Overcoming rituximab drug-resistance by the genetically engineered anti-CD20-hIFN-α fusion protein: Direct cytotoxicity and synergy with chemotherapy

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Abstract. Treatment of patients with B-NHL with rituximab and CHOP has resulted in significant clinical responses. However, a subset of patients develops resistance to further treatments. The mechanism of unresponsiveness *in vivo* is not known. We have reported the development of rituximab-resistant clones derived from B-NHL cell lines as models to investigate the mechanism of resistance. The resistant clones exhibit hyper-activated survival/anti-apoptotic pathways and no longer respond to a combination of rituximab and drugs. Recent studies reported the therapeutic efficacy in mice bearing B-cell lymphoma xenografts following treatment with the anti-CD20-hIFNα fusion protein. We hypothesized that the fusion protein may bypass rituximab resistance and inhibit survival signaling pathways. Treatment of the ritux-imab-resistant clones with anti-CD20-hIFNα, but not with rituximab, IFNα, or rituximab+IFNα resulted in significant inhibition of cell proliferation and induction of cell death. Treatment with anti-CD20-hIFNα sensitized the cells to apoptosis by CDDP, doxorubicin and Treanda. Treatment with anti-CD20-hIFNα inhibited the NF-κB and p38 MAPK activities and induced the activation of PKC-δ and Stat-1.

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These effects were corroborated by the use of the inhibitors SB203580 (p38 MAPK) and Rottlerin (PKC-δ). Treatment with SB203580 enhanced the sensitization of the resistant clone by anti-CD20-hIFNα to CDDP apoptosis. In contrast, treatment with Rotterin inhibited significantly the sensitiza-tion induced by anti-CD20-hIFNα. Overall, the findings demonstrate that treatment with anti-CD20-hIFNα reverses resistance of B-NHL. These findings suggest the potential application of anti-CD20-hIFNα in combination with drugs in patients unresponsive to rituximab-containing regimens.

Introduction

Non-Hodgkin's lymphoma (NHL) is the fifth or sixth most common cancer in the US, and diffuse large B-cell lymphoma (DLBCL) is the most commonly occurring lymphoma in the Western world (1,2). Currently, the standard front-line therapy for DLBCL is the combination of rituximab and chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) (R-CHOP), with expected 5- and 10-year overall survival (OS) rates of 58 and 43.5%, respectively (3). Rituximab is a chimeric monoclonal antibody (mAb) targeting CD20. Rituximab acts, in part, by engaging Fc receptors on immune effector cells, such as NK and macrophages, and induces cyto-toxicity by antibody-dependent cellular cytotoxicity (ADCC). It also activates complement-dependent cytotoxicity (CDC) and rarely induces apoptosis (4). While therapeutic outcomes have improved in the post-rituximab era, there is evidence of patients exhibiting rituximab-resistance (RR). Thus, attempts to overcome RR have been a major focus of novel therapeutic developments. The mechanisms of resistances *in vivo* are not clear. Several mechanisms underlying RR have been postulated. These included resistance to antibody-mediated cytotoxicity mechanisms (ADCC, CDC, and induction of apoptosis), Fc-receptors polymorphisms, downregulation or loss of CD20 expression, altered antibody pharmacokinetics and altered molecular signaling pathways through CD20 (5).

We have explored the potential mechanisms of rituximab resistance by developing *in vitro* clones of rituximab-resistant

(RR) variants in several B-NHL cell lines and characterized their properties. Briefly, unlike the parental wild-type, the RR clones express CD20 but no longer respond to treatments with rituximab or combination of rituximab and cytotoxic drugs. Further, the RR clones overexpressed the activity of several survival/anti-apoptotic pathways. Interference in the activity of these hyper-activated pathways reversed resistance (6). In the hope of overcoming rituximab resistance alternative therapies such as the use of HDAC or Bcl-2 inhibitors have also been demonstrated to enhance sensitization of tumor cells to rituximab (7). The efficacy of rituximab has also been shown to be augmented when used in combination with biological agents such as interferon-α-2a (IFN-α), specific interleukins, bortezomib and lenalidomide (8).

An alternative strategy for the management of patients with lymphoma has been to use biologic agents instead of chemotherapy in relapsed and refractory lymphoma patients. Clinical trials using rituximab alone or in combination with IFN-α have shown that T-cells are important for the survival for lymphoma patients (9). Preclinical studies have suggested a synergistic activity by the combination of IFN-α and rituximab and phase II clinical trials exploring the use of this combination yielded promising results (10,11). Due to the good results of this randomized phase clinical II trial, the priming effect of INF-α on malignant B cells and immune-cells was evaluated in a large randomized phase III trial with preliminary promising results (12).

IFN-α is a cytokine that affects diverse biologic functions as antiviral activity, immunomodulatory action, cell differentiation, and cell survival or death, in a variety of cell types (13,14). IFN-α has been employed for the treatment of certain tumors including hairy cell leukemia, chronic myelogenous leukemia, melanoma and renal cancer (15,16). In some cases, the antitumor action of IFN-α has been shown to involve the induction of apoptosis through the activation of JNK via PKC-δ, leading to upregulation of TRAIL and activation of Stat-1 (17).

An alternative approach to tumor immunotherapy is the development and application of fusion proteins. Fusion proteins have been employed to deliver cytokines, radioisotopes and toxins for cancer therapy (18). Recent studies have demonstrated that a fusion protein consisting of anti-CD20 antibody and IFN-α (anti-CD20-hIFN-α) exhibited superior activity over rituximab, IFN-α or the combination, with significant anti-proliferative and apoptotic effects *in vitro* against several B-NHL cell lines. *In vivo*, anti-CD20-hIFN-α showed a significant antitumor activity against xenografts (19). Hence, we hypothesized that anti-CD20-hIFN-α treatment may also be effective against the RR clones.

To test the above hypothesis, the followings were investigated: i) do the RR B-NHL clones respond to anti-CD20-hIFN-α treatment with a decrease in both cell viability and cell recovery? ii) Does the drug resistance of the RR clones reverse following treatment by the combination of chemotherapeutic drugs with anti-CD20-hIFN-α? iii) Does treatment of the RR clones with anti-CD20-hIFN-α signal the cells and modify their survival/anti-apoptotic pathways? and iv) Does the treatment of the RR clones with inhibitors of gene products modified by anti-CD20-hIFN-α result in the reversal of drug resistance? The findings corroborated the above hypothesis. It was found that the RR clones responded to anti-CD20-hIFN-α treatment, but not to anti-CD20, hIFNα or combination, and treatment with anti-CD20-hIFN-α sensitized the drug-resistant RR clones to drug-induced apoptosis.

Materials and methods

Cell lines and reagents. The human B-NHL cell lines Ramos and 2F7 were purchased from the ATCC (Manassas, VA, USA). Clones were developed as previously reported (20). Briefly, the cells were grown in the presence of step-wise increasing concentrations of rituximab (5-20 µg/ml) for 10 weeks. Single cells were then isolated and subjected to three consecutive rounds of limiting dilution analyses. The cell lines were cultured as described previously (21). All cells used in this study were within 15 passages after resuscitation. The cells were checked routinely by morphology and tested for mycoplasma contamination with the CELLshipper® Mycoplasma Detection kit (Bionique® Testing Laboratories, Saranac Lake, NY, USA). Rituximab was commercially obtained. CDDP was purchased from Sigma (St. Louis, MO, USA) and was diluted in DMSO. Treanda® (bendamustine hydrochloride) was purchased from Cephalon, Inc., (Teva Pharmaceutical Industries Ltd. Frazer, PA, USA). Adriamycin® (doxorubicin) was purchased from Sun Pharma Global FZE. The PE-labeled anti-active caspase-3 antibody and the corresponding IgG1 isotype controls were obtained from BD Pharmingen (San Diego, CA, USA). The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and were directed against Bcl-$_{XL}$, Bcl-6, p65, phospho-p65 (Ser 536), p38, phospho-p38 (Thr180-Tyr182), Bax, PKC-δ, phospho-PKC-δ (Thr 505), Stat-1, phospho-Stat-1 (Tyr701 and Ser727), and phospho-JNK (Thr183/Tyr185). The genetically engineered anti-CD20-hIFNα was developed as described by Xuan *et al* (19) and kindly provided by Dr Sherie L. Morrison, UCLA. Human IgG (Sigma) was used as control. IFN-α2a was purchased from Sigma-Aldrich Co. (USA), the PKC-δ inhibitor rottlerin was obtained from Sigma-Aldrich Co.. The p38 Map kinase inhibitor SB203580 was purchased from Cell Signaling Technology, Inc. (USA).

Viability assay. Cell viability was assessed by either the trypan blue dye exclusion assay by microscopy or by the XTT dye absorbance according to the manufacturer's instructions (Roche Diagnostic GmbH, Nonnenwald, Germany) as previously described (21). The viability of the untreated cells was set at 100% and total cell recovery was recorded. Each experimental condition was performed in triplicate and the SD was calculated.

Apoptosis determination. Apoptosis was assessed in tumor cells by flow cytometry for activated caspase-3 as previously described (21). Briefly, B-NHL cell cultures were preincubated with various concentrations of the anti-CD20-hIFNα fusion protein (30, 50 or 100 pM), or rituximab, rhIFN-α (equivalent range of concentrations) or combination of rituximab and rhIFN-α for 18 h and CDDP, Treanda or doxorubicin (10, 5 and 5 µg/ml), respectively, were added for an additional 18 h at at 37°C. The cells were stained intracellularly for activated caspase-3 and the samples were analyzed by flow cytometry.

Population data were acquired on a Flow Centre EPICSR XL-MCL (Coulter, Co.) with System II software and the percentage of positive cells was recorded.

Western blot analysis for protein expression. B-NHL cell lines were incubated with or without 100 pM of anti-CD20-hIFNα or rituximab at 37˚C for 18 h. Western blot analysis was performed as previously described (21). Briefly, cell extracts for protein analysis were prepared by lysing $2x10^6$ cells on ice with cold 200 μl of radioimmunoprecipitation assay buffer [1% NP40, 0.1% SDS, 0.5% deoxycholic acid, complete protease inhibitor cocktail tablets (Roche Diagnostic Co.), and 1X PBS]. Lysates were transferred to microcentrifuge tubes and sonicated in the Sonicator, model W-220F (Heat-System Ultrasonic, Inc.) for 10 sec. Lysates were centrifuged at 12,000 x g at 4˚C for 5 min. Protein concentration was quantified using the Bio-Rad protein assay (Bio-Rad Laboratories). Gel loading buffer Bio-Rad (Bio-Rad Laboratories) was added to the cell lysates at a 1:1 volume. Samples were boiled for 5 min and were separated on 12% SDS-polyacrylamide mini-gels and transferred to a nitrocellulose membrane Hybond enhanced chemiluminescence (Amersham Pharmacia Biotech) in Trans-Blot SD semidry Transfer Cell System (Bio-Rad).

Statistical analysis. All results were expressed as the mean ± SD of data obtained from three triplicate, independent and separate experiments. The statistical significance of differences between group means was determined using one-way ANOVA to compare variance. Significant differences were considered for probabilities <5% (p<0.05).

Results

Effects of anti-CD20-hIFN-α treatment on the proliferation and viability of various B-NHL cell lines. The 2F7 and Ramos B-NHL cell lines and their rituximab-resistant variants, 2F7R and Ramos R, were treated with different concentrations of anti-CD20-hIFN-α and equimolar concentrations of rituximab (30, 50 and 100 pM), IFN-α, or rituximab+IFN-α or for 18 h and examined for cell recovery, cell viability and apoptosis. Treatment with anti-CD20-hIFN-α, in contrast to treatment with rituximab, IFN-α, or rituximab+IFN-α, resulted in significant inhibition of cell recovery in 2F7 at a concentration >50 pM. With 2F7R, there was a significant inhibition of cell recovery at the concentration of 30 pM and an augmented inhibition at 50 and 100 pM of anti-CD20-hIFN-α (Fig. 1A, upper right panel). With Ramos cells, there was inhibition at >50 pM of anti-CD20-hIFN-α treatment. Likewise, there was significant inhibition of Ramos R by anti-CD20-hIFN-α at concentrations ≥50 pM (Fig. 1A, lower panels). These findings demonstrate that, in contrast to treatments with rituximab, IFN-α or rituximab+IFN-α, treatment with anti-CD20-hIFNα inhibited significantly the cell recovery in both the 2F7R and Ramos R cell lines.
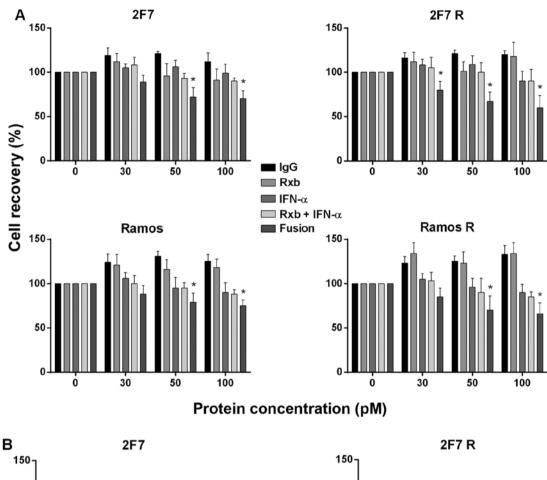
The viability of the cell lines treated with the above agents was determined microscopically by trypan blue dye exclusion. In contrast to the treatments with rituximab, IFN-α or rituximab+IFN-α, treatment with anti-CD20-hIFN-α induced significant cytotoxicity in all the cell lines (2F7, 2F7R, Ramos, and Ramos R) at concentrations ≥50 pM (Fig. 1B). These

findings suggested that the inhibition of the cell recovery shown in Fig. 1A was the result, in part, of cell loss induced by anti-CD20-hIFN-α.

The cytotoxic activity exhibited by anti-CD20-hIFN-α above in Fig. 1B by dye exclusion was also examined for apoptotic activity as assessed by the activation of procaspase-3 as described in Materials and methods. The findings showed that treatment with anti-CD20-hIFN-α, but not with rituximab, IFN-α or rituximab+IFN-α, induced apoptosis in all four of the cell lines tested (Fig. 1C). With 2F7, there was significant apoptosis induction following treatment with anti-CD20-hIFNα at 50 pM, whereas, in 2F7R, there was significant apoptosis at ≥30 pM. Also, a significant number of cells undergoing apoptosis was recorded in both Ramos and Ramos R cell clones treated with anti-CD20-hIFN-α at ≥30 pM.

Overall, the above findings demonstrated clearly that the response of RR clones to treatment with anti-CD20-hIFN-α could not be mimicked by the treatment with single agents or the combination of anti-CD20 and hIFN-α. Further, the findings also supported the contention that treatments with anti-CD20-hIFN-α signalled and triggered the RR cells leading to cell death and apoptosis. This cell signaling in the RR clones was conditioned on the fusion protein and was not induced by the combination of rituximab and hIFN-α.

Chemosensitization of 2F7R and Ramos R cells following treatment with anti-CD20-hIFN-α and chemotherapeutic drugs. Previous reports have demonstrated that drug resistance of the wild-type B-NHL cell lines, but not the rituximab-resistant variants, can be reversed following treatment with rituximab (22,23). These findings are corroborated here for the 2F7, 2F7R, Ramos and Ramos R B-NHL cell lines treated with rituximab and CDDP (Fig. 2A, upper and bottom left panels). Noteworthy, treatment with anti-CD20-hIFN-α and CDDP, in contrast to rituximab+CDDP, resulted in significant induction of apoptosis in 2F7R and Ramos R at anti-CD20-hIFN-α concentrations of ≥30 pM (Fig. 2A). The chemosensitization-induced apoptosis by anti-CD20-hIFN-α was detected at 24, 48 and 72 h following treatment (Fig. 2B). These findings suggested that signaling by anti-CD20-hIFN-α on 2F7R and Ramos R must have altered the anti-apoptotic pathways and thus, resulting in the sensitization of the cells to CDDP apoptosis. Similar sensitizations had been observed by the combination treatment of rituximab+CDDP on the wild-type Ramos and 2F7 cell lines.

In addition to CDDP, we also examined the sensitizing activity of the anti-CD20-hIFN-α treatment on Treanda-induced apoptosis. Treatment of the wild-type cell lines 2F7 and Ramos with rituximab+Treanda resulted in significant apoptosis as compared to treatment with single agents (Fig. 2C, left panels). The combination of rituximab+Treanda did not result in increased apoptosis for either the 2F7R or Ramos R cell lines (Fig. 2C, right panels). In contrast, treatment with anti-CD20-hIFN-α in combination with Treanda resulted in significant apoptosis in both the 2F7R and Ramos R cell lines (Fig. 2C, right panels). Similar findings were obtained with the combination of anti-CD20-hIFN-α and doxorubicin (Fig. 2D). Thus, treatment with anti-CD20-hIFN-α sensitized the RR cell lines, findings that could not be achieved by the combination of rituximab and chemotherapeutic agents.
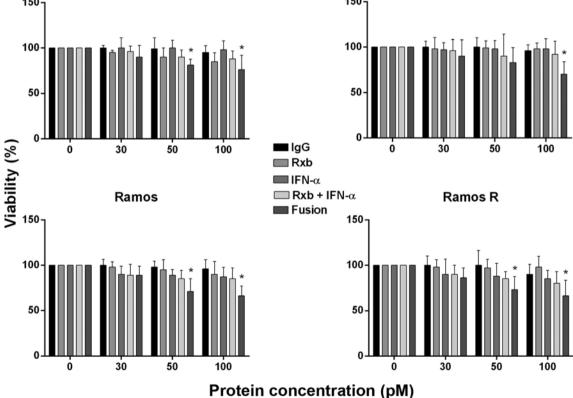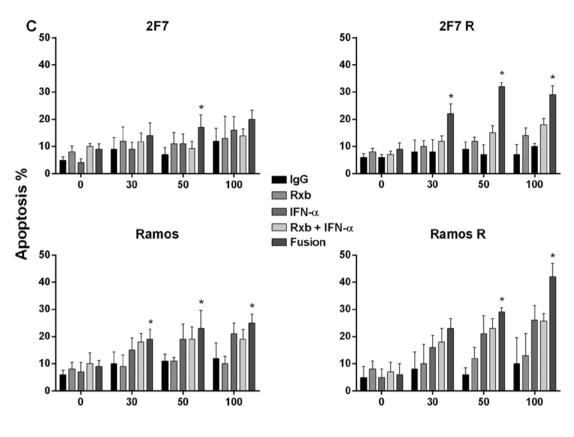
Figure 1. Anti-CD20-hIFN-α induces inhibition of cell recovery and viability and induction of apoptosis in rituximab-resistant (R) B-NHL cell lines. (A) The B-NHL cell lines 2F7, 2F7R, Ramos and Ramos R were treated with various concentrations of anti-CD20-hIFN-α (30, 50 or 100 pM) or equimolar concentrations of rituximab, rhIFN-α or the combination and incubated for 18 h. The total cell recovery was determined by trypan blue dye-exclusion. The B-NHL cell lines treated with normal IgG represent 100% cells recovered. The data represent the mean±SD from triplicate values, *p<0.05. (B) The B-NHL cell lines 2F7, 2F7R, Ramos and Ramos R were treated with various concentrations of anti-CD20-hIFN-α (30, 50 or 100 pM) or equimolar concentrations of rituximab, rhIFN-α or the combination and incubated for 18 h and cell viability was determined by the XTT assay. B-NHL cell lines treated with normal IgG represent 100% cells recovered. The data represent the mean±SD from triplicate values, *p<0.05.

Figure 1. Continued. (C) The B-NHL cell lines 2F7, 2F7 R, Ramos and Ramos R were treated with various concentrations of anti-CD20-hIFN-α (30, 50 or 100 pM) or equimolar concentrations of rituximab, rhIFN-α or the combination and incubated for 18 h and apoptosis was determined as assessed by activated caspase-3, as described in Materials and methods. The data represent the mean±SD from triplicate values, *p<0.05.
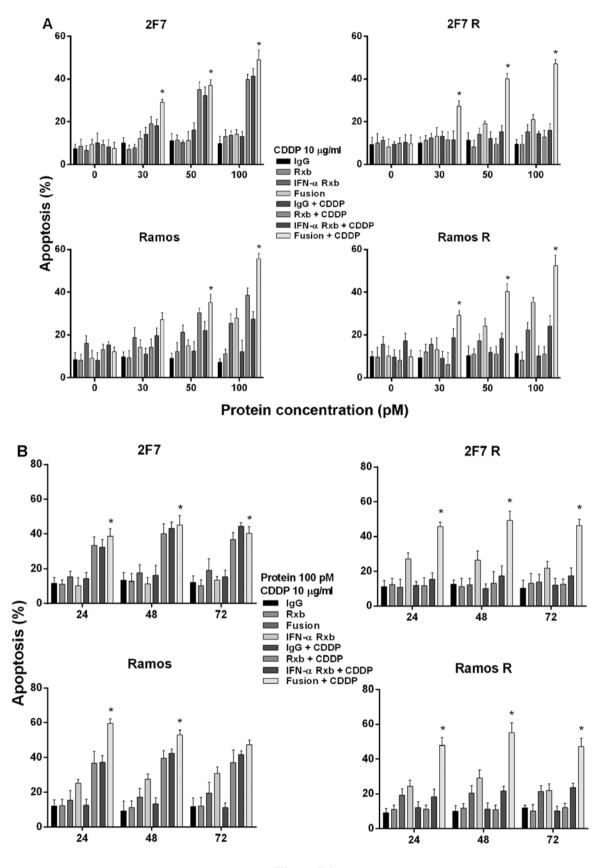
Cell signaling mediated by anti-CD20-hIFN-α following treatment of the Ramos R and 2F7R cell lines. Previously, we have shown that treatment of wild-type B-NHL cell lines with rituximab resulted in the inhibition of several survival and anti-apoptotic pathways (23). These modifications were responsible, in part, for the chemosensitization of the cells to drug-induced apoptosis. Based on the present findings that treatment of the resistant variants with anti-CD20-hIFN-α resulted in the inhibition of cell recovery, the induction of cell apoptosis and the sensitization to drugs, we deduced that treatment with anti-CD20-hIFN-α must have altered cell survival pathways. We analyzed by western blotting several proteins involved in survival following treatment with either rituximab or anti-CD20-hIFN-α. Treatment of Ramos with either rituximab or anti-CD20-hIFN-α resulted in similar inhibition of p-p38, p-p65 and Bcl-$_{XL}$ (Fig. 3A). In addition, treatment with anti-CD20-hIFN-α resulted in the upregulation of Bax. As expected, consistent with previous studies, treatment of Ramos R with rituximab did not have any effect on the expression of these proteins. In marked contrast, treatment of Ramos R with anti-CD20-hIFN-α resulted in significant inhibition of p-p38, p-p65, Bcl-$_{XL}$ and the induction of Bax (Fig. 3A). These findings demonstrated that treatment with anti-CD20-hIFN-α signaled the Ramos R cells similarly to the signaling observed following treatment of the wild-type Ramos cells with rituximab.

In addition to the mentioned gene products, we also examined other signaling pathways that may be induced by IFN-α and that may have contributed to the signaling by anti-CD20-hIFN-α. Treatment of Ramos with anti-CD20-hIFN-α resulted in the activation of PKC-δ (p-PKC-δ) and Stat-1 but had no effect on Bcl-6, and p-JNK (Fig. 3B). However, treatment of Ramos R with anti-CD20-hIFN-α, but not with rituximab, resulted in significant overexpression of p-PKC-δ and p-Stat-1 (Fig. 3B) and inhibition of Bcl-6 expression: there was no effect on p-JNK. Treatment of Ramos or Ramos R with hIFN-α induced p-PKC-δ (Fig. 3C), suggesting that hIFN-α in the fusion protein contributed to the activation of p-PKC-δ.

The above findings demonstrated that treatment of the Ramos R cell line with anti-CD20-hIFNα impacted pathways already observed following treatment of wild-type Ramos with rituximab alone as well as by IFN-α alone. Thus, rituximab and IFN- α in the anti-CD20-hIFN-α fusion protein each contributed to the signaling observed in Ramos R.

Roles of the p-p38 and PKC-δ by anti-CD20-hIFN-α in chemosensitization to CDDP and doxorubicin
a) Effect of the PKC-δ inhibitor Rotterin on the sensitization of 2F7 R and Ramos R cell lines by anti-CD20-hIFN-α to CDDP-induced apoptosis. Since treatment of Ramos R with anti-CD20-hIFN-α induced p-pKC-δ (Fig. 3B) we examined the role of p-pKC-δ induction on chemosensitization by

Figure 2. Anti-CD20-hIFN-α sensitizes rituximab-resistant B-NHL cell lines to apoptosis by chemotherapy. (A) The indicated B-NHL cell lines cells were treated with various concentrations of anti-CD20-hIFN-α (30, 50 or 100 pM) or equivalent concentrations of rituximab or rhIFN-α for 18 h and then treated with CDDP (10 μg/ml) for an additional 18 h. Apoptosis was determined as described in Materials and methods. The data represent the mean±SD from three independent experiments, *p<0.05. (B) Treatment of rituximab-resistant B-NHL cell lines with anti-CD20-hIFN-α and CDDP for various times. The indicated B-NHL cell lines were treated with anti-CD20-hIFN-α (100 pM) or equivalent concentrations of rituximab, rhIFN-α or the combination for 24, 48 or 72 h and then treated with CDDP (10 μg/ml) for an additional 18 h. Apoptosis was determined as described. The data represent the mean±SD from three values, *p<0.05.
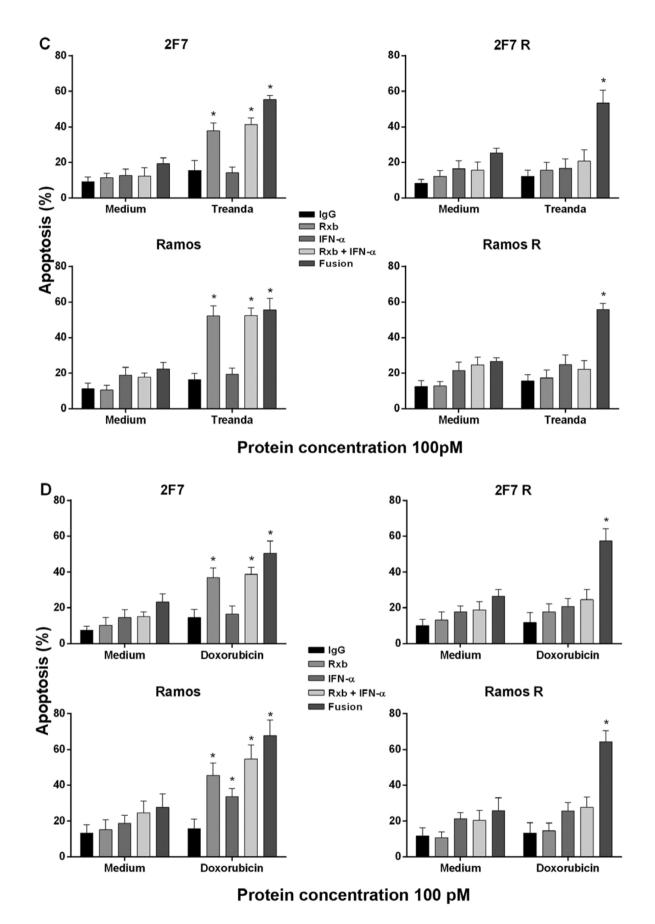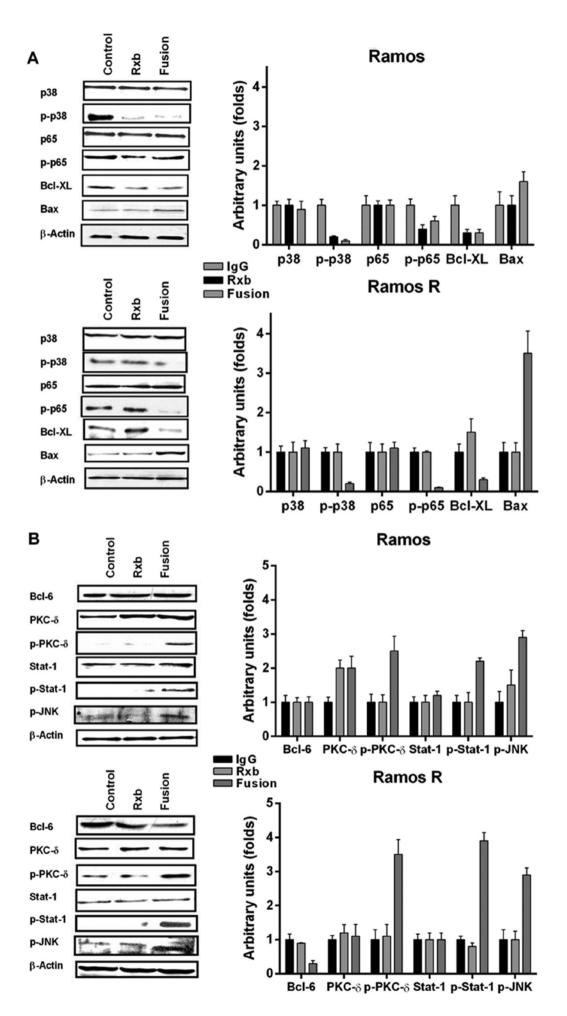
Figure 2. Continued. (C) B-NHL cell lines were treated with anti-CD20-hIFN-α (100 pM) or equivalent concentrations of rituximab, rhIFN-α or the combination for 18 h and then treated with Treanda (5 μg/ml) for an additional 18 h and apoptosis was determined. The data represent the mean±SD from three values, *p<0.05. (D) B-NHL cell lines were treated with anti-CD20-hIFN-α (100 pM) or equivalent concentrations of rituximab, rhIFN-α or the combination for 18 h and then treated with doxorubicin (5 μg/ml) for an additional 18 h. Apoptosis was determined as described. The data represent the mean±SD from three values, *p<0.05.
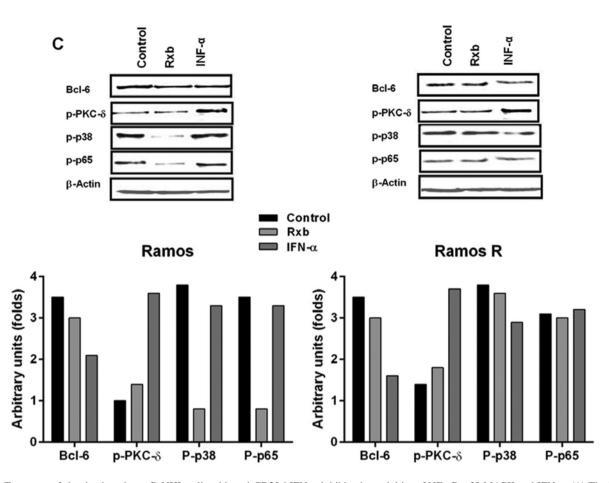
Figure 3. Treatment of rituximab-resistant B-NHL cells with anti-CD20-hIFN-α inhibits the activities of NF-κB, p38 MAPK and IFN-α. (A) The B-NHL cell lines Ramos and Ramos R were treated with anti-CD20-hIFN-α (100 pM) for 18 h and total cell lysates were prepared and p38, p-p38, p65, p-p65 Bcl$_{XL}$ and Bax proteins levels were determined by western blotting. β-actin was used as a loading control (left panel). Densitometry is presented in the right panel. (B) The B-NHL cell lines Ramos and Ramos R both treated and not, with anti-CD20-hIFN-α (100 pM) for 6 h were analyzed by western blotting for Bcl-6, PKC-δ, p-PKC-δ, Stat-1, p-Stat-1 and p-JNK. β-actin was used as a loading control (right panel). Densitometry is presented on the right panel. (C) Ramos and Ramos R were treated with rituximab or hIFN-α for 24 h and lysates were analyzed by western blotting for the expression of gene products compared to untreated controls (top images). The western blots were also analyzed by densitometry.

anti-CD20-hIFN-α. Treatment with the PKC inhibitor rottlerin significantly inhibited the chemosensitization induced by anti-CD20-hIFN-α in 2F7 (p<0.005), Ramos (p<0.001), and Ramos R (p<0.001) (Fig. 4A). PKC-δ is activated by IFN-α and there was also significant inhibition by any combination containing IFN-α. These findings suggest the participation of IFN-α in anti-CD20-hIFNα induced chemosensitization of Ramos R cells.

b) Effect of p38 MAPK inhibition by anti-CD20-hIFN-α in chemosensitization. We have reported that treatment of B-NHL cells with rituximab inhibited p-p38 MAPK activity and sensitized the cells to drug apoptosis (24). Treatment of Ramos R with anti-CD20-hIFN-α, but not with rituximab alone, inhibited p-p38 MAPK (Fig. 3A). Thus, we examined the role of anti-CD20-hIFNα-induced inhibition of p-p38 MAPK in chemosensitization of 2F7 R and Ramos R to doxoru-bicin-induced apoptosis. Treatment with the p-p38 MAPK inhibitor SB203580 was found to significantly augment the apoptosis induced by the combination of anti-CD20-hIFN-α and doxorubicin in all four cell lines (Fig. 4B). There was also augmentation of apoptosis by doxorubicin alone and doxorubicin+IFN-α+ rituximab. The augmented apoptosis by

treatment with anti-CD20-hIFN-α and doxorubicin suggested that it is regulated, in part, by p-p38 MAPK and that inhibition p-p38 MAPK by anti-CD20-hIFNα in 2F7 R and Ramos R participated in the chemosensitization observed.

Discussion

The present standard therapy for B-NHL is anti-CD20 mAb, rituximab, plus CHOP. Although it has been shown that the combination treatment with chemotherapy and rituximab improved the remissions and the overall survival in indolent B cell lymphomas (25), the majority of patients remains incurable and new therapeutic approaches are needed. The present study reports, for the first time, the novel finding demonstrating that treatment by anti-CD20-hIFN-α of rituximab-resistant (RR) B-NHL clones results in the inhibition of cell proliferation, induction of apoptosis and sensitization to drug-induced apop-tosis. These findings are reminiscent of our previous studies that demonstrated that intracellular inhibitors of survival pathways sensitized the RR B-NHL clones to apoptosis by various chemotherapeutic drugs. The observed response of the RR clones to anti-CD20-hIFN-α was specific to the fusion protein as neither anti-CD20, hIFN-α nor the combination was
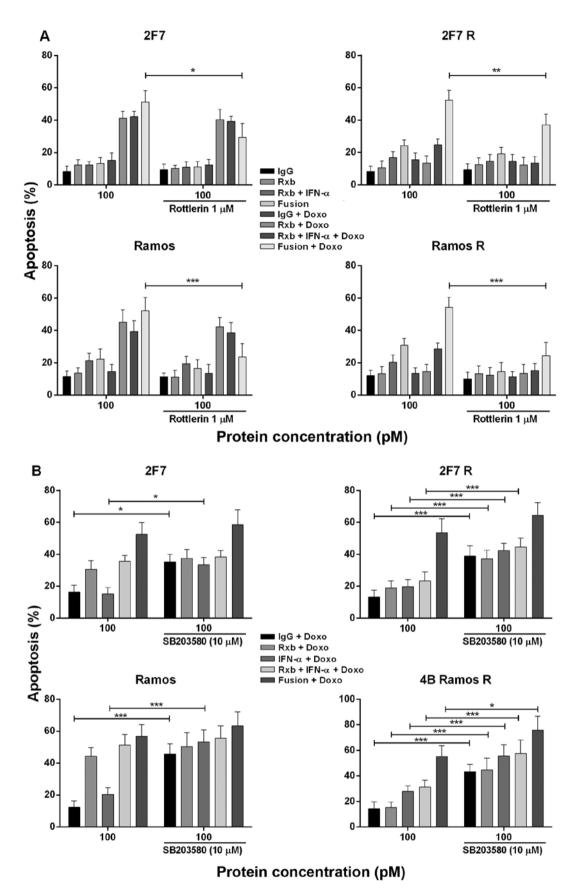
Figure 4. Effect of treatment of Ramos and Ramos R with the inhibitors Rottlerin or SB203580 on chemosensitization by anti-CD20/hIFN-α to drug apoptosis. (A) Rottlerin-mediated inhibitory effect on chemosensitivity of B-NHL cells by anti-CD20/IFN-α and CDDP. 2F7, 2F7 R, Ramos and Ramos R cells were cultured in the presence or absence of rotterin (1 μM) in combination with normal IgG control, rituximab or anti-CD20/IFN-α at indicated concentrations for 12 h and the treated with or without CDDP (10 μg/ml) for an additional 24 h and apoptosis was determined as described in Materials and methods. The graph displays the mean±SD of three independent experiments. (B) Treatment with SB203580 sensitizes the cells to apoptosis by doxorubicin. The B-NHL cell lines 2F7, 2F7 R. Ramos and Ramos R cells were treated with SB203580 (10 μM) for 24 h in the presence or absence of normal IgG, rituximab, anti-CD20/IFN-α and doxorubicin was added for an additional 24 h. Apoptosis was determined as described in Materials and methods. The data represent the mean±SD of 3 experiments.
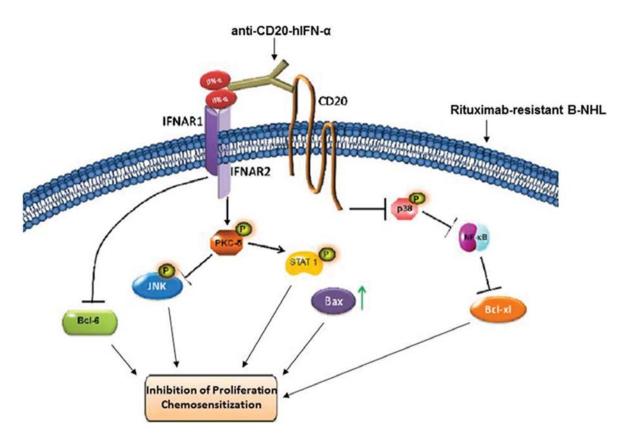
Figure 5. Schematic diagram representing the mechanism by which the anti-CD20-hIFN-α fusion protein sensitizes rituximab-resistant B-NHL cells to chemotherapeutic drugs. Treatment of the RR clones with hIFN-α resulted in the cell signaling mediated by both antiCD20 and by hIFN-α. The cell signaling by anti-CD20-hIFN-α resulted in the inhibition of p38 MAPK activity and inhibition of NF-κB activity. These resulted in the downstream inhibition of Bcl-XL and resulting in the inhibition of cell proliferation and chemosensitization. The cell signaling by hIFN-α resulted in the inhibition of PKC-δ activity and in the inhibition of JNK activity and induction of Stat 1 and Bax, thus resulting in the inhibition of proliferation and chemosensitization. Clearly, the effects of both anti-CD20 and hIFN-α merged and potentiated the inhibition of proliferation and chemosensitzation.

effective. The anti-CD20-hIFN-α-mediated antitumor effects on the RR clones resulted from the independent cell signaling pathways triggered by the fusion protein whereby both anti-CD20 and hIFN-α contributed to the antitumor activity. These findings provide a new potential therapeutic application of anti-CD20 IFN-α for the treatment of patients who are initially unresponsive or become refractory to treatment with rituximab monotherapy or combination of rituximab with chemotherapy.

As previously observed, the anti-CD20-hIFN-α fusion protein was shown to exert greater anti-proliferative and cytotoxic effects on rituximab sensitive lines compared with treatment with either single agent alone or with the combination of anti-CD20 and IFN-α (19). Of interest, the level of apoptosis achieved by anti-CD20-hIFN-α on the RR clones was higher than that achieved on the parental wild-type cells. Previously, we have reported that the potent activity of the fusion protein against human lymphoma cells is dependent on targeting IFN-α to the IFN-α receptor on the tumor cell surface. In addition, we have shown that the RR clones have high levels of IFN-αR compared to the wild-type parental cell lines (19).

We have reported that treatment of sensitive, but drug-resistant B-NHL cell lines, with rituximab were sensitized to various chemotherapeutic drugs and synergy was achieved (20). We now demonstrate that the combination of

anti-CD20-hIFN-α and chemotherapeutic drugs [CDDP, doxorubicin and bendamustine (Treanda)] resulted in reversal of rituximab and drug resistance of RR clones to apoptosis.

Examination of the intracellular pathways, that may be implicated in the sensitization, we showed that the treatment of RR clones with anti-CD20-hIFN-α resulted in the inhibition of both the p38 MAPK and NF-κB pathways. These findings are reminiscent to those previously observed following treatment of wild-type cells with rituximab (20,23,24). These findings suggested that treatment of RR cells with anti-CD20-hIFN-α resulted in the recovery of the cell signaling mediated by rituximab in the parental wild-type cells: this recovery, however, required that anti-CD20 is physically linked to IFN-α.

The interaction of IFN-α with its receptor results in the phosphorylation of receptor-associated janus kinases (JAK1 and Tyk2) and leading to the activation of signal transducer activators of transcription (STAT) (26,27) and in B lymphoma cells it induced the activation of JNK1 via PKC-δ (17). In addition, recent results identified type I IFNs as the first group of cytokines that can downregulate Bcl-6 expression directly in germinal center (GC) B cells (28). In B cells, Bcl-6 modulates both the activation and apoptosis, in addition to controlling DNA-damage sensing and response (29). The phosphorylation of Bcl-6 protein induces its subsequent degradation by the ubiquitin-proteasome pathway (30,31). Inhibition of Bcl-6 arrests the proliferation and induces apoptosis in B-NHL

cell lines (32), through the regulation of MAPK and NF-κB pathways; thus, this link between the inhibition of Bcl-6 and NF-κB can explain, in part, the inhibition of NF-κB mediated by the fusion protein.

We also explored the role IFN-α in cell signaling by the fusion protein. We observed induction of p-PKC-δ in both the wild-type and the RR cells by the fusion protein, but not by rituximab. Treatment with the PKC inhibitor rottlerin reversed the chemosensitizing effects of anti-CD20-hIFN-α, findings that are consistent with p-PKC-δ playing a role in the reversal of resistance. Clearly, IFN-α signals the cells by activation of p-JNK, however, in the present findings, there was no effect by the fusion protein on this activity in either the wild-type or the RR clones. In addition, treatment with anti-CD20-hIFN-α resulted in the inhibition of Bcl-XL and Bcl-6 and the induction of Bax, gene products that regulate apoptosis and that might play a direct role in the chemosensitization observed by the treatment of the RR clones with anti-CD20-hIFN-α.

In addition, the role of p38 MAPK inhibition by anti-CD20-hIFN-α on chemosensitization was corroborated by the use of the p38 inhibitor, SB203580, which mimicked anti-CD20-hIFN-α in the reversal of drug resistance in the RR clones. Therefore, the anti-CD20-hIFN-α-mediated inhibition of p38 MAPK and NF-κB and target genes such as Bcl-XL, Bcl-6, p-Stat-1 and p-PKC-δ appear to play a role in the reversal of drug resistance.

CD20 is a membrane-associated non-glycosylated phosphoprotein expressed on the surface of all mature B-cells, and it plays a key role in the development and differentiation of B-cells into plasma cells. The natural CD20 ligand is still unknown, its function is suspected to be similar to a calcium channel in the cell membrane (33). Recently, it was suggested that CD20 may play a central role in the generation of the T-cell-independent antibody response (34). In addition, recent data suggest that CD20-Ab or rituximab potentiates B lymphocytes for the production of interferon (35). In some studies, an additive or synergistic activity of INF with rituximab has been reported in the treatment of lymphomas (10). This relationship between CD20 and IFN may contribute to the efficacy of the anti-CD20-hIFN-α fusion protein. Alternatively, crosslinking with CD20 may prevent the internalization or downregulation of the IFN receptors, resulting in a more prolonged and effective IFN-α-induced signal. The mechanism by which anti-CD20-hIFN-α may be acting on the RR clones is not completely understood.

CD20 is constitutively associated to lipid rafts and this association depends on cholesterol and a short membrane-proximal cytoplasmic sequence (36). The presence of a dynamic interplay between the neutral glycosphingolipid CD77 and CD20 in B cell lymphomas has been reported (37). Cross-linking of CD77 with SLT-1 induces colocalization with BCR and CD20 in Ramos cells resulting in a regulation of Lyn kinase activity and an increase of the accessibility of the monoclonal Ab to CD20 (38). The above findings could indicate the possible interaction at the cell surface of CD20 and CD77 and this interaction can modulate the accessibility and the CD20 molecular signaling-mediated antibodies. CD77 has been implicated to play a role in IFN-α signal transduction (39). The roles of CD77 in IFN and CD20 signaling may be mediated through interactions between

CD77, the intracellular domains of CD20 and the IFNR-1 of the IFN-α receptor as observed on other proteins, such as CD19 (40). This observation suggests a role for the IFNR/CD77/CD20 interactions on the cytotoxic effect of the anti-CD20-hIFN-α on B-NHL cells. Anti-CD20-hIFN-α is more potent and effective than either IFN-α or anti-CD20 alone or their combination, suggesting that the cross-linking between the IFN receptor and CD20 signaling can potentiate its effect, probably mediated by CD77 interactions. We and others have previously reported that targeting IFN-α to CD20 on B-cell lymphomas resulted in high potency and efficacy *in vitro* and *in vivo* models (19,41,42).

The anti-CD20-IFN-α fusion protein induced the activation of PKC-δ, which is involved, in part, in the chemosensitization as shown here in the RR B-NHL cells treated with the PKC-δ inhibitor rottlerin. Furthermore, since PKC-δ was hyper-phosphorylated in Ramos R cells, its inhibition only partially decreased the drug-induced apoptosis suggesting that PKC-δ activation alone is not sufficient for the antiproliferative and proapoptotic actions of the anti-CD20-IFN-α fusion protein. Related studies have demonstrated that PKC-δ has multiple targets in response to apoptotic stimuli, including IFN-α (43-46). For example, it has been shown that PKC-δ mediated the activation of caspase-3 (45) and activation of Bax (47). Our results show that the treatment with the anti-CD20-IFNα fusion protein induced high expression of Bax in Ramos R cells. The classical type 1 IFNs pathway included JAK/STAT activation (26). For instance, IFN-α induced prolonged JNK1 activation (48) with subsequent Stat-1 Ser 727 phosphorylation at least through PKC-δ signaling (17,49). Stat-1 activation favors the induction of apoptosis (50). We analyzed the activation of JNK/Stat-1 pathway after fusion protein treatment and the activation of these proteins was observed, further suggesting that the classical JNK/Stat-1 activation plays a role in the action of the anti-CD20-IFN-α against RR B-NHL cells.

Clearly, the present findings (schematically diagrammed in Fig. 5) using RR B-NHL cell lines need to be validated with tumor derived RR B-NHL cells in both untreated patients and patients resistant to treatment. In addition, the findings need also to be validated *in vivo* on the antitumor effect of anti-CD20 in mice bearing RR tumor xenografts as monotherapy and in combination with drug therapy. The findings suggest new therapeutic options for the treatment of refractory B-NHL cells or CD20-mediated diseases that no longer respond to rituximab and its combination with chemotherapeutic drugs. Such an application clearly would be targeted and possibly less toxic overall.

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