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Review

Molecular Cancer Research

Direct Effect of Rituximab in B-Cell–Derived Lymphoid Neoplasias: Mechanism, Regulation, and Perspectives

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Abstract

The anti-CD20 monoclonal antibody rituximab is the backbone of treatment for the B-cell malignancies non-Hodgkin lymphoma and chronic lymphocytic leukemia. However, there is a wide variability in response to rituximab treatment, and some patients are refractory to current standard therapies. Rituximab kills B cells by multiple mechanisms of action, including complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity, which are immune-mediated mechanisms, as well as by direct effects on cell signaling pathways and cell membranes following CD20 binding. A large number of events that are affected by rituximab binding have been identified, including lipid raft modifications, kinase and caspase activation, and effects on transcription factors and apoptotic/antiapoptotic molecules. Studies on cell lines and isolated tumor cells have shown that by targeting these pathways, it may be possible to increase or decrease susceptibility to rituximab cell killing. An increased understanding of the direct effects of rituximab may therefore aid in the design of new, rational combinations to improve the outcome of CD20-based therapy for patients who currently have suboptimal outcome following standard treatments. *Mol Cancer Res; 9(11); 1435–42. ©2011 AACR.*

Introduction

The anti-CD20 monoclonal antibody rituximab has revolutionized the treatment of non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukemia (CLL) in the last decade, improving overall survival and even cure rates in many of these malignancies (1). However, the clinical response to rituximab-based regimens can vary substantially, and some patients fail to respond to rituximab even though they express CD20 on their malignant cells. This review considers the different mechanisms of action of rituximab, and how patient and disease factors may affect them, with a particular focus on the direct mechanisms of action and the multiple pathways that may contribute to the variability of response. The article also addresses how the combination of rituximab with novel agents that target rituximab signaling pathways or their regulatory mechanisms might affect the clinical activity of the antibody.

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Rituximab: Immune-Mediated Mechanisms of Action

Rituximab kills B cells by multiple mechanisms that have been studied extensively *in vitro*. The most widely studied mechanisms are antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), which are immune-mediated mechanisms. The importance of these mechanisms is well established (2–4). Although the relative contributions of these mechanisms to clinical efficacy remain unclear, *in vitro* studies have suggested ways in which the sensitivity of B cells to rituximab may be enhanced or reduced. For example, preclinical models show that expression of the complement-inhibitory cell surface proteins CD55 and CD59 by B cells can reduce rituximab cell killing (5, 6), whereas ADCC may be enhanced by agents such as bromohydrin (7) and lenalidomide (8, 9). However, lenalidomide has also been shown to downregulate CD20 and reduce ADCC *in vitro* (10). *In vivo*, the inhibitory FcγRIIb molecule has been shown to regulate ADCC, with mice deficient in this molecule showing enhanced ADCC in response to rituximab (2). In a study of the influence of the *FCGR3A* polymorphism in the FcγRIIIa receptor on the therapeutic response to rituximab, patients with a homozygous *FCGR3A*-158V genotype showed the best clinical response to rituximab. The FcγRIIIa receptor 158V allotype displays a higher affinity for rituximab and increased ADCC (3). Di Gaetano and colleagues (4) showed that CDC was crucial for rituximab therapeutic activity *in vivo* using a nonimmunodeficient mouse model in which C1q and classical C activation was shown to be fundamental to the action of rituximab.

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Rituximab: Direct Cellular Effects

Apart from the immune-mediated mechanisms CDC and ADCC, it has become increasingly clear that rituximab also targets B cells through direct mechanisms that involve cell membrane and intracellular signaling effects (11, 12). These mechanisms are induced by binding of rituximab to CD20 on the cell surface, resulting in direct induction of cell death, inhibition of B-cell survival, and/or enhanced sensitivity to chemotherapeutic agents.

Effects of rituximab on lipids and rafts

Lipid components seem to play an important role in rituximab signaling through CD20. Binding of rituximab results in redistribution of CD20 to lipid microdomains or "rafts" in the membranes of cells from a Burkitt lymphoma cell line (11). In addition, deletion analyses have identified a membrane-proximal sequence in the cytoplasmic carboxyl tail of CD20 as being responsible for CD20 relocalization into rafts (12). This redistribution modifies raft stability and organization, and it modulates the associated signaling pathways (11). The clustering of CD20 into raft microdomains results in an apoptotic response mediated through an *src* kinase-dependent pathway (13). The integrity of lipid rafts and their association with CD20 and consequent activation of *src* kinases are all dependent on cholesterol, with cholesterol depletion inhibiting rituximab-induced apoptosis (14). In follicular lymphoma (FL) cell lines, we found that rituximab inhibits B-cell–receptor signaling by preventing B-cell–receptor relocalization into rafts and decreasing raft-associated cholesterol content (15).

CD20 translocation into rafts seems to be necessary for calcium mobilization and for rituximab-induced apoptosis in Burkitt lymphoma cell lines (16). However, it has also been shown that anti-CD20 antibodies induce cell death through a caspase- and raft-independent mechanism (17), and a recent study showed that, at supersaturating doses, calcium release induced by rituximab (without crosslinking) is independent of both rafts and *src* kinases (18).

In the absence of crosslinking, rituximab binding to CD20 can also induce a rapid, transient increase in acid-sphingomyelinase activity and ceramide generation in raft microdomains in Burkitt lymphoma and FL cell lines (19). The consequent increase in intracellular ceramide concentration can result, through protein kinase C ζ (PKCζ)-mTOR module inhibition, in a decrease in growth and survival (20).

Effects of rituximab on intracellular signaling pathways

Rituximab has been shown to exert its direct killing effects *in vitro* and *in vivo* through various intracellular signaling pathways via its F(ab′)2 fragment (21). Multiple signaling pathways that are affected by rituximab binding to CD20 have been identified. These pathways include activation or downregulation of protein kinases, phosphatases, caspases, and Bcl-2 family members; effects on transcription factors and gene expression; modulation of B-cell–receptor signaling; and changes in the lipid distribution in the cell membrane (20, 22–31). The biologic effects of rituximab include inhibition of cell survival and proliferation, induction of apoptosis and macroautophagy, and sensitization to chemotherapeutic drugs and radiation. Table 1 lists the intracellular signaling pathways upon which rituximab has been shown to act.

Mechanisms of Rituximab Resistance

Immunotherapy with rituximab, alone or in combination with chemotherapy, has greatly improved treatment outcomes in patients with NHL and CLL. However, a subpopulation of patients will relapse and/or become unresponsive to rituximab. This suggests that some NHL cells may develop *in vivo* resistance to rituximab. Some of these mechanisms have been identified based on *in vitro* models, although for most of them, the *in vivo* relevance remains uncertain. Clinical trials including combinatory therapies with rituximab are presented in Table 2.

Regulation of rituximab's direct effects

The signaling pathways that mediate the direct cellular effects of rituximab are regulated by a variety of intracellular and extracellular factors. This presents the possibility that perturbations in these regulatory mechanisms may affect the ability of rituximab to kill B cells, leading to resistance. Given the multiple pathways that are involved in rituximab signaling, the ability to target these pathways may offer the potential to overcome the mechanisms of resistance to rituximab that are observed *in vitro* and possibly increase clinical efficacy in refractory or poorly responsive patients.

Lipids. As described above, rituximab activates the sphingomyelin cycle, and ceramide, the product of sphingomyelin degradation, acts as a secondary messenger for transducing growth-inhibitory signals, such as p27 accumulation. Theoretically, this complex cascade opens a large variety of regulation mechanisms, including the amount of sphingomyelin that is disposable for hydrolysis (in this case, the sphingomyelin fraction associated with the plasma membrane outer leaflet), the magnitude of the activation of the sphingomyelinase (in this case, an acid that is not yet fully characterized), the correct enzyme routing for translocation, and the metabolism of ceramide (which can be metabolized in several ways). Very little is known about the influence of these parameters on NHL cell response to rituximab. However, it seems that the membrane lipid organization itself plays a role. Thus, the amount of GM1, a ganglioside and important sphingolipid constituent of lipid rafts, correlates with the susceptibility of NHL and CLL to rituximab *in vitro*, irrespective of the level of CD20 expression (32). GM1 may therefore represent a predictive factor for the response of lymphoma cells to rituximab (32). With the widespread use of cholesterol-lowering statins, which can interfere with the formation of cholesterol-rich lipid rafts (33), inhibit expression of GM1 (34), and induce conformational changes in CD20 *in vitro* (35), there has been some concern that patients who have been treated with statins may have an impaired response to rituximab (36). However,

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Table 1. Effects of rituximab on intracellular signaling pathways

Targeted pathway	Cell line model	Biologic effect	Reference
PKCζ/Raf/MEK/ERK/mTOR	FL cell lines	Inhibition of survival	(20)
A-SMase/CER/p27(Kip1)	FL and BL cell lines	Inhibition of proliferation	(19)
Caspase 3/9	FL cell lines	Apoptosis	(22)
CD20 relocalization in raft/calcium influx/caspases 3/7	BL cell lines		(16)
Src/calcium influx/caspases	NHL cell lines		(23)
Calcium influx	BL cell lines	CI-PCD	(24)
ΔΨm	BL cell lines		(25)
LC3II	BL cell lines	Macroautophagy	(26)
p38/SP1/IL10/STAT3/Bcl-2	BL cell lines	Chemosensitization	(27)
RKIP/Raf1/MEK/ERK/AP1/Bcl-xL	NHL cell lines		
RKIP/NIK/TAK/IKK/NF-κB/Bcl-xL	BL cell lines		
PI3K/PDK1/Akt/Bad/IKK/NF-κB/Bcl-xL	B-NHL cell lines		
Src/Raf1/MEK1/2/ERK1/2/AP-1/Bcl-xL	FL-NHL cell lines		
p38/MAPK/STAT3/NF-κB/SP1/Bcl-2	FL-NHL cell lines		
PI3K/Akt/NF-κB	B-NHL cell lines		
YY1, NF-κB, Bcl-xL	NHL cell lines		
p38 MAPK/NFκB/YY1/Fas	B-NHL cell lines	Sensitization to Fas	
Fas translocation in raft/DISC formation	BL cell lines	Sensitization to Fas	(28)
YY1	B-NHL cell lines	Sensitization to Apo2L/TRAIL	(29)
Caspase 3/7	NHL cell lines and xenograft	Sensitization to Apo2L/TRAIL	(30)
Survivin/AIF/XIAP/Hsp70	FL and BL cell lines	Sensitization to radiation	(31)

Abbreviations: AIF, apoptosis-inducing factor; A-SMase, acid-sphingomyelinase; BL, Burkitt lymphoma; B-NHL, B-cell non-Hodgkin lymphoma; CER, ceramide; CI-PCD, caspase-independent programmed cell death; DISC, death-inducing signaling complex; IKK, inhibitor of nuclear factor κβ kinase; IL10, interleukin-10 protein; LC3II, light chain 3-II protein; NIK, NFκB-inducing kinase; PDK1, pyruvate dehydrogenase kinase 1; RKIP, Raf kinase inhibitory protein; SP1, specific protein 1; STAT3, signal transducer and activator of transcription 3 factor; TAK, transforming growth factor β activated kinase; XIAP, X-linked inhibitor of apoptosis protein; YY1, Yin Yang 1.

recent clinical studies indicate that statin use in patients with NHL does not adversely affect the outcome following rituximab-based therapy (37–39).

Antiapoptotic proteins. As with many other antitumor agents, rituximab's direct effect is greatly influenced by a large variety of kinases and antiapoptotic proteins. For example, it has been reported that antiapoptotic proteins, such as Bcl-2 and Bcl-xL (which are regulated by NF-κB), largely contribute to resistance to rituximab (40). Furthermore, silencing of Bfl-1, a transcriptional target of NF-κB, resulted in enhanced sensitivity to rituximab of diffuse large B-cell lymphoma (DLBCL) cells (41). However, most of these protective pathways are also targets for rituximab signaling. It is thus not surprising that the complete disappearance of these proteins also confers upon NHL cells a relative resistance to rituximab. For example, reduced expression of Bcl-2 family members, such as Bax and Bak, results in decreased direct killing by rituximab (42, 43). The dual effect of the antiapoptotic protein expression level is well illustrated by Bcl-2 itself: the lack of this protein is a negative factor for clinical efficacy (44), whereas very high expression is protective.

The latter should be considered in the context of FL, with dramatic Bcl-2 accumulation due to the t(14;18) translocation being a molecular hallmark of FL cells. This molecular rationale has been exploited through Bcl-2 inhibitory agents as candidates for stimulating rituximab efficacy in Bcl-2–expressing NHL cells. Thus, the small-molecule Bcl-2 inhibitor ABT-263 (navitoclax) has been shown to enhance the efficacy of rituximab in DLBCL tumor models (45, 46), and the BH3 mimetic AT-101 increased cell killing by rituximab plus cyclophosphamide in cell lines and animal models (47). The pan-bcl-2 inhibitor ABT-737 has also been shown to sensitize resistant B-cell lines to rituximab killing (48). The Bcl-2 antisense oligonucleotide oblimersen and the small-molecule Bcl-2 inhibitors navitoclax and obatoclax are currently under evaluation in combination with rituximab in clinical trials in both NHL and CLL.

To conclude this section dealing with rituximab and apoptosis regulators, we should mention the intriguing observation made by Bonavida (27) of increased expression of the apoptotic Fas molecule in NHL cells treated with rituximab. The author showed that rituximab interferes with Fas expression at a transcriptional level through accumulation of the Yin Yang 1 (YY1) transcription factor, which binds to Fas gene regulatory sequences. In these cells, increased Fas expression correlated with increased Fas-induced apoptosis (27, 28). This observation may have some implications regarding the *in vivo* effect of rituximab.

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Table 2. Experimental combination therapies with rituximab to address rituximab resistance and sensitivity

Agent	Mechanism	Therapeutic partners	Patient population	Clinical trial registry[a]
Oblimersen	Bcl-2 antisense oligonucleotide	Rituximab, fludarabine	CLL	NCT00078234
		Rituximab	NHL	NCT00054639
Navitoclax	Small-molecule Bcl-2 inhibitor	Fludarabine, cyclophosphamide, rituximab	CLL	NCT00868413
		Bendamustine, rituximab		
		Rituximab	CD20-positive lymphoid malignancies	NCT00788684
		Dose-intense rituximab	CLL	NCT01087151
Obatoclax	Small-molecule Bcl-2 inhibitor	Fludarabine, rituximab	CLL	NCT00612612
		Rituximab, bendamustine	NHL	NCT01238146
		Rituximab	FL	NCT00427856
CAL-101	PI3K inhibitor	Rituximab	CLL or SLL	NCT01203930
		Rituximab, bendamustine, ofatumumab, fludarabine	NHL or CLL	NCT01088048
Everolimus	mTOR inhibitor	Rituximab	DLBCL	NCT00869999
		Rituximab, chemotherapy	DLBCL	NCT01334502
Enzastaurin	PKC inhibitor	R-CHOP	DLBCL	NCT00451178
		R-GEMOX	DLBCL	NCT00436280
Dasatinib	BCR/ABL and Src family tyrosine kinase inhibitor	Rituximab	CLL	NCT00949988
		Rituximab, fludarabine	CLL or SLL	NCT01173769

Abbreviations: ABL, Abelson protein; BCR, breakpoint cluster region protein; SLL, small lymphocytic leukemia.
[a]http://www.clinicaltrials.gov.

Indeed, Fas accumulation on the membrane should facilitate NHL cell clearing by the immune system, notably by cytotoxic T cells present in FL tissue as previously shown (49). Moreover, Fas plays a role in the cellular response to cytotoxic drugs by transducing an apoptotic signal through death-inducing signaling complex formation and caspase-8 activation (50). If Fas plays a role in the antitumor effect of rituximab, one can speculate that the loss of Fas at the cell surface or abnormal Fas signaling should contribute to rituximab resistance when used alone or in combination with drugs. In this context, it is interesting to note that, in NHL cells, the Fas gene may be mutated (51). Fas could not be the sole proapoptotic molecule involved in rituximab's mechanism of action. Indeed, rituximab cooperates with rhApo2L/TNF-related apoptosis-inducing ligand (TRAIL), another death receptor, in NHL cell lines to promote apoptosis (30).

Phosphatidylinositol 3-kinase inhibitors. Phosphatidylinositol 3-kinase (PI3K) plays an important role in cell survival by regulating several enzymes, the most critical being Akt and its downstream targets, which include potent antiapoptotic proteins of the Bcl-2 family. Activation of PI3K in some cell lines results in loss of sensitivity to rituximab cell killing; however, in these cells, the addition of a small-molecule PI3K inhibitor can restore sensitivity to rituximab (51). Perifosine and CAL-101 are PI3K inhibitors in clinical development for hematologic malignancies. No information on their efficacy in combination with rituximab is available, but combination studies with rituximab and CAL-101 are currently underway.

Mammalian target of rapamycin inhibitors. Mammalian target of rapamycin (mTOR) is a serine-threonine kinase that is involved in cell growth signaling via cell surface receptors. We previously showed that rituximab inhibits mTOR function (20). It is therefore conceivable that any mechanism that results in mTOR stimulation should render NHL cells resistant to rituximab. This hypothesis is a major concern because we have shown that, in biopsy specimens, FL cells display constitutive activation of the mTOR pathway. This pathway is highly sensitive to a variety of regulators that act both upstream [e.g., mitogen-activated protein kinase (MAPK), PI3K, TSC1, and TSC2] and downstream (Pim kinases) of mTOR. All of these proteins, including mTOR itself, are thus potential candidates for mediating resistance to rituximab. Targeting these proteins or (more directly) mTOR should improve the efficacy of rituximab efficacy. The mTOR inhibitor everolimus has been shown to have synergistic cell-killing activity with rituximab in mantle cell lymphoma and DLBCL cell lines (52, 53). A clinical trial combining everolimus with rituximab for the treatment of DLBCL is underway.

Protein kinase C. The PKC family contains several isoforms, including the so-called "atypical" PKCζ enzyme. PKCζ is an important regulator of canonical pathways such as NF-κB and MAPK, and through this signaling, the enzyme promotes mitogenesis and survival. PKCζ is also

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the target of critical lipid messengers such as phosphatidic acid and ceramide. These are all reasons for considering this enzyme as an excellent target for antitumor therapy. In a previous work, we noted that rituximab decreased PKCζ activity in FL cells and that PKCζ inhibition was responsible for the inhibition of a MAPK/mTOR module (20). Of interest, expression of constitutively active mutated forms of PKCζ confers significant protection to FL cells against rituximab (20). Thus, PKCζ expression or activity should be an indicator of rituximab activity. For the moment, the value of PKCζ as a biomarker is dampened by the lack of a specific antibody available for immunohistochemistry.

Other regulators. Other PKC isoforms and some tyrosine kinases are believed to interfere with rituximab activity. Therefore, agents that target PKC (enzastaurin), *syk* kinase (fostamatinib), and *src* kinases (dasatinib) are currently being evaluated in combination with rituximab in NHL and/or CLL. Fenretinide (4HPR) is a synthetic retinoid that has been shown to induce apoptosis in a variety of cancer cell lines, including human Burkitt lymphoma cell lines. In an *in vivo* model, concurrent administration of 4HPR and rituximab prevented disease progression in a minimal disease model and induced complete response in 80% of animals with established tumors (54). Similarly, combining lysophosphatidic acid acyltransferase-β inhibitors with rituximab in NHL cell lines resulted in a 2-fold increase in apoptosis compared with either agent alone and induced complete response in athymic mice bearing subcutaneous human Ramos lymphoma xenografts (55). Inhibition of survivin, a protein involved in inhibition of apoptosis and cell-cycle regulation, using antisense oligonucleotides in combination with rituximab has been shown to decrease the viability of DoHH2 cells in culture compared with either agent alone. In a xenograft model, survivin inhibition in combination with rituximab significantly inhibited tumor growth compared with control cohorts (56).

Rituximab target availability

CD20 shaving. CD20 shaving is a phenomenon whereby rituximab-CD20 complexes are removed from rituximab-opsonized B cells by monocytic cells in an FcγR-mediated reaction. This has been shown *in vitro* in mantle cell lymphoma and CLL cells, with the THP-1 cell line or peripheral blood monocytes used as the source of mononuclear cells (57). In CLL patients, the appearance of CD20-negative malignant B cells in the circulation was also observed following single-agent rituximab, suggesting that this shaving reaction may also occur *in vivo* (58). Shaving is believed to occur when the density of rituximab-opsonized malignant B cells in the circulation exceeds the capacity of the mononuclear-phagocytic system (59). It has not been reported in NHL, where relatively few malignant cells are seen in the circulation. Frequent (thrice-weekly) low dosing of rituximab was shown to clear CLL cells from the peripheral blood without inducing shaving and/or the appearance of CD20-negative B cells (59). The relevance of shaving with regard to the clinical efficacy of rituximab in CLL, particularly in the context of immunochemotherapy, remains unclear.

Proteasomes. The ubiquitin-proteasome system may influence CD20 expression, which in turn influences sensitivity to rituximab. Therefore, Czuczman and colleagues (60) investigated the role of proteasomes in resistance to rituximab by using lymphoma cell lines that were rendered insensitive to rituximab. In these cells, proteasome inhibition resulted in an increased expression of the carboxyl region of CD20 and subsequent partial restoration of sensitivity to rituximab. Similarly, the proteasome inhibitor bortezomib in combination with cytotoxic drugs may reverse insensitivity to rituximab (27). However, this issue remains controversial. Indeed, in other cellular models, inhibition of the ubiquitin-proteasome system actually resulted in down-regulated CD20 and decreased sensitivity to rituximab, perhaps due to compensatory action by the lysosomal/autophagosomal system (61).

Alternatively spliced CD20 transcript. A truncated CD20 splice variant, called ΔCD20, has been detected in malignant or Epstein–Barr virus–transformed B cells but not in resting B lymphocytes isolated from healthy donors. In Raji and Ramos B-cell lines that were rendered insensitive to rituximab through repeated exposure to escalating doses of the drug, the expression of ΔCD20 increased with increasing rituximab exposure (62). There is some indication that increased expression of ΔCD20 is associated with lower expression of wild-type CD20 and that ΔCD20 is upregulated in patients with lowered response to rituximab (62). Manipulating the ratio of ΔCD20 to wild-type CD20 in malignant B cells could therefore be of benefit; however, the factors that regulate the expression of these 2 transcripts are currently unknown.

Controversial Issues

Although our knowledge about the mechanism responsible for the direct antitumor effect of rituximab is increasing, several major concerns are still debated, as discussed below.

How does rituximab act in combination with chemotherapy?

The important question of how rituximab acts in combination with chemotherapy is still unresolved. It is possible that the direct effect of rituximab coincides with chemotherapy-induced DNA damage through an additive but mechanistically distinct mechanism of action. Alternatively, some investigators have proposed that rituximab may act as a chemosensitizer. Previous studies from Bonavida's laboratory provided some evidence that rituximab may indeed increase sensitivity to drugs by interfering with protective signaling pathways (27). For example, rituximab inhibits p38, MAPK, and NF-κB modules, resulting in inhibition of the IL-10/IL-10 receptor autocrine/paracrine loop, down-regulation of STAT3 activity, decreased Bcl-2 expression, and subsequent sensitization to drug apoptosis. Rituximab has also been shown to upregulate Raf kinase inhibitor protein, thus decreasing the activity of the ERK1/2 pathway, reducing Bcl-xL expression, and subsequently increasing the sensitivity of NHL cells to chemotherapy (27).

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Of interest, rituximab has been implicated in the sensitization of NHL cells to radiation-induced apoptosis through caspase-dependent and caspase-independent mechanisms (31). Moreover, rituximab can also reverse P-glycoprotein–mediated multidrug resistance mediated by the induction of translocation of P-glycoprotein out of lipid rafts *in vitro* (63). However, the significance of these mechanisms in the clinical setting remains to be shown. Moreover, any synergistic effects could be mediated by many other mechanisms. For example, it is possible that chemotherapy could improve antibody diffusion into tumors by reducing tumor size or priming NHL cells to direct rituximab apoptotic effects. One also cannot exclude the possibility that chemotherapy elicits an immune response by stimulating cellular effectors that have adoptive or innate immunity and are able to recognize and eliminate rituximab-targeted cells. These questions should be of interest for optimizing the modalities of immunochemotherapy, with the doses and timing of administration currently being somewhat empirical.

How can the direct effects of anti-CD20 antibodies be increased?

All of the data converge toward the notion that although rituximab is effective in patients, it exerts a relatively modest direct inhibitory effect *in vitro* that can be counteracted by several potent signaling pathways. This observation explains why much more attention has been given to other mechanisms of action, such as ADCC. However, some investigators have hypothesized that by targeting other CD20 epitopes, it should be possible to elicit a more favorable cellular response, eventually leading to cell death. Thus, GA101, a type II, humanized, glycoengineered anti-CD20 antibody, was rationally designed to build on the scientific platform created by rituximab. Specifically, the glycoengineering process gave GA101 a strong affinity for the FcγRIIIa receptor, which in preclinical studies has been shown to result in significantly enhanced ADCC activity compared with rituximab (64). Similarly, it is thought that type II antibodies such as GA101 exhibit increased direct cell death rates compared with type I antibodies (65), due to their fundamentally different way of binding to CD20 (66). At present, the precise intracellular signaling pathways that are influenced by the binding of GA101 to CD20-positive cells remain to be fully determined. However, very recently the mechanism of action was described as nonapoptotic lysosomal-dependent cell death (67). *In vitro*, GA101 seems to show higher antitumor activity against CD20-positive cells compared with rituximab (64, 67–70). Therefore, through its ability to bypass the apoptotic machinery, GA101 may help to eradicate tumors that are resistant to apoptosis and respond poorly to chemotherapy or immunotherapy. In phase I trials, GA101 has been shown to be well tolerated and active in patients with heavily pretreated, end-stage B-cell malignancies (66–73) and to induce rapid B-cell depletion and substantial tumor response in patients with relapsed-refractory CLL (74). Ongoing phase III trials will confirm whether GA101 efficacy is improved versus current rituximab-based standards of care.

What should we look for in novel anti-CD20 antibodies?

One of the major challenges for antibody development is to create a screening system that has the simplicity of a cell-line culture but also reasonably mimics the clinical situation, including spatial 3D organization. The xenograft model has not been largely used in this context. The lack of a spheroid model for NHL probably reflects the fact that, in contrast to carcinoma cells, NHL cells lack cadherin and thus cannot create cell-to-cell contacts. However, on the basis of recent studies that established that 3D spatial organization can interfere with intracellular signaling and the direct effects of the antibody trastuzumab (75), we can conclude that some sort of 3D model is required. The trastuzumab experience suggests that the direct antitumor effect of monoclonal antibodies can be underestimated unless it is explored in a 3D culture model. The main challenge for developers of novel anti-CD20 antibodies, however, will be to show a clinical benefit over rituximab. Evidence of greater cell killing in preclinical studies is not meaningful without a demonstration of benefit in clinical trials. However, the bar is currently set high by rituximab, so large, well-designed trials with long follow-up would be required. In the shorter term, it may be possible to show a benefit, if it exists, in selected groups of patients, such as those who are in high-risk categories or are already refractory to rituximab.

Conclusions

It is clear that rituximab exerts a direct effect on NHL cells by activating a complex network of signaling pathways. However, these pathways are efficiently counterregulated, resulting in a relatively limited antitumor activity by direct mechanisms. Beyond these observations, the general concept that an anti-CD20 antibody may act as a more effective antitumor agent paves the way to a wide range of promising approaches to facilitate the direct effect of anti-CD20 antibodies. Among these different approaches, the most direct (and probably the most efficient) would be to create new antibodies directed against different epitopes that are capable of triggering potent negative signals, converging in cell death. Thus, one can speculate that new anti-CD20 antibodies, such as GA101, will not only challenge the first generation of anti-CD20 antibodies but may even ultimately replace chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Research

Direct Effect of Rituximab in B-Cell–Derived Lymphoid Neoplasias: Mechanism, Regulation, and Perspectives

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