CD20 as a target for therapy

MAGDALENA WINIARSKA1, JACEK BIL2, URSZULA DEMKOW1, MARIA WĄSIK1

1Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, The Medical University of Warsaw, Warsaw, Poland; 2Department of Immunology, Center of Biostructure Research, The Medical University of Warsaw, Warsaw, Poland

Abstract

Rituximab is a chimeric human-mouse monoclonal antibody directed against CD20 antigen. CD20 antigen is characteristic for precursors and mature B lymphocytes. Rituximab is thought to induce complement-mediated cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and apoptosis. Rituximab is mainly used to treat non-Hodgkin’s lymphomas, however it may prove an optimal treatment in various diseases related to autoantibody production. Beside rituximab there are other anti-CD20 antibodies: ibritumomab, tositumomab (both conjugated with radioisotopes), ofatumumab (human antibody undergoing clinical studies). The other strategies to target lymphoma cells include small peptides (mimotopes) that mimic CD20 antigen and thus force patient’s organism to produce anti-CD20 antibodies.

Key words: rituximab, non-Hodgkin’s lymphoma, CD20 antigen, complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity.

Introduction

It is more than one hundred years ago that Paul Ehrlich invented the targeted therapy. His magic ball was supposed to target selectively every disease. Nowadays, this magic ball consists of therapeutics that inhibit many molecular pathways, such as monoclonal antibodies, small-molecule inhibitors, peptide mimetics and antisense oligonucleotides.

The monoclonal antibodies were introduced into the medicine quite a long time ago. At the beginning polyclonal anti-D antibodies were used to prevent the hemolytic disease of the newborns. In 1975 Georges Kohler and Cesar Milstein were the first to invent and describe the production of monoclonal antibodies. In 1984 they were awarded a Nobel Prize for this groundbreaking invention. Since then, monoclonal antibodies were improved to be more efficient and less immunogenic. The production of chimeric and humanized antibodies has started. Nowadays, human antibodies (produced in transgenic mice or by means of phage peptide libraries) are undergoing clinical studies [1].

In 1980 for the first time the monoclonal antibody (Ab89) directed against lymphoma antigen was used to treat a patient [2]. In 1986 the FDA (Food and Drug Administration) registered first monoclonal antibody-muromonab (anti-CD3 antibody) to treat acute kidney rejection. However, the last decade is connected with a huge development, improvement and practical application of monoclonal antibodies in medicine. 21 monoclonal antibodies are registered at the moment and 10 of them are widely used in oncology. Others are used in many fields of medicine, such as transplantology, rheumatology, dermatology, ophthalmology.

CD20 antigen

The CD20 antigen is the surface molecule characteristic for precursors (pre-B lymphocytes) and mature B lymphocytes. CD20 is neither expressed on plasma cells [3], nor on hematopoietic stem cells and pro-B lymphocytes (figure 1). However, IFN-γ was reported to induce CD20 expression on plasma cell [4].

Because CD20 antigen is not expressed by either plasma cells or B-lymphoid stem cells, therapy with rituximab does not affect significantly immunoglobulin serum concentrations. Also, after a conventional regimen therapy with rituximab B lymphocyte count recovers in 9-12 months [5]. The CD20 expression on the surface of B lymphocytes increases with age [6].

Certain characteristics make the CD20 antigen an appealing target for monoclonal antibody (MAb) therapy.
The CD20 antigen is one of the most stable lymphocyte antigen. It does not circulate in the plasma as a free protein that could competitively inhibit MAb binding to lymphoma cells, does not shed from the surface of CD20 positive cells after antibody binding [7] and does not seem to be internalized [8] or, subsequently, downregulated on antibody binding. It is also expressed on the surface of lymphocytes in the large number of copies (approximately 10000 molecules per cell). It was also reported that plasma from lymphoma-or leukemia-bearing patients did not block the CD20 – rituximab binding [9]. However, in some studies the decrease in CD20 expression after rituximab treatment of CLL patients was reported [10, 11]. To sum up, in the majority of in vivo and in vitro studies no internalization nor down-regulation in CD20 expression was detected [12].

CD20 structure

No CD20 ligand has been known so far. Therefore, the exact CD20 functions remain largely unknown. Some data indicate that CD20 plays an important role in B lymphocytes signalling, growth and differentiation [3]. It could also function as a Ca$^{2+}$ membrane channel that sustains intracellular Ca$^{2+}$ concentration and allows the activation of B cells. CD20 is a nonglycosylated phosphoprotein of 33, 35 and 37 kDa. The predicted CD20 structure consists of 4 membrane-spanning domains with both amino and carboxy termini located within the cytoplasm (hence, CD20 is also called MS4A1 – membrane spanning 4 domain subfamily A, member 1) [13].

CD20 has two extracellular loops. The smaller one (a segment of 7 aminoacids, between the first and second transmembrane regions) probably does not extent beyond the cellular membrane. This loop is identical in every member of MS4A family [13, 14]. The bigger loop, a segment of 43 aminoacids, between the third and fourth transmembrane regions has a disulfide bond [13, 14] (figure 2) and is recognized by the majority of anti-CD20 antibodies.

Phosphorylation and dephosphorylation of proteins is recognized as a major process of regulation of cellular functions and serves a prominent role in signal transduction. No tyrosine residues or recognized signaling motifs occur in any of the cytoplasmic regions of CD20 molecule, although there are number of consensus sites for serine and threonine phosphorylation.

It has been reported that CD20 relocates into lipid rafts upon binding with antibodies [15]. The association between CD20 and lipid rafts is dependent on cholesterol and on a short cytoplasmic sequence. Lipid rafts are membrane microdomains enriched in cholesterol and sphingolipids, that serve as platform for signal transduction, allowing the colocalization of different proteins. Thus, CD20 may exist as dimers and tetramers in complex with at least one additional protein component [3]. The CD20 protein has been reported to be closely associated with the transmembrane adapter protein p75/80 (also named C-terminal src kinase-binding protein Cbp), CD40 and major histocompatibility complex class II proteins (MHC II) [16]. CD20 antigen undergoes conformational changes during B lymphocytes differentiation. At least two conformational isoforms of CD20 exist.

**Fig. 1.** CD20 expression on different stages of B lymphocyte development

**Fig. 2.** CD20 molecule with marked aminoacids essential for rituximab binding
IL-4 and CD40 signaling both can influence the conformation of CD20 [17].

Some evidence indicates that various cytokines including IFN-α, GM-CSF, IL-4, TNF upregulate CD20 expression on lymphoma cells isolated from patients with CLL in vitro and probably in vivo [18]. However, the mechanism of this up-regulation is unknown. Altogether, some of these cytokines may increase the therapeutic activity of rituximab and do not increase the CD20 expression [19, 20].

Anti-CD20 therapeutics

Rituximab was approved by FDA in 1997 for treating low-grade non-Hodgkin’s lymphoma. Beside rituximab there are two other anti-CD20 antibodies, approved by FDA. Also other therapeutics directed against CD20 are currently undergoing clinical studies. In Non-Hodgkin’s lymphomas murine monoclonal antibodies conjugated with radioisotopes are approved: ibritumomab (Zevalin) and tositumomab (Bexxar). Ibritumomab is an IgG1 murine antibody that recognizes the same epitope as rituximab. Ibritumomab is conjugated with metal chelator – tiuxetan that binds with radioactive yttrium (^90Y). Both radioimmunoconjugates eradicate tumor cells by the antibody-mediated effects (CDC, ADCC) as well as by the cytotoxic activity of the radiation. Their main disadvantages are toxicity against the bone marrow and immunogenicity that results in HAMA (human anti-mouse antibodies) production. Ibritumomab and tositumomab are used to treat rituximab-resistant and refractory non-Hodgkin’s lymphomas.

Over recent years much of the effort has focused on decreasing immunogenicity of antibodies by creating humanized and human antibodies. Ocrelizumab is a humanized monoclonal antibody directed against CD20 antigen. It is currently undergoing phase II clinical studies in patients with non-Hodgkin’s lymphomas and rheumatoid arthritis.

Ofatumumab (2F2-HuMax-CD20) is a human anti-CD20 antibody generated in human Ig transgenic mice. It recognizes a unique epitope of CD20, which is located probably in both loops of CD20 antigen [21]. It is currently undergoing phase III studies in the treatment of non-Hodgkin’s lymphomas, chronic lymphocytic leukemia and rheumatoid arthritis. In in vitro studies 2F2 antibody was shown to have high affinity for CD20 antigen as well as slow off-rates (after 3 hours dissociation rate of rituximab fluctuates around 70-80%, while that of 2F2 is only about 20-30%). CDC activity of 2F2 antibody (as well as other human antibodies) is unusually potent, also against rituximab – resistant lymphoma cells with low expression of CD20.

One-chain polypeptide directed against CD20 is also currently undergoing phase II studies in patients with rheumatoid arthritis. It belongs to the SMIP group of drugs (small modular immunopharmaceutical drugs). It strongly binds with CD20, activates ADCC, although has no CDC activity.

The other strategy to target lymphoma cells is to force the patient’s organism to produce anti-CD20 antibodies. In order to obtain this effect the small peptides (mimotopes) are created that mimic CD20 antigen. It is only hard to predict the results of such an autoagression.

Rituximab

Mechanisms of action

Rituximab is a chimeric human-mouse monoclonal antibody directed against CD20 molecule. The mechanism of its action in vivo is not entirely clear. However, rituximab is thought to induce complement-mediated cytotoxicity (CDC) or phagocytosis and antibody-dependent cellular cytotoxicity (ADCC). CDC is triggered by C1q component that binds to Fc portion of the antibody, ADCC is mediated by Fcγ receptors (FcγR) that are widely expressed on the surface of granulocytes, macrophages and NK lymphocytes.

Besides, rituximab binding may result in direct effects on cell growth and viability by inducing apoptosis in the absence of complement and effector cells (figure 3). It was also showed that rituximab may sensitize tumor cells to cytotoxic therapies (doxorubicin, cisplatin, fludarabine, retinoids) and glycoprosthetic steroids (dexamethasone) [22, 23].

Some results suggest that rituximab also has a ‘vaccinal’ effect. Several studies have indicated that maximal clinical and molecular responses to rituximab may occur after several months. It suggests that not only short-term cytolytic
mechanisms (CDC, ADCC, apoptosis) are involved in the activity of rituximab. Rituximab-induced lysis of tumor cells may promote an uptake and presentation of lymphoma-derived peptide by dendritic cells. As a result an increase in number of specific cytotoxic T lymphocytes appears [24]. The specific antitumor response after therapy with monoclonal antibodies was demonstrated in murine experiments, although it is hard to demonstrate it in humans [25, 26].

Despite many in vitro studies still little is known about which mechanism is the most important for antitumor activity of rituximab in vivo.

**Complement-dependent cytotoxicity**

The complement system that consists of about 30 proteins may be activated by 3 different pathways – classical, lectin and alternative. The classical pathway requires the presence of immunoglobulins, while alternative pathway is activated spontaneously on every accessible cellular membrane. Collectins that bind unspecifically sugar groups on the microorganisms’ surface trigger lectin pathway.

The first step in the activation of the classical pathway is the binding of C1q component to conformationally changed Fc portion of IgG (except IgG4) or IgM immunoglobulin. As a result a proteolytic cascade is triggered that generates large amounts of C3b, the main effector molecule of the complement system. The final effect of complement activation is a generation of MAC (membrane attacking complex). MAC kills target cells by C9 polymerization, pore formation and disrupting the cellular membrane. Consequently, the water and ions influx leads to the cell swelling and necrosis. Microorganisms, virus-infected cells, tumor cells and erythrocytes are main targets for MAC. C3b component may also bind to complement receptors (CRs) expressed on many effector cells such as granulocytes, macrophages or NK-cells and induce cell-mediated lysis or phagocytosis (opsonization).

IgG1 and IgG3 are the most effective in inducing complement-dependent cytotoxicity. Rituximab was shown to induce CDC in many B-lymphoma cell lines, fresh B-lymphoma cells and in in vivo studies with Macaque cynomolgus monkeys [28, 29, 30]. The increase in complement activation products during therapy with rituximab suggests that the process of complement activation is indeed involved in vivo [31]. However, it is only recently that experiments with C1q-deficient mice were performed and confirmed this hypothesis. Rituximab was ineffective in C1q-deficient mice inoculated with syngenic lymphoma cells transduced with human CD20 [32]. The similar effect was observed in SCID mice that had the complement cascade inhibited with cobra venom factor CVF [33].

Complement inhibitors are membrane proteins that protect cells from complement activation. Thus, the effectiveness of rituximab-induced CDC may also depend on the expression of complement inhibitors. Among them the most important are glycosylphosphatidylinositol (GPI) – anchored proteins: CD46, CD55, CD59. CD46 (membrane cofactor protein MCP) acts as a cofactor for the cleavage of C3b and C4b. CD55 (decay accelerating factor DAF) accelerates the decay of C3 and C5 convertases. CD59 prevents MAC deposition and pore formation.

While the positive correlation between CD20 expression level and rituximab – induced CDC has been found [34, 35], the relationship between complement inhibitors and the effectiveness of rituximab has not been established [29, 34, 36]. However, it was demonstrated in vitro that rituximab induces CDC that is related to the ratio of CD20 and complement inhibitors [35]. The in vitro functional block of CD55 and to some extent of CD55 and CD46 enhanced the ability of rituximab to induce CDC [37, 38]. In CLL patients, treated with rituximab for 8 weeks, an increase in CD59 expression in lymphoma cells was observed [39]. In CLL, resistant cells with low density of CD20 on the cell surface, along with high expression of CD59 persist and reexpand. This may explain the need for higher doses of rituximab to achieve clinically meaningful responses in patients with CLL.

Contrary to these studies analysis of tumor cells obtained from patients with follicular lymphoma showed no significant correlation between the expression of CD46, CD55 and CD59 and response to rituximab [36].

In lymphomas that are not characterized by large numbers of circulating malignant cells, such as follicular lymphoma, rituximab mainly reaches lymphoma cells outside the intravascular compartment and exerts its effects through ADCC. This observation is confirmed by experiments with SCID mice [40]. ADCC was a main mechanism of rituximab activity in mice implanted subcutaneously with Burkitt’s lymphoma [41], while in mice injected with lymphoma cells intravenously rituximab mainly exerted complement-related effects [32].

**Antibody-dependent cellular cytotoxicity**

Many effector cells, such as NK cells, monocytes and macrophages, play an important role in the mechanism of cellular cytotoxicity. ADCC is an important effector mechanism in the eradication of intracellular pathogens and tumor cells. Tumor cells are mainly killed by lymphoid cells, poorly by phagocytic cells (macrophages, monocytes, granulocytes). IgG1 and IgG3, that bind to FcγRI (CD64) and FcγRIII (CD16), respectively, are the most effective in inducing ADCC. Cross-linking of activating FcγR by IgG-coated cells induces effector cells activation and phagocytosis, granule exocytosis and ADCC. Activating receptors such as FcγRIa, FcγRIIa, FcγRIIc and FcγRIIIa (CD16a) possess the ITAM sequences (immunoreceptor tyrosine-based activation motif) that are phosphorylated upon binding with IgG antibodies. FcγRIIB is the only one
inhibitory Fc receptor that contains ITIM sequences (immunoreceptor tyrosine-based inhibitory motif). ADCC mediated through cross-linking of rituximab is considered as a major antitumor mechanism of rituximab activity in vivo. This was supported by studies with FcγRI and RIIa-deficient mice [41]. It was also shown that FcγRIIIa on macrophages are critical to the ability of rituximab to control subcutaneous B-cell lymphoma. The impairment of rituximab-induced ADCC was observed in a model with FcγRIIa neutralizing antibody [42].

However, it seems that not only FcγR activating receptors play an important role in rituximab activity. FcγRIIb-deficient mice displayed a better response to antitumor activity of rituximab [41]. It was also shown that response to rituximab, especially ADCC, is associated with FcγRIIIa polymorphism. Human FCG3A gene displays a polymorphism at 559 nucleotide that results in amino acid substitution at position 158 (with phenylalanine (F) or valine (V)). Patients with follicular lymphoma expressing the high-affinity 158V variant of the FCG3A gene (homozygous 158VV) had the best clinical and molecular responses to rituximab when compared with heterozygotes VF or homozygous FF [43, 44, 45]. However, this correlation was not observed in patients with CLL [44]. It can not be excluded that also other Fcγ receptors play a pivotal role in mediating the antitumor activity of rituximab. Rituximab-sensitized lymphoma cells in in vitro studies were shown to be phagocytosed by macrophages with the help of FcγRIIIa [35].

The interaction between the FcγR and Fc portion of rituximab is established as an important mechanism of antitumor rituximab activity. Some potential ways to increase it include the improvement by molecular engineering of affinity of rituximab for FcγRIIIa. It is widely known that glycosylation of IgG is necessary for its interaction with FcγR [46]. Hence, the modification of glycosylation such as addition of N-acetylgalactosamine or removal of fucose could potentiate the monoclonal antibody-induced ADCC [47]. Also cytokines such as IL-2, IL-12, IFN-γ, GM-CSF (granulocyte-macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor) potentiate ADCC and phagocytosis by stimulation and expansion of NK cells and macrophages [48, 49, 50, 51]. IL-2 (in patients with non-Hodgkin’s lymphoma), IL-12 [52] or IFN-γ [53] were shown to potentiate the antitumor activity of rituximab.

Apoptosis

Binding of antibody with antigen may result in arrest of cell growth (usually G0-G1 arrest) or in apoptosis. Non-Hodgkin’s lymphomas are especially sensitive to anti-idiotypic antibodies directed against the surface immunoglobulins. There was a case of 64 years old man reported in advanced stage of lymphoma with long-term remission after the treatment with anti-idiotypic antibodies. Rituximab in many studies has been shown to induce apoptosis in certain B-cell lymphomas in vitro [54]. However, mechanism of its action remains still elusive. This effect is probably achieved through modulation of intracellular proteins resulting in increase of proapoptotic Bax and decrease of antiapoptotic Bcl-xL [55]. It has also been reported that rituximab-mediated activation of Src kinases (Fyn, Lyn, Lck) [56] results in phosphorylation of phospholipase C [57]. This activation leads to Ca2+ influx and caspase activation [58].

It has been also reported that rituximab stimulated CD20 translocation into lipid rafts [59]. Upon binding with rituximab CD20 complexes translocate into lipid rafts that are rich in cell signaling proteins, such as Src kinases. This leads to a higher concentration and cross-linking of CD20. Consequently, this translocation down-modulates the activity of Lyn kinase and thus leads to susceptibility to apoptosis. Lck and Fyn inhibitors, calcium chelators and caspase inhibitors were shown to attenuate the rituximab-induced apoptosis [58, 55]. Whereas CD20 hypercrosslinking was reported to increase Fas expression. It is not a general rule, although it is thought that CD20 cross-linking potentiates the apoptosis and in some cases it is essential to trigger apoptosis.

In one study caspase activation after treatment with rituximab was reported in patients with CLL, that support that rituximab-induced apoptosis may take place in vivo [60]. However, these results were not confirmed by other groups. In fact, even different cell lines vary in sensitivity to anti-CD20 – triggered apoptosis in vitro. This sensitivity also depends on the type of antibody used [33].

However, in contrast with previously mentioned studies, it was also reported that CD20-induced apoptosis is associated with membrane changes (phosphoserine translocation), but not with DNA fragmentation and chromatin condensation. In addition, in that study apoptosis seemed to be caspase-independent, as not blocked by caspase inhibitors [61, 62].

Chemosensitizing effects

Chemotherapy is so far the most important and the most frequently used therapy of lymphomas. In many studies rituximab was shown to exert chemosensitizing effects. It synergizes with alkylating agents (e.g. cyclophosphamide [63], chlorambucil [64], cisplatin [65]), antimetabolites (e.g. methotrexate [66], fludarabine [67], doxorubicin [63]), paclitaxel [68] or etoposide [69] in vitro. Moreover, rituximab also potentiates chemotherapy regimens such as CHOP (cyclophosphamid + doxorubicin + vincristine + prednisolone) [70, 71]. The mechanism of chemosensitization is probably connected with proapoptotic activity of rituximab and influence on intracellular proteins. Among them the most important are those implicated in regulation of cell death, such as Bcl-2, Bcl-xL, XIAP (X-linked inhibitor of apoptosis protein) and Mcl-1.

There is also another hypothesis, so far confirmed in in vitro studies. After CD20 binding rituximab leads through
Src kinases to decrease in IL-10 transcription and secretion. IL-10 in B lymphocytes plays an important role in maintaining the viability of lymphocytes, even when stimulated with proapoptotic signals. IL-10 is essential for STAT3 constitutive expression, that in turn is responsible for expression of antiapoptotic Bcl-2 [72]. Hence, rituximab by decreasing Bcl-2 expression sensitizes lymphoma cells to chemotherapy. Moreover, rituximab was also reported to synergize with novel therapeutics such as anti-CD19 immunotoxin in the treatment of Burkitt’s lymphoma [73] or bortezomib in CLL treatment [74].

**Rituximab in other diseases**

Rituximab is widely used in the treatment of B-cell lymphoproliferative disorders. However, the accumulating data suggest that it may prove an optimal treatment in various diseases related to autoantibody production, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), dermatomyositis, idiopathic thrombocytopenia purpura, essential mixed cryoglobulinemia, hemolytic anemias. B lymphocytes appear to play a central role in the immunopathogenesis of autoimmune diseases. Rituximab was shown to be effective in RA [75], SLE [76, 77, 78] and hemolytic anemia [79, 80]. The effectiveness of rituximab in autoimmune disease is related not only to the decrease in the production of autoantibodies, but also to the inhibition of B lymphocyte-mediated antigen presentation [81].

In recent studies immunotherapy with anti-CD20 antibodies demonstrated also some efficacy in the treatment of posttransplant lymphoproliferative disorders (PTLD). PTLD are serious complication arising in solid organ transplant recipients. The incidence varies from 1-10%, depending on the type of organ transplanted, EBV infection and doses of immunosuppression [82]. Most patients develop CD20-positive malignancies. Rituximab was well tolerated by patients with PTLD when compared to standard chemotherapy [83]. Therefore rituximab appears to be beneficial as first-line therapy for PTLD.

References

43. Koene HK, Kleijer M, Algra J et al. (1997): Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIa, independently of the Fc gammaRIIa-48L/R/H phenotype. Blood 90: 1109-1114.