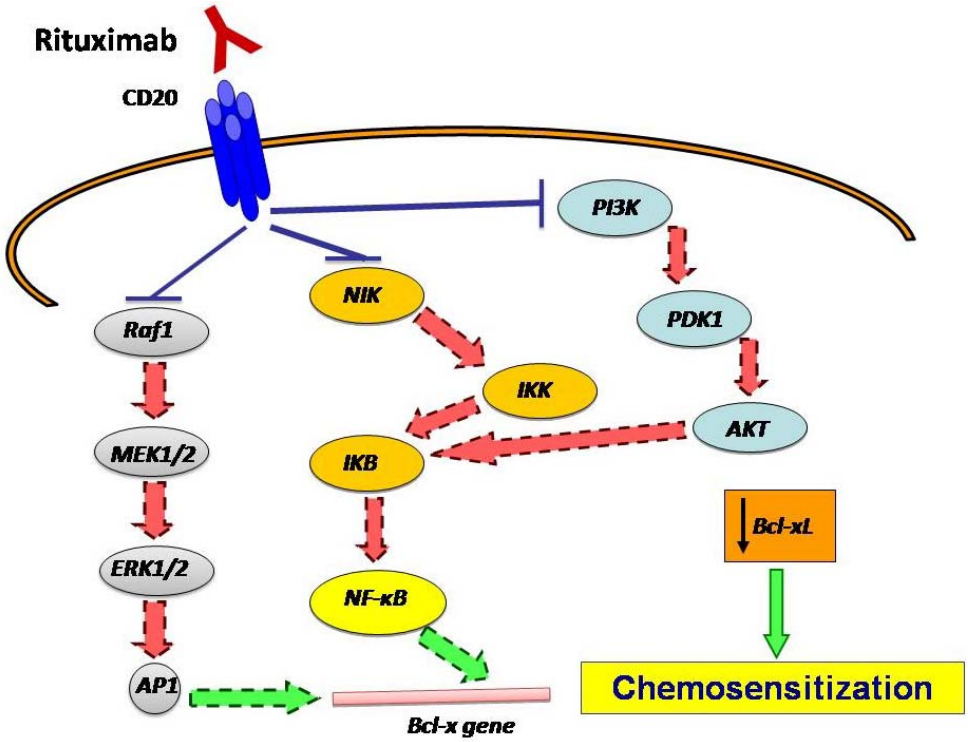

Figure 2. Rituximab mediated inhibition of the ERK1/2, NF-kB and AKT signaling pathways. Schematic representation of the effects of Rituximab treatment on the major survival signaling pathways, all leading to downregulation of the transcription of Bcl-xL and chemosensitization.

amplification of Rel genes or through aberrant activation of the upstream regulators (80-82, 88).

Nuclear factor kB (NF-kB) is a cytokine-inducible transcription factor playing an essential role in a variety of physiological processes including infammatory responses, stress, immune responses, apoptosis, and cellular proliferation (89-91). In mammalian cells, this family consists of five members: p50, p65 (Rel A), p52, c-Rel and Rel-B which share conserved DNA-binding and dimerization domains, and form various homo- and heterodimers (92-94). NF-kB is normally sequestered in the cytoplasm by the association with members of the IkB protein family, which bind NF-kB and prevent its nuclear localization. Upon stimulation by different agents, IkB molecules are rapidly phosphorylated and degraded, allowing the NF-kB dimers to translocate to the nucleus and regulate transcription by binding to the kB site (95-96). The poly-ubiquitination of IkB is regulated upstream by IkB kinase (IKK) complex which is phosphorylated and activated by the upstream NF-kB-inducing kinase NIK. Activation of NF-kB occurs through the signaling cascade of NIK/IKK/IkB-alpha.

The authors investigating on Rituximab have shown that the treatment of Ramos and Daudi cells is able to induce a significant and rapid decrease in the phosphorylation-dependent activated state of NF-κB–inducing kinase (NIK), IκB kinase (IKK) and IκB-α (NIK/IKK/IκB-α), as well as of the DNA-binding activity of NF-κB, starting 3–6 hours post treatment (87). Such inhibition of the NF-κB pathway induced by Rituximab has been shown to determine the downregulation of Bcl-xL expression (Figure 2) (80-81).

Moreover, Rituximab significantly upregulates RKIP expression. The induction of RKIP augments its physical association with endogenous NIK, IKK, and transforming growth factor beta–activated kinase 1 (TAK1), resulting in decreased activity of the NF-κB pathway and diminishing NF-κB DNA-binding activity (97). These findings established that Rituximab inhibits the activity of the NF-kB pathway and the inhibition of this pathway is in part regulated by the induction of RKIP expression.

3.1.4.4. Inhibition of the PI3K/AKT/Bcl-xL pathway

Akt is a serine/threonine protein kinase that mediates various downstream effects of PI3-Kinase. It plays a central role in signaling by the PI3-K pathway, by regulating many biological processes, such as proliferation, cell growth and apoptosis (98). In addition, the activated PI3K-Akt pathway provides major survival signals to lymphoma cells and many other cancer cells (99-100). Akt controls a variety of mechanisms that inhibit apoptosis and prolong cell survival, exerting a positive effect on NF-kB functions (101-102). The Akt pathway is constitutively activated in most tumor cells, in B-NHL cell lines and in B-NHL cells derived from patients (103). Bcl-xL expression and/or activity can be regulated by the Akt pathway (104).

The regulation of Bcl-xL transcription and translation by the Akt pathway is indirect and under the control of NF-kB; NF-kB is in turn regulated by the Akt pathway (105).

Treatment of Ramos and Daudi cells with Rituximab inhibited the PI3K/Akt pathway by the inhibition of phosphorilated PI3K, PDK-1 and Akt, with no effects on non-phosphorylated proteins. Inhibition of the Akt pathway also inhibited the NF-κB pathway and suppressed Bcl-xL expression. The role of the Akt pathway in the regulation of chemoresistance was corroborated by the use of the Akt inhibitor Ly-294002, and by transfection with small interfering RNA (siRNA) (66). These findings revealed another pathway inhibited by Rituximab and identified the Akt pathway as a target for therapeutic intervention (Figure 2).

The inhibition of various signaling pathways by Rituximab treatment results in the inhibition of anti-apoptotic gene products such as Bcl-2 and Bcl-xL and in the reversal of drug resistance (106-107). The signaling pathways modified by Rituximab represent potential targets for several therapeutic strategies aiming to mimic Rituximab-mediated chemosensitizing effects. (65).

In studies on the 2F7 cell line, in which Rituximab induces inhibition of Bcl-2 expression, it has been shown that the treatment with pharmacologic inhibitors of p38 MAPK (SB203580), NF-kB (Bay 11-7085) and STAT3 (piceatannol), all resulted in the inhibition of Bcl-2 expression and sensitization to apoptosis by various chemotherapeutic drugs (73, 108). In addition, neutralization of secreted IL-10 in 2F7 cells by a monoclonal anti-IL- 10 antibody also sensitized cells to drug-induced apoptosis. Further, inhibition of STAT3 activity mimicked Rituximab induced chemosensitization (50, 70, 109). In addition, direct inhibition of Bcl-2 function with an inhibitor of Bcl-2 family members, 2-methoxyantimycin-A3 (2MAM-A3), resulted in sensitizing the tumor cells to drug-induced apoptosis (73, 85). In studies with the NHL cell lines Ramos and Daudi, in which Rituximab lead to downregulation of Bcl-xL expression by inhibition of the of the Raf-1/ERK/MEK and NF-kB pathways, it has been shown that the treatment with pharmacologic inhibitors of the ERK 1/2 pathway (GW-5074, PD-8098059 and UO-126) and of the NF-kB pathways (Bay 11-7085, DHMEQ and SN-50) all sensitized the drug-resistant cell lines to drug-induced apoptosis (85). The above findings revealed that the down-regulation of Bcl-xL/Bcl-2 by Rituximab is a result of Rituximab-mediated inhibition of the p38 MAPK, NF-kB (73), and ERK1/2 signaling pathways (110).

It is also known that Rituximab up-regulates Fas expression and sensitizes B-NHL cell lines to Fas-induced apoptosis via inhibition of Yin-Yang 1 (YY1) (107). The transcription repressor YY1 negatively regulates surface and total Fas expression and confers resistance to Fas-induced apoptosis through binding to the silencer region of the Fas promoter (111). Three elements potentially responsive to YY1 were found to cluster in a very narrow sequence within the Fas promoter silencer region, between –1619 and –1533 base pairs relative to the transcription initiation site. Because YY1 is downstream of NF-κB and is regulated by NF-κB activity, Rituximab, which inhibits NF-κB, may also inhibit YY1 and sensitize NHL cell lines to FASL-induced apoptosis.

Other studies have reported that inhibition of YY1 sensitizes tumor cells to TRAIL-induced apoptosis via upregulation of DR5 (112). Therefore, it has been suggested that Rituximab mediated inhibition of NF-κB and YY1 may also result in the upregulation of DR5, and consequently in the sensitization to TRAIL.

4. MECHANISMS OF RESISTANCE TO RITUXIMAB

A great number of NHL patients treated with Rituximab monotherapy become resistant to the drug. In a re-treatment study of patients with relapsed follicular or low-grade NHL, only 40% of patients who had a prior partial or complete response to Rituximab as single-agent responded to re-treatment at the time of relapse (113). The mechanism of resistance has not been clearly defined yet, but most probably the development of resistance is a result of multiple events. Several mechanisms of resistance have been postulated, including reduction or loss of CD20 cell surface expression, deregulation of intracellular signal transduction pathways, and inhibition of CDC by complement inhibitors such as CD55 and CD59, and alteration of cell mediated immunity; the clinical significance of these mechanisms, however, still remains to be assessed. Thus, by exploring the molecular mechanisms of resistance to Rituximab in NHL, promising strategies to overcome drug-resistance may be developed.

4.1. Preceding CD20 binding

Approaches to increase CD20 expression on B-cells are limited, but in some studies (114-116) it has been reported that a CD20-negative phenotype of primary or relapsed NHL after treatment with Rituximab can regain the CD20 expression, indicating that the downmodulation of CD20 may not be rare, both at the protein and RNA level. Although these reports contain important information from clinical experiences, the frequency of occurrence and detailed molecular biologic information about the CD20-negative phenotype remain to be elucidated.

Some authors have claimed that some epigenetic mechanisms can be responsible for CD20 modulation, as it has been found that the histone deacetylase inhibitor suberoylanilide hydroxamic acid is able to modulate the expression of apoptosis-related genes (117), and another histone, deacetylase inhibitor trichostatin A (TSA), was able to increase CD20 mRNA and protein expression in an established CD20-negative cell line, thus sensitizing the line to Rituximab therapy (118).

In another study using the B cell lines DB and RAMOS, as well as tumor cells derived from a chronic lymphocytic leukemia patient, it was demonstrated that bryostatin-1 enhanced the expression of both CD20 mRNA

and protein (45). The enhanced expression of CD20 was associated with increased transcriptional activity of the CD20 gene, whereas the stability of CD20 mRNA was not affected. The effect of bryostatin-1 on CD20 expression in non-Hodgkin's lymphoma cells was mediated through the ERK/MAPK signal transduction pathway and involved protein kinase C (PKC), but was independent from p38 MAPK and was insensitive to dexamethasone. Cells pretreated with bryostatin-1 were more susceptible to the proapoptotic effect of anti-CD20 Abs. Overall, these data demonstrate for the first time that ERK phosphorylation is required for the CD20 up-regulation on B cell malignancies. The findings also suggest that bryostatin-1 and Rituximab could be a valuable combined therapy for B cell malignancies.

Genetic mutations in the CD20 coding sequence were also observed and may cause resistance or relapse after Rituximab therapy. These mutations led to aminoacid alterations located at the second transmembrane domain and at the C-terminal intracellular domain, respectively. A recent report showed that the binding capacity *in vitro* of Rituximab to the CD20 extracellular portion was strongly reduced by mutations in two aminoacid sequences, ANPS and YCYSI, at positions 170 to 173 and 182 to185 (119).

4.2. After CD20 binding

Molecular mechanisms of acquired resistance to Rituximab have been analysed *in vitro* using Rituximab-resistant cell lines. These clones were described to exhibit upregulation of proliferative and anti-apoptotic signalling pathways like hyperactivation of NF-kB, PI3K/Akt and ERK1/2 (66, 120-121).

Molecular dissection of Rituximab-resistant B-NHL cells identified the simultaneous expression of multiple antiapoptotic Bcl-2 family proteins, as well as the up-regulation of Mcl-1 by deregulated phosphatidylinositol-3-kinase (PI3K) signaling pathway, as independent resistance mechanisms, which were successfully reversed by molecularly targeted pharmacotherapies. Mutations within the catalytic domain p110 of PI3K, or the loss of the PI3K negative regulator and of the tumor suppressor PTEN are found in most of the cancers (122-123). Moreover, the up-regulation of the anti-apoptotic Bcl-2 protein family members Bcl-2, Bcl-xL and Mcl-1, as well as the down-regulation of the essential pro-apoptotic Bak and Bax proteins have been observed (66, 118, 120, 124).

5. CONCLUSION

Many investigators have demonstrated that Rituximab has significant clinical activity in low-grade or high-grade lymphoma patients. However, many patients relapse after treatment with Rituximab or don't obtain any clinical response. In this paper the most significant studies reporting Rituximab molecular function have been reported. Additionally, mechanisms of resistance to Rituximab has been discussed. Understanding of these mechanisms may be useful for the rational design of new strategies to overcome resistance in patients refractory with standard regimens.

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Abbreviations: ADCC: antibody-dependent cell-mediated citotoxicity, HAMA: human anti-mouse antibody, HARA: human anti-rat antibody,FL: follicular lymphoma, SLL: small lymphocytic lymphoma, mCRP: membrane complement regulatory protein, DAF: decay accelerating factor (CD55), CVF: cobra venom factor

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