Irradiation of peripheral blood mononuclear cells with 7.5 Gy X-rays prior to donor lymphocyte infusion inhibits proliferation while preserving cytotoxicity, and improves the effectiveness of HSCT in patients with hematological malignancies

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Abstract. The aim of the present study was to explore the effect of different X-ray doses on the proliferation and cytotoxic activity of peripheral blood mononuclear cells (PBMCs), particularly lymphocytes, in order to assess whether this reduces the incidence of graft vs. host disease (GVHD) while preserving the graft vs. tumor (GVT) effect in patients with hematological malignancies following hematopoietic stem cell transplantation (HSCT). PBMCs from healthy donors were irradiated with X-rays at doses of 0, 2.5, 5, 7.5, 10, 15, 25 or 50 Gy, and their proliferative activity was determined using a WST-8 assay kit. The cytotoxic activity of non-irradiated PBMCs and PBMCs irradiated with 7.5 Gy X-rays was tested in the leukemic cell line K562 and its Adriamycin-resistant strain K562A using a lactate dehydrogenase assay. The clinical data of 7 patients who received 7.5 Gy X-ray-irradiated PBMC infusions following autologous HSCT were analyzed. PBMCs irradiated with ≥7.5 Gy X-rays exhibited a complete inhibition of proliferation. PBMCs irradiated with 7.5 Gy X-rays exhibited significantly increased cytotoxic activity towards K562 cells compared with K562A cells (P<0.05). There was no significant difference in cytotoxicity between irradiated and non-irradiated PBMCs, irrespective of the target cell, K562 or K562A (P>0.05). Based on the in vitro data, clinical data from patients who received 7.5 Gy X-ray-irradiated PBMC infusions following HSCT between January 2005 and January 2013 were assessed retrospectively. A total of 7 patients were included in the current study. The majority achieved various degrees of remission following donor lymphocyte infusion (DLI) and none suffered from GVHD. This indicates that 7.5 Gy-irradiated PMBCs have a potential application in DLI for the treatment of patients following HSCT. However, further studies on larger patient cohorts are required to assess the clinical potential of 7.5 Gy-irradiated PBMCs for preserving the GVT effect while avoiding GVHD following HSCT.

Introduction

Donor lymphocyte infusion (DLI) has been demonstrated to promote disease regression in selected patients with refractory and relapsed hematological malignancies, either by inducing a direct graft-versus-tumor (GVT) effect or by indirectly destroying the tumor cells (1-4). Several studies have demonstrated that DLI can promote the formation of donor-type chimerism, in addition to preventing or treating the relapse following bone marrow stem cell transplantation (5,6), particularly for the treatment of chronic myeloid leukemia. Thus, this technique has gradually gained a wide application in the management of other malignancies (7,8). Unfortunately, the severe graft vs. host disease (GVHD) that may be induced by DLI limits its application as a routine treatment for the prevention of tumor relapse. It has been demonstrated that irradiation of peripheral blood mononuclear cells (PBMCs) with ≥25 Gy prior to infusion decreases the probability of GVHD (9); however, the beneficial effects of DLI are impaired. The irradiation of PBMCs has been performed in certain clinical applications for over a decade (10-13).

Previous study from our group determined the optimum dose of X-rays required to inhibit PBMC proliferation using an MTT assay (7.5 Gy; Yu-Hua Sun, unpublished data). In the present study, an additional proliferation assay using a WST-8 kit was performed to confirm the optimum dose of X-rays, and then the cytotoxic activity of irradiated and non-irradiated PBMCs was measured. Additionally, the safety and efficacy of irradiated PBMCs was assessed in patients following autologous hematopoietic stem cell transplantation (HSCT) to avoid confounding factors from GVHD owing to HLA-haploidentical bone marrow transplantation.
Materials and methods

Preparation of PBMCs. PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation of blood samples from three healthy donors at the Peking University First Hospital (Beijing, China) between January 2015 and March 2015, and separation of the samples using a continuous automated flow cell separator (14). Granulocyte colony-stimulating factor was not administered to the donors prior to PBMC collection. The cells were stained with a MultiTEST™ IMK kit (BD Biosciences, Franklin Lakes, NJ, USA) (cluster of differentiation (CD)3/CD16/CD56/CD45/CD19 and CD3/CD8/CD45/CD4) and analyzed by using FACSCalibur flow cytometer (BD Biosciences) and CellQuest Pro software v 6.0 (BD Biosciences). PBMCs were irradiated with 0, 2.5, 5, 7.5, 10, 15, 25, or 50 Gy using an X-ray source (Varian 21iEX Linear Accelerator; Varian Medical Systems, Inc., Palo Alto, CA, USA) prior to the in vitro experiments. The non-irradiated group (0 Gy) served as the control.

Cell proliferation assay. Irradiated or non-irradiated PBMCs were seeded at a concentration of 1x10^6 cells/ml in Roswell Park Memorial Institute-1640 medium containing penicillin and streptomycin, supplemented with 10% fetal bovine serum (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), at 37°C with 5% CO_2 in a humidified incubator, and all cells were cultured in interleukin (IL)-2 (Peprotech, Inc., Rocky Hill, NJ, USA) at a concentration of 200 IU/ml for 2 weeks. The IL-2 medium was replenished each alternate day. Cell proliferation was analyzed using a WST-8 assay kit (Cell Counting Kit-8 (CCK-8); Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The cells (100 µl cells/well), at a concentration of 1x10^6 cells/ml, were seeded onto 96-well plates and incubated at 37°C with 5% CO_2. At the indicated time points (days 1 and 15), 10 µl CCK-8 solution was added to each well and the plates were incubated for 2 h. The absorbance was measured at 450 nm using a MultiSkam™ microplate reader (Thermo Fisher Scientific, Inc.). The results were obtained from three independent experiments performed in triplicate. The proliferation inhibition ratio was calculated using the equation illustrated below. The control group consisted of non-irradiated cells (0 Gy) while the blank control was fresh medium alone.

(%)Proliferation inhibition ratio = \frac{(Control group OD - Experimental group OD)}{(Control group OD - Blank control group OD) \times 100%}

Cytotoxicity assay. Cytotoxicity was assessed using a lactate dehydrogenase (LDH) assay following irradiation. Effector PBMC cells were isolated from the peripheral blood of healthy donors (4x10^6 cells/ml). Target (K562) and Adriamycin-resistant (K562A) cells (100 µl/well at a concentration of 2x10^5 cells/ml), in addition to the serial dilutions of effector cells, were incubated in a 96-well U-bottomed plate at 37°C for 4 h. Three independent assays were performed in triplicate at each effector:target (EF:TA) ratio used. The target or effector cells alone were used as spontaneous LDH release controls. To measure the maximum target cell LDH release, lysis solution was added to the target cells and the supernatants were assayed for their LDH concentration with the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega Corporation, Madison, WI, USA), with measured at 492 nm. The percentage of cytotoxicity was determined using the equation described below.

\[
\text{Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100\%
\]

Patients. The study was approved before 2005 by the Ethics Committee of Peking University First Hospital (Beijing, China; approval no. 808), and the clinical data were assessed retrospectively. Written informed consent was obtained from all participants. The methodology used for risk stratification was according to the World Health Organization classification (15). A total of 7 patients with hematological malignancies who received X-ray-irradiated PMBCs from day 9-330 following autologous HSCT at Peking University First Hospital between January 2005 and January 2013 were included in the present study. The PBMCs used were from HLA-haploidentical sibling or offspring. Confounding factors from GVHD owing to allogeneic stem cell transplantation could be avoided in these patients, in order to accurately evaluate the effects of DLI. A total of 21 infusions were administered (range, 1-5 infusions/patient). The median interval and average time were 34 and 80.6 days after HSCT, respectively (range, 9-330 days). The dose and the time to HCST of each infusion are shown in Table I. Prior to all the infusions, cells were irradiated with a 7.5 Gy X-ray dose, as this was determined to be the optimum dose to inhibit PBMC proliferation (Yu-Hua Sun, unpublished data). All patients were followed up to assess the outcome of their treatment and GVHD occurrence. A total of 5 patients received 7.5 Gy-irradiated infusions upon complete remission (CR) in an effort to prevent relapse. Another patient was treated for partial remission (PR), while the other patient relapsed with acute myeloid leukemia (AML) following HSCT and received infusions to reinforce the GVT effect. Patient clinicopathological characteristics and outcomes are provided in Table I.

Statistical analysis. SPSS software (version 19.0; IBM SPSS, Armonk, NY, USA) was used to compare the cytotoxicity of fresh and irradiated PBMCs. A paired t-test was used to compare cytotoxicity between dependent samples (fresh PBMCs to K562 vs. K562A cells; irradiated PBMCs to K562 vs. K562A cells). The Wilcoxon signed-rank test was used to compare cytotoxicity between paired samples (non-irradiated vs. irradiated PBMCs). P<0.05 was considered to indicate a statistically significant difference.

Results

PBMC subpopulation analysis. The phenotypes and ratios of PBMCs were analyzed using flow cytometry. The proportion of CD3+ cells was 71.67±3.06%, the proportion of CD3+CD8+ cells 28.33±2.89%, the proportion of CD3+CD4+ cells 42.33±5.86%, the proportion of CD19+ cells 13.00±4.58%, and the proportion of CD3–CD16–CD56+ cells 14.00±2.65% (Fig. 1). The majority of the PBMCs were lymphocytes [T cell-B cell-natural killer (NK) cell ratio, ~98.67%].

Determination of the optimum X-ray dose for the inhibition of PBMC proliferation. To evaluate the effect of irradiation on the proliferation of PBMCs, the cells were irradiated with eight
Table I. Patient characteristics and treatment outcome.

| Patient no. | Disease | Risk stratification | Age (years)/sex | Cell donor | Disease status pre-HSCT | Disease status pre-infusion | No. of infusions/cell dose, $x10^7$/kg (days after HSCT) | aGVHD status | Outcome |
|-------------|---------|---------------------|-----------------|------------|------------------------|---------------------------|-------------------------------------------------|-------------|---------|
| 1           | NHL (ENK/T) | Lugano II2 | 57/male | Haploidentical (son) | CR1 | CR | 4.32 (+9), 7.57 (+12) and 4.55 (+16) | No | CR for 9 years to present |
| 2           | NHL (DLBCL) | Ann arbor IVB | 48/male | Haploidentical (son) | PD | PR | 19.4 (+13), 14.1 (+20), 7.1 (+29) and 8.4 (+36) | No | PR for 7 months and died with PD |
| 3           | NHL (AITL) | Ann arbor IVB | 45/male | Haploidentical (daughter) | PR | Relapse | 8.7 (+120), 6.7 (+150), 7.3 (+180), 7.1 (+240) and 8.1 (+330) | No | CR at 2 years followed by secondary malignancy |
| 4           | MM | DS-IIIB/ISS III IgA $\kappa$ | 58/male | Haploidentical (son) | PR | CR | 3.23 (+106) | No | CR for 4 years to present |
| 5           | AML M2 | Medium | 34/male | Haploidentical (sister) | CR1 | CR | 8.72 (+14), 10.59 (+24) and 11.76 (+34) | No | CR for 7 years to present |
| 6           | AML M4EO | Favorable prognosis | 46/male | Haploidentical (son) | CR1 | CR | 7.6 (+26), 6.1 (+37) and 4.5 (+243) | No | Reinforced with CIK, and CR for 2 years to present |
| 7           | AML M2a | Medium | 38/male | Haploidentical (brother) | CR1 | CR | 7.7 (+20) and 5.4 (+34) | No | CR at 5 months followed by relapse |

NHL, non-Hodgkin lymphoma; DLBCL, diffuse large B cell lymphoma; ENK/T, extranodal NK/T cell lymphoma; AITL, angioimmunoblastic T cell lymphoma; MM, multiple myeloma; AML, acute myeloid leukemia; aGVHD, acute graft vs. host disease; CR, complete remission; CR1, achieving CR for the first time; PR, partial remission; PD, progressive disease; HSCT, hematopoietic stem cell transplantation.
Table II. Proliferation inhibition rates of PBMCs irradiated with different doses of X-rays.

| Dose (Gy) | Inhibition rate (%) | CI, confidence interval | SD, standard deviation |
|-----------|---------------------|-------------------------|------------------------|
| 0         | 0.0000              | 0.00                    | 0.0000                 |
| 2.5       | 76.0667             | 71.80-79.80             | 4.02658                |
| 5.0       | 78.8667             | 75.60-82.00             | 3.20208                |
| 7.5       | 98.6667             | 95.60-103.80            | 4.47363                |
| 10.0      | 98.9000             | 93.20-107.70            | 7.73111                |
| 15.0      | 98.1667             | 93.60-105.10            | 6.10437                |
| 25.0      | 103.1000            | 92.50-110.90            | 9.51420                |
| 50        | 111.1500            | 103.10-119.20           | 11.38442               |

CI, confidence interval; SD, standard deviation.

**Infusion of irradiated cells.** As 7.5 Gy was determined as the optimum X-ray dose in vitro, 7 patients who received 7.5 Gy X-ray-irradiated PBMCs following HSCT were followed up to assess the outcome of their treatment and GVHD occurrence. Differences in survival rates were not significant because of the small cohort size (data not shown). Therefore, each patient’s condition is described individually below.

Patient 1 suffered from extranodal NK/T-cell lymphoma that originated from the small intestine, with lymph node metastases in the popliteal and groin (Lugano II2). The patient did not progress into a CR state following 7 courses of chemotherapy; however, the patient achieved CR following a course of regular chemotherapy and HSCT for a long duration. Following PBMC infusion, the patient remained in continuous CR without GVHD for 9 years up to the present.

Patient 2 suffered from diffuse large B-cell lymphoma that originated from the small intestine, and was diagnosed with multiple tumor metastases in the ileum, urinary bladder and blood vessels during their initial consultation, where the patient presented with emaciation and night sweats (Ann Arbor IVB). Following 8 courses of chemotherapy, the disease continued to progress. The patient received salvage HSCT and irradiated PBMCs from their haploidentical son. The patient remained at PR for 7 months without GVHD prior to succumbing to the progressive disease.

Patient 3 suffered from angioimmunoblastic T-cell lymphoma and was diagnosed with multiple tumor invasions spread throughout the body on their first visit to the clinic. Following 7 courses of chemotherapy accompanied with low sugar consumption, the patient achieved PR, which was
determined by positron emission tomography-computed tomography; however, their lymph nodes remained enlarged. The patient relapsed 7 weeks subsequent to HSCT. Following the infusion of irradiated PBMCs, the patient remained in CR without GVHD for 2 years, and then suffered from a secondary malignancy.

Patient 4 suffered from multiple myeloma IIIB IgA κ (Durie and Salmon), with acute renal insufficiency as the first clinical manifestation. The patient achieved PR following 6 courses of chemotherapy, then received 1 infusion with haploidentical lymphocytes following HSCT, and has remained in continuous CR without GVHD for >4 years.

A total of 3 patients (5, 6 and 7) suffered from AML without a suitable matched donor for allogeneic hematopoietic stem cell transplantation prior to achieving CR at the first time (CR1), and received ASCT following CR1, with irradiated PBMCs preventing relapse. Only 1 patient remained in continuous CR without GVHD for 7 years. Another sustained CR without GVHD for >2 years, while the other relapsed at 5 months following 2 PBMC infusions without GVHD, but

Figure 2. (A) The inhibition ratios of the eight groups. The inhibition ratios of the seven irradiated groups stabilized over time. (B) The proliferation inhibition ratios for all seven irradiated groups were significantly different compared with the non-irradiated peripheral blood mononuclear cells, which was set as a reference (the proliferation inhibition was 0).

Figure 3. (A and B) PBMCs isolated from the peripheral blood of 3 healthy donors were tested as effector cells in the cytotoxicity assay. Irradiated or non-irradiated PBMCs did not show significant difference in cytotoxic activity against either of the leukemic cell lines, (C) K562 and (D) K562A, when mixed in a 1:1 to 20:1 EF:TA ratio. PBMCs, peripheral blood mononuclear cells; EF, effector cell; TA, target cell.
WEI et al.: 7.5 Gy X-RAYS INHIBIT LYMPHOCYTE PROLIFERATION BUT PRESERVE CYTOTOXICITY

Table III. Comparison of cytotoxic activity between irradiated and non-irradiated peripheral blood mononuclear cells against K562 cells.

| EF:TA ratio          | Median  | 95% CI      | SD    | P-value (irradiated vs. non-irradiated) |
|----------------------|---------|-------------|-------|----------------------------------------|
| 1:1 (non-irradiated) | 15.60   | 10.85-19.92 | 4.55  |                                        |
| 1:1 (irradiated)     | 13.73   | 10.51-17.10 | 3.30  | P>0.05                                 |
| 5:1 (non-irradiated) | 37.59   | 31.56-43.86 | 6.15  |                                        |
| 5:1 (irradiated)     | 38.94   | 34.32-46.60 | 6.68  | P>0.05                                 |
| 10:1 (non-irradiated)| 59.48   | 56.34-62.57 | 3.12  |                                        |
| 10:1 (irradiated)    | 62.69   | 59.78-66.87 | 3.71  | P>0.05                                 |
| 20:1 (non-irradiated)| 82.33   | 80.92-85.14 | 2.43  |                                        |
| 20:1 (irradiated)    | 81.90   | 80.41-82.26 | 0.96  | P>0.05                                 |

Table IV. Comparison of cytotoxic activity between irradiated and non-irradiated peripheral blood mononuclear cells against K562A cells.

| EF:TA ratio          | Median  | 95% CI      | SD    | P-value (irradiated vs. non-irradiated) |
|----------------------|---------|-------------|-------|----------------------------------------|
| 1:1 (non-irradiated) | 9.46    | 6.91-11.65  | 2.39  |                                        |
| 1:1 (irradiated)     | 8.10    | 5.92-9.99   | 2.05  | P>0.05                                 |
| 5:1 (non-irradiated) | 25.32   | 23.52-27.66 | 2.12  |                                        |
| 5:1 (irradiated)     | 22.22   | 21.28-23.49 | 1.14  | P>0.05                                 |
| 10:1 (non-irradiated)| 44.85   | 44.60-45.11 | 0.26  |                                        |
| 10:1 (irradiated)    | 47.98   | 46.33-50.05 | 1.90  | P>0.05                                 |
| 20:1 (non-irradiated)| 62.99   | 59.50-65.89 | 3.23  |                                        |
| 20:1 (irradiated)    | 61.20   | 54.02-64.81 | 6.22  | P>0.05                                 |

EF, effector cell; TA, target cell; CI, confidence interval; SD, standard deviation.

succumbed to the complications of transplantation from a related donor.

Patients were closely monitored for signs and symptoms of GVHD development following PBMC infusion, and no acute or chronic GVHD occurred in any of the 7 patients.

Discussion

DLI is one of the most effective treatment strategies for patients with hematological malignancies, and markedly improves survival rates. Allogeneic lymphocytes can induce a beneficial GVT effect or detrimental GVHD (16-19). Previous studies have primarily focused on minimizing the incidence of GVHD while preserving the GVT effect, such as the infusion of allogeneic donor T cells, delayed DLI time, gradually increasing the infusion dose, gene modification, the infusion of specific Epstein-Barr virus cytotoxic lymphocytes, and the infusion of granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells; however, these could not effectively prevent GVHD (10,20-24).

Previous studies have demonstrated that transfusion-associated GVHD can be entirely prevented with the infusion of lymphocytes irradiated with X-rays or γ-rays at a dose of ≥25 Gy. Lymphocytes do not perish immediately following irradiation; hence, there is no sustained GVHD with a rising proliferation inhibition rate (9,11,12). As a result, the transfusion of irradiated PBMCs has been performed in certain clinical applications for over a decade (9,11,12,25,26). The aim of the present study was to confirm the optimum dose of X-rays, under appropriate conditions, that can reduce the proliferation of lymphocytes while preserving or enhancing their cytotoxic activity, therefore improving the effectiveness of irradiation in the clinic.

The in vitro data from the present study demonstrated that PBMCs irradiated by different doses of X-ray exhibit various levels of proliferation inhibition, and PBMCs irradiated with ≥7.5 Gy exhibited a complete inhibition of proliferation. No significant difference was observed in the cytotoxicity between the irradiated or non-irradiated groups, irrespective of the target cell, K562 or K562A. For PBMCs irradiated

EF, effector cell; TA, target cell; CI, confidence interval; SD, standard deviation; n.s., not significant.
with 7.5 Gy X-rays, the cytotoxicity activity towards K562 cells was markedly stronger compared with that for K562A cells (P<0.05). However, PBMCs still exhibited some cytotoxicity towards K562A cells, indicating that they may have a potential application for the treatment of drug-resistant tumors.

Additionally, the in vitro data from the present study demonstrated that the proliferation of lymphocytes irradiated with 7.5 Gy was inhibited effectively without any significant decrease in cytotoxicity. Thus, 7.5 Gy may be the appropriate dose to irradiate lymphocytes with in order to rescue their GVT effect while avoiding GVHD.

Based on the in vitro data of the current study, the safety and efficacy of 7.5 Gy-irradiated haploidentical lymphocytes were reviewed in 7 patients. To test for the occurrence of GVHD caused by DLI, 7 patients that had received an autologous HSCT were chosen to avoid GVHD owing to HLA-haploidentical bone marrow transplantation. Notably, none of the patients in the present study developed GVHD following infusion with PBMCs irradiated with a 7.5 Gy dose. The patients benefitted from the 7.5 Gy-irradiated PBMC infusion regardless of whether their tumors were previously resistant to chemotherapy or not. Therefore, despite the small cohort size, the data suggest that infusion with PBMCs pre-irradiated with a 7.5 Gy dose of X-rays could be widely used as a treatment for a variety of patients. However, further studies with larger cohorts are required to verify the data from the present study.

In conclusion, the proliferation capacity of PBMCs was demonstrated to be inhibited by an X-ray dose of ≥7.5 Gy. There was no significant difference between the cytotoxicity activities for PBMCs in the 7.5 Gy-irradiated and non-irradiated groups; however, there was a significant difference between the cytotoxicity activities of the K562 and K562A cells, no matter the irradiation status of PBMCs. Patients that received infusions of PBMCs irradiated with a 7.5 Gy dose of X-rays achieved CR or had an extended overall survival time, with a low incidence of GVHD in the autologous HSCT/haploidentical DLI setting. Further studies with larger cohorts are required to assess the potential for 7.5 Gy X-irradiated PBMCs to rescue the GVT effect of HSCT while avoiding GVHD.

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