Breaking Chemoresistance and Radioresistance with [^{213}Bi]anti-CD45 Antibodies in Leukemia Cells

Claudia Friesen,[1] Gerhard Glatting,[1] Bernd Koop,[1] Klaus Schwarz,[2] Alfred Morgenstern,[4] Christos Apostolidis,[4] Klaus-Michael Debatin,[3] and Sven N. Reske[1]

[1]Nuclear Medicine Clinic, [2]Department of Transfusion Medicine, and [3]University Children's Hospital, University of Ulm, Ulm, Germany and [4]European Commission, Joint Research Centre, Institute for Transuranium Elements, Karlsruhe, Germany

Abstract

Chemoresistance and radioresistance are considered one of the primary reasons for therapeutic failure in leukemias and solid tumors. Targeted radiotherapy using monoclonal antibodies radiolabeled with α-particles is a promising treatment approach for high-risk leukemia. We found that targeted radiotherapy using monoclonal CD45 antibodies radiolabeled with the α-emitter ^{213}Bi ([^{213}Bi]anti-CD45) induces apoptosis, activates apoptosis pathways, and breaks β-irradiation–, γ-irradiation–, doxorubicin-, and apoptosis-resistance in leukemia cells. In contrast to β-irradiation–, γ-irradiation–, and doxorubicin-mediated apoptosis and DNA damage, [^{213}Bi]anti-CD45–induced DNA damage was not repaired, and apoptosis was not inhibited by the nonhomologous end-joining DNA repair mechanism. Depending on the activation of caspase-3, caspase-8, and caspase-9, [^{213}Bi]anti-CD45 activated apoptosis pathways in leukemia cells through the mitochondrial pathway but independent of CD95 receptor/CD95 ligand interaction. Furthermore, [^{213}Bi]anti-CD45 reversed deficient activation of caspase-3, caspase-8, and caspase-9, deficient cleavage of poly(ADP-ribose) polymerase, and deficient activation of mitochondria in chemoresistant and in radioresistant and apoptosis-resistant leukemia cells. These findings show that [^{213}Bi]anti-CD45 is a promising therapeutic agent to break chemoresistance and radioresistance by overcoming DNA repair mechanisms in leukemia cells and provide the foundation for discovery of novel anticancer compounds. [Cancer Res 2007;67(5):1950–8]

Introduction

One of the primary causes for therapeutic failure in radiotherapy and chemotherapy of leukemia and solid tumors is the resistance to radiation and chemotherapeutic drugs (1). Attempts to improve the results of chemotherapy and radiotherapy by increasing the total radiation absorbed dose, by increasing the concentration of chemotherapeutic drugs, or by changing chemotherapeutic drugs have only partly been successful (1). Various approaches using native monoclonal antibodies, immunotoxins and radioimmunoconjugates have emerged as promising treatment strategies. Selective targeting of tumor cells with radiolabeled antibodies for systemic radioimmunotherapy was developed, which reduces toxicity, the limiting factor in treatment of high-risk leukemia and lymphoma (2–6). Development of effective strategies for radioimmunotherapy depends on the radionuclide and particularly on the type of emitted radiation (4). Selective targeting of tumor cells with radiolabeled antibodies for systemic radioimmunotherapy was developed using β-emitting nuclides, such ^{131}I, ^{90}Y, ^{188}Re, or ^{177}Lu, and antibodies, such as anti-CD45, anti-CD66, and anti-CD33 (4). These seem to provide a well-tolerated, safe, and effective therapeutic option for patients with high-risk leukemia (4). There has been an emerging interest in using α-particles, such as ^{213}Bi, for radioimmunotherapy (7–9). Because of the short path length (50-80 μm) and high linear energy transfer (~100 keV/μm) of α-particles emitting radioisotopes, targeted α-particle therapy offers the potential for higher biological effectiveness and more specific tumor cell kill with less damage to surrounding normal tissues compared with β-emitters and external radiation (9). These properties make targeted α-particle therapy ideal for elimination of minimal residual or micrometastatic disease (8, 9). Another potential application is in treating lymphoma and leukemia (9). However, the mechanism of α-particle–induced cell kill in leukemia cells is poorly understood at the molecular level.

Anticancer drugs, β-irradiation, and γ-irradiation have been shown to activate apoptosis pathways in leukemias and solid tumors (10–18). Caspases play a critical role in apoptosis induction (18, 19). The apoptotic caspases are divided in two classes: effector caspases, such as caspase-3, caspase-6, and caspase-7, and initiator caspases, such as caspase-8 and caspase-9 (18). Effector caspases are responsible for the cleavages that disassemble the cell, and initiator caspases initiate the proteolytic cascade (19). Two distinct pathways upstream of the caspase cascade have been identified: the ligand/receptor–driven amplifier pathway, such as the CD95 receptor/CD95 ligand system, and the direct mitochondrial activation pathway without ligand/receptor interaction resulting in cleavage and activation of effector caspases (20). Anticancer drugs, β-irradiation, and γ-irradiation have been shown to activate the ligand/receptor pathway and the mitochondrial activation pathway in leukemia and tumor cells (10–14, 18, 20). The mitochondrial pathway can be activated through the ligand/receptor system or directly by apoptotic stimuli (19, 20). Caspase-9, which is released during apoptosis from mitochondria into the cytosol, triggers caspase-3 activation through the formation of a cytochrome *c*/Apaf-1/caspase-9–containing apoptosome complex (19, 21). Bcl-2 family members regulate the mitochondrial pathway (19, 22). Antiapoptotic Bcl-2 family members, such as Bcl-2 or Bcl-x_L, inhibit the cytochrome *c* release by modulating the ability of proapoptotic family members, such as Bax and Bak, to open the voltage dependent ion channels in the outer mitochondrial membrane (19, 22). The effects of the Bcl-2 family members depend on the balance between proapoptotic and antiapoptotic family members (19, 22).

Radiation and anticancer drugs, such as cyclophosphamide, cisplatin, doxorubicin, or etoposide, induce DNA damage (23–25).

Requests for reprints: Claudia Friesen, Nuclear Medicine Clinic, University of Ulm, Robert-Koch-Straße 8, D-89081 Ulm, Germany. Phone: 49-731-500-61342; Fax: 49-731-500-61302; E-mail: claudia.friesen@uni-ulm.de.

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The nonhomologous end-joining pathway (NHEJ) is the predominant mechanism to repair DNA double-strand breaks (DSBs) in mammalian cells, with DNA-PK and ligase IV playing critical roles (26–28). The first step in DSB repair by NHEJ is the binding of Ku70/80 to the damaged DNA followed by the recruitment of the catalytic subunit DNA-PK, thereby forming the active DNA-PK holoenzyme (23). One of the targets of DNA-PK is XRCC4, which forms a stable complex with DNA ligase IV (ligase IV), joining the ends of broken DNA strands (23). In the absence of DNA-PK or ligase IV, NHEJ fails to repair DSBs (26–31).

In the present study, we investigate the molecular mechanisms by which anti-CD45 antibodies labeled with ^{213}Bi ([^{213}Bi]anti-CD45) induced cell death in leukemia cells and the effect of [^{213}Bi]anti-CD45 on β-irradiation–, γ-irradiation–, apoptosis-, and doxorubicin-resistant leukemia cells. We show that ^{213}Bi bound to CD45 antibodies breaks radioresistance and chemoresistance in leukemia cells, and we provide the molecular requirements for overcoming this resistance and for α-particle–induced cell killing. Understanding the molecular mechanisms induced by α-irradiation, such as ^{213}Bi, in sensitive and resistant leukemia cells may provide important implications for the further development of novel treatment options in leukemia therapy.

Materials and Methods

Cell Culture

The human myeloid leukemia cell line HL-60, the human lymphoblastic leukemia T-cell line CEM, and the human pre-B-lineage leukemia cell line Nalm6 were grown in RPMI 1640 (GIBCO, Invitrogen, Karlsruhe, Germany) containing 10% FCS (Biochrom, Berlin, Germany), 10 mmol/L HEPES (pH 7.3; Biochrom), 100 units/mL penicillin (GIBCO), 100 μg/mL streptomycin (GIBCO), and 2 mmol/L L-glutamine (Biochrom) at 37°C and 5% CO_2. HL-60^{gammaR} cells are resistant to radiation absorbed dose up to 10 Gy of γ-irradiation (14); HL-60^{betaR} cells are resistant to activities up to 876 kBq/mL of β-irradiation (^{90}Y); CEM^{CD95R} cells are resistant to 1 μg/mL anti-CD95 (12); and CEM^{DOXOR} cells are resistant to 0.1 μg/mL doxorubicin (13). HL-60, HL-60^{gammaR}, HL-60^{betaR}, CEM, CEM^{DOXOR}, and CEM^{CD95R} are CD45 positive. Nalm6 (DNA ligase IV +/+, +/−, −/−) cells were obtained from M. Lieber (USC, Los Angeles, CA) and were grown as described above for Nalm6; 1.5 mg/mL G418 (GIBCO) was added to the medium of the DNA ligase IV heterozygotic (+/−) cells; 1.5 mg/mL G418 and 4 μg/mL puromycin (Sigma, Taufkirchen, Germany) were used for the cultivation of the DNA ligase IV–deficient (−/−) cells.

All cell lines used in this study were suspension cell lines.

Cells (2×10^5/mL) were α-irradiated with ^{213}Bi or [^{213}Bi]anti-CD45, β-irradiated with [^{90}Y]anti-CD45, and γ-irradiated in 150 mL flasks (100 mL). Twenty-four hours after treatment with different activities of ^{213}Bi, [^{213}Bi]anti-CD45, and [^{90}Y]anti-CD45, cells were washed twice with RPMI 1640. After washing, cells were resuspended in medium and incubated at 37°C for 24, 48, or 72 h.

Dosimetry, Radiation Exposure, and Radionuclides

For α-particles, ^{213}Bi was used with a half-life of 45.6 min and decays by a branched pathway by α- and β-emissions to stable ^{209}Bi (32). Of the emitted energy, 80% is deposited by α-particles with energies of 8.4 MeV (79%) and 5.9 MeV (1%; ref. 32). Activities were applied in 100 mL within 24 h using 0.75, 2.25, 7.5, 15, 22.5, and 74.6 MBq of ^{213}Bi, respectively, and correspond to radiation absorbed doses of 0.05, 0.15, 0.5, 1, 1.5, and 5 Gy.

For β-particles, ^{90}Y was used with a half-life of 64 h and decays by β-emission (100%, 2.3 MeV) to stable ^{90}Zr (33). Activities were applied in 100 mL within 24 h using 8.8, 26.3, and 87.6 MBq of ^{90}Y, respectively, and correspond to radiation absorbed doses of 1, 3, and 10 Gy.

γ-Irradiation of cells were done with a Philips MG320, operated at 300 kV with a 0.6-mm copper filter (1.9 mm copper half value layer). The dose rate was ~0.4 Gy/min; the inhomogeneity within the field was lower than ±1%. The applied doses were measured with a transmission chamber (PTW, Freiburg, Germany), which was referenced against a Farmer 2570 device equipped with a 0.6-cc sensor (Nuclear Enterprises, Edinburgh, United Kingdom).

Monoclonal CD45 Antibody (anti-CD45)

The rat IgG2a monoclonal antibody YAML568 recognizes the CD45 antigen present on human leukocytes and was provided by Dr. G. Hale (Sir William Dunn School of Pathology, Oxford, United Kingdom; refs. 34, 35).

Conjugation of Chelate to Anti-CD45 and Radiolabeling with ^{213}Bi or ^{90}Y

Anti–CD45-diethylenetriaminepentaacetic acid (antibody conjugate). Anti-CD45 in HEPES-buffer (50 mmol/L, pH 9.5) was conjugated with *p*-SCN-CHX-A″-diethylenetriaminepentaacetic acid (DTPA) and *p*-SCN-MX-DTPA (Macrocyclics, Dallas, TX), respectively, using an initial molar ratio of 1:5 for 20 h at 25°C. After the reaction, excessive chelator was removed via PD10 desalting column chromatography. The average number of chelates per antibody was determined by the yttrium-arsenazo(III) spectrophotometric method and found to be 3 to 4 (36).

[^{213}Bi]anti-CD45. ^{213}Bi was produced from a ^{225}Ac/^{213}Bi generator system (Institute for Transuranium Elements, Karlsruhe, Germany; ref. 37) and was incubated with antibody conjugate for 5 min. The radiolabeled antibody was purified by PD10 desalting column chromatography.

[^{90}Y]anti-CD45. [^{90}Y]YCl_3 in 0.05 mol/L HCl (AEA, Braunschweig, Germany) was diluted with 0.05 mol/L sodium citrate/sodium acetate buffer (pH 5.5) followed incubation with antibody conjugate for 5 min. The antibody was diluted with 7.5% HSA-PBS buffered solution.

CD45 Binding

Cells were stained with 2 μg of anti-CD45, anti-CD45-DTPA (conjugated antibody), and [^{213}Bi]anti-CD45 followed by a secondary antibody goat-$F(ab')_2$ anti-rat IgG-phycoerythrin (1:20, Southern Biotechnology Associates, Inc., Birmingham, AL). Immunofluorescence analysis was done on a FACSCalibur flow cytometer (BD, Heidelberg, Germany). Rat IgG2a isotype control antibody (Southern Biotechnology Associates) was used for detecting unspecific binding of anti-CD45.

Induction of Apoptosis and Cell Cycle

Quantification of apoptosis and cell cycle analysis were done by flow cytometry as described (14, 38, 39). To determine apoptosis, cells were lysed with Nicoletti buffer containing 0.1% sodium citrate plus 0.1% Triton X-100 and 50 μg/mL propidium iodide at 4°C (38). The percentage of apoptotic cells was measured by hypodiploid DNA ($subG_1$) by the Nicoletti method (38) or forward scatter/side scatter analysis (39). Propidium iodide–stained nuclei (38) or forward scatter/side scatter profile of cells (39) were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany).

Western Blot Analysis

Western blot analyses were done as described (13, 14). Immunodetection of poly(ADP-ribose) polymerase (PARP), caspase-3, active caspase-9, XIAP, CD95, CD95-L, Bax, Bcl-x_L, and β-actin was done using rabbit anti-PARP polyclonal antibody (1:5,000; Enzyme Systems Products, Dublin, CA), mouse anti-caspase-3 monoclonal antibody (1:500; Cell Signaling Technologies, Beverly, MA), mouse anti-caspase-8 monoclonal antibody (1:1,000; Cell Signaling), rabbit anti–active caspase-9 polyclonal antibody (1:200; Chemicon International, Temecula, CA), mouse anti-XIAP monoclonal antibody (1:1,000; Transduction Laboratories, Lexington, KY), mouse anti-Fas (anti-CD95) monoclonal antibody (1:1,000; Transduction Laboratories), mouse anti–Fas ligand (anti–CD95 ligand) monoclonal antibody (1:250; BD), rabbit anti-Bax polyclonal antibody (1:250; Oncogene, Cambridge, MA), rabbit anti-Bcl-$X_{S/L}$ polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-p21 polyclonal antibody (1:1,000; Santa Cruz Biotechnology), and mouse anti-β-actin monoclonal antibody (Sigma). Peroxidase-conjugated goat anti-mouse IgG or peroxidase-conjugated goat anti-rabbit IgG (1:5,000; Santa Cruz Biotechnology) as secondary antibody was used for

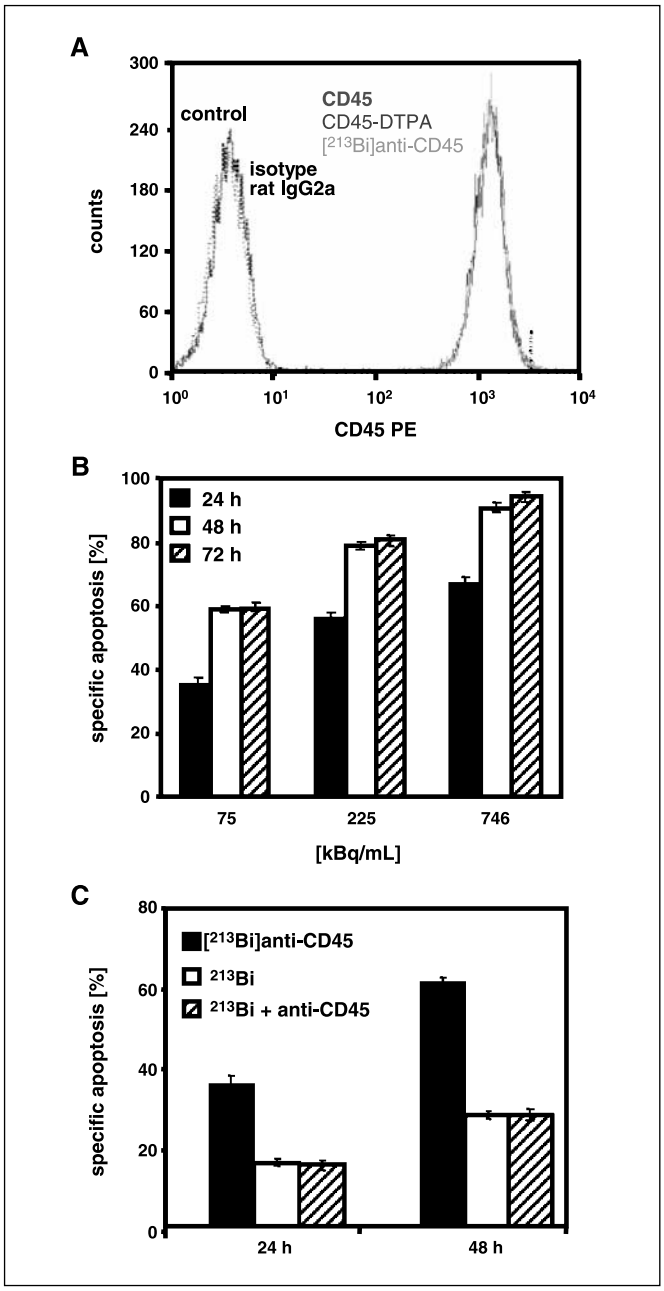

Figure 1. [^{213}Bi]anti-CD45 induced apoptosis in leukemia cells. *A,* anti-CD45 binding on HL-60 cells. HL-60 cells were stained with anti-CD45 (CD45), anti-CD45-DTPA (CD45-DTPA), or [^{213}Bi]anti-CD45-DTPA ([^{213}Bi]anti-CD45) followed by secondary antibody antirat IgG-phycoerythrin (*antirat IgG-PE*) and analyzed by flow cytometry. Isotype-matched controls isotype rat IgG2a and control are exhibited as broken curves. *B,* [^{213}Bi]anti-CD45 induced apoptosis in HL-60 cells. HL-60 cells were treated with different activities of [^{213}Bi]anti-CD45 as indicated. The different activities of [^{213}Bi]anti-CD45 were applied over 24 h. After 24 h (*black columns*), 48 h (*white columns*), and 72 h (*hatched columns*), the percentages of apoptotic cells were measured by hypodiploid DNA analysis. The percentage of specific cell death was calculated as follows: 100 × [experimental dead cells (%) − spontaneous dead cells in medium (%)] / [100 % − spontaneous dead cells in medium (%)]. *Columns,* mean of triplicates; *bars,* SD <10%. Similar results were obtained in three independent experiments. *C,* [^{213}Bi]anti-CD45 and ^{213}Bi induced apoptosis in HL-60 cells. HL-60 cells were treated with 75 kBq/mL [^{213}Bi]anti-CD45 (*black columns*), 75 kBq/mL ^{213}Bi (*white columns*), or 4 μg/mL anti-CD45 with addition of 75 kBq/mL ^{213}Bi (^{213}Bi + anti-CD45, *hatched columns*). The activities of [^{213}Bi]anti-CD45 or ^{213}Bi were applied over 24 h. After 24 and 48 h, the percentage of apoptotic cells was measured by hypodiploid DNA analysis. The percentage of specific cell death was calculated as described in (*B*). *Columns,* mean of triplicates; *bars,* SD <10%. Similar results were obtained in three independent experiments.

the enhanced chemoluminescence system (Amersham Pharmacia, Freiburg, Germany). Equal protein loading was controlled by β-actin detection.

Measurement of DNA Damage

DNA damage (DNA breaks) was measured by the alkaline Comet assay as described (40). Ten microliters of cell suspension (10,000 cells) from treated and untreated cells were resuspended in 120 μL low-melting agarose at 37°C. One hundred microliters of this suspension were spotted onto a microscope slide and covered with a coverslide. After keeping on ice for 5 min in the dark, the coverslide was removed, and the slides were incubated overnight in lysis buffer containing 2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, and 1% Na-laurylsarcosinate (pH 10). Thereafter, slides were preincubated in electrophoresis buffer [300 mmol/L NaOH, 1 mmol/L EDTA (pH > 13)] for 25 min, and electrophoresis was done for 25 min (25 V, 300 mA). Slides were neutralized in 0.4 mol/L Tris (pH 7.5), washed in aqua dest, fixed in 100% ethanol for 5 min, and air-dried overnight. Analysis was done after staining with ethidium bromide (20 μg/mL) on a fluorescence microscope using a CCD camera connected to a personal computer and analysis software. Relative DNA breakage was expressed as Olive tail moment, which was determined by measuring the fluorescence intensity of tail and nucleus using Kinetic Imaging Komet 5.0 Software (BFI-Optilas, Puchheim, Germany).

Results

[^{213}Bi]anti-CD45 and ^{213}Bi induce apoptosis in leukemia cells. Anticancer drugs, β-irradiation, and γ-irradiation have been shown to activate apoptosis pathways in leukemias and solid tumors (10–18). To analyze which type of cell death (apoptosis or necrosis) can be induced by targeted α-irradiation in HL-60 leukemia cells, we used ^{213}Bi for α-irradiation, and as antibody, we chose anti-CD45. At first, we proved the binding of [^{213}Bi]anti-CD45 to the surface of HL-60 cells with flow cytometry analysis (Fig. 1*A*). We found that anti-CD45, anti-CD45-DTPA, and [^{213}Bi]anti-CD45 bound strongly with identical intensities to HL-60 cells, which express CD45 on the surface. This indicates that HL-60 cells are target cells for CD45 antibodies. Next, we investigated apoptosis induction by [^{213}Bi]anti-CD45 in HL-60 cells (Fig. 1*B*). For activities of 75, 225, and 746 kBq/mL [^{213}Bi]anti-CD45, a strong induction of apoptosis was detected in HL-60 cells after 24, 48, and 72 h (Fig. 1*B*). In addition, [^{213}Bi]anti-CD45, when bound to CD45 of HL-60 cells, induced higher apoptosis rates in HL-60 cells after 24 and 48 h when compared with ^{213}Bi, which was diluted in medium and not bound to CD45 antibody, or compared with ^{213}Bi diluted in medium in combination with nonradioactive anti-CD45 (Fig. 1*C*), indicating that targeted α-particle radioimmunotherapy with [^{213}Bi]anti-CD45 kills preferred single-targeted leukemia cells.

[^{213}Bi]anti-CD45 and [^{90}Y]anti-CD45 induce apoptosis with different efficiencies at comparable activities in leukemia cells. We recently found that β-irradiation used in radioimmunotherapy and external γ-irradiation induce apoptosis in leukemia cells with different apoptosis rates and efficiencies (14). We therefore compared apoptosis induction of an α-emitter (^{213}Bi) bound to the CD45 antibody ([^{213}Bi]anti-CD45) and a β-emitter (^{90}Y) bound to the CD45 antibody ([^{90}Y]anti-CD45), which is used during radioimmunotherapy of high-risk leukemia *in vivo*, for different activities. At comparable activities and time points, [^{90}Y]anti-CD45 and [^{213}Bi]anti-CD45 also exhibited differences in apoptosis rates (Fig. 2*A* and *B*). [^{213}Bi]anti-CD45 induced a higher apoptosis rate in contrast to [^{90}Y]anti-CD45. At activities < 37.5 kBq/mL, [^{213}Bi]anti-CD45 killed HL-60 leukemia cells up to 60% and CEM leukemia cells up to 95%. In contrast, HL-60 and CEM leukemia cells survived after treatment with comparable activities

applied by [^{90}Y]anti-CD45, suggesting that [^{213}Bi]anti-CD45 is much more potent in killing leukemia cells than [^{90}Y]anti-CD45 using during radioimmunotherapy *in vivo*.

[^{213}Bi]anti-CD45 breaks doxorubicin-resistance, CD95-resistance, and radioresistance to β-irradiation and γ-irradiation in leukemia cells. Resistance to cytotoxic drugs and external radiation is a major problem and a limiting factor in treatment of tumor patients (14, 41). Cross-resistance has been noted between radiotherapy and chemotherapy and involves defects in apoptosis signaling or an increased ability to repair DNA (14, 41). We have found that an α-emitter bound to anti-CD45 is much more effective in cell killing than that of a β-emitter bound to anti-CD45 or γ-emitter. Therefore, we investigated if [^{213}Bi]anti-CD45 can induce cell killing in radioresistant and in chemoresistant and apoptosis-resistant leukemia cells. We treated doxorubicin-, CD95-, γ-irradiation–, and β-irradiation–resistant leukemia cells with different activities of [^{213}Bi]anti-CD45 (Fig. 3). After different time intervals, cell killing was measured by flow cytometry. After treatment activities of 75, 225, and 746 kBq/mL using [^{213}Bi]anti-CD45, we detected that α-particle irradiation with [^{213}Bi]anti-CD45 induced apoptosis in β-irradiation–resistant HL-60 (HL-60^{betaR}; Fig. 3*A*) and in γ-irradiation–resistant HL-60 (HL-60^{gammaR}) cells up to 80% (Fig. 3*B*) after 48 h. In addition, we treated doxorubicin-resistant (CEM^{DoxoR}), CD95-resistant (CEM^{CD95R}), and parental sensitive CEM cells with different activities (7.5, 22.5, 75, 150, and 225 kBq/mL) of [^{213}Bi]anti-CD45 (Fig. 3*C*). After 24, 48, and 72 h, a strong induction of apoptosis up to 95% was determined in doxorubicin-resistant CEM cells (CEM^{DoxoR}; Fig. 3*C*) similar to that in sensitive CEM cells (CEM; Fig. 3*C*). Sixty-five percent of apoptosis was detected for equal radiation absorbed doses and at the same time points in CD95-resistant CEM cells (CEM^{CD95R}; Fig. 3*C*). These findings provide evidence that [^{213}Bi]anti-CD45 is able to induce apoptosis in β-irradiation–, γ-irradiation–, doxorubicin-, and CD95-resistant leukemia cells, indicating that [^{213}Bi]-anti-CD45 breaks radioresistance, chemoresistance, and apoptosis resistance in these cells.

[^{213}Bi]anti-CD45 activates caspases and PARP cleavage in HL-60 cells through the mitochondrial pathway independent of CD95 ligand/receptor system. β-Irradiation and γ-irradiation activates apoptosis pathways and caspases involving ligand receptor–driven pathways and the mitochondrial pathway (14). Defects in apoptosis signaling are due to resistance to radiation and chemotherapy (14, 41). To identify effector molecules that may be altered by [^{213}Bi]anti-CD45 in sensitive and resistant leukemia cells, we examined differences of caspase activation by Western blot analysis. After incubation with activities of 75, 225, and 746 kBq/mL using [^{213}Bi]anti-CD45, a strong activation of caspase-3 (Fig. 4*A*), activation of caspase-8 (Fig. 4*B*), and PARP cleavage (Fig. 4*A*) were observed in HL-60 cells after 24 and 48 h. In addition, [^{213}Bi]anti-CD45 reversed defective activation of caspases and PARP cleavage in radioresistant and chemoresistant leukemia cells completely. This suggests that after irradiation with [^{213}Bi]anti-CD45, caspases were activated, and PARP, the prototype caspase substrate, was processed in sensitive and in resistant leukemia cells. In contrast to [^{213}Bi]anti-CD45, activation of caspase-3 and caspase-8 and PARP cleavage were strongly reduced when ^{213}Bi was diluted in medium and not bound to CD45 antibody (Fig. 4*A* and *B*), showing that ^{213}Bi activates caspases preferred in target cells.

Caspase activation is central in apoptosis induction (13, 14). To examine the role of caspase activation in α-particle irradiation–induced apoptosis, we incubated HL-60 cells with [^{213}Bi]anti-CD45

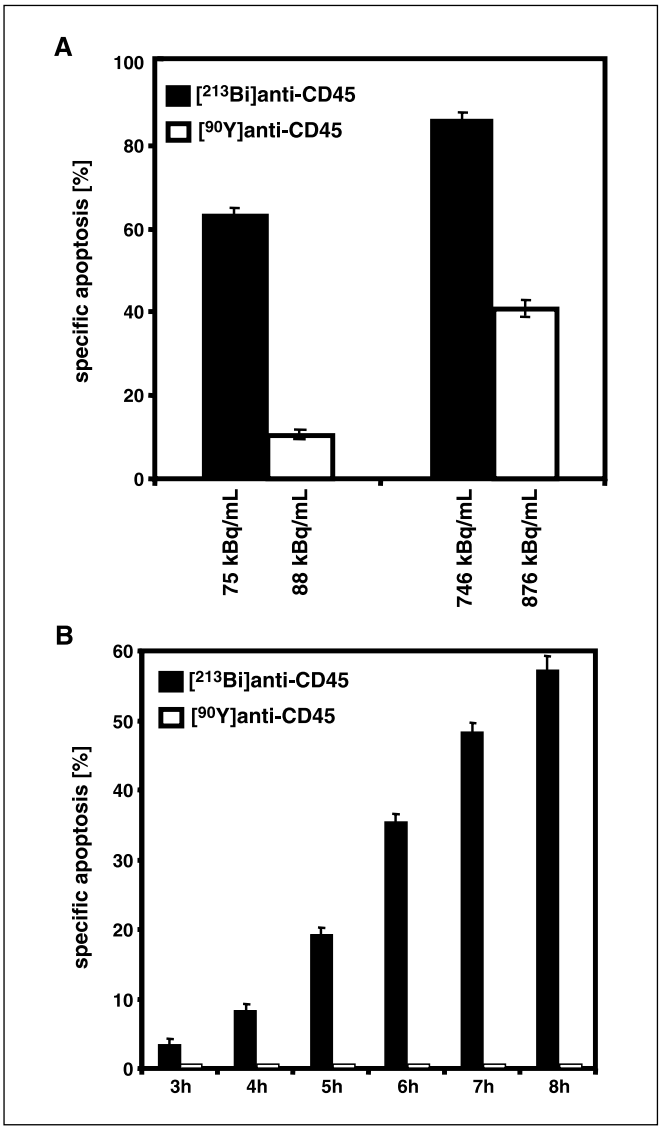

Figure 2. Comparison of [^{213}Bi]anti-CD45 and [^{90}Y]anti-CD45 induced apoptosis in HL-60 cells. *A,* comparison of [^{213}Bi]anti-CD45– and [^{90}Y]anti-CD45–induced apoptosis in HL-60 cells. HL-60 cells were treated with different activities of [^{213}Bi]anti-CD45 (*black columns*) or [^{90}Y]anti-CD45 (*white columns*) as indicated. The different activities of α-particles (^{213}Bi) and β-particles (^{90}Y) were deposited within 24 h. Twenty-four hours after irradiation, the percentage of apoptotic cells was measured by hypodiploid DNA analysis. The percentage of specific cell death was calculated as described in Fig. 1*B*. *Columns,* mean of triplicates; *bars,* SD <10%. Similar results were obtained in three independent experiments. *B,* [^{213}Bi]anti-CD45 induced apoptosis in HL-60 cells at earlier time points than [^{90}Y]anti-CD45. HL-60 cells were treated with [^{213}Bi]anti-CD45 (746 kBq/mL, *black columns*) or [^{90}Y]anti-CD45 (876 kBq/mL, *white columns*). After 3, 4, 5, 6, 7, and 8 h, the percentages of apoptotic cells were measured by hypodiploid DNA analysis. The percentage of specific cell death was calculated as described in Fig. 1*B*. *Columns,* mean of triplicates; *bars,* SD <10%. Similar results were obtained in three independent experiments.

(75, 225, and 746 kBq/mL) with or without the broad-spectrum inhibitor of caspases zVAD-fmk (benzoylcarbonyl-Val-Ala-Asp-fluoromethyl-ketone). After 24 and 48 h, inhibition of [^{213}Bi]anti-CD45–induced apoptosis was almost complete (Fig. 4*C*) after inhibition of caspase activation, showing that caspases are central in [^{213}Bi]anti-CD45–induced cell death.

Mitochondria are important in apoptosis induction. Formation of the cytochrome *c*/Apaf-1/caspase-9–containing apoptosome complex plays a critical role in mitochondrial activation (19, 21).

The apoptosome complex itself activates caspase-3 (19). To assess the role of mitochondria in [^{213}Bi]anti-CD45–induced apoptosis, we examined caspase-9 activation and XIAP expression, an inhibitor of caspase activation, after [^{213}Bi]anti-CD45 treatment. After incubation with different activities (75, 225, and 746 kBq/mL) using [^{213}Bi]anti-CD45, a strong activation of caspase-9 and down-regulation of XIAP were seen in HL-60 cells (Fig. 4*D*). This indicates that mitochondria play a role in [^{213}Bi]anti-CD45–induced apoptosis in leukemia cells. In addition, defective activation of caspase-9 was reversed completely after [^{213}Bi]anti-CD45 treatment in radioresistant and chemoresistant leukemia cells (data not shown), showing that [^{213}Bi]anti-CD45 reversed deficient activation of mitochondria in chemoresistant and radioresistant leukemia cells. Proapoptotic and antiapoptotic members of the Bcl-2 family play an important role in the regulation of mitochondrial changes (22). After application of [^{213}Bi]anti-CD45 (75, 225, and 746 kBq/mL), a reduction of Bcl-x_L and an induction of Bax (Fig. 4*D*) were found, suggesting that proapoptotic and antiapoptotic members are involved in [^{213}Bi]anti-CD45–induced cell death in leukemia cells. In contrast to [^{213}Bi]anti-CD45, activation of caspases-9, XIAP

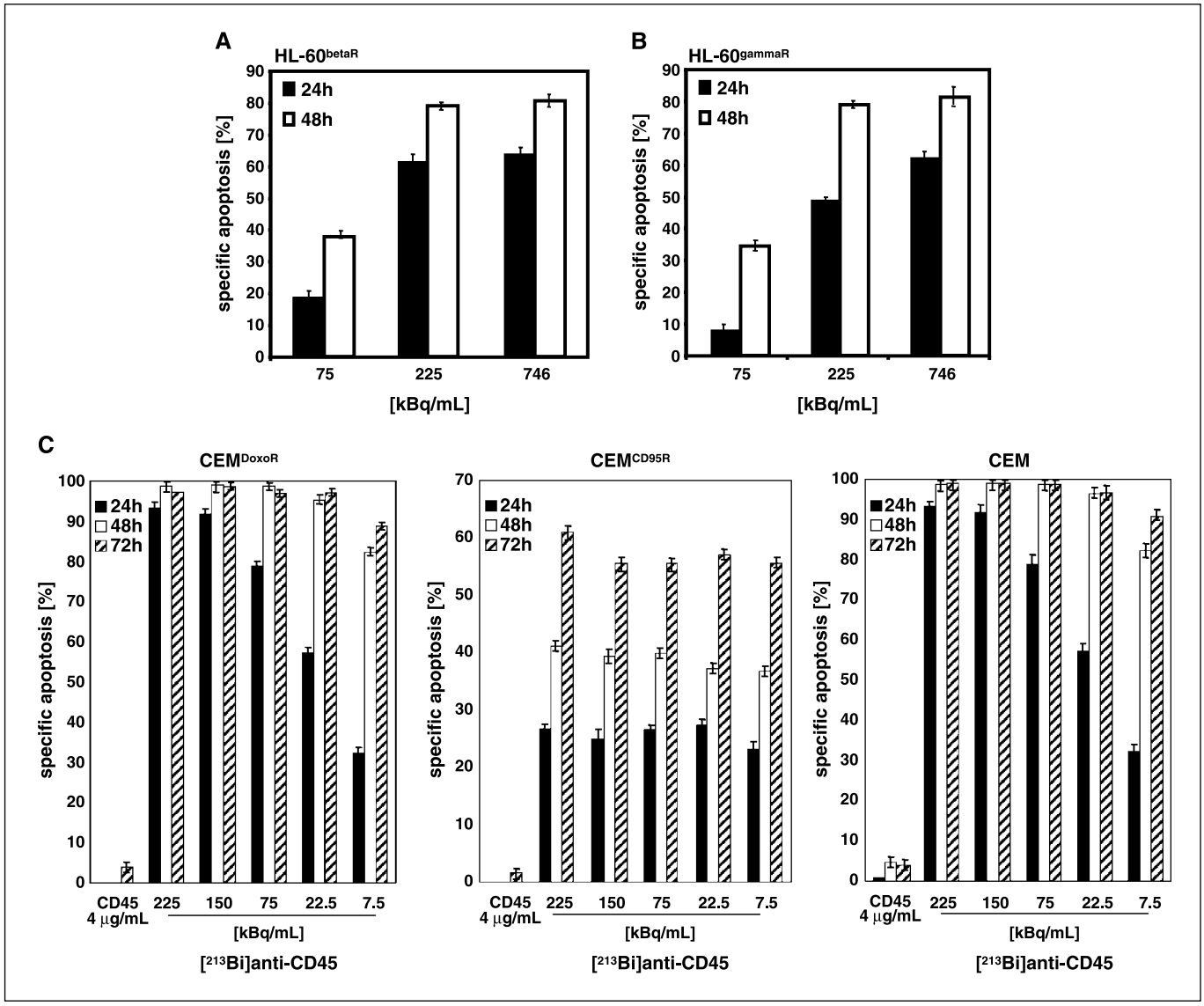

Figure 3. [^{213}Bi]anti-CD45 induced apoptosis in β-irradiation–, γ-irradiation–, doxorubicin-, and CD95-resistant leukemia cells. *A,* [^{213}Bi]anti-CD45 induced apoptosis in β-irradiation–resistant HL-60 leukemia cells (HL-60^{betaR}). HL-60^{betaR} (resistant up to 876 kBq/mL of ^{90}Y and [^{90}Y]anti-CD45) cells were treated with different activities of [^{213}Bi]anti-CD45 as indicated. The different activities were applied over 24 h. After 24 h (*black columns*) and 48 h (*white columns*), the percentage of apoptotic cells was measured by the hypodiploid DNA analysis. The percentage of specific cell death was calculated as described in Fig. 1*B*. *Columns,* mean of triplicates; *bars,* SD <10%. Similar results were obtained in three independent experiments. *B,* [^{213}Bi]anti-CD45 induced apoptosis in γ-irradiation–resistant HL-60 leukemia cells (HL-60^{gammaR}). HL-60^{gammaR} (resistant up to 10 Gy of γ-irradiation) cells were treated with different activities of [^{213}Bi]anti-CD45 as indicated. The different activities were applied over 24 h. After 24 h (*black columns*) and 48 h (*white columns*), the percentage of apoptotic cells was measured by the hypodiploid DNA analysis. The percentage of specific cell death was calculated as described in Fig. 1*B*. *Columns,* mean of triplicates; *bars,* SD <10%. Similar results were obtained in three independent experiments. *C,* [^{213}Bi]anti-CD45 induced apoptosis in doxorubicin-resistant (CEM^{DoxoR}), CD95-resistant (CEM^{CD95R}), and parental sensitive CEM leukemia cells. CEM^{DoxoR} (*left*), CEM^{CD95R} (*middle*), and CEM (*right*) leukemia cells were treated with different activities of [^{213}Bi]anti-CD45 as indicated or with 4 μg/mL anti-CD45-DTPA (CD45). The different activities were applied over 24 h. After 24 h (*black columns*), 48 h (*white columns*), and 72 h (*hatched columns*), the percentage of apoptotic cells was measured by the hypodiploid DNA analysis. The percentage of specific cell death was calculated as described in Fig. 1*B*. *Columns,* mean of triplicates; *bars,* SD <10%. Similar results were obtained in three independent experiments.

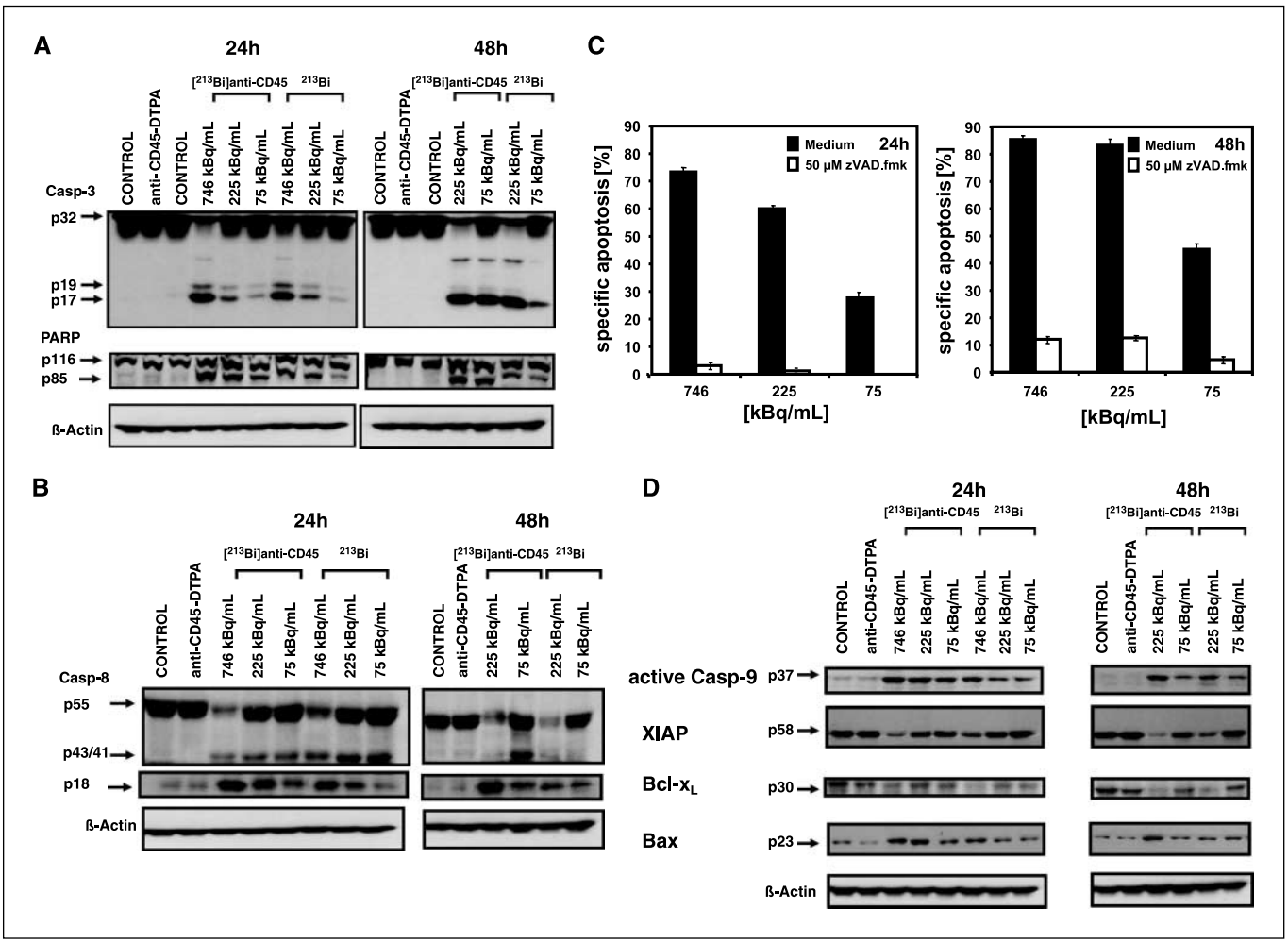

Figure 4. *A,* [^{213}Bi]anti-CD45 induced activation of caspase-3 (*Casp-3*) and PARP cleavage in HL-60 cells. HL-60 cells were treated with different activities of [^{213}Bi]anti-CD45 or ^{213}Bi as indicated, with 2 μg/mL anti-CD45-DTPA or left untreated (*CONTROL*). The different activities were applied over 24 h. After 24 and 48 h, Western blot analyses for caspase-3 and PARP were done. The active fragment of caspase-3 was detected at ~17 kDa, and the cleaved product of PARP was detected at ~85 kDa. Equal protein loading was controlled by anti-β-actin antibody. *B,* [^{213}Bi]anti-CD45 induced activation of caspase-8 (*Casp-8*) in HL-60 cells. HL-60 cells were treated with different activities of [^{213}Bi]anti-CD45 or ^{213}Bi as indicated, with 2 μg/mL anti-CD45-DTPA or left untreated (*CONTROL*). The different activities were applied over 24 h. After 24 and 48 h, Western blot analyses for caspase-8 was done. The active fragment of caspase-8 was detected at ~18 kDa. Equal protein loading was controlled with anti-β-actin antibody. *C,* inhibition of [^{213}Bi]anti-CD45 induced apoptosis by z-VAD.fmk in HL-60 cells. HL-60 cells were treated with different activities of [^{213}Bi]anti-CD45 as indicated in the absence (*black columns*, *Medium*) or presence (*white columns*, *50 μM z-VAD.fmk*) of 50 μmol/L z-VAD.fmk. The different activities were applied over 24 h. After 24 and 48 h, the percentages of apoptotic cells were measured by hypodiploid DNA analysis. The percentage of specific cell death was calculated as described in Fig. 1*B*. *Columns,* mean of triplicates; *bars,* SD <10%. Similar results were obtained in three independent experiments. *D,* [^{213}Bi]anti-CD45 induced activation of caspase-9 (*Casp-9*), down-regulation of XIAP, down-regulation of Bcl-x_L, and up-regulation of Bax in HL-60 cells. HL-60 cells were treated with different activities of [^{213}Bi]anti-CD45 or ^{213}Bi as indicated, with 2 μg/mL anti-CD45-DTPA or left untreated (*CONTROL*). The different activities were applied over 24 h. After 24 and 48 h, Western blot analyses for active caspase-9, XIAP, Bcl-x_L, and Bax were done. The active fragment of caspase-9 was detected at ~37 kDa; XIAP was detected at ~58 kDa; Bcl-x_L was detected at ~30 kDa; and Bax was detected at ~23 kDa. Equal protein loading was controlled by anti-β-actin antibody.

down-regulation, Bax up-regulation, and Bcl-x_L down-regulation were reduced when ^{213}Bi was diluted in medium and not bound to CD45 antibody (Fig. 4*D*), showing that ^{213}Bi activates mitochondria preferred in target cells.

We found that the CD95 receptor/CD95 ligand system is involved in β-irradiation– and in γ-irradiation–induced apoptosis that mediated cell death via activation of caspases in leukemia cells (14). Therefore, we assessed the involvement of the CD95 receptor/CD95 ligand system in α-irradiation–induced apoptosis in HL-60 cells as well. Treatment of HL-60 cells with [^{213}Bi]anti-CD45 (75 and 225 kBq/mL) induced up-regulation of the CD95 receptor (Fig. 5). However, upon treatment with [^{213}Bi]anti-CD45 (75 and 225 kBq/mL), induction of the CD95 ligand did not seem to account for the activation of caspases (Fig. 5), suggesting that the CD95 receptor/CD95 ligand system is not involved in α-irradiation–induced apoptosis.

[^{213}Bi]anti-CD45 induces DNA damage and overcomes DNA repair. DNA repair mechanisms play a critical role in radioresistance and in chemoresistance (23, 41). Increased DNA repair was shown in tumor cells resistant to radiation and anticancer drugs in comparison with sensitive tumor cells (42). Inhibition of DNA repair with DNA repair enzymes inhibitors overcomes radioresistance, chemoresistance, and apoptosis resistance in leukemia cells (14). [^{213}Bi]anti-CD45 induces DNA damage in leukemia cells (Fig. 6*A* and *B*). After the application of [^{213}Bi]anti-CD45 (225 kBq/mL), a strong induction of DNA damage was found in HL-60 cells as measured by the Comet assay (Fig. 6*A*) and quantified by the fluorescence intensity of tail and nucleus

(Fig. 6*B*). NHEJ is the predominant mechanism in DSBs repair in mammalian cells, with DNA-PK and ligase IV playing critical roles (26–28). In the absence of DNA-PK or ligase IV, NHEJ fails to repair DSBs (26–31). β-Irradiation– and γ-irradiation–induced DNA damage is repaired by the NHEJ DNA repair pathway (Fig. 6*C*). After treatment with β-irradiation (^{90}Y; Fig. 6*C*) or γ-irradiation (Fig. 6*C*), a strong reduction of apoptosis was found in DNA repair–proficient ligase IV$^{+/+}$ Nalm6 and ligase IV$^{+/-}$ Nalm6 cells, and strong induction of apoptosis was seen in NHEJ DNA repair–deficient ligase IV$^{-/-}$ Nalm6 cells. We investigated whether α-irradiation–induced apoptosis and DNA damage are suppressed by NHEJ DNA repair. We treated ligase IV$^{+/+}$, ligase IV$^{+/-}$, and ligase IV$^{-/-}$ Nalm6 cells with ^{213}Bi (Fig. 6*C*). In contrast to β-irradiation (Fig. 6*C*), γ-irradiation (Fig. 6*C*), and doxorubicin (data not shown), we could not detected differences in apoptosis induction and DNA damage after treatment with ^{213}Bi in DNA repair–proficient ligase IV$^{+/+}$ Nalm6, ligase IV$^{+/-}$ Nalm6, and DNA repair–deficient ligase IV$^{-/-}$ Nalm6 cells (Fig. 6*C*), indicating that [^{213}Bi]anti-CD45–induced apoptosis was not inhibited by the DNA repair mechanism NHEJ.

Discussion

Resistance to chemotherapy or radiation therapy is one of the primary causes for treatment failure of malignomas (1, 41). Therefore, new options are needed to improve therapeutic success in the treatment of cancer. Various approaches using native monoclonal, immunotoxins, and radioimmunoconjugates have emerged as promising strategies. Clinical studies of radioimmunotherapy have used β-emitters, such as ^{90}Y and ^{131}I bound to anti-CD45, anti-CD33, or anti-CD66 antibodies (2, 4–6). When labeled with the β-emitters ^{90}Y or ^{131}I, the antibodies destroys cells via cross-fire effects, leading to the destruction of surrounding tumor cells, but β-irradiated target cells may survive without the cross-fire effect from neighboring cells. This effect can also result in the killing of normal bystander cells and produce significant toxicity (7). In contrast, α-particles, such as ^{213}Bi and ^{225}Ac, with their short path lengths destroy mainly target cells with limited side effects (8, 9). Therefore, radioimmunotherapy with α-particle–emitting isotope–labeled antibodies may produce more efficient cell killing of individual target cells with little damage to surrounding normal tissue (7). In addition, targeted α-particles may be well suited for the treatment of micrometastatic and minimal residual disease and for treatment of leukemia and lymphoma (7–9). In our study, we show that ^{213}Bi bound to CD45 antibodies break radioresistance and chemoresistance in leukemia cells, and we provide the molecular requirements for overcoming this resistance and for α-particle–induced cell killing. Our findings are crucial and fundamental for the development of novel treatment options in leukemia therapy.

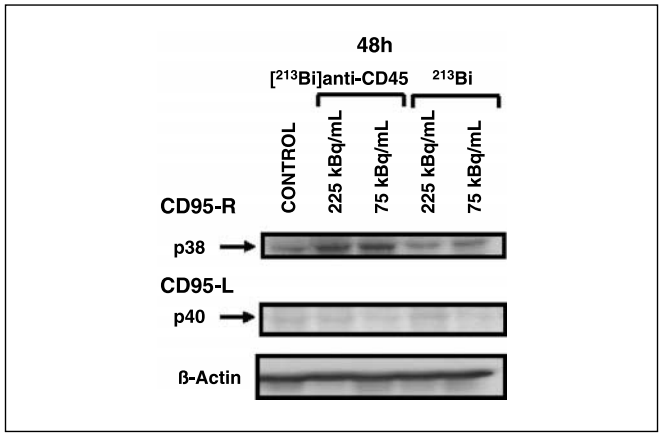

Figure 5. CD95 receptor (*CD95-R*) and CD95 ligand (*CD95-L*) analyses in [^{213}Bi]anti-CD45–treated HL-60 cells. HL-60 cells were treated with different activities of [^{213}Bi]anti-CD45 or ^{213}Bi as indicated or left untreated (*CONTROL*). The different activities were applied over 24 h. After 48 h, Western blot analysis for CD95 receptor and CD95 ligand was done. The CD95 receptor was detected at ~38 kDa, and the CD95 ligand was detected at ~40 kDa. Equal protein loading was controlled by anti-β-actin antibody.

We have recently published that γ-irradiation and β-irradiation differed in apoptosis induction, indicating that different types of radiation may play a different role in apoptosis induction (14). At comparable activities and time points, the β-emitter ^{90}Y bound to anti-CD45 ([^{90}Y]anti-CD45) and the α-emitter ^{213}Bi bound to anti-CD45 ([^{213}Bi]anti-CD45) also exhibited differences in apoptosis rates. [^{213}Bi]anti-CD45 induced a higher apoptosis rate in contrast to [^{90}Y]anti-CD45 at comparable activities. At activities <37.5 kBq/mL, [^{213}Bi]anti-CD45 killed HL-60 leukemia cells up to 60% and CEM leukemia cells up to 95%. In contrast, HL-60 and CEM leukemia cells survived after treatment with comparable activities applied by [^{90}Y]anti-CD45, suggesting that targeted α-irradiation using [^{213}Bi]anti-CD45 is much more potent in killing leukemia cells than targeted β-irradiation with [^{90}Y]anti-CD45, which is used during radioimmunotherapy of high-risk leukemia *in vivo*.

Resistance to cytotoxic drugs and radiation is a major problem and a limiting factor in treatment of tumor patients (14, 41). Cross-resistance has been noted between radiotherapy and chemotherapy and involves defects in apoptosis signaling or an increased ability to repair DNA (14, 41). We provide evidence that [^{213}Bi]anti-CD45 is able to induce apoptosis in β-irradiation–, γ-irradiation–, doxorubicin-, and CD95-resistant leukemia cells, indicating that [^{213}Bi]anti-CD45 breaks radioresistance, chemoresistance, and apoptosis resistance in leukemia cells. Our findings suggest that targeted α-particle therapy is a promising approach for the treatment of leukemias resistant to chemotherapeutic drugs and radiation.

β-Irradiation and γ-irradiation activates apoptosis pathways and caspases involving ligand receptor–driven pathways and the mitochondrial pathway (14). Interestingly, α-irradiation induced apoptosis in leukemia cells involving the mitochondrial pathway independent of the CD95 receptor/ligand interaction. After irradiation with [^{213}Bi]anti-CD45, caspase-3 and caspase-8 were activated, and PARP, the prototype caspase substrate, was processed in leukemia cells. In addition, deficient activation of caspases was reversed after irradiation with [^{213}Bi]anti-CD45 in chemoresistant and radioresistant leukemia cells. Formation of the cytochrome *c*/Apaf-1/caspase-9–containing apoptosome complex plays a critical role in mitochondrial activation (19, 21). The apoptosome complex itself activates caspase-3 (19). [^{213}Bi]anti-CD45 induced caspase-9 activation and XIAP (19, 43) down-regulation, indicating that mitochondria are involved in [^{213}Bi]anti-CD45–triggered apoptosis in leukemia cells. In addition, deficient activation of mitochondria was reversed in chemoresistant and radioresistant leukemia cells after [^{213}Bi]anti-CD45 treatment. The effect of apoptosis induction and caspase activation was strongly reduced when ^{213}Bi was not bound to CD45, suggesting that ^{213}Bi induces cell kill and activates apoptosis pathways specifically in target cells.

β-Irradiation and γ-irradiation induced apoptosis involved induction of CD95 ligand expression that mediated cell death via

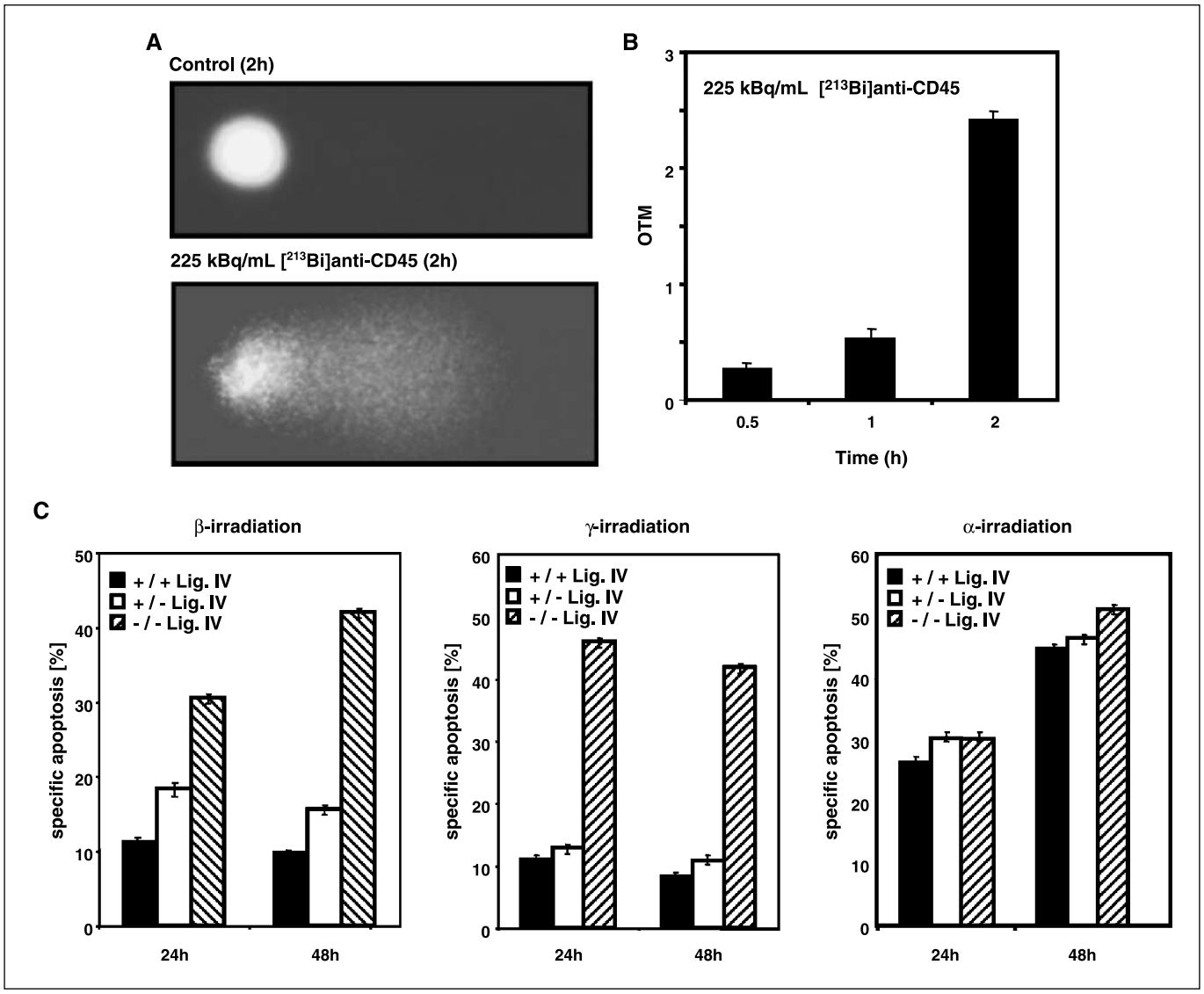

Figure 6. *A* and *B,* [^{213}Bi]anti-CD45 induced DNA damage in leukemia cells (HL-60). HL-60 cells were treated with 225 kBq/mL [^{213}Bi]anti-CD45 or left untreated (*CONTROL*). After 0.5, 1, and 2 h, alkaline electrophoresis (Comet assay) was done. DNA damage was measured (*A*) by Comet assay and (*B*) was quantified by the fluorescence intensity of the tail and nucleus (Olive tail moment, *OTM*). *C,* α-irradiation–induced apoptosis in leukemia cells is not inhibited by NHEJ DNA repair mechanism in contrast to β-irradiation– and γ-irradiation–induced apoptosis. Ligase IV$^{+/+}$ Nalm6 (+/+ *Lig. IV, black columns*), ligase IV$^{+/-}$ Nalm6 (+/− *Lig. IV, white columns*), and ligase IV$^{-/-}$ Nalm6 (−/− *Lig. IV, hatched columns*) were irradiated with 263 kBq/mL (3 Gy) β-irradiation using ^{90}Y (*left*), 3 Gy γ-irradiation (*middle*), and 225 kBq/mL (1.5 Gy) α-irradiation using ^{213}Bi (*right*). After 24 and 48 h, the percentages of apoptotic cells were measured by the hypodiploid DNA analysis. The percentage of specific cell death was calculated as described in Fig. 1*B*. *Columns,* mean of triplicates; *bars,* SD <10%. Similar results were obtained in three independent experiments.

activation of caspases (14). Upon treatment with [^{213}Bi]anti-CD45, induction of the CD95 ligand did not seem to account for the activation of caspases. However, up-regulation of the CD95 receptor was found after treatment with [^{213}Bi]anti-CD45 in HL-60 cells. Posovsky et al. showed that cytotoxic drugs sensitized acute lymphoblastic leukemia cells to apoptotic signaling by up-regulation of the CD95 receptor (44). We hypothesize that up-regulation of CD95 receptor by [^{213}Bi]anti-CD45 may sensitize leukemia cells towards apoptotic signaling, such as CD95, and cytotoxic drugs, such as doxorubicin, which up-regulate CD95 ligand in leukemia cells (12). Bcl-2 family members regulate the mitochondrial pathway (22). After treatment with [^{213}Bi]anti-CD45, we detected a strong up-regulation of Bax and down-regulation of Bcl-x_L, suggesting that proapoptotic and antiapoptotic members are involved in [^{213}Bi]anti-CD45–induced cell death in leukemia cells.

Increased DNA repair was shown in tumor cells resistant to radiation and anticancer drugs in comparison with sensitive tumor cells (42). In addition, inhibition of DNA repair with DNA repair enzymes inhibitors overcomes radioresistance, chemoresistance, and apoptosis resistance in leukemia cells (14). NHEJ is the predominant mechanism in DSBs repair in mammalian cells (42). We have found that NHEJ inhibits doxorubicin-, γ-irradiation–, and β-irradiation–induced apoptosis. [^{213}Bi]anti-CD45–induced apoptosis was not inhibited by NHEJ, suggesting that α-irradiation overrides DNA repair in leukemia cells. Thus, our findings provide evidence that [^{213}Bi]anti-CD45 breaks chemoresistance and radioresistance by overcoming DNA repair mechanisms.

In conclusion, our study highlights the potential of using [^{213}Bi]anti-CD45 in leukemia therapy to improve therapeutic success. [^{213}Bi]anti-CD45 is a promising radiotherapeutic agent to break chemoresistance and radioresistance in leukemias by overcoming the cellular DNA repair. These findings are crucial and fundamental for the development of novel treatment options in leukemia and solid tumor therapy and provide the foundation for discovery of novel anticancer compounds.

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