Rituximab Enhances Radiation-Triggered Apoptosis in Non-Hodgkin’s Lymphoma Cells Via Caspase-dependent and – Independent Mechanisms

Ira SKVORTSOVA1*, Sergej SKVORTSOV2, Bela-Andre POPPER1, Alfred HAIDENBERGER1, Maria SAURER1, Andreas R. GUNKEL3, Heinz ZWIERZINA2 and Peter LUKAS1

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Rituximab (RTX), a chimeric human anti-CD20 monoclonal antibody, is currently employed in the treatment of malignant non-Hodgkin’s lymphoma (NHL) either alone or in combination with other cytotoxic approaches. The present study examines the effects of ionizing radiation in combination with RTX on proliferation and apoptosis development in B-lymphoma RL and Raji cells. RTX was used at a concentration of 10 μg/mL 24 hours prior to irradiation at a single dose of 9 Gy. CD20 expression, cell viability, apoptosis, mitochondrial membrane potential and apoptosis-related proteins were evaluated in the treated B cells. The constitutive level of CD20 expression in RL and Raji lymphoma cells did not play an essential role in RTX-induced cell growth delay. Both lymphoma cells showed similar inhibition of cell proliferation without apoptosis development in response to RTX treatment. Exposure to ionizing radiation induced cell growth delay and apoptosis in RL cells, whereas Raji cells showed moderate radio-resistance and activation of cell growth at 24 hours after irradiation, which was accompanied by increased radiation-triggered CD20 expression. The simultaneous exposure of lymphoma cells to ionizing radiation and RTX abrogated radioresistance of Raji cells and significantly enhanced cell growth delay and apoptosis in RL cells. X-linked inhibitor of apoptosis protein (XIAP) and the inducible form of heat shock protein 70 (Hsp70) were positively modulated by RTX in combination with ionizing radiation in order to induce apoptosis. Furthermore, it was demonstrated that mitochondrial membrane potential dissipation is not an essential component to induce apoptosis-inducing factor (AIF) maturation and apoptosis. Our results show that RTX-triggered enhancement of radiation-induced apoptosis and cell growth delay is achieved by modulation of proteins involved in programmed cell death.

INTRODUCTION

Radiation therapy (RT) plays an essential role in the management of non-Hodgkin’s lymphoma (NHL). RT alone may be used as a curative treatment for stages I and II in patients with indolent NHL. For more extensive and aggressive histologies, RT is used in combination with chemotherapeutic substances. While indolent and aggressive NHLs are responsive to RT and chemotherapy, 50–70% of patients relapse.1,2) As a result, there is a need for novel therapeutic strategies that may improve the outcome of NHL patients.

CD20, a 33- to 37-kDa surface non-glycosylated phosphoprotein, is expressed during early pre-B cell development until plasma cell differentiation.3) Similarly, the majority of human B cell-lineage malignancies express CD20.4) Therefore, CD20 is suggested as an attractive candidate to be a possible target for NHL treatment.5,6) The role of CD20 in B cell function and development is not completely known. It was demonstrated that CD20 participates in the regulation of B cell cycle progression7) and plays an important role in Ca2+ influx across the plasma membrane, sustaining intracellular Ca2+ concentration and allowing B cell activation.8)

Rituximab (RTX) is a genetically engineered chimeric murine-human monoclonal antibody (mAb) to the CD20 antigen and has demonstrated antitumor activity in various types of NHL.9,10) The mechanism of action of RTX has not been clearly elucidated. However, RTX has been shown in vitro to have anti-proliferative effects on lymphoma cells by cell cycle regulation11) with or without induction of apoptosis.12–14) Additionally, the efficacy of RTX in vivo appears to involve immune effector mechanisms such as antibody-
dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity.3,15)

Most importantly, it has been demonstrated that cells with increased CD20 expression are more sensitive to RTX16) and that the more prominent clinical benefit from RTX treatment is obtained by patients with higher expression of CD2017).

The strategy of using RTX in combination with conventional cytotoxic approaches is not completely new. Recent clinical and in vitro data have shown that RTX synergizes with chemotherapy by sensitizing lymphoma cells to the cytotoxic effects of chemotherapeutic compounds such as fludarabine, doxorubicin, cisplatin, glucocorticoids and retinoids.11,18–22) The first evidence of RTX-stipulated potentiation of anti-proliferative activities of ionizing radiation in lymphoma cell lines was recently demonstrated.23) Our results showed that RTX modulates radiation-triggered cell cycle distribution followed by apoptosis development. RTX potentiates radio-sensitivity of lymphoma cells by modulating the expression of the inhibitors of cyclin-dependent kinases p21/WAF1 and p27/KIP, and also down-regulation of bcl-XL and c-myc expression. However, the precise cellular mechanisms of apoptosis development are not yet examined. Here, we consider (1) the role of the CD20 receptor in cell growth delay stipulated by combined radiation and RTX treatment; (2) involvement of caspase-dependent and -independent pathways of apoptosis caused by ionizing radiation in combination with RTX; (3) participation of mitochondria and mitochondria-related apoptosis-inducing factor (AIF) in lymphoma cell death in response to combination treatment; (4) the modulation of the expression pattern of proteins associated with apoptosis induced by RTX and irradiation (p53, mdm-2, heat shock protein 70 (Hsp70), survivin, X-linked inhibitor of apoptosis protein (XIAP), Smac/DIABLO).

**MATERIALS AND METHODS**

**Cell culture**

CD20-positive follicular lymphoma cells (RL) carrying the t(14;18) and CD20-positive Burkitt EBV-infected lymphoma cells (Raji) were obtained from ATCC (Rockville, MD, USA) and cultured in RPMI 1640 medium ( Gibco BRL, Grand Island, NY, USA) at 37°C in 5% CO2. Also RL and Raji cells express mutant p53 protein [24,25]). Culture medium was supplemented with heat-inactivated fetal calf serum (10%), antibiotics (100 U/mL penicillin/streptomycin) and 2 mM L-glutamine ( all from Sigma-Aldrich, Vienna, Austria).

**Chemicals and irradiation treatment**

RTX (MabThera®), a murine-human chimeric anti-CD20 IgG1 class mAb (Roche Austria, Vienna, Austria), was used at a concentration of 10 μg/mL 24 hours prior to irradiation. This concentration of RTX was used because a further increase in RTX concentration to up to 100 μg/mL did not result in the enhancement of growth inhibition of either RL or Raji lymphoma cells (data not shown). Since our previous publication demonstrated that the combination of RTX at a dose of 10 μg/mL and ionizing radiation at a dose of 9 Gy resulted in ~50% cell growth delay in Raji cells, this dose of irradiation was selected for further experiments.23) Cells were irradiated with a single dose of 9 Gy (16 MV x-rays) using an Elekta Precise Linear Accelerator (Elekta Oncology Systems, UK) at a dose rate of approximately 1.8 Gy/min. Lymphoma cells treated with RTX and/or ionizing radiation were incubated at 37°C for 72 hours.

**Cell viability assay**

Cell viability was evaluated with the WST-1 Assay (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer’s instructions and as described previously.23) In brief, lymphoma cells were incubated in 96-well plates and treated with RTX, ionizing radiation or their combination as described above. WST-1 solution (20 μL) was added to each well containing lymphoma cell suspension in 200 μL of RPMI-1640 medium. Plates were then incubated at 37°C for 3 hours, and the optical density of each well was read at 430 nm using a microplate reader (SPECTRAFluor Plus, Tecan, Vienna, Austria).

**Apoptosis detection using flow cytometry**

To evaluate the induction of apoptosis, samples of cells were taken at relevant time points, resuspended in hypotonic fluorochrome solution (50 μg/mL propidium iodide (PI), 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100), stored in the dark for 30 minutes and kept at 4°C until FACS analysis. To determine DNA fragmentation, PI fluorescence of individual nuclei was performed using flow cytometry (FACScan; Becton Dickinson, San Jose, CA, USA) with an excitation wavelength of 488 nm and an emission wavelength of 670 nm. Results were represented as DNA histograms for further analysis using CellQuest software (Becton Dickinson, San Jose, CA, USA). The percentage of apoptotic cells was calculated by gating the sub-G1 region on the DNA content histogram.

**Inhibition of caspase activity by the substrate inhibitor z-VAD-fmk**

The pan-caspase inhibitor z-VAD-fmk (50 mM stock solution in DMSO, VWR International GmbH, Vienna, Austria) was added to the cultures at a concentration of 50 μM at the onset of the experiments, and again at 24 and 48 hours because of z-VAD-fmk degradation. DMSO (0.1% v/v) was used as a solvent control.

**Analysis of CD20 expression**

For analysis of CD20 expression by flow cytometry, 1 × 10⁶ cells per tube were pelleted at 200 ×g, washed twice with

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PBS and stained with 50 μg/mL mouse monoclonal IgG1 anti-CD20 PE-conjugated antibodies (BD Biosciences, San Jose, CA, USA). An equivalent amount of PE-conjugated IgG1 mAb (BD Biosciences, San Jose, CA, USA) was used as an isotype control. Propidium iodide (Sigma Chemical Co., St. Louis, MO, USA) was added to all assays to exclude dead cells. From each sample, ten thousand life events were collected by FACScan. Results are reported as the mean fluorescence intensity (MFI) of CD20 staining in lymphoma cell lines.

**Measurements of mitochondrial membrane potential difference**

Changes in mitochondrial membrane potential (ΔΨm) were evaluated using an ApoAlert™ Mitochondrial Membrane Sensor Kit (BD MitoSensor™ Reagent, BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions and using a FACScan cytometer. Briefly, lymphoma cells (1 × 10⁶ / mL) were incubated for 20 minutes at 37°C, with BD MitoSensor™ Reagent. From each sample, twenty five thousand events were collected to measure fluorescence on the FL-2 channel after suitable compensation. In healthy cells, MitoSensor™ is taken up by the mitochondria where it forms aggregates exhibiting intense red/orange fluorescence (FL-2 channel). In dysfunctional cells, the MitoSensor™ cannot aggregate in the mitochondria due to alterations in the membrane potential. The stain remains as monomers in the cytoplasm and exhibits green fluorescence (FL-1 channel) leading to reduced fluorescence on the FL-2 channel.

**Western blot analysis**

Western blot analysis was performed essentially as described previously [23]. In brief, from each sample, 40 μg of protein per lane was separated by 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Protein loading was controlled by Ponceau red staining of membranes. After blocking for one hour in Tris-buffered saline (TBS) supplemented with 5% nonfat milk and 0.1% Tween 20 (Sigma-Aldrich Vienna, Austria), membranes were incubated for one hour at room temperature with antibodies specific for either p53, PARP, Hsp70 (NeoMarkers, Fremont, CA, USA), Hsp72 (Stressgen Bioreagents, Victoria, BC, Canada), caspase-8, caspase-9, caspase-7, caspase-3, AIF, p53 phosphorylated at serine-15, Mdm-2 phosphorylated at serine-166, survivin, XIAP, Smac/DIABLO (Cell Signaling Technology, Inc., Beverly, MA, USA) or α-tubulin (Oncogene Research, Cambridge, MA, USA) as a loading control. Membranes were washed three times in TBS-Tween and incubated for one hour with horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham, Les Ulis, France). Immunodetection was performed using a chemiluminescence system (ECL, Amersham Biosciences, Vienna, Austria). The bands were measured with a computerized digital imaging system using GelScan 5.1 software (Serva Electrophoresis, Heidelberg, Germany). The Integrated Density Value (IDV) was obtained as a ratio of protein band density to α-tubulin band density after background correction.

**Fluorescence microscopy**

Treated and control cells were cytocentrifuged onto slides at 700 r.p.m. for 2 min (Cytospin; Shandon, Pittsburgh, PA, USA) and then fixed for 15 minutes with 4% paraformaldehyde in PBS. Cells were then washed with PBS and permeabilized for 15 minutes with 0.1% saponin in PBS containing 1% bovine serum albumin and 0.1% sodium azide. Cells were incubated at room temperature for one hour with rabbit anti-AIF (Cell Signaling Technology, Inc., Beverly, MA, USA) at a concentration of 5.0 μg/mL, washed twice then incubated with anti-rabbit immunoglobulin/FITC swine F(ab’)2 (DakoCytomation GmbH, Vienna, Austria) secondary antibody for 30 minutes. All incubations were performed at room temperature. After washing, cells were analyzed by fluorescence microscopy (Olympus IX71, Olympus Optical GmbH, Hamburg, Germany) using a blue filter of 470–490 nm.

**Transfection of short interfering RNA and treatment with RTX and ionizing radiation**

Synthesized AIF- and Hsc70-specific 20–25 nucleotide short RNA and scrambled siRNA (negative) controls (siRNA-A: sc-37007) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Cells (25 × 10⁴ per well) were seeded and cultured for 24 hours in RPMI 1640 medium without antibiotics at 37°C in 5% CO₂. Just prior to transfection, cells were washed with Transfection Medium (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and pelleted by centrifugation at 200 × g for 5 minutes. Either AIF-, Hsc70-specific siRNA (10 μM) or scrambled siRNA (10 μM), siRNA Transfection Medium and siRNA Transfection Reagent (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were mixed and added to the cell pellets. After 7 hours at 37°C, cells were cultivated in the appropriate medium containing twice the normal concentration of FCS and antibiotics. Twenty-four hours after transfection, the knock-down levels of AIF and Hsc70 were determined by Western blot analysis and then cells were treated with RTX at a concentration of 10 μg/mL and 24 hours later with ionizing radiation (9 Gy) and then incubated for 72 hours at 37°C. Apoptosis development was determined as described in the Apoptosis detection using flow cytometry section.

**Statistical analysis**

All experiments were repeated at least 3 times. Results were expressed as the mean ± standard deviation of the
mean. All laboratory data were evaluated according to standard statistical methods and using commercially available computer programs such as Microsoft Excel 2000 (Microsoft Inc.) and SigmaPlot 8.0 (SPSS Inc., Chicago, IL, USA). Statistical differences were studied using the Student t-test. In all tests, \( p \) values less than 0.05 were considered statistically significant.

**RESULTS**

The modulation of CD20 expression in RL and Raji lymphoma cells followed by ionizing radiation

The constitutive expression of CD20 on the cell surface of RL and Raji lymphoma cells was various (Fig. 1). The MFI for CD20 staining of untreated RL cells averaged 214.04 ± 6.49. Raji cells showed a lower CD20 expression with the MFI measured as 165.39 ± 5.23.

To further determine whether the CD20 antigen in lymphoma cells was modulated by ionizing radiation, levels of CD20 expression were analyzed during 96 hours after radiation application. Maximum CD20 expression was registered after 24 hours of incubation following radiation exposure and demonstrated ~1.58-fold higher MFI than in untreated Raji cells. At 48 hours after irradiation, and after CD20 staining, Raji cells revealed an MFI comparable to the constitutive level of expression (179.11 ± 7.35 MFI in irradiated cells versus 165.39 ± 5.23 in untreated Raji cells). Whereas at 72 hours, the MFI became markedly lower (117.16 ± 4.29) than before treatment.

In contrast, ionizing radiation did not enhance CD20 expression in RL cells. Moreover, CD20 continuously decreased after treatment (178.52 ± 6.17, 108.06 ± 7.03 and 121.58 ± 2.63 MFI in radiation-treated RL cells at 24, 48 and 72 hours, respectively, versus 214.04 ± 6.49 in untreated RL cells).

**RTX enhances radiation-induced apoptosis**

Recently, we have shown that ionizing radiation was ~2.5-fold more active in RL cells than in Raji cells to initiate cell growth delay, whereas RTX alone caused a similar anti-proliferative effects in both NHL cell lines. Furthermore, stimulation of cell growth was observed at 24 hours in the irradiated Raji cells. Enhancement of Raji cell growth at this time point corresponded to the increased CD20 expression at 24 hours after irradiation (Fig. 1). RL and Raji cell viabilities significantly declined after RTX pretreatment followed by irradiation. Thus, the combination of RTX and radiation resulted in 14.9% ± 2.87 cell viability for RL cells and 54.3% ± 9.75 of cell surviving for Raji cells at 72 hours.

In order to determine whether ionizing radiation and RTX influenced apoptosis development in RL and Raji lymphoma cells, we analyzed the rate of DNA fragmentation by flow cytometry as described in the Materials and Methods section. As shown in Fig. 2, irradiation alone induced apoptosis in both RL and Raji lymphoma cell lines (40.79% ± 3.44 and 25.93% ± 4.22, respectively). In contrast, no significant apoptosis induction was observed in the RL and Raji cells incubated with RTX alone (11.01% ± 2.46 versus 6.17% ± 3.61 in untreated cells; 9.35% ± 2.54 versus 6.77% ± 2.91 in untreated cells, respectively). Pretreatment with RTX enhanced the radiation-induced apoptosis response in each cell line. Of the RL and Raji cells, 55.95% ± 5.22 and 44.68% ± 5.44 were seen to be apoptotic, respectively.

To determine whether RTX- and radiation-triggered apoptosis was caspase-dependent or caspase-independent, the effect of each cell treatment mode was analyzed in RL and Raji lymphoma cells pretreated with pan-caspase inhibitor z-VAD-fmk. The percentage of apoptosis did not change considerably in response to RTX in either the RL or Raji cells pre-treated with 50 \( \mu \)M z-VAD-fmk. Cell death was markedly, but not completely inhibited by z-VAD-fmk at 72 hours following irradiation in RL and Raji cells (21.02% ± 1.27 in z-VAD-fmk pre-treated RL cells versus 40.79% ± 3.44 apoptosis in the irradiated RL cells; 13.67% ± 6.22 in z-VAD-fmk pre-treated Raji cells versus 25.93% ± 4.22 apoptosis in the irradiated Raji cells). Cell mortality stipulated by the combined treatment in the presence of z-VAD-fmk was also decreased in both cell lines at 72 hours (35.33% ± 4.19 % in RL cells and 15.69% ± 5.44 % in Raji cells).

**RTX modulates the expression of radiation-induced apoptosis-related proteins, caspase-initiators and caspase-effectors**

As indicated in Fig. 3, activation of caspase-8 upon treat-
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ment with RTX and ionizing radiation was characterized by the release of the intermediate cleaved forms (p41/p43). Caspase-8 was completely but marginally poorly processed until its active subunit p18 in RL and Raji cells only after RTX exposure or combination treatment. RTX in combination with irradiation did not show an enhanced cleavage of caspase-8 compared to treatment with only RTX. The activation of procaspase-9 and the cleaved form of caspase-9 was more active than caspase-8 in response to RTX, irradiation and combination treatment in lymphoma cells. RL cells demonstrated more effective caspase-9 activation than was observed in Raji cells. Both RL and Raji cells revealed no difference in caspase-9 cleavage after pretreatment with RTX and following irradiation compared to only radiation exposure.

Next, the activation of caspase-effectors, such as caspase-3 and caspase-7, were investigated in RL and Raji lymphoma cells. RTX did not induce caspase-7 cleavage in RL cells. Ionizing radiation alone and the combination of RTX and radiation gently up-regulated the expression of the cleaved

![Fig. 2. RTX potentiates radiation-induced apoptosis in lymphoma cells. Apoptosis development in RL and Raji lymphoma cells treated with RTX (10 μg/mL) and/or ionizing radiation (9 Gy) and then incubated over 72 hours without or with z-VAD-fmk. Control samples were allowed to grow in complete medium RPMI 1640 alone. Cells were analyzed for DNA content by staining with PI. The percentage of apoptotic cells were determined by quantifying the sub-G1 population by FACS analysis. Results are shown as mean and standard deviation obtained from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001](image-url)
form of caspase-7 with combination treatment being slightly higher than irradiation alone. In contrast to RL cells, Raji cells demonstrated an impressive and significantly more prominent caspase-7 activation in response to the administration of RTX and irradiation alone or to the combination of the two.

When RL cells were treated with either RTX or ionizing radiation alone, they exhibited caspase-3 activation at 48 hours with further enhancement at 72 hours. In the Raji cells, caspase-3 cleavage was registered at 72 hours after RTX or radiation exposure. The combination of RTX and ionizing radiation in the RL cells resulted in enhanced cleavage of caspase-3 at the same times as did irradiation alone. Raji cells revealed accelerated caspase-3 activation 24 hours earlier than single agents did. It is necessary to note that in Raji cells caspase-3 cleavage was slower, but more active than in RL cells.

We also documented PARP cleavage accompanying caspase-3 activation in both investigated lymphoma cell lines. Similar to caspase-3 activation, PARP cleavage was slightly delayed and simultaneously more evidenced in Raji than in RL cells. Thus, RL cells revealed both full and cleaved forms of PARP, whereas Raji cells showed almost completely cleaved PARP.

Effects of RTX and ionizing radiation on mitochondrial pathway of apoptosis

Next, we analyzed changes in mitochondrial membrane potential (Δψm) in the treated lymphoma cells. As it is shown in Fig. 4, neither RTX, ionizing radiation nor the combination of RTX and irradiation significantly altered mitochondrial membrane potential in RL cells. In contrast, Raji cells revealed detectable breakdown of Δψm during treatment with RTX and ionizing radiation. The combination of RTX and irradiation showed more prominent Δψm dissipation than was observed in Raji cells after either RTX or radiation exposure.

RTX regulates the expression of survivin, XIAP, AIF and Hsp70 participating in the radiation-triggered apoptosis

Following RTX, radiation or combination treatment of RL cells an up-regulation of p53 was evident at 24 hours and more pronounced at 48 hours, while declining slightly at 72 hours (Fig. 5A). The delicate phosphorylation of p53 at serine-15 (pSer15-p53) was registered in RL cells at 24 hours after RTX exposure. The combination of RTX and ionizing radiation strikingly up-regulated pSer15-p53 in the RL follicular lymphoma cells. In contrast, Raji cells showed no evident changes in total p53 expression after RTX treatment, whereas the level of pSer15-p53 was enhanced. While ionizing radiation slightly up-regulated both total p53 and pSer15-p53 in Raji cells, the combination of ionizing radiation and RTX caused weak down-regulation of the total p53 and significant up-regulation of pSer15-p53 at 72 hours.

Mdm-2, an ubiquitin ligase for p53, plays a central role in regulation of the stability of p53 via Mdm-2 phosphorylation at serine-166 (pSer166-Mdm-2).26 It was found that pSer166-Mdm-2 expression was just slightly induced following RTX in RL and Raji cells. Ionizing radiation caused more prominent up-regulation of pSer166-Mdm-2 in both cell lines. The combination of RTX and ionizing radiation
led to the progressive decreasing of pSer166-Mdm-2 with the reciprocal enhancement of pSer15-p53 expression in both lymphoma cell lines.

Next, it was found that RTX temporarily enhanced expression of survivin at 24 hours, whereas ionizing radiation and combination treatment triggered the constant up-regulation of survivin in RL cells. Raji cells revealed increased expression of survivin only in response to ionizing radiation and the combination of RTX with irradiation. Unlike survivin, XIAP expression was down-regulated at 72 hours after treatment.
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Fig. 6. Attenuation of AIF and Hsc70 expression by siRNA treatment modulates apoptosis development in lymphoma cells treated with radiation in combination with RTX. RL and Raji cells were pre-treated with either siRNA-AIF, siRNA-Hsc70 or scrambled siRNA. The knock-down levels of AIF and Hsc70 were determined by Western blot analysis: lane 1, untreated cells; lane 2, cells treated with scrambled siRNA; lane 3, cells treated with the appropriate siRNA. Twenty-four hours after transfection cells were treated with RTX (10 µg/mL) in combination with ionizing radiation (9 Gy) as described in the Materials and Methods section. After combination treatment, cells were incubated over 72 hours and apoptosis development was evaluated. Cells treated with scrambled siRNA were used as control. *p < 0.05; **p < 0.01; ***p < 0.001 versus scrambled siRNA-treated cells.

Fig. 5. Effects of ionizing radiation and/or RTX on apoptosis-related protein expression. (A) RL and Raji cells were treated with RTX (10 µg/mL), ionizing radiation (9 Gy), or a combination of RTX and irradiation. P53, pSer15-p53, pSer166-Mdm2, survivin, XIAP, Smac/DIABLO, AIF and Hsp 72 expression was assessed after appropriate treatment. Untreated cells were used as a control (C). Lane 1, RTX, 10 µg/mL (24 h); Lane 2, RTX 10 µg/mL (48 h); Lane 3, RTX 10 µg/mL (72 h); Lane 4, ionizing radiation, 9 Gy (24 h); Lane 5, ionizing radiation, 9 Gy (48 h); Lane 6, ionizing radiation, 9 Gy (72 h); Lane 7, RTX plus ionizing radiation (24 h); Lane 8, RTX plus ionizing radiation (48 h); Lane 9, RTX plus ionizing radiation (72 h). α-tubulin was used as loading control. (B) Densitometric analysis of the immunoreactive bands showing AIF and Hsp72 levels in RL and Raji lymphoma cells after treatment with RTX (10 µg/mL), ionizing radiation (9 Gy) or combination of RTX (10 µg/mL) and irradiation (9 Gy). IDV was calculated as a ratio of either AIF or Hsp72 protein band density to α-tubulin band density after background correction. Data are given as mean and standard deviation obtained from three independent experiments. (C) AIF translocation from mitochondria to nuclei in lymphoma cells after combination treatment. The cells were treated with combination of RTX (10 µg/mL) and irradiation (9 Gy) and then incubated for 6 hours, fixed and incubated with anti-AIF antibody. RL and Raji cells showed translocation of AIF into the nuclei.
initial level of the expression of the AIF-precursor in the untreated RL cells was ~1.4-fold higher in RL cells than in Raji cells (Fig. 5B). The continuously increasing expression of the mature form of AIF (57 kDa) was registered after RTX treatment in RL cells (0.832, 1.064, 1.170 IDV at 24, 48 and 72 hours, respectively). Ionizing radiation also triggered the mature form of AIF (0.724, 0.891 and 1.230 IDV at 24, 48 and 72 hours, respectively). RTX in combination with ionizing radiation caused a more rapid and enhanced AIF maturation in comparison with treatments using RTX and irradiation alone (0.919, 1.083, 1.436 IDV at 24, 48 and 72 hours, respectively). Raji cells did not reveal the same level of AIF maturation in response to treatment. Thus, in Raji cells maximum expression of the mature AIF was documented at 72 hours after RTX (0.559 IDV), ionizing radiation (0.671 IDV) and RTX in combination with irradiation (0.684 IDV). RL and Raji cells demonstrated mitochondrial nuclear translocation of AIF already at 6 hours after treatment with RTX in combination with ionizing radiation (Fig. 5C).

Hsp70, which is a protein that protects cells from caspase-dependent and caspase-independent cell death, was also analyzed in the untreated and treated RL and Raji cells. The level of the inducible form of Hsp70 (Hsp72) were more pronounced in the untreated Raji cells than in RL cells (Fig. 6B). RTX or ionizing radiation just slightly suppressed the expression of Hsp72 at 72 hours following treatment. However, the combination of RTX and irradiation markedly decreased the isoform of Hsp70 in lymphoma cells (0.089 IDV at 72 hours in treated RL cells versus 0.140 IDV in control RL cells and 0.038 IDV at 72 hours in treated Raji cells versus 0.398 IDV in control Raji cells).

Treatment by AIF or Hsc70 short interfering RNA resulted in a modulation of apoptosis induced by ionizing radiation in combination with RTX

To determine whether AIF or Hsc70 inhibition by the appropriate siRNA modulates apoptosis induced by irradiation either alone or in combination with RTX, we compared the apoptosis rate in RL and Raji lymphoma cells transfected with specific siRNA or scrambled control siRNA. Transfection of the scrambled control siRNA showed no effect on the level of either AIF or Hsc70 when compared with the non-transfected control. Treatment with siRNA-AIF and the subsequent irradiation resulted in a significant reduction of apoptosis development compared to irradiated lymphoma cells transfected with scrambled control siRNA (Fig. 6).

Thus, at 72 hours after irradiation the level of apoptosis was ~ 2.8-fold and ~ 2.6-fold less in siRNA-AIF-transfected RL and Raji cells, respectively, than in control siRNA-transfected lymphoma cells. Similar results were observed when AIF-specific- and non-specific-transfected cells were treated with irradiation in combination with RTX. siRNA-AIF transfected RL and Raji cells showed a reduced apoptosis rate at 72 hours after combination treatment by ~ 2.3-fold and ~ 3.7-fold, respectively. Cell transfection with siRNA-Hsc70 significantly enhanced apoptosis development in both cell lines after radiation and combination treatment exposure. Radiation-induced apoptosis was enhanced by ~ 1.4-fold and ~ 1.9-fold in siRNA-Hsc70-transfected RL and Raji cells, respectively. Combination treatment revealed a similar apoptosis increasing in siRNA-Hsc70-transfected compared to control siRNA-transfected lymphoma cells (~ 1.2-fold for RL cells and ~ 1.3-fold for Raji cells at 72 hours after combination treatment).

**DISCUSSION**

In this report, we have presented in vitro evidence which shows that the combination of RTX and ionizing radiation results in a radiosensitization of lymphoma cells. The cellular mechanisms of apoptosis and cell growth delay that follow ionizing radiation and RTX interaction in CD20-positive RL and Raji lymphoma cells were examined.

We explored the use of follicular and Burkitt lymphoma cell lines, RL and Raji, as representatives of indolent and aggressive forms of lymphoma, respectively. Our results showed that the stimulation of Raji cell proliferation was closely related to the radiation-triggered CD20 up-regulation also observed at 24 hours after ionizing radiation exposure. In contrast, RL cells revealed neither CD20 up-regulation nor enhancement of cell proliferation after irradiation. Since CD20 significantly contributes to the cell cycle progression and proliferation, it is possible to assume that CD20 plays an important role in the radiation-caused intracellular events leading to the increased cell survival and proliferation. Despite the possible negative regulation of radiosensitivity, CD20 is considered as a positive prognostic factor for RTX efficacy in lymphoma patients. Our results did not support this suggestion. Thus, the constitutive level of CD20 expression was approximately 1.3-fold higher in RL than in Raji cells. However, RTX caused a similar delay in cell proliferation in both lymphoma cell lines. As regards RTX-triggered modulation of radiosensitivity of lymphoma cells, we here show that RTX markedly enhances the radio-induced anti-proliferative effect in RL and Raji cells by approximately 2.4-fold and 1.6-fold, respectively.

Unlike RTX, which did not induce apoptosis in RL or Raji cells, radiation caused apoptosis in both lymphoma cell lines. Thus, we demonstrated that the level of radiation-mediated apoptosis was ~1.57-fold higher in RL than in Raji cells. RTX in combination with irradiation significantly increased radio-triggered apoptosis in RL and Raji cells by ~1.4-fold and ~1.7-fold, respectively. It is generally accepted that radiation-induced apoptosis develops via both caspase-dependent and caspase-independent pathways. We have confirmed that both mechanisms of cell death are presented in lymphoma cells treated with either ionizing...
radiation or RTX in combination with irradiation.

It was demonstrated that z-VAD-fmk, a broad-spectrum inhibitor of caspases, partially prevented cell death triggered by irradiation alone or RTX in combination with ionizing radiation in both lymphoma cell lines. Raji cells revealed more prominent apoptosis inhibition in response to z-VAD-fmk application than RL cells. This suggests that the caspase-dependent pathway of cell death is more pronounced in Raji cells, whereas RL cells exhibit a more caspase-independent pathway of apoptosis. In our experimental model in response to treatment with RTX and irradiation, caspase-3 activation and subsequent PARP cleavage were preceded by the cleavage of caspase-9, the apical caspase of the intrinsic pathway. These findings suggest that caspase-9 may play the main role in the activation of the caspase executors, such as caspase-3 and caspase-7, while caspase-8 plays a marginal role in the development of cell death after exposure to RTX and radiation.

Our results are in accordance with other publications describing the dominating role of mitochondrial events in RTX- and radiation-triggered apoptosis.\(^{22,29,30}\) RTX especially markedly enhanced radiation-mediated activation of caspase-3 in RL and Raji cells. Since we have shown here that the induction of apoptosis triggered by ionizing radiation in combination with RTX was stipulated by mitochondrial events in the investigated lymphoma cells, we studied changes in mitochondrial membrane potential (\(\Delta \Psi_m\)). \(\Delta \Psi_m\) dissipation has been originally postulated to be among the early and obligate events in the apoptotic signaling pathway.\(^{31–33}\) However, our results showed invariable \(\Delta \Psi_m\) in RL cells despite the significant enhancement apoptosis rate after treatment with RTX in combination with irradiation. Perhaps such observations depend on the constitutive overexpression of the anti-apoptotic protein bcl-2 in RL cells, which is characterized by the t(14;18) translocation.\(^{24}\) Thus, it was reported that bcl-2 overexpression blocks only mitochondrial permeability changes, but not apoptosis.\(^{34,35}\) At that time, in Raji cells RTX, ionizing radiation or the combination of RTX and irradiation markedly dissipated \(\Delta \Psi_m\).

Loss of the mitochondrial membrane potential in Raji cells did not correlate with the apoptosis rate caused by RTX or ionizing radiation. Such phenomenon has already been described. Thus, Ly et al. (2003) affirm that changes in \(\Delta \Psi_m\) do not necessarily lead to apoptosis in all systems.\(^{31}\) Nevertheless, lymphoma cell treatment with RTX in combination with ionizing radiation resulted in mitochondrial events that preceded caspase activation. We found down-regulation of XIAP in RL cells, whereas in Raji cells it was continuously up-regulated upon combination of RTX and irradiation.

Survivin, another protein from the family of inhibitors of apoptosis, was overexpressed in both lymphoma cell lines during any kind of treatment. It is known, overexpression of human inhibitors of apoptosis, such as XIAP or survivin, suppress apoptosis in response to a number of stimuli, including those which activate the intrinsic pathway of apoptosis.\(^{36,37}\) Perhaps differences in XIAP down-regulation in the RTX-pretreated and irradiated lymphoma cells led to a reduced apoptosis rate in Raji cells. Another mitochondria-related protein, Smac/DIABLO, is able to interact with inhibitors of apoptosis and remove their inhibition of caspas-\(\text{es}^ {37–39}\).

It is considered that AIF, a 57-kDa flavoprotein, locating in the mitochondrial intermembrane space,\(^{40}\) translocates from mitochondria concurrently with the dissipation of \(\Delta \Psi_m\).\(^{41}\) The authors noted that overexpression of bcl-2, which blocks the opening pores in the mitochondrial membrane and thereby the loss of \(\Delta \Psi_m\), prevents the release of AIF. Despite bcl-2 overexpression and the absence of \(\Delta \Psi_m\) dissipation in RL cells, enhanced expression of the mature form of AIF and translocation of AIF from mitochondria to nucleus were registered after treatment with RTX and ionizing radiation. Raji cells also showed maturation and mitochondrial-nuclear translocation of AIF, but it was not as pronounced as in RL cells. It has been suggested that the loss of \(\Delta \Psi_m\) is not a determining factor for AIF maturation in RL cells. Recently, it was proposed that the release of AIF befalls to the caspase-independent\(^ {42,43}\) pathway of apoptosis. Indeed, our results confirmed this fact given that RL cells revealed a high apoptosis rate in response to the combination of RTX and irradiation that was not only partially abrogated after z-VAD-fmk application. Apoptosis development was accompanied by simultaneously enhanced AIF maturation.

The p53 tumor suppressor gene plays an important role during induction of apoptosis in cancer.\(^ {44}\) Our previous study has provided an evidence that up-regulation of p21/WAF1 expression and apoptosis development after treatment with RTX in combination with ionizing radiation are p53-independent in NHL cells.\(^ {23}\)

In this report, we have complementarily studied the phosphorylation of p53 at serine-15. Ser-15 is a functionally important residue within the p53 N-terminal region,\(^ {45,46}\) and phosphorylation of p53 at Ser-15 characterizes an early cellular response to a variety of genotoxic stresses.\(^ {47–49}\) Indeed, despite the mutations in p53 in both lymphoma cell lines,\(^ {24,25}\) an active phosphorylation at Ser-15 accompanied by the suppressed phosphorylation of Mdm-2 was observed after irradiation in combination with RTX, whereas neither RTX nor ionizing radiation revealed such levels of p53 phosphorylation. Activated p53 contributes to apoptosis by down-regulation of survivin\(^ {40}\) and transcription-independent mechanisms, likely via translocation to the mitochondria, where p53 binds to and inhibits bcl-2 family members.\(^ {51,52}\) Although we have demonstrated bcl-XL down-regulation in lymphoma cells upon combination treatment with RTX and ionizing radiation, the modulation of expression of survivin, bcl-2 and bax was not observed.\(^ {23}\) Our results showed that mutant p53 protein is considered as a non-obligatory component in the sequence of events leading to apoptosis devel-
opment in NHL cells in response to combination of RTX and ionizing radiation.

Heat shock proteins have been shown to disturb apoptosis signaling, suggesting that they might play a role as mediators of resistance to ionizing radiation.53,54) The inducible isoform of Hsp70 (Hsp72) has been shown to inhibit apoptosis by preventing the recruitment of pro-caspase-9 and pro-caspase-3 to the apoptosome complex,55,56) binding and sequestering AIF, preventing the release of AIF,57,58) forming stable immunocomplexes with the mutant form of p53 and sequestering the wild form of p5359) and binding with c-myc.60) Indeed, Raji cells revealing an increased expression of both forms of Hsp70 simultaneously showed the delayed cleavage of the caspase-initiators and caspase-effectors and p53 phosphorylation and decreased AIF maturation compared to RL cells. RTX treatment in combination with irradiation markedly down-regulated expression of the inducible form Hsp72 by ~10.5-fold in Raji cells and ~1.6-fold in RL cells, which possibly contributed to the abrogation of radioresistance of Raji cells and the enhancement of the apoptosis rate in both lymphoma cell lines. The implications regarding the role of AIF and Hsp70 in the development of radiation-stipulated cell death were proven in our experiments with siRNA. Attenuation of AIF expression in lymphoma cells reduced but did not abolish apoptosis induced by irradiation or combination treatment. Transfection with Hsp70 (constitutive form of Hsp70) siRNA significantly increased the apoptosis rate in lymphoma cells treated with ionizing radiation alone or in combination with RTX. These observations demonstrated that both proteins, AIF and Hsp70 seemed to be important in the RTX-triggered modulation of radio-induced apoptosis. The previous and current work allowed us to propose the possible mechanisms of RTX-stipulated potentiation of radiation-induced lymphoma cell death (Fig. 7). Despite the demonstrated modulation of various proteins upon combination treatment of lymphoma cells, the possible underlying mechanisms by which RTX potentiates cytotoxic effects of ionizing radiation still need to be carefully investigated.

The present results highlight the value of the combination of ionizing radiation with RTX. We have shown a strong synergism between RTX and irradiation to cause cell growth delay and apoptosis in both radiosensitive and radioresistant lymphoma cell lines. The RTX-triggered enhancement of radiation-induced apoptosis is achieved by modulation proteins involved in programmed cell death. The results of this study have a potential clinical relevance because they suggest that it may be possible to potentiate the anti-tumor activity of ionizing radiation by combining radiotherapy with RTX in NHL patients.

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