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 ~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, $^{3}$ 40–70% 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 radioisotopes. $^{67}$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-$N,N',N''$’-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 G2-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 G2-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

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3 The abbreviations used are: NHL, non-Hodgkin’s lymphoma; RIT, radioimmunotherapy; MoAb, monoclonal antibody; 2IT, 2-iminothiolane; BAT, 6-[p-(bromoacetamido)benzyl]-1,4,7,11-tetraazacyclotetradecane-$N,N',N''$’-tetraacetic acid; RT-PCR, reverse transcription-PCR.
jugate 2IT-BAT-Lym-1 was prepared by conjugating BAT to
transported for molecular biology studies but never beyond the size
treatment with ~7Cu-2IT-BAT-Lym-1 or until tumors were har-
tumors were >20 mm 3, mice were sorted and either left as
untreated controls or injected by tail vein with 67Cu-2IT-BAT-
treatment. Subgroups of mice treated with 335 pCi of
of therapy was attributed to the radiation side effects of the
2 106 to 5 106 of Raji cells in the lower abdomen. Once the
Three days later, each mouse received bilateral s.c. injection of
Radiolabeling of 2IT-BAT-Lyme-1 with 6VCu was performed by previ-
you previously described methods (10). 67Cu-2IT-BAT-Lym-1 was ex-
tained by cellulose acetate electrophoresis, molecular sieving
lymphoma (HLA-DR) antigen located on the neutrophil surface in
rat is an IgG2a mouse MoAb with high affinity against a discon-
magnitude of RNA was determined with a spectrophotometer. One Ixg of

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
amplification in each PCR run. Ten µl of RT-PCR
product were 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 (PDSI;
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
level of the RT-PCR products for BCL-2 to the endogenous
standard, N-RAAS as previously reported (28).

Western Blot Analysis. Protein (150 µg) isolated from the
Raji xenograft was separated in a 15% SDS-polyacrylamide mini-
gen and then electrotransferred to nitrocellulose membrane (Bio-
Rad Laboratories, Hercules, CA) at a constant 120 V for 1 h (31).
The membrane was washed in Tris-buffered saline, incubated with horseradish pero-
idase-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 (com-
plete 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
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 xen-
ografts 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 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 $^{67}$Cu-21T-BAT-Lym-1. At doses translatable to patients, $^{67}$Cu-21T-BAT-Lym-1 provided a therapeutic, and frequently curative (33%), dose of radiation to tumored 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 $^{67}$Cu-21T-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 xenografts treated with $^{67}$Cu-21T-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.

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tumors after 335 μCi of ⁶⁷Cu-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 ⁶⁷Cu-2IT-BAT-Lym-1 RIT suggests that the events were related. These gene and protein expression events occurred within 24 h of administration of the ⁶⁷Cu-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 ⁶⁷Cu-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 ⁶⁷Cu-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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