Effect of $^{67}$Cu-2IT-BAT-Lym-1 Therapy on BCL-2 Gene and Protein Expression in a Lymphoma Mouse Model

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Abstract

Radioimmunotherapy using monoclonal antibodies against tumor-associated antigens has been particularly promising in the treatment of radiosensitive malignancies such as lymphoma. $^{67}$Cu has excellent physical and biochemical properties for radioimmunotherapy. $^{67}$Cu-2IT-BAT-Lym-1 has been used in preclinical and clinical trials, where an exceptionally long residence time of $^{67}$Cu on tumor was observed. BCL-2, a proto-oncogene that promotes cell survival by blocking apoptotic cell death, is overexpressed in most B-cell lymphomas including Raji human Burkitt's lymphoma cells. In this study, therapeutic efficacy and BCL-2 gene and protein expression levels were examined in Raji xenografts in mice after $^{67}$Cu-2IT-BAT-Lym-1 radioimmunotherapy. $^{67}$Cu-2IT-BAT-Lym-1 therapy induced a response rate (complete and partial responses) of $\sim 50\%$. BCL-2 gene expression was decreased 3 h after radioimmunotherapy, followed by a decrease in Bcl-2 protein by 24 h. Decreases in BCL-2 gene and protein expression preceded observations of $^{67}$Cu-2IT-BAT-Lym-1 therapeutic effect suggest that down-regulation of BCL-2 leaves cells more likely to be killed by low dose-rate radiation from radioimmunotherapy.

Introduction

Despite advances in conventional therapy for NHL, 30-70% of patients with intermediate and high grade NHL fail to achieve long-term disease-free survival, and no curative treatment has been established for patients with low grade NHL (1, 2). RIT using MoAbs against tumor-associated antigens to deliver cytotoxic radionuclides to tumors has been particularly promising in the treatment of radiosensitive malignancies such as lymphoma (3-8). $^{67}$Cu has excellent physical and biochemical properties for RIT. Its half-life (62 h) is well matched to the uptake and residence time of antibodies in the tumor (9). $^{67}$Cu has $\beta$ emissions (mean energy, 141 keV; $e_{\beta}$max = 577 keV) for therapy, and $\gamma$ emissions (185 keV, 47%; 93 keV, 17%) that are excellent for imaging but contribute minimal radiation exposure to medical personnel. An advantage of $^{67}$Cu is its enhancement of the therapeutic index compared with other radionuclides. $^{67}$Cu-radiolabeled MoAbs have been shown in preclinical studies to deliver higher doses to tumor and higher tumor to nontumor dose ratios when compared with their iodinated counterparts (10, 11). These results have been substantiated by data from clinical trials of $^{67}$Cu-2IT-BAT-Lym-1, in which an exceptionally long residence time of $^{67}$Cu in tumors was observed (12, 13). The macrocyclic chelating agent, 1,4,7,11-tetraazacyclotetradecane-$N,N',N'',N'''$-tetraacetic acid, specifically designed to stably bind copper (14), is the chelating moiety in the immunoconjugate 2IT-BAT-Lym-1. Under well-characterized conditions, 2IT-BAT-Lym-1 binds $^{67}$Cu rapidly and selectively, providing $^{67}$Cu-2IT-BAT-Lym-1 with high specific activity and complete retention of structural and functional integrity (15). $^{67}$Cu-2IT-BAT-Lym-1 radiolabeling has been optimized to the extent that product yield and quality are comparable to those for iodinated MoAbs.

BCL-2 is the acronym for the B-cell lymphoma/leukemia-2 gene, which was first discovered in association with B-cell malignancies in which chromosomal translocations were found to activate the gene in the majority of follicular NHLs (16). BCL-2 is a proto-oncogene that regulates cell survival by blocking a common pathway leading to apoptotic cell death (17). It is overexpressed, and thus oncogenic, in most B-cell lymphomas, including Raji human Burkitt's lymphoma cells (18). Cells blocked at the $G_2$-M interface appear to be much more radiosensitive to radiation (19-22). Macklis et al. (23) studied radiation-induced cell cycle changes and demonstrated that Raji cells are efficiently blocked at the $G_2$-M interface despite overexpression of BCL-2.

The goal of this study was to examine modulation of BCL-2 in the response of Raji xenografts to $^{67}$Cu-2IT-BAT-Lym-1 therapy. In these studies, nude mice bearing Raji tumors were treated with 335, 400, and 500 $\mu$Ci of $^{67}$Cu-2IT-BAT-Lym-1 and were followed for efficacy and toxicity (10). Selected mice that received 335 $\mu$Ci of $^{67}$Cu-2IT-BAT-Lym-1 were sacrificed, and their tumors were analyzed for BCL-2 gene expression and Bcl-2 protein levels.

Materials and Methods

Cell Lines. The Raji human lymphoma cell line was obtained from American Type Culture Collection (Rockville, MD). The human renal carcinoma cell line, G2101, was isolated from a rib metastasis of a renal cell carcinoma and has been shown to be positive for BCL-2 expression (24). Cells were...
jugate 2IT-BAT-Lym-1 was prepared by conjugating BAT to treatment with ~'7Cu-2IT-BAT-Lym-1 or until tumors were harvested using calipers two times per week for 84 days after untreated controls or injected by tail vein with 67Cu-2IT-BAT-Lym-I (335,400, or 500 μCi). Tumors were measured in three dimensions using calipers acetate electrophoresis, molecular sieving high-performance liquid chromatography, and radioimmunoreactive assay. All preparations passed quality control tests.

Animal Studies. Female athymic BALB/c-nu/nu mice (Harlan Sprague Dawley, Frederick, MD) weighing 20–25 g were maintained according to University of California Animal Care guidelines on a normal diet ad libitum and under pathogen-free conditions. Prior to tumor implantation, mice received a total body radiation dose of 400 rad to suppress B-cell function. Three days later, each mouse received bilateral s.c. injection of 2IT-BAT-Lym-1 was prepared by conjugating BAT to Lym-1 via 2IT (Sigma Chemical Co., St. Louis, MO). Radiolabeling of 2IT-BAT-Lym-1 with 67Cu was performed by previously described methods (10). 67Cu-2IT-BAT-Lym-1 was examined by cellulose acetate electrophoresis, molecular sieving of therapy was attributed to the radiation side effects of the total body radiation dose of 400 rad to suppress B-cell function. 67Cu-2IT-BAT-Lym-1 had tumors harvested at 3 and 24 h after treatment for 3 and 24 h after treatment with 67Cu-2IT-BAT-Lym-1 or until tumors were harvested for molecular biology studies but never beyond the size stipulated in the approved animal protocol. Death within 30 days of therapy was attributed to the radiation side effects of the treatment. Subgroups of mice treated with 335 μCi of 67Cu-2IT-BAT-Lym-1 had tumors harvested at 3 and 24 h after treatment for BCL-2 analysis. Harvested tumors were flash frozen and stored at −70°C until analyzed.

RNA Extraction and cDNA Synthesis. Total cellular RNA was extracted from Raji tumor specimens and the G2101 cell line by guanidine thiocyanate extraction followed by cesium chloride purification as described previously (28, 29). The RNA was removed from the gradient and precipitated twice at −70°C by the addition of 5 M NaCl (to a concentration of 0.3 M) and twice the volume of ice-cold 100% ethanol. The RNA was pelleted by microcentrifugation and resuspended in Tris-EDTA [10 mM Tris-HCl and 1 mM EDTA (pH 7.6)]. The concentration of RNA was determined with a spectrophotometer. One μg of extracted RNA was converted to cDNA by reverse transcription using random hexamer primers (Promega, Madison WI) as described previously (28, 29).

Quantitative RT-PCR Analysis. The procedure for using RT-PCR to quantitate levels of mRNA has been published previously (28). cDNA was diluted 1:4 in Tris-EDTA buffer and subjected to PCR amplification using specific primer pairs for BCL-2 (30). Triplicate PCR of cDNA was performed on each tumor specimen and on the control cell line. The maximum acceptable variation from the mean of the normalized triplicate RT-PCR values for each tumor was set at 20%; otherwise PCR was repeated. PCR conditions for mRNA amplification were as follows: the cDNA was amplified 34 cycles of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C. A 10-min extension at 72°C was performed after cycling in each PCR run. Ten μl of RT-PCR product was resolved by electrophoresis on a 2% agarose gel with the positive control. The ethidium bromide-stained gels (0.5 μg/ml) were photographed under UV light, and the photographic negative was scanned using a laser densitometer (PDScan; Molecular Dynamics, Sunnyvale, CA). Integrations and band intensities were performed using the ImageQuaNT Software Program (Molecular Dynamics). Absolute area integrations under the curves representing each specimen were compared. Levels of mRNA expression were determined by comparing the ratio of the RT-PCR products for BCL-2 to the endogenous standard, N-RAS as previously reported (28).

Western Blot Analysis. Protein (150 μg) isolated from the Raji xenograft was separated in a 15% SDS-polyacrylamide gel and then electrotransferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) at a constant 126 V for 1 h (31). The membrane was incubated with 4% nonfat dry milk in 25 mM Tris-HCl pH 8.0, 0.125 M NaCl, 0.025% Tween 20 for 1 h and incubated with a 1:800 dilution of hamster anti-Bcl-2 antibody (PharMingen, San Diego, CA) for 16 h at 4°C. The membrane was washed in Tris-buffered saline, incubated with horseradish peroxidase-conjugated antihamster IgG for 1 h at room temperature followed by incubation with streptavidin peroxidase (Boehringer Mannheim, Indianapolis, IN) for 30 min. Signal was detected by chemiluminescence (ECL reagents; Amersham, Arlington Heights, IL). The membrane was exposed to X-ray film (XAR; Kodak, Rochester, NY), developed, and scanned with a laser densitometer supported by ImageQuaNT software.

Results

Tumor Response to 67Cu-2IT-BAT-Lym-1. Tumor volume in untreated mice increased rapidly, whereas tumor volume in treated mice characteristically decreased after 5 days. The overall response rate (complete and partial response and cure) for tumors in all three treated groups of mice was 47%, and 33% of the tumors were cured (Fig. 1). In mice that received 335 μCi of 67Cu-2IT-BAT-Lym-1, 5 of 18 (28%) tumors disappeared and did not regrow during the 84-day study (Fig. 1, Cure), 1 of 18 (6%) tumors disappeared but later regrew (complete response; Fig. 1, CR) and 3 of 18 (17%) tumors decreased by at least 50% (partial response; Fig. 1, PR). In mice that received 400 μCi of 67Cu-2IT-BAT-Lym-1, 5 of 12 (42%) tumors were cured. In mice that received 500 μCi of 67Cu-2IT-BAT-Lym-1, two of six (33%) tumors were cured, and one of six (17%) tumors decreased by at least 50%. Toxicity was modest at all three dose levels of 67Cu-2IT-BAT-Lym-1 (335, 400, or 500 μCi) with 11, 20, and 9% mouse mortality occurring within 30 days of therapy, respectively.

Modulation of BCL-2 Expression following RIT. To study the effect of RIT on BCL-2 expression, 4–5 Raji xenografts were harvested at each time point (3 and 24 h) after 67Cu-2IT-BAT-Lym-1 therapy and were evaluated for changes in gene expression. Untreated tumors were used as controls for gene expression. BCL-2 expression was significantly decreased.
BCL-2 in Raji Xenografts after ⁶⁷Cu-2IT-BAT-Lym-1

**Fig. 1** Tumor responses to ⁶⁷Cu-2IT-BAT-Lym-1 (335, 400, or 500 μCi) or no treatment. The response rates were similar at all dose levels. Overall response rate for all treated groups and cure rate were 47 and 33%, respectively.

**Fig. 2** Gene expression of BCL-2 by untreated tumors and tumors obtained from mice 3 and 24 h after 335 μCi of ⁶⁷Cu-2IT-BAT-Lym-1. mRNA expression was obtained by scanning the photographic negative of the ethidium bromide-stained agarose gels and normalizing to N-RAS and G2101 cell expression levels. Decreased expression of BCL-2 is seen at 3 h with diminished expression persisting at 24 h. Bars, SD.

3 h after RIT (P = 0.006) and remained decreased at 24 h (P = 0.005) when compared with the BCL-2 expression of the control tumors (Fig. 2).

**Bcl-2 Protein Expression.** Four Raji xenografts harvested at 3 and 24 h after ⁶⁷Cu-2IT-BAT-Lym-1 therapy were evaluated for Bcl-2 protein expression by Western blotting and compared to Bcl-2 protein levels in untreated tumors. Western blot analysis revealed that Bcl-2 protein expression in xenografts treated with ⁶⁷Cu-2IT-BAT-Lym-1 was unchanged at 3 h but decreased to 64% of the control level at 24 h (Fig. 3).

**Discussion**

BCL-2 is a proto-oncogene that regulates cell survival by blocking a common pathway leading to apoptotic cell death (17). BCL-2 is overexpressed in most B-cell lymphomas, including the Raji cells used for these studies (18). Additionally, Burkitt’s lymphoma cells have p53 mutations that cluster in two regions (codons 209–216 and 234–243). Raji Burkitt’s cells harbor two abnormal p53 alleles, one at codon 213 and one at codon 234 (32). p53 protein promotes apoptosis in DNA-damaged cells, such as those that might be induced by radiation.

To assess therapeutic efficacy, mice bearing Raji tumors were treated with ⁶⁷Cu-2IT-BAT-Lym-1. At doses translatable to patients, ⁶⁷Cu-2IT-BAT-Lym-1 provided a therapeutic, and frequently curative (33%), dose of radiation to tumor mice with modest toxicity (10). Lym-1 antibody by itself has no therapeutic effect on lymphoma in patients (27). A major low dose-rate radiation effect, such as that induced by ⁶⁷Cu-2IT-BAT-Lym-1 RIT, is abrupt intermitotic cell death, referred to as apoptosis, through mediation of cell cycle processes and the activation of endogenous endonucleases (19, 33–35). Although some have observed this effect in response to antibody alone (36), the combination of antibody binding and low dose-rate radiation appear to be synergistic in inducing this effect (19, 37). In studies on human B-lymphoma cells, low dose-rate radiation has been associated with arrest of cells in the G₂-M phase of the cell cycle and ultimately cell death by necrosis or apoptosis (19, 23, 38).

To examine a potential factor contributing to RIT efficacy, BCL-2 gene and protein expression was determined in Raji
tumors after 335 μCi of $^{67}$Cu-2IT-BAT-Lym-1, a dose associated with substantial efficacy and minimal toxicity. BCL-2 gene expression was decreased at 3 h with a subsequent decrease in Bcl-2 protein by 24 h. This orderly decrease in BCL-2 gene and protein expression after $^{67}$Cu-2IT-BAT-Lym-1 suggests that the events were related. These gene and protein expression events occurred within 24 h of administration of the $^{67}$Cu-2IT-BAT-Lym-1 and preceded evidence of tumor regression. Additional evidence that the decrease in BCL-2 proto-oncogene represented a significant mechanism for the therapeutic effect of RIT on the tumor is provided by the description of an analogous decrease in BCL-2 expression associated with response to RIT in a breast cancer model (30).

The oncogene BCL-2 acts to decrease the probability of apoptosis because the protein encoded by the BCL-2 gene blocks programmed cell death. Despite inherent overexpression of BCL-2 and mutant p53 in Raji cells, we found decreases in BCL-2 and subsequent decreases in Bcl-2 protein after RIT with $^{67}$Cu-2IT-BAT-Lym-1 that induced responses and even cures of Raji tumors. In the absence of an alternative explanation, we speculate that the mutant p53 gene maintained some functional capacity to produce p53 protein. In summary, decreases in BCL-2 gene and protein expression preceding observations of $^{67}$Cu-2IT-BAT-Lym-1 therapeutic effect suggest that down-regulation of BCL-2 leaves cells more likely to be killed by low dose-rate radiation from radioimmunotherapy. This mechanism may play a significant role in the effect of low dose-rate radiation on cancer.

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References

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