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P53-Based Strategy for Protection of Bone Marrow from Y-90 Ibritumomab Tiuxetan

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Abstract

Purpose—The main drawbacks of radioimmunotherapy have been severe hematological toxicity and potential development of myelodysplastic syndrome and secondary leukemia. p53 activation is a major pathway by which normal tissues respond to DNA damaging agents such as chemotherapy and radiotherapy, resulting in injuries and pathological consequences. This pathway is separate from the tumor suppressor pathway of p53. We have previously reported that the use of low-dose arsenic (LDA) temporarily and reversibly suppresses p53 activation, thereby ameliorating normal tissue toxicity from 5-FU and X-ray. We have also demonstrated that LDA-mediated protection requires functional p53 and thus is selective to normal tissues, as essentially every cancer cell has dysfunctional p53. Here, we tested the protective efficacy of LDA for bone marrow tissue against radioimmunotherapy through animal experiments.

Method and Materials—Mice were subject to LDA pretreatment for three days, followed by Y-90 ibritumomab tiuxetan treatment. Both dose-course (10, 25, 50, 100 and 200 μ Ci) and time-course (6h, 24h, 72h, 1wk and 2wk) experiments were performed. The response of bone marrow cells to LDA was examined by examining the expression of NF κ B, Glut1 and Glut3. H&E, γ -H2AX, and TUNEL staining was employed to examine morphology, DNA damage response and apoptotic cell populations.

Results—Elevated levels of NF κ B, Glut1 and Glut3 were observed in bone marrow cells after LDA treatment. Bone marrow damages induced by Y-90 ibritumomab tiuxetan were greatly reduced by LDA pretreatment. Consistent with this observation, significantly less DNA damage and fewer apoptotic cells were accumulated after Y-90 ibritumomab tiuxetan treatment in LDA-pretreated mice. Furthermore, in the mouse xenograft model implanted with human Karpas-422

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lymphoma cells, LDA pretreatment did not have any detectable effect on either tumor growth or Y-90 ibritumomab tiuxetan (200 μ Ci)-induced tumor suppression.

Conclusions—LDA pretreatment protected bone marrow without compromising tumor control caused by Y-90 ibritumomab tiuxetan.

Introduction

Radioimmunotherapy has a niche in the management of B-cell lymphoma and its role continues to evolve. Its main drawbacks have been grade 3 and 4 hematological toxicity and potential contribution to the development of myelodysplastic syndrome (MDS) and secondary leukemia especially in heavily pretreated patients [1]. Agents that ameliorate radiation-induced toxicity in bone marrow could improve the therapeutic ratio of radioimmunotherapy.

Arsenic trioxide is currently used to treat acute promyelocytic leukemia and is known as a cytotoxic agent. Arsenic is also known as a carcinogen. However, epidemiological data suggest certain threshold cumulative total dose needs to be reached for carcinogenic effect[2,3]. It has been well documented in vitro that arsenic has different biology and induces expression of different sets of genes depending on the dose[2,4].

p53 activation is a major pathway by which normal tissues respond to DNA damaging agents such as chemotherapy and radiotherapy, resulting in injuries and pathological consequences [5–8]. This pathway is separate from the tumor suppressor pathway of p53 [9,10]. We have previously reported that the use of low-dose arsenic (LDA) for 3 days before chemotherapy or external beam radiation therapy temporarily and reversibly suppresses p53 activation for about 5 days, thereby ameliorating the toxicity of the treatments [11–13]. We found that treatment with LDA in vitro and in vivo elicited a p53/NF κ B-mediated metabolic shift from oxidative phosphorylation to glycolysis, accompanied by increased expression of glucose transporter 1 (Glut1) and 3 (Glut3). As functional p53 is required in LDA-induced protection [12,13], and essentially every cancer cell has dysfunctional p53 [14,15], this LDA-mediated protection is thought to be preferential to normal cells but not cancer cells. Consistent with this notion, experiments using mouse models bearing SW-480 colon carcinoma or A549 lung cancer xenografts did show that LDA selectively protects small intestine and bone marrow from 5-FU or X-ray radiation without affecting their antitumor efficacy[12,13]. However, it has not been tested yet whether LDA protects the normal bone marrow against cytotoxicity of radioimmunotherapy by eliciting similar molecular responses.

Here, we report a preclinical animal model where LDA pretreatment has resulted in protection of the bone marrow from radioimmunotherapy without protecting lymphoma by using Y-90 ibritumomab tiuxetan as a model for radioimmunotherapy.

Methods and Materials

Animal study

Female Balb/c mice were purchased from Harlan Laboratories (Indianapolis, IN, USA) and were housed in the Department of Laboratory Animal Resources facility at our institute. All animal procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee. Mice were housed under pathogen-free conditions and maintained in a 12h light/12h dark cycle, with food and water supplied ad libitum. LDA pretreatment in mice has been described previously [12,13]. For Y-90 ibritumomab tiuxetan treatment, a dose of 10, 25, 50, 100 or 200 μ Ci of Y-90 ibritumomab tiuxetan was diluted with PBS resulting in a total volume of 100 μ L, and was administered to mice via tail vein injection. Ibritumomab tiuxetan was a gift from the Spectrum Pharmaceuticals (Henderson, NV, USA). Yttrium-90 was purchased through Eckert & Ziegler Radiopharma, Inc (Hopkinton, MA, USA). Product labeling was performed at the Cardinal Health (San Antonio, TX).

For mouse xenograft experiments, athymic nude mice (Balb/Cnu/nu, 5–6 weeks old) from Harlan laboratories were used. Inoculums of 5×10^6 Karpas-422, a human B-cell lymphoma cell line, in 0.1 ml of diluted Matrigel (1:1 in PBS) at 4°C were injected into the subcutaneous space on the right flank of mice. When tumors reached 0.1 cm in diameter, twenty mice were randomized into four treatment groups – control, LDA only, Y-90 ibritumomab tiuxetan only and Y-90 ibritumomab tiuxetan with LDA pretreatment. For Y-90 ibritumomab tiuxetan treated groups, 200 μ Ci of Y-90 ibritumomab tiuxetan was injected via the tail vein.

Tissue procurement and immunohistochemistry

Mice were sacrificed 6h, 24h, 72h, 1wk and 2wk after treatment. Cleaned femurs and tibias were fixed in 10% buffered formalin solutions and decalcified in 14% EDTA for 1 week. Decalcified samples were washed with tap water and placed into 70% ethanol. The high levels of radioactivity from Y-90 prevented us from using any instrument such as flow cytometer or fluorescent microscope, and so we had to pursue the following procedures after the radioactivity diminished.

Paraffin-embedded bones were cut into 4- μ m slides with bone marrow exposure. Bone marrow morphology was assessed by hematoxylin and eosin (H&E) staining according to standard procedure.

For Glut1, Glut3, NF κ B and γ -H2AX staining, a standard procedure was followed and has been described previously [12] except that the following primary antibodies were used: rabbit anti-Glut1 (Abcam, 1:500), anti-Glut3 (Abcam, 1:200), anti-NF κ B p65 (Abcam, 1:500) and anti- γ -H2AX (Cell Signaling Technology, 1:100). γ -H2AX staining was used as an indicator of DNA damage, as formation of γ -H2AX foci is widely used as a surrogate marker of double-strand DNA breaks. Results from γ -H2AX staining were quantitated by counting cells in three randomly chosen high power fields (400 $\times$) from each stained section via a double-blinded manner. All data represented the mean calculated from each mouse at

each time point, and the standard error of the mean (S.E.M.) was derived from the mean value from each mouse.

Assessment of apoptosis

Apoptotic cell death in bone marrow was detected using TUNEL staining. TUNEL assay was performed as described previously [16]. Briefly, after deparaffinization, nuclei of tissue sections were stripped from proteins by incubation with 20 mg/mL proteinase K for 15 min. Endogenous peroxidase was inactivated by incubation with 2% hydrogen peroxide for 5 min. The sections were then immersed in TdT buffer and incubated with TdT and biotinylated dUTP (Thermo Scientific, Hudson, NH) for 1h. Reaction was terminated by transferring the slides to TB buffer. The sections were then covered with 2% BSA for 10 min, followed by incubation with Streptavidin Horseradish Peroxidase (BD Biosciences, San Jose, CA), and stained with DAB Chromogen (Biocare medical, LLC, Concord, CA) for about 10 min. Slides were counterstained with 1% aqueous methyl green. Results were quantitated by counting cells in three randomly chosen high power fields (400×) from each stained section via a double-blinded manner. All data represented the mean calculated from each mouse at each time point, and the S.E.M. was derived from the mean value from each mouse.

Statistical analysis

Two-tailed paired Student's t-test was performed to find the significance between two groups. Where appropriate, results are expressed as the mean $\pm$ S.E.M. Values of $P < 0.05$ were considered to represent statistically significant differences.

Result

LDA ameliorates bone marrow damage by Y-90 ibritumomab tiuxetan in mouse

As LDA mediates normal tissue protection by eliciting NF κ B-mediated glycolysis [12], it was of interest to determine whether bone marrow cells respond to LDA by increasing expression of factors involved in glycolysis. We firstly assessed the expression level of NF κ B, Glut1 and Glut3 in bone marrow tissues collected from control mice and LDA-treated mice. Indeed, all of them were up-regulated in LDA-treated group (Fig. 1), indicating a possibility for bone marrow protection by LDA.

To study the protective effect of LDA from bone marrow damage induced by Y-90 ibritumomab tiuxetan, we examined the morphological changes after a single dose of Y-90 ibritumomab tiuxetan. Mice were treated with different doses of Y-90 ibritumomab tiuxetan and bone marrow tissues were harvested 24 hours later for morphological examination by H&E staining. As shown in Sup. Fig. 1, a single dose treatment with 10 μ Ci or 25 μ Ci of Y-90 ibritumomab tiuxetan inflicted little, if any, damage to bone marrow cells in our mouse model. However, bone marrow damages were readily observed when mice were subject to 50 μ Ci of Y-90 ibritumomab tiuxetan, and the damages got progressively more severe as the dose increased to 100 μ Ci and 200 μ Ci. To test whether LDA has protective effect on bone marrow against Y-90-induced damage, mice were pretreated with LDA for three days and

then treated with Y-90 ibritumomab tiuxetan. Pretreatment with LDA ameliorated the bone marrow damage by Y-90 ibritumomab tiuxetan (Sup. Fig. 1).

LDA protects bone marrow from Y-90 ibritumomab tiuxetan-induced DNA damage and apoptosis

Y-90 induced tissue damages in bone marrow are mainly caused by radiation-induced DNA damage and subsequent apoptotic cell death. To understand the protective effect of LDA on bone marrow, we conducted a time-course experiment to track DNA damage and apoptosis after Y-90 ibritumomab tiuxetan treatment. As shown in Fig. 2A–B, γ -H2AX expression level, an indicator of DNA damage [17], rose with time after Y-90 ibritumomab tiuxetan treatment. The percentage of γ -H2AX positive cells was $43.7 \pm 0.3\%$ 6h after treatment and reached a peak at 72h ($82.4 \pm 4.7\%$). However, DNA damages were significantly attenuated in mice pretreated with LDA. Only $28.4 \pm 1.8\%$ cells were γ -H2AX stained 6h after treatment ($P < 0.05$), and $61.5 \pm 3.2\%$ at 72h ($P < 0.001$). Remarkably, while DNA damage was largely eliminated in LDA-pretreated mice 2 weeks after Y-90 ibritumomab tiuxetan treatment ($1.9 \pm 1.9\%$), there was still fair amount of DNA damage ($16.8 \pm 2.1\%$, $P < 0.05$) in mice that had no LDA pretreatment.

Consistent with γ -H2AX staining result, TUNEL staining and quantification of TUNEL positive cells indicating apoptosis showed significantly lower counts in LDA-pretreated group than the control group 24h, 72h and 1 week after Y-90 ibritumomab tiuxetan treatment (Fig. 2C–D). Notably, at 72h when more than 80% of the cells underwent apoptotic cell death in Y-90 ibritumomab tiuxetan-treated group, the apoptosis rate in the LDA-pretreated group was 47% lower ($P < 0.001$). Damage in bone marrow persisted for at least 2 weeks after Y-90 ibritumomab tiuxetan treatment. Further morphological examination revealed that the time course of the DNA damage and apoptosis (Fig. 2A, C) paralleled the histological damage (Sup. Fig. 2), as all of them were detected 6 hours after Y-90 ibritumomab tiuxetan administration and peaked at 72 hours.

LDA does not protect malignant cells from Y-90 ibritumomab tiuxetan treatment

To test the hypothesis that LDA does not protect malignant cells, a mouse xenograft model was generated using a CD20-expressing lymphoma cell line, Karpas-422, in which p53 is mutant [18]. When tumors reached 0.1 cm in diameter, mice were randomly assigned to one of four groups: control, LDA pretreatment only, Y-90 ibritumomab tiuxetan treatment only, and LDA followed by Y-90 ibritumomab tiuxetan. No mice died during this experiment. As shown in Fig. 3, tumor size in the control group increased with time, and reached $(2.25 \pm 0.47) \times 10^3 \text{ mm}^3$ by the end of the experiment. LDA pretreatment had little effect on the growth of the implanted tumors ($(2.32 \pm 0.48) \times 10^3 \text{ mm}^3$). As expected, treatment with a single dose of Y-90 ibritumomab tiuxetan resulted in marked tumor growth suppression (average tumor size of $(0.41 \pm 0.10) \times 10^3 \text{ mm}^3$). And importantly, LDA pretreatment did not compromise the radiation-induced tumor growth suppression, as Y-90 ibritumomab tiuxetan-induced tumor regression is indistinguishable in mice that were pretreated with or without LDA ($(0.49 \pm 0.07) \times 10^3 \text{ mm}^3$ with LDA vs. $(0.41 \pm 0.10) \times 10^3 \text{ mm}^3$ without LDA). To verify this finding, this experiment was repeated independently, and similar result was

obtained that LDA had little effect on tumor growth and the anti-cancer role of Y90 ibritumomab tiuxetan (Sup. Fig. 3).

In summary, our results demonstrated that a brief pretreatment with LDA is associated with a marked protection of bone marrow from Y-90 ibritumomab tiuxetan without compromising the tumor control.

Discussion

Targeting of the bone marrow by Y-90 ibritumomab tiuxetan can result in cytopenia not only through damage in the CD20-expressing cells but also through adverse effects from radiation on normal cells [1]. In our mouse model, bone marrow damages were observed when mice were subject to as little as one dose of 50 μ Ci of Y-90 ibritumomab tiuxetan. The damages got progressively more severe when the dose increased to 100 μ Ci and then 200 μ Ci. Notably, pretreatment of mice with LDA provided marked resistance to Y-90 ibritumomab tiuxetan-induced damage (Sup. Fig. 1 and Sup. Fig. 2). This protection was also evident at the level of DNA damage, measured with the surrogate marker γ -H2AX (Fig. 2A–B). The lower percentage of cells with DNA damage and apoptosis (Fig. 2C–D) in LDA-pretreated group was not due to a delay in accumulation of γ -H2AX or apoptotic process, as mice with and without LDA pretreatment both showed similar temporal pattern of DNA damage and cell death.

The protective effect of LDA has been indicated by several lines of evidence. Epidemiological studies indicate that exposure to arsenic in drinking water at concentrations less than approximately 0.8 μ M is associated with risks of bladder or lung cancer that are below control values [3,19]. Although long-term exposure to LDA is thought to induce changes in the gene expression profile and neoplastic transformation, a brief use of LDA has been indicated as cytoprotective [20,21]. Our mouse model also found no detectable cancer development in both arsenic-treated and control mice during 12-month period, indicating that such a brief use of LDA in mice did not detectably increase cancer risk [12]. An in vitro study using human keratinocyte and fibroblast cells showed that treatment with 0.1–1 μ M arsenite for 24h produced protective effect against oxidative stress and DNA damage [22]. Moreover, LDA (0.1–10 μ M) treatment for 24h had no impact on the viability of UROtsa cell, a human urothelial cell line [23], but stimulated its mitochondrial activity. Consistent with these results, we recently reported that a 12h-exposure to 0.1 μ M arsenite protected human fibroblast cells against 5-FU or X-ray induced-DNA damage [12,13]. Remarkably, all these in vitro data come from non-cancerous cell lines, pointing toward selective protection of normal cells by LDA.

Though the mechanisms of LDA-conferred cytoprotection have not been fully understood, several studies using in vitro models have shed some light on it. It has been reported that LDA treatment potentiates the clearance of reactive oxygen species (ROS). As reported by Schuliga et al., this was attributed to a multicomponent response after LDA treatment in human keratinocytes that involves upregulation of redox enzymes including glutathione reductase, glutathione S-transferase, and glutathione [24]. Moreover, base excision repair (BER) pathway was enhanced after LDA treatment. At least three enzymes in BER pathway

have been shown to be upregulated by LDA: apurinic/aprimidinic (AP) endonuclease, DNA polymerase β (Pol β), and DNA ligase I. [22,25]. Recently, we have shown that brief treatment of untransformed cells with LDA induced concerted p53 suppression and NF κ B activation, which elicited a marked induction of glycolysis [12]. This metabolic shift provided cells with effective protection against cytotoxic chemotherapy or external beam radiation, coupling the metabolic pathway to cellular resistance. Co-treatment of LDA with 2-deoxyglucose, an inhibitor of glycolysis, abolished the protection of small intestine from 5-FU-induced damage, strongly supporting the notion that glycolysis induction is critical for LDA-mediated protection. [12] In the current study, we expanded our work by exploring the potential of LDA in protecting the bone marrow from radioimmunotherapy. We evaluated the expression level of several factors involved in glycolysis, and found marked induction of NF κ B, Glut1 and Glut3 by LDA treatment, as indicated by immunohistochemistry showing increase in these protein levels in LDA-treated mice (Fig. 1). These in vivo data suggested that bone marrow cells' response to LDA might include acquisition of survival advantage. Indeed, bone marrow damage induced by Y-90 ibritumomab tiuxetan was largely reduced in LDA-pretreated mice, as evidenced by the decreased number of DNA-damaged cells and apoptotic cells (Fig. 2). Importantly, given that functional p53 is required in LDA-mediated protection [12], and that essentially every cancer cell has dysfunctional p53 [14,15], LDA-mediated protection is very likely selective to normal cells. It is of significance that our LDA regimen that protected bone marrow did not compromise the tumor control by Y-90 ibritumomab tiuxetan in our B-cell lymphoma xenograft mouse model (Fig. 3).

For the past several decades, researchers have been investigating the use of drugs to decrease the side effects of radiotherapy, ultimately increasing the therapeutic ratio of radiotherapy. Although numerous compounds were tested for this purpose [26,27], amifostine is the only radioprotector that has been officially approved by the FDA for human use [28]. Unfortunately, this drug has not been widely used due to a variety of reasons such as cost, limited efficacy, and significant side effects (including hypotension, serious cutaneous reactions, nausea, vomiting, and anaphylaxis) and the need for daily IV administration [28]. We have previously shown that pretreatment with LDA for three days protects small intestine and bone marrow from toxicity induced by 5-FU and external beam radiation therapy in a mouse model, and no clear toxicity by LDA was observed during the experimental period [12,13]. And herein, we have demonstrated that LDA also significantly reduces bone marrow damage caused by Y-90 ibritumomab tiuxetan. Our work has also demonstrated the protective effect of the LDA against DNA damage from radioimmunotherapy, raising the speculation that this approach might reduce the incidence of MDS attributable to radioimmunotherapy. A recently completed pilot phase II clinical trial (<http://clinicaltrials.gov>: NCT01428128) conducted by our group has defined the dose of arsenic trioxide that suppresses the activation of p53 and demonstrated protection of the white blood cell and absolute neutrophil counts from myelosuppressive chemotherapy (data not shown). A clinical trial is under development to assess the bone marrow protective effect of the LDA pretreatment in patients to be treated with Y-90 ibritumomab tiuxetan based on our present data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary

Previous studies have shown that low-dose arsenic (LDA) treatment temporarily and reversibly suppresses p53 activation which is a major pathway leading to normal tissue injury from DNA damaging agents. Main toxicities of radioimmunotherapy such as Y-90 ibritumomab tiuxetan are myelosuppression and secondary myelodysplastic syndrome/acute myeloid leukemia. We herein demonstrate that LDA pretreatment can reduce DNA damage and bone marrow suppression from Y-90 ibritumomab tiuxetan without compromising tumor control in a xenograft B-cell lymphoma model.

Figure 1. NFκB, Glut1 and Glut3 staining of bone marrow sections of mice with or without low dose arsenic (LDA) treatment

Balb/c mice were treated with or without LDA for three days. Bone marrows were harvested for immunohistostaining with the indicated antibodies. Original magnification $\times 400$ for all panels.

Figure 2. γ -H2AX and TUNEL staining of bone marrow sections at different time points after Y-90 ibritumomab tiuxetan treatment

Balb/c mice were pretreated with or without low-dose arsenic (LDA) for three days, followed by administration of 200 μ Ci Y-90 ibritumomab tiuxetan (Y90) via tail vein injection. At the indicated time points, bone marrow tissues were harvested for staining. (A) Representative photomicrographs showing γ -H2AX staining of bone marrow sections at 400 $\times$ magnification. (B) Quantification of γ -H2AX positive cells. (C) Representative photomicrographs showing TUNEL staining of bone marrow sections. (D) Quantification of TUNEL positive cells in bone marrow sections. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3. Tumor growth after Y-90 ibritumomab tiuxetan treatment in mice with or without low-dose arsenic (LDA) pretreatment

Five million human B-cell Non-Hodgkin lymphoma Karpas-422 cells were injected subcutaneously at the right flank of each Balb/c nude mice in 100 μ L as a 50% suspension in Matrigel. When tumors reached 0.1 cm in diameter, mice were randomly assigned to the following groups: control, LDA only, Y-90 ibritumomab tiuxetan only (Y90), LDA pretreatment followed by Y-90 ibritumomab tiuxetan (LDA+Y90). Tumor sizes were measured with a Fisherbrand Traceable digital caliper (Fisher Scientific), and tumor volume was calculated using the equation: volume = length $\times$ width $\times$ depth $\times$ 0.5236 mm³. The tumor volumes are means $\pm$ S.E.M from one representative experiment. Photographs of tumors collected from mice (A) and the tumor growth curve (B) from one representative experiment are shown.