Dysregulation of the cell survival/anti-apoptotic NF-κB pathway by the novel humanized BM-ca anti-CD20 mAb: Implication in chemosensitization

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Received June 2, 2009; Accepted July 21, 2009

DOI: 10.3892/ijo_00000446

Abstract. Treatment of patients with relapsed or refractory low grade follicular B-NHL lymphoma with rituximab (chimeric anti-CD20 mAb) has resulted in ~50% response rate. The mechanism underlying the failure of rituximab to affect the remaining 50% of the patients is not clear, though their tumors express CD20. The *in vivo* effector functions of rituximab include ADCC, CDC and seldom apoptosis. In addition, we have reported that rituximab signals the cells and inhibits several intracellular cell survival pathways that are responsible for the immuno and chemo-sensitizing effects of rituximab on resistant B-NHL cell lines. The objective of this study was to develop novel and fully humanized anti-CD20 monoclonal antibodies with enhanced effector functions and molecular signaling that may potentiate their therapeutic efficacy. Novel humanized anti-CD20 monoclonal antibodies were derived from a chimerized form of murine anti-CD20 1K11791, shown to exert a more potent ADCC, CDC and apoptotic activities compared to rituximab. A representative humanized monoclonal antibody, BM-ca was used to examine its biological effect and molecular signaling using Ramos B-NHL cell line as a model. The studies were also performed in parallel with rituximab treatment for comparison. Ramos cells were treated with various concentrations of BM-ca monoclonal antibody. Inhibition of cell proliferation was observed in a concentration-dependent manner, suggesting cell signal perturbations must have occurred. Compared to untreated cells, treatment with BM-ca inhibited both the constitutively activated NF-κB and p38 MAPK pathways, as assessed by inhibition of both phospho-p65 and phospho-IκBα and phospho-p38, respectively, but not the unphosphorylated forms. BM-ca significantly induced the expression of the metastasis suppressor and immune surveillance cancer gene product, Raf-1 kinase inhibitor protein (RKIP). These alterations resulted in inhibition of anti-apoptotic gene products and sensitized Ramos cells to apoptosis by CDDP. In comparison with rituximab, BM-ca showed qualitative and quantitative differences in the above analyses. These findings demonstrate that BM-ca triggers CD20 expressing B-NHL cells resulting in a significant alteration of several gene products that regulate cell growth and chemoresistance.

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Key words: BM-ca humanized anti-CD20 mAb, NF-κB, apoptosis, chemosensitization, Raf-1 kinase inhibitor protein, Snail

Introduction

The anti-CD20 chimeric monoclonal antibody (mAb) rituximab is the most widely used therapeutic antibody for B-cell malignancies (1,2). CD20 is a member of the membrane-spanning 4A gene family (MS4A). The size of the molecule is 33-35 kDa but with only about 43 amino acids exposed on the extracellular surface. It is highly expressed on the plasma membrane of almost all plasma B cells but not on hematological stem cells; it is not shed from the surface after antibody binding and is not shed into the circulation (3). The exact function of the CD20 molecule is unknown. It has no known ligand and is purported to act as an ion channel to facilitate re-entry of intracellular calcium following BCR-induced efflux (4). The importance of CD20 as a target for mAb immunotherapy is irrefutable, and anti-CD20 monoclonal antibodies appear to be ideal for B-cell diseases.

It has been reported that rituximab can mediate antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and apoptosis both *in vitro* and *in vivo*, and the target epitope is critical in determining which of these various mechanisms predominates (1,5,6). In the case of anti-CD20, evidence suggests that Fc/Fc-receptor

(FcR) interactions are critical, as determined in both animal models and humans (7). However, determining whether such interactions are required for classical ADCC-mediated by NK or myeloid cells or whether they provide cross-linking which promotes apoptosis has been difficult to resolve (8). The role of complement in the depletion of malignant cells is less convincing and a number of anti-tumor mAbs appear to operate in the absence of lytic complement (9-11). A strong correlation between the level of CD20 expression and therapeutic outcome for rituximab has been reported, although there is also contrary evidence where no correlation was found (12,13). Chan *et al* (14) have reported a CD20 mAb which is potent in CDC and less effective in apoptosis, whereas a different antibody was ineffective in CDC with potent induction of apoptosis. Both were equally effective in ADCC. Rituximab is currently being used in the management of NHL patients as a single agent or in combination with CHOP. It also shows clinical response in patients with chronic lymphocytic leukemia (15). Rituximab is also used in auto-immune diseases such as rheumatoid arthritis and has recently been approved for treatment of patients with moderate to advanced rheumatoid disease (16).

In addition to ADCC and CDC, when CD20 is engaged by mAb, it can trigger transmembrane signaling directly and inhibit cell growth and trigger cell death in certain tumors. Further, it has also been shown to sensitize tumor cells to both chemotherapy and immunotherapy (17). Different anti-CD20 mAbs have been reported to have different properties and epitope specificities, and mediate differential effects on CDC and cell death. All of the monoclonal antibodies described to date recognize the extracellular loop and partially or completely cross-block each other's binding (18-20).

The overall response rate (complete response plus partial response) of B-NHL patients treated with rituximab was ~50%. It is not clear why the remaining patients, though their tumors express CD20, do not sufficiently respond to treatment. In a recent study, we have reported on the generation of several murine anti-CD20 mAbs and demonstrated that many exhibited higher functional activities compared to 2B8 (the murine parental mAb for rituximab) or rituximab (21). We hypothesized that such novel anti-CD20 mAbs with enhanced effectors functions and different biological activities might potentiate the clinical response. One in particular, murine mAb 1K1791 showed a significant inhibition of growth in several malignant B-cell lines. We assumed, therefore, that the generation of chimeric and humanized mAbs forms of 1K1791 would maintain or exceed the superior activities observed with murine 1K1791. Humanized mAbs derived from 1K1791 such as BM-ca were designed using four different humanization techniques and characterized. In contrast to rituximab or 2F2 (human anti-CD20 mAb), several of these exhibited superior ADCC, CDC, inhibition of cell growth and cell death (6).

The objective of the present study was to investigate the effect of BM-ca mAb on cell signaling modification in B-NHL cells, in particular, on the constitutive activity of the NF-κB and p38 MAPK cell survival/anti-apoptotic pathways and its comparison with rituximab.

Based on the superior activity of BM-ca in ADCC, CDC and apoptosis over those observed by rituximab, we hypothesized that BM-ca may be more effective in inhibiting the NF-κB and p38 MAPK pathways, which are responsible, in part, for the regulation of cell growth and the response to cytotoxic therapy. We used the drug-resistant Ramos B-NHL cells as a model and the comparisons between BM-ca and rituximab were investigated in the following: i) cell growth survival and proliferation; ii) inhibition of the constitutively activated NF-κB and p38 MAPK pathways; iii) modulation of gene products that regulate apoptosis and resistance; and iv) sensitization to apoptosis of Ramos cells by CDDP. The findings herein demonstrate that BM-ca is superior to rituximab in the above studies.

Materials and methods

Cell line and reagents. The $CD20^+$ human Burkitt's lymphoma B-cell line Ramos was obtained from the American Type Culture Collection (Bethesda, MD). The cells were propagated and maintained in RPMI-1640 supplemented with 10% (v/v) heat-inactived fetal bovine serum (FBS). Cultures were incubated in a controlled atmosphere incubator at 37˚C with saturated humidity and 5% CO_2.

The humanized mAb derived from 1K1791 was designed using four different humanization techniques and characterized. The resultant mAb BM-ca was used in this study (1 mg/ml) and was kindly provided by Biomedics Inc., Tokyo, Japan. Rituximab (10 mg/ml) was obtained commercially. Cisplatin (CDDP) was purchased from Sigma (St. Louis, MO) and was diluted in DMSO. The concentration of DMSO did not exceed 0.1% in any experiment. Rabbit anti-p65, anti-phospho-p65, anti-IκBα, anti-phospho-IκBα, anti-p38, anti-phospho-p38 MAPK, anti-Mcl-1, anti-Bax mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-RKIP (antibody) was purchased from Zymed Laboratories (South San Francisco, CA) and anti-Snail was purchased from Abcam (Cambridge, MA).

Assessment of cell growth proliferation (XTT). Inhibition of proliferation was assessed using the XTT assay kit (Roche, Indianapolis. IN). This measures the metabolic activity of viable cells. The percentage of proliferation was calculated using the background-corrected reading as follows: Proliferation (%) = [(absorbance of sample wells/absorbance of untreated cells)] x 100.

Western blot analysis. Whole cellular protein was extracted by incubation in lysis buffer (Cell Signaling Technology, Danvers, MA) for 30 min in ice and then centrifuged to remove cellular debris. The protein in the resulting supernatant was quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Gel loading buffer (Bio-Rad) was added to the cell lysates at a 1:2 ratio. Samples were boiled for 5 min separated on 12% SDS-polyacrylamine minigels, and transferred to nitro-cellulose membrane Hybond ECL (Amersham Biosciences, Sunnyvale, CA) in a Trans-Blot SD semidry transfer cell system (Bio-Rad). The blots were developed by the LumiGlo reagent and peroxide (Cell Signaling Technology).

Determination of apoptosis by activation of caspase-3. Apoptosis was assessed using the active caspase-3 assay.

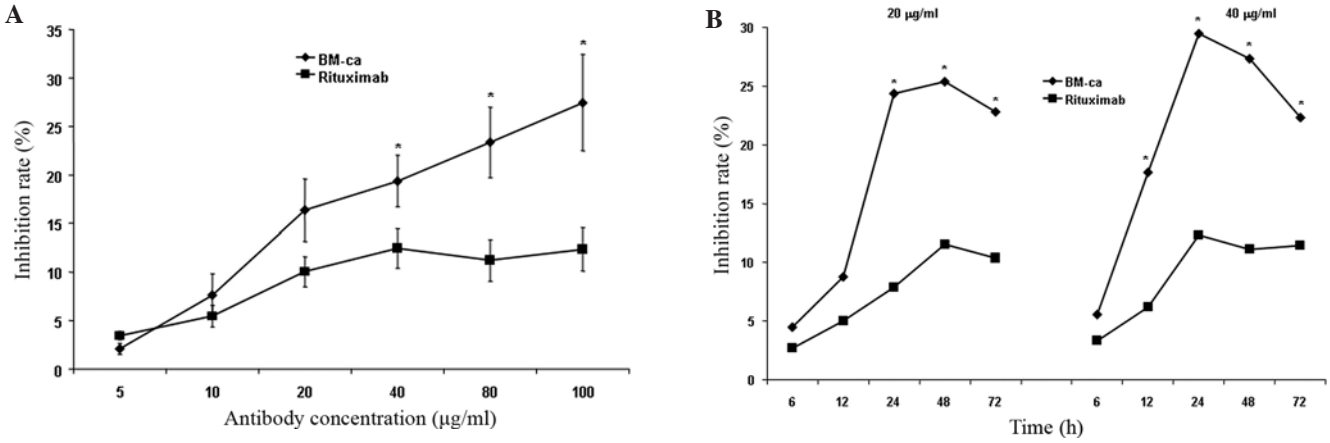

Figure 1. Inhibition of Ramos cell growth by BM-ca. Ramos cells were treated with various concentrations of BM-ca or rituximab for 24 h. The cells were then tested for cell growth inhibition by the XTT assay as described in Materials and methods. Cell growth inhibition was calculated as follows: cell growth without mAb (100%) - cell growth rate (%) in the presence of mAb. *p<0.05 compared with treatment with rituximab (A). Time kinetic analysis of treatment of Ramos cells with BM-ca or rituximab with two different concentrations (20 and 40 μg/ml) and incubated for 6-72 h. Inhibition of cell growth was determined by the XTT assay and percentage inhibition was calculated as above. *p<0.05 compared with treatment with rituximab (B).

The cells were stained for intracellular active caspase-3 with PE-conjugated anti-caspase-3 mAb (Pharmingen, San Jose, CA) and analyzed by flow cytometry. As a negative control, the cells were stained with pure (Isotope) IgG. Population data were acquired on a Flow Epics XL-MCL (Coulter) with System II Software, and the percentage of apoptotic cells was recorded.

Statistical analysis. All results were expressed as the mean ± SD of data obtained from three or four independent and separate experiments. The statistical significance of differences between group means was determined using One-way ANOVA to compared variance, using Graphpad Prism for Windows (GraphPad Software, San Diego, CA USA). Significant differences were considered when P-value was ≤0.05.

Results

The novel humanized anti-CD20 BM-ca mAb was examined for its biological and cell signaling effects using the CD20 positive Ramos B-NHL cell line as model. For comparison, rituximab was analyzed in parallel with BM-ca.

BM-ca-induced inhibition of cell growth. The effect of BM-ca on Ramos cell growth was determined by treating the cells with various concentrations of the antibody or control isotype for 24 h. Cell recovery was determined by the XTT assay. Shown in Fig. 1A, treatment of Ramos with BM-ca resulted in significant inhibition of viable cell recovery in a concentration-dependent manner. At the concentration of 10 μg/ml <25% inhibition of cell growth was observed. By comparison, treatment with rituximab resulted in less inhibition of cell growth and at the concentration of 10 μg/ml there was about 10% inhibition of cell growth and higher concentrations did not augment the inhibition and a plateau was achieved. These findings demonstrate that BM-ca inhibits cell growth and its inhibition is more pronounced than rituximab when used at equal concentrations.

We then determined the time kinetics of BM-ca-mediated inhibition of cell growth. Ramos cells were treated with two concentrations of BM-ca, 20 and 40 μg/ml, for different periods of time (0-72 h) and cell growth was determined by the XTT assay. The data in Fig. 1B show that beginning of 12 h treatment, significant inhibition of cell growth is observed with BM-ca and the inhibition was more pronounced at 24 h and a plateau was observed thereafter. At the concentration of 40 μg/ml there was significant inhibition at 12 and 24 h. Rituximab, however, was not as a potent inhibitor of cell survival as BM-ca and there was steadily increase in cell growth inhibition as a function of time from 12 to 48 h for both the 20 and 40 μg/ml concentrations (Fig. 1B). These findings demonstrate that treatment of Ramos cells with BM-ca exerts its inhibitory activity on cell growth that is maintained for longer periods of time.

Inhibition of constitutively activated NF-κB and p38 MAPK cell survival pathways by BM-ca. The above findings demonstrated that BM-ca inhibits Ramos cell growth, suggesting that the antibody must have signaled the cells and perturbed the cell survival pathways. We have previously reported that rituximab inhibits both the constitutively activated NF-κB and p38 MAPK survival pathways in B-NHL cells (17,22) (see pathways in Fig. 2A). Hence, we examined the effect of BM-ca treatment on the NF-κB and p38 MAPK-activated pathways. Ramos cells were treated with BM-ca (25 μg/ml) or rituximab (25 μg/ml) for 24 h and cell lysates were prepared as described in Materials and methods. Western blot analysis was performed to determine the effect of treatment on key mediators of the NF-κB and p38 MAPK pathways. The NF-κB-mediated activation occurs through the phosphorylation of p65 (RelA) and IκBα and resulting in the proteasome degradation of phospho-IκBα and translocation of NF-κB from the cytosol to the nucleus where it binds to corresponding DNA-binding sites in the promoter of various gene products, namely, anti-apoptotic gene products. Inhibition of p65 and IκBα phosphorylation results in the inhibition of NF-κB translocation and hence, inhibition of NF-κB-mediated gene transcription. Treatment of Ramos cells with BM-ca resulted in significant inhibition of phospho-p65 and phospho-IκBα and no effect on the unphosphorylated forms (Fig. 2B). By comparison,

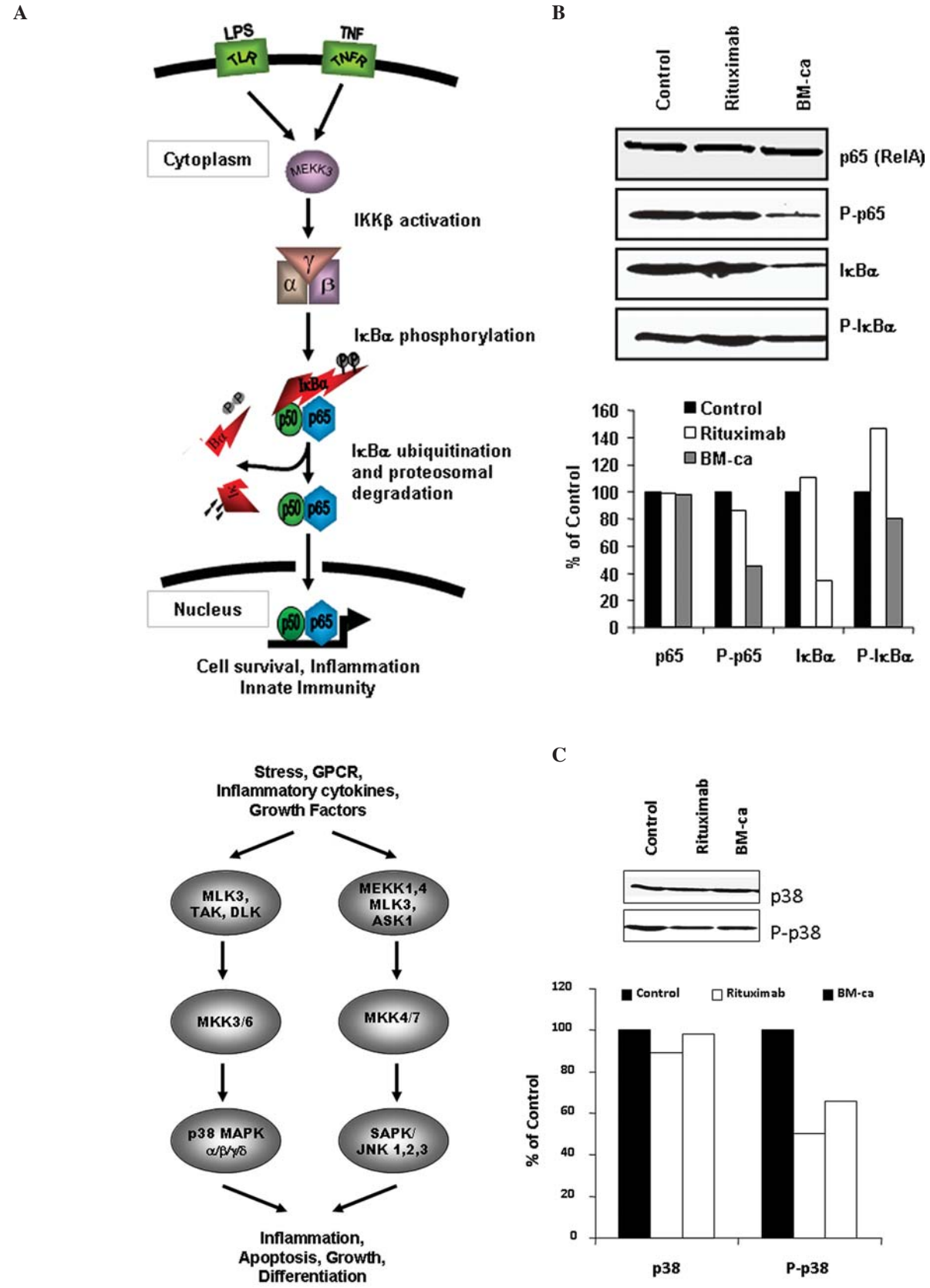

Figure 2. Inhibition of the constitutively activated NF-κB and p38 MAPK pathways in Ramos cells by BM-ca. (A) Schematic diagrams of the NF-κB and p38 MAPK pathways. (B) Ramos cells were treated with BM-ca or rituximab (25 μg/ml) for 24 h and total cell lysates were treated for various gene products by Western blot analysis and by densitometric analysis for the inhibtion of NF-κB. (C) Inhibition of p38 MAPK by BM-ca analyzed by both Western blotting and densitometry.

rituximab mediated less inhibitory activity. The p38 MAPK pathway, when activated, exhibits its downstream effects on the phosphorylation of various kinases and results in gene activation for inflammation, apoptosis, growth, and

differentiation (23). Inhibition of phosphorylation inhibits these various manifestations. Treatment with BM-ca resulted in significant inhibition of phospho-p38 and no effect on the unphosphorylated p38 (Fig. 2C). Both BM-ca and rituximab exerted similar inhibitory activity on phospho-p38. These findings demonstrate that BM-ca inhibits both the NF-κB and p38 MAPK survival pathways and these effects may be responsible, in part, for the observed anti-proliferative activity shown above in Fig. 1.

Induction by BM-ca of the metastasis suppressor/immune surveillance cancer gene product, RKIP. The above findings demonstrated that BM-ca inhibits NF-κB activity in Ramos cells. The mechanism by which BM-ca inhibits the constitutively activated NF-κB pathway is not clear. Previous findings by Yeung *et al* (24) reported that Raf-1 kinase inhibitor protein (RKIP) inhibits the NF-κB pathway by the direct physical association with NF-κB-inducing kinase (NIK), tumor growth factor-B activating kinase 1 (TAK1) and IκB kinase (IKK), thus rendering them incapable of relaying the signal to downstream molecules. Most tumor cells express low levels of RKIP as one underlying mechanism to maintain tumor cell survival and growth through the constitutive activation of the Raf-1/MEK/ERK1-2 and NF-κB pathways. We hypothesized, therefore, that BM-ca-mediated inhibition of NF-κB activity may be due, in part, to the induction of RKIP expression. This hypothesis was tested by treating Ramos cells with BM-ca for 24 h and cell lysates were examined by Western blot analysis for RKIP expression. The data in Fig. 3 show that, indeed, treatment with BM-ca significantly induced the expression of RKIP in Ramos cells. By comparison, rituximab also induced RKIP expression although at a much lower level than BM-ca.

The transcription regulation of RKIP has not been studied in detail. A recent report by Beach *et al* (25) demonstrated that the transcription factor and metastasis-inducer Snail negatively regulates RKIP transcription. Further, Snail was reported to be transcriptionally regulated, in part, by NF-κB (26). Thus, it was possible that BM-ca-induced inhibition of NF-κB results downstream in the inhibition of Snail and consequently, depression of RKIP transcription leading to upregulation of RKIP expression. Upregulation of RKIP, in turn, will inhibit NF-κB and regulates the NF-κB-Snail-RKIP loop. Indeed, treatment with BM-ca resulted in the inhibition of Snail expression (Fig. 3). Like BM-ca, rituximab also inhibited Snail expression. The above findings demonstrate that one potential mechanism by which BM-ca inhibits NF-κB is through the inhibition of the RKIP transcriptional repressor Snail.

Chemosensitization of CDDP-resistant Ramos cells by BM-ca monoclonal antibody. The above findings demonstrated that BM-ca inhibits the cell survival and anti-apoptotic NF-κB and p38 MAPK pathways. These pathways regulate the transcription of many genes that regulate the apoptotic pathways. Thus, it was possible that dysregulation of such apoptotic pathways by BM-ca may reduce the anti-apoptotic threshold and sensitizes the cells to drug-induced apoptosis. This hypothesis was tested by using the chemotherapeutic drug CDDP as model since Ramos cells were resistant to

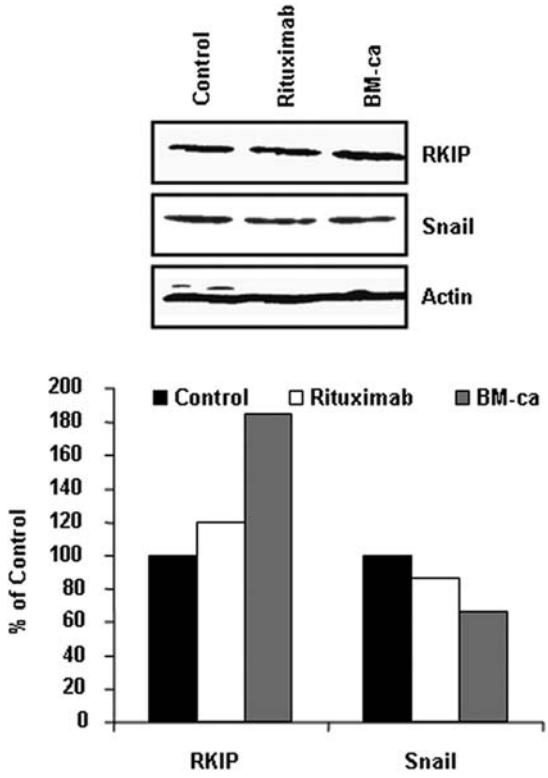

Figure 3. Induction of RKIP and inhibition of Snail by BM-ca. Ramos cells were treated with BM-ca or rituximab (25 μg/ml) for 24 h and cell lysates were tested for RKIP and Snail expression by Western blotting. Actin was used as loading control. The Western blot was analyzed for expression by densitometric analysis.

CDDP-induced apoptosis at higher concentrations. The cells were treated with BM-ca (25 μg/ml) for 24 h and then treated with various concentrations of CDDP (0-40 μg/ml) for an additional 24 h. The cells were harvested and analyzed for apoptosis by flow cytometry for activated caspase-3 as described in Materials and methods. The findings in Fig. 4A demonstrate that treatment with BM-ca sensitizes cells to CDDP apoptosis and the extent of apoptosis was a function of the CDDP concentration used. Significant apoptosis was initially observed at the concentration of 5 μg/ml of CDDP and the level of apoptosis increased up to 25 μg/ml CDDP and a plateau reached at 40 μg/ml of CDDP. By comparison, rituximab also sensitized the cells to CDDP apoptosis, although at lesser levels, at CDDP concentrations of 5-20 μg/ml. However, at 40 μg/ml of CDDP, rituximab sensitizes cells to the same maximum level achieved by BM-ca at a 10 μg/ml CDDP.

The chemosensitization of Ramos cells to CDDP apoptosis by BM-ca may have resulted from BM-ca-induced dysregulation of gene products involved in apoptosis under the regulation of both NF-κB and p38 MAP kinase signaling pathways. We examined the expression of representative anti-apoptotic (example Mcl-1) and pro-apoptotic (example Bax) gene products that might have been regulated by BM-ca. The data in Fig. 4B demonstrate that BM-ca-induced inhibition of Mcl-1 and induction of Bax. Rituximab also modified the expression of these gene products to a lower extent consistent with the above findings with rituximab.

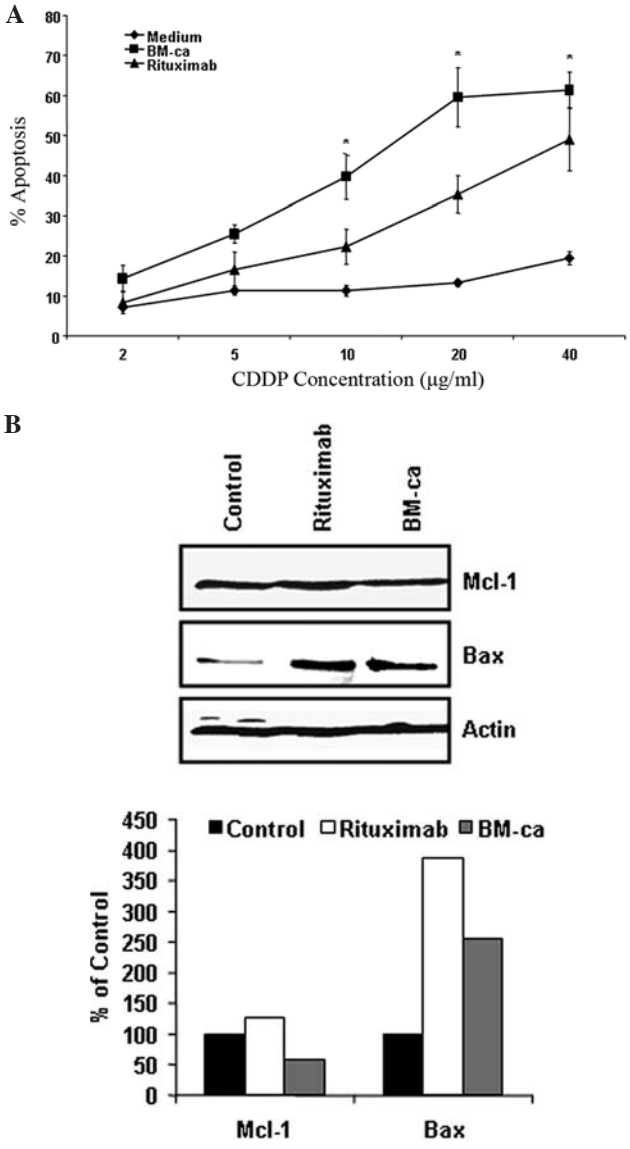

Figure 4. Chemosensitization of Ramos cells to CDDP apoptosis by BM-ca and underlying mechanisms. (A) Chemosensitization of Ramos cells to CDDP. Ramos cells were treated with BM-ca or rituximab (25 μg/ml) for 24 h and then treated with various concentrations of CDDP (2-40 μg/ml) for an additional 18 h. The cultures were then examined for apoptosis by flow cytometry for activated caspase-3 as described in Materials and methods. (B) Modulation of gene products that regulate apoptosis by BM-ca. Ramos cells were treated with BM-ca or rituximab (25 μg/ml) for 24 h and total cell lysates were tested for various gene products by Western blot analysis. The Western blot was also analyzed for expression levels by densitometric analysis.

Discussion

This study provides evidence that the novel humanized anti-CD20 BM-ca monoclonal antibody is able to inhibit significantly cell proliferation and the constitutively activated NF-κB and p38 MAPK survival/anti-apoptotic pathways. These effects resulted in the dysregulation of gene products involved in apoptosis and thus, treatment with BM-ca sensitized the B-NHL cells to drug-induced apoptosis. The BM-ca-induced inhibition of NF-κB activation was the result, in part, of the downstream inhibition of the RKIP transcription repressor Snail and subsequently induction of RKIP. Thus, BM-ca disrupts the regulation of the NF-κB-Snail-RKIP loop, which is dominated in cancer cells by overexpression of NF-κB and Snail activities and downregulation of RKIP expression. Hence, through BM-ca-induced inhibition of NF-κB and Snail, RKIP is induced, which in turn inhibits NF-κB and Snail in the regulatory loop. Thus, BM-ca treatment results in the dominant RKIP induction and inhibition of NF-κB and Snail. These events lead to downstream inhibition of anti-apoptotic gene products and upregulation of pro-apoptotic gene products, all of which result in the sensitization of B-NHL cells to drug-induced apoptosis. The BM-ca-induced effects were superior to those obtained with rituximab, both qualitatively and quantitatively. We suggest that BM-ca might have superior activity in the treatment of patients with B-NHL and other B-cell-mediated diseases, used alone or in combination with therapeutics.

BM-ca is a novel humanized monoclonal antibody that was generated from a murine anti-CD20 monoclonal antibody, 1K1791, and humanized due to its superior ADCC, CDC and apoptosis activities when compared to rituximab (6). BM-ca induced a significant inhibition of cell growth following treatment and the inhibition was noticeable at 12 h post treatment. The inhibition of cell proliferation was maximal for Ramos B-cells at 20-40 μg/ml of BM-ca and was maintained up to 48 h following treatment and no more inhibition was observed thereafter. The mechanism of inhibition of cell proliferation by BM-ca is not clear and will be the subject of another report. It is possible that BM-ca treatment affected one or more survival pathways and inhibited several gene products that regulate cell proliferation. A prominent cell survival pathway that regulates cell growth and proliferation is the NF-κB pathway, which is constitutively activated in most B-NHL tumors (27). Therefore, we examined whether BM-ca interferes with the NF-κB activated pathway through analysis of key gene products in the NF-κB activation cascade.

Constitutive activation of the NF-κB/Rel transcription factors has been observed in various malignancies including B-cell lymphoma (28). Likewise, the NF-κB pathway is constitutively activated in Ramos and Daudi cells (17). Constitutive activation of NF-κB/Rel either through the amplification of Rel genes or through aberrant activation of the upstream regulators contributes to pathologic conditions including cancer (29). In mammals, the NF-κB family contains five members: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100), the most abundant form being the p65/p50 heterodimer. In normal cells, NF-κB activity is tightly controlled by IκB inhibitory proteins. NF-κB activation can be induced by a plethora of extracellular stimuli resulting in phosphorylation of IκB at two conserved serines in the NH2-terminal regulatory region, which in IκBα correspond to Ser32/36. This phosphorylation step is rapidly followed by polyubiquitination and IκB degradation by the 26S proteasome, allowing stable translocation of NF-κB to the nucleus and activation of gene transcription. IκB phosphorylation is catalyzed by the multiprotein IKK complex, which is phosphorylated and activated by the upstream NIK (29).

Our findings demonstrate that BM-ca inhibited the phosphorylation of both p65 and IκBα and thus, interfered with the activation of NF-κB and these led to inhibition of NF-κB

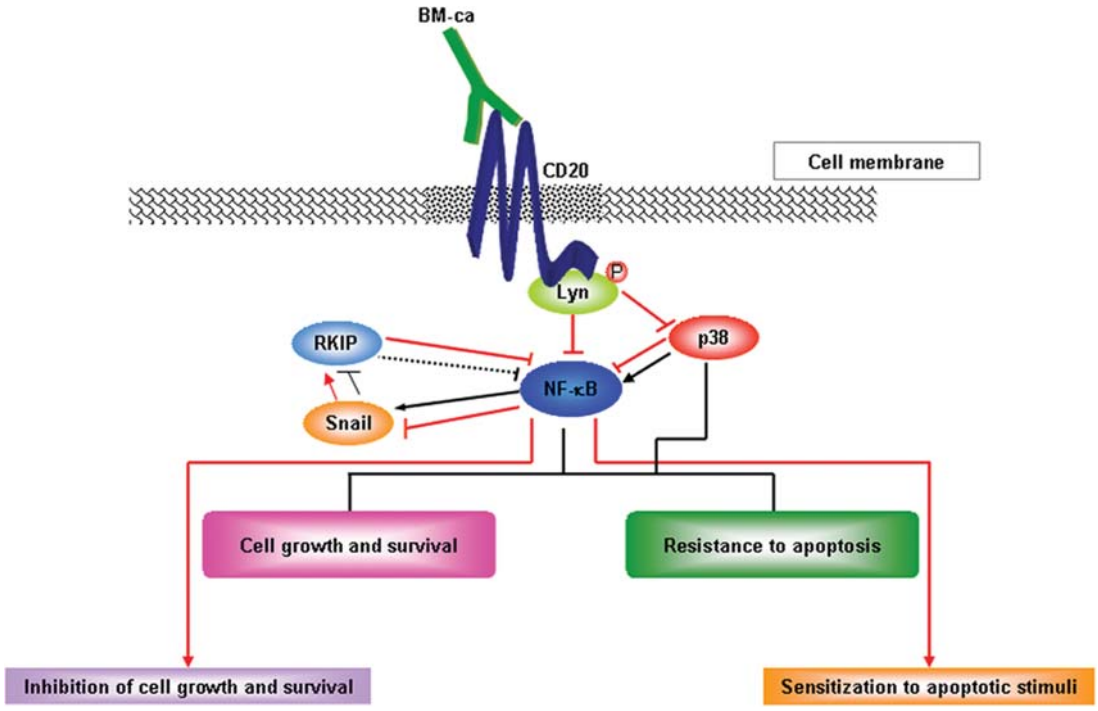

Figure 5. Schematic diagram on the potential mechanism by which BM-ca inhibits NF-κB and p38 MAPK and reverses resistance. This schematic diagram demonstrates that Ramos cells constitutively express activated NF-κB and p38 MAPK pathways. These pathways regulate cell growth and survival and through the transcription of various anti-apoptotic gene products, regulate the resistance to apoptotic stimuli. In addition, constitutively activated NF-κB induces the expression of Snail which, in turn, represses RKIP expression. Treatment with BM-ca results in the inhibition of both the NF-κB and p38 MAPK pathways, resulting downstream in the inhibition of Snail and gene products that regulate apoptosis. In addition, inhibition of Snail derepresses RKIP transcription and results in the upregulation of RKIP which in turn inhibits NF-κB. These resulted in the inhibition of cell growth and survival and sensitization of tumor cells to apoptotic stimuli.

translocation from the cytosol into the nucleus. In addition, BM-ca inhibited upstream the p38 MAPK which is involved in the activation of NF-κB (30). We have reported using the AIDS-derived B-NHL cell line, 2F7, that rituximab treatment resulted in significant inhibition of the p38 MAPK and NF-κB pathways and in the selective inhibition of the anti-apoptotic gene products Bcl-2 and Bcl-xl and resulted in the sensitization to apoptosis by various chemotherapeutic drugs (22). The present findings with BM-ca corroborate these findings in the Ramos B-NHL cell line. Inhibition of p38 MAPK and NF-κB activities has been shown to be mediated by upstream inhibition of Src kinases (22,31). The present findings corroborate these studies and demonstrate that treatment of Ramos cells with BM-ca inhibited the phosphorylation of p38 and inhibited the transcription factor NF-κB leading to inhibition of Mcl-1 and induction of Bax.

Treatment of Ramos cells with BM-ca sensitizes the cells to CDDP-induced apoptosis. Previous findings by us and others have reported that inhibition of NF-κB activity reverses drug resistance when used in combination with subtoxic levels of drugs (32). BM-ca-induced inhibition of NF-κB and sensitization, thus, corroborate these findings and establish BM-ca as a chemosensitizer of drug-resistant B-NHL cells. The sensitization was the result, in part, of BM-ca-induced downregulation of genes that regulate apoptosis. Various studies demonstrated that inhibition of the anti-apoptotic Bcl-2 family members (example Bcl-2, Bcl-xl, Mcl-1, and survivin) or upregulation of pro-apoptotic family members (example Bax and Bad) result in the perturbation of the mitochondrial membrane potential and its depolarization and the release of cytochrome c and Smac/DIABLO into the cytosol, leading to activation of caspases-9, -7, and -3 and resulting in apoptosis (33). In this study, we show that BM-ca inhibited Mcl-1 through upstream inhibition of NF-κB and p38 MAPK. Inhibition of the expression of these anti-apoptotic gene products might have been responsible, in part, for BM-ca-mediated sensitization to CDDP apoptosis.

The findings obtained with BM-ca were compared with those obtained by rituximab under similar conditions and concentrations. In all of the reported findings herein, BM-ca was superior over rituximab. For instance, there was ~2-fold less concentration of BM-ca to achieve the same level of cell growth inhibition. Likewise, BM-ca was more potent in inhibiting NF-κB and p38 MAPK activities than rituximab. BM-ca-induced significantly higher level of RKIP than rituximab. The level of induction of RKIP by BM-ca may explain, in part, the higher NF-κB inhibitory activity observed with BM-ca as compared to rituximab. RKIP inhibits both the Raf/MEK and NF-κB pathways (24,34). The induction of RKIP by BM-ca was due, in part, to inhibition of its transcription repressor factor Snail. Clearly, Snail which is downstream of NF-κB was downregulated by BM-ca and established Snail as a target for BM-ca-induced sensitization.

The present findings establish BM-ca in targeting the NF-κB-Snail-RKIP loop, which is normally present in cancer cells in the form of high NF-κB and Snail and low RKIP. This dominance is maintained for tumor cell maintenance of survival and growth and for the regulation of mechanisms of

resistance to apoptotic stimuli. BM-ca interferes with this loop and through inhibition of NF-κB and Snail and induction of RKIP, the cells are inhibited for growth and replication and a sensitive phenotype is established to respond to a variety of apoptotic stimuli. Schematically diagramed in Fig. 5, the NF-κB and p38 MAPK pathways are constitutively activated in B-NHL cells. Activation of these pathways results downstream in the induction of Snail and repression of RKIP. Anti-apoptotic gene products are transcribed and regulate the resistance to drug-induced apoptosis. Following treatment with BM-ca, the P38 MAPK and NF-κB pathways are inhibited resulting downstream in the inhibition of Snail transcription and derepression of RKIP. Derepression of RKIP, in turn, accentuates the inhibition of NF-κB and Snail, manifested by the inhibition of anti-apoptotic gene products and/or induction of pro-apoptotic gene products, leading to sensitization to apoptosis by drugs. In addition, the present findings unravel a new set of therapeutic targets that can be explored in cases whereby tumor cells become resistant to CD20 antibodies.

Acknowledgements

This work was supported by the Jonsson Comprehensive Cancer Center (MV), the UCLA AIDS Institute (MV) and Fogarty International Center Fellowship (D43 TW00013-14) (MIV), UC-MEXUS-CONACYT (SH-Y). This work was supported by various donors. The authors acknowledge the assistance of Erica Keng in the preparation of the manuscript.

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