Radiosensitivity of thymic interleukin-7 production and thymopoiesis after bone marrow transplantation

Brile Chung, Lucia Barbara-Burnham, Lora Barsky, and Kenneth Weinberg

Interleukin-7 (IL-7) is the major thymopoietic cytokine. Injections of IL-7 after murine bone marrow transplantation (BMT) correct defects in thymic differentiation, including thymic hypocellularity, abnormal differentiation of CD3+ CD4+ CD8- (triple-negative [TN]) thymocytes into CD4+ CD8+ (double-positive [DP]) cells, and antigen-specific nature T-lymocyte proliferation. To determine whether IL-7 production is decreased in BMT recipients, BMT was performed with congenic murine donor-recipient strains and escalating doses of pre-BMT conditioning. Increasing doses of radiation resulted in decreased thymic cellularity and maturation from the TN to the DP stage. Quantitative reverse transcription–polymerase chain reaction analyses demonstrated that intrathymic production of IL-7 was significantly decreased in irradiated mice than in nonirradiated controls. Decline in IL-7 transcript levels was correlated with the dose of radiation administered. Analyses of the numbers of CD45+ major histocompatibility complex class II- thymic stromal cells suggested that the mechanism for the decreased IL-7 production was loss of IL-7–producing thymic stromal cells. Experiments indicated that pre-BMT conditioning with radiation led to decreased stromal production of IL-7 and consequent blocks in the maturation of thymocytes. They provided a mechanism for both the abnormal thymopoiesis observed after BMT and the previously observed beneficial effects of IL-7 administration in murine models. Impaired production of IL-7 by thymic stroma may be a general model for the clinically observed adverse effects of cytotoxic therapy on thymopoiesis.

Introduction

The immune deficiency observed after bone marrow transplantation (BMT) is a major cause of morbidity and mortality in patients who undergo transplantation and results in prolonged susceptibility to infection. Some of the immunologic defects observed after BMT have included abnormalities of thymopoiesis, activation of T lymphocytes, and antibody production. The thymus has been demonstrated to be a target of graft-versus-host disease (GVHD), and GVHD is associated with decreased thymopoietic capacity after BMT, resulting in decreased thymic output. However, abnormal numbers of circulating T lymphocytes have been observed in patients without GVHD, suggesting that other mechanisms besides GVHD suppress the production of new T lymphocytes. Thymopoietic defects may be due to the effects of radiation or chemotherapy on the thymic microenvironment. In addition, these effects may be age related. Analyses of patients undergoing either high-dose chemotherapy or BMT have shown an age-related decline in the production of new T lymphocytes. Abnormal numbers of T lymphocytes are especially evident in adult recipients of T-cell–depleted, matched, unrelated donor transplants, suggesting that the combined effects of age, alloreactivity, and high-dose cytotoxic therapy result in clinically significant defects in thymopoiesis.

We have been studying the thymopoietic defects in BMT using syngeneic or congenic mice as a model. In a previous study, the administration of interleukin-7 (IL-7) after BMT resulted in the normalization of thymic numbers, subpopulations, and T-lymphocyte proliferative responses to mitogens and antigens. The pattern of thymic subpopulations in the control animals that received BMT but not IL-7 suggested a block in thymic differentiation. Control BMT animals had increased frequency of immature triple-negative (TN) thymocytes and decreased frequency of double-positive (DP) and single-positive (SP) CD4+ and CD8+ thymocytes representing later stages of thymic differentiation. Defects in thymopoiesis were similar to those observed in X-linked severe combined immune deficiency (X-SCID), caused by inherited defects of the γc component of the IL-7 receptor (IL-7R). IL-7 is a stimulus for proliferation, survival, and differentiation of immature thymocytes. IL-7 is normally made by a subset of thymic epithelial cells that express major histocompatibility complex (MHC) class II. The similarities between the thymic defects observed after BMT and in X-SCID led us to examine whether IL-7 production is normal in BMT recipients. We have previously demonstrated increased circulating levels of IL-7 after BMT. However, these studies measured steady-state levels of IL-7 that were probably a function of IL-7 production by many stromal and peripheral dendritic cell sources and of consumption by IL-7R-bearing cells. Thus, analysis of the intrathymic production of IL-7 is necessary to determine whether the loss of IL-7 production contributes to the thymopoietic defects seen after BMT. In the present paper, we used a murine BMT model to test the hypothesis that pre-BMT radiotherapy inhibits IL-7 production and consequently interferes with post-BMT thymopoiesis. These studies...
demonstrated that IL-7–producing stromal cells, IL-7 mRNA production, and post-BMT thymopoiesis are radiosensitive.

Materials and methods

Animals

Recipient 4- to 6-week-old C57BL/6j mice expressing Ly5.2 (CD45.2) and B6.SJL mice congenic for Ly5.1 (CD45.1) (Jackson Laboratory, Bar Harbor, ME) were reared for 2 weeks after receipt. Untransplanted normal control mice were littermates of the recipient mice and were the same ages at the time they were killed. The animals were maintained in laminar flow cages with acidified water and antibiotics. Mice were killed with CO2 narcosis. All work was performed in accordance with protocols approved by the Animal Care Committee of the Children’s Hospital Los Angeles.

Bone marrow transplantation procedure

Recipient CD45.2+ mice were prepared for transplantation with radiation given in 2 divided doses (650 cGy on day −1 and either 0, 350, 550, or 750 cGy on day 0). The radiation source was a linear accelerator with blocks to given in 2 divided doses (650 cGy on day 0). The radiation source was a linear accelerator with blocks to

CAT-3’ (bp 905-885, exon 4) using the probe FAM–5’ AGGCTCTTTTC-CAGCCTCTTCTCTGG-3’–TAMRA (bp 856-882). PCR amplification parameters were 95°C for 15 seconds and 60°C for 60 seconds. Amplification of an IL-7 cDNA clone over a range from 1.5 × 102 to 2.9 × 103 copies per reaction was used as a standard. Values obtained from the linear range of the PCR reaction of each experimental sample were first standardized to the β-actin signal to normalize loading and then compared to the signals from the cloned IL-7 cDNA to determine copy numbers of IL-7 per microgram RNA.

Isolation of CD45+ MHC class II+ thymic stromal cells

The intact thymus was suspended in cold RPMI 1640 medium with 50 µg/mL DNase I (Sigma, St Louis, MO) in a 35-mm tissue culture dish and cut into small fragments. The fragments were then gently stirred in RPMI 1640 containing 12.5% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Nonadherent cells were removed by gentle washing. Adherent cells were detached with Cell Dissociation Buffer (Gibco/BRL). To quantify the numbers of CD45+ MHC class II+ cells, the cells were stained with fluorescein anti–murine CD45 and PE-labeled anti–I-Ab (A3) antibodies (Pharmingen). The number of CD45+ MHC class II+ cells was determined with the FACSCalibur flow cytometer and CellQuest software using previously defined parameters.19,20

Results

Inverse relationship between radiation dose before and thymocyte numbers after bone marrow transplantation

To test the effects of radiation on thymic reconstitution, mice were irradiated with total doses of either 1000, 1200, or 1400 cGy. The mean absolute number of thymocytes in normal mice was 142 × 106 ± 21 × 106 and was significantly greater than that seen in all transplantation groups on day 28 after BMT (P < .000 001). As shown in Figure 1, the total number of thymocytes on day 28 after BMT was inversely related to the pre-BMT dose of radiation.
and Thy1 antibodies established that the observed increase in TN cells resulted from the increased frequency of donor-derived thymocytes, not recipient (CD45.1\(^+\)) TN cells or non-T (Thy1\(^-\)) lineage cells. The increased frequency of TN cells and the decreased frequency of DP cells are consistent with a defect in the microenvironment that affects maturation of donor TN cells. Overall reduced cellularity of the thymus after higher doses of radiation is also consistent with such a block in differentiation.

Results were similar to those we previously described in animals that received BMT but not exogenous IL-7.\(^\text{12}\)

### Radiosensitivity of IL-7 transcript levels

The increase in thymic immaturity observed with higher radiation doses was consistent with a block in differentiation between the TN and the DP stages. Because IL-7 is critical for the maturation of TN cells, we investigated the effects of increased radiation doses on IL-7 production. Real-time RT-PCR was used to quantitatively measure the levels of IL-7 mRNA. Levels of IL-7 produced 5 and 28 days after radiation were determined by extraction of total thymic RNA immediately after they were killed, then reverse transcription and amplification. In initial experiments, animals received radiation without subsequent BMT. Thymic levels of IL-7 mRNA were significantly decreased in irradiated mice compared to normal mice (Figure 4A-B). On day 5 after irradiation, the mean level of IL-7 transcripts was 55% ± 19% of normal after 650 cGy, 36% ± 24% after 1000 cGy, and 17% ± 7% after 1400 cGy. Thus, radiation caused a rapid decrease in intrathymic IL-7 mRNA levels in a dose-dependent manner.

Because developing thymocytes have been shown to influence the thymic stroma, levels of IL-7 mRNA were then compared between mice that received radiation only (no BMT) and mice that also underwent transplantation. For the first 5 days after radiation treatment, there was no difference in the levels of IL-7 mRNA in irradiated mice not receiving transplants, and BMT mice. Because of hematopoietic toxicity, it was only possible to analyze later time points for IL-7 expression in the BMT mice. Defects in IL-7 production persisted for at least 1 month after BMT. On day 28
The absolute number of CD45 different doses of irradiation and at different time points after BMT. 5) It was also evident that regeneration of the CD45 actually killed by the pre-BMT irradiation, we analyzed the number of CD45 numbers of CD45 2 MHC class II 1 antigens.19,20 To determine whether IL-7–producing cells were adherent cells that do not express CD45 but do express MHC class II cells on day 5 after BMT, all the BMT groups had equivalent levels of IL-7 transcripts that were less than half of normal but were not statistically significant (Figure 4B).

Loss of CD45– MHC class II+ thymic stromal cells

In the mouse, thymic IL-7 production resides in a subset of adherent cells that do not express CD45 but do express MHC class II antigens.19,20 To determine whether IL-7–producing cells were actually killed by the pre-BMT irradiation, we analyzed the number of CD45– MHC class II+ adherent cells in the thymus after different doses of irradiation and at different time points after BMT. The absolute number of CD45– MHC class II+ cells on day 5 after BMT was significantly lower than normal. Numbers of CD45– MHC class II+ cells increased by days 15 and 28 but were still significantly less than normal in all transplantation groups (Figure 5). It was also evident that regeneration of the CD45– MHC class II+ stromal cells was greater after 650 cGy than after 1000 or 1400 cGy, but no statistically significant difference between 1000 and 1400 cGy was found. Results indicate that pre-BMT radiation decreases the number of CD45– MHC class II+ stromal cells in a dose-dependent manner and that recovery after BMT is also dose dependent.

Discussion

Reconstitution of immunity after BMT ultimately depends on the production of new lymphocytes from donor-derived hematopoietic stem cells. The difference in infectious complications and time to immune reconstitution in patients receiving unmanipulated marrow and T-cell–depleted marrow suggests that there is a transient role for adoptive transfer of mature T lymphocytes from the donor as a source of immune function early after BMT.23 Eventually, new T lymphocytes are produced from donor-derived hematopoietic stem cells and prothymocytes that mature in the host thymus. Thus, immune recovery after BMT is dependent on the maturational capacity of donor cells (transplanted hematopoietic stem cells) and host cells (thymic microenvironment).

In the present study, we have demonstrated that the dose of pre-BMT radiation has profound quantitative and qualitative effects on post-BMT thymopoiesis. Increasing doses of radiation decreased the capacity of the thymus to regenerate. There was an inverse relationship between the pre-BMT dose of radiation and the cellularity of the thymus. Decreased thymic cellularity was mainly attributed to decreased numbers of donor-derived thymocytes, not to increased destruction of recipient thymocytes. Furthermore, higher doses of radiation led to decreased maturation of the donor thymocytes, as evidenced by an increased proportion of immature TN cells and a decreased proportion of DP cells. In addition, the capacity of the thymic stroma to produce IL-7 was inversely related to the radiation dose given, providing at least one mechanism for the impaired thymopoiesis was observed. By day 28 after BMT, IL-7 transcript levels and numbers of IL-7–producing stromal cells were still abnormally low. We demonstrated that one mechanism of decreased thymic IL-7 production is the destruction of the stromal population that produces IL-7.

IL-7 is secreted by stromal cells from fetal liver, thymus, and bone marrow. In the murine thymus, IL-7 is produced by a subset of CD45– MHC class II+ epithelial cells distributed in both the cortex and the medulla.19,20 In murine thymic reaggregation assays, CD45– MHC class II+ stromal cells allow immature hematopoietic progenitors to undergo T-lymphoid differentiation.20 Specifically,
CD45<sup>−</sup> MHC class II<sup>+</sup> stromal cells permit the maturation of fetal liver–derived progenitors with germline T-cell receptor β (TCR-β) and TCR-α loci into cells with appropriate TCR-β and TCR-α rearrangements. IL-7 production by the CD45<sup>−</sup> MHC class II<sup>+</sup> stromal cells is critical in these reaggregation assays because thymic differentiation is not observed when MHC class II<sup>+</sup> stromal cells from IL-7–knock-out mice are used. In our analyses of the CD45<sup>−</sup> MHC class II<sup>+</sup> adherent cells after BMT, we found at least a 1-log decrease in this cell population, even at the lowest dose (650 cGy) of radiation used. Furthermore, higher doses resulted in greater losses of the CD45<sup>−</sup> MHC class II<sup>+</sup> adherent cells, suggesting that radiation killed these cells in a dose-dependent manner.

Besides intrathymic secretion of IL-7, other functions of stromal cells—eg, expression of c-kit ligand or integrins—are important for thymopoiesis. However, the defects seen in BMT mice after radiation are most consistent with a loss of IL-7–mediated signaling. IL-7 has been shown in various genetic experiments to be essential for the survival, proliferation, and differentiation of immature thymocytes. The relative increase in the proportion of TN thymocytes and the decrease in DP thymocytes in the irradiated mice are similar to those seen in X-SCID dogs that lack the γc subunit of the IL-7 receptor. It is likely that damage to the thymic microenvironment by pre-BMT conditioning led to decreased maturation of TN to DP thymocytes. In previous experiments, we have shown that the administration of recombinant IL-7 improves the thymopoietic defects seen after BMT, indicating that the loss of IL-7 production alone is sufficient to explain much of the impaired thymopoiesis we observed. Furthermore, cotransplantation of bone marrow stromal cells transduced with the IL-7 gene can also largely correct the thymopoietic defects (E. Bolotin et al, manuscript submitted, 2001).

RT-PCR data indicate that transcript levels for IL-7 decreased within 5 days of radiation treatment. The decrease in IL-7 production is likely to be greater than that observed by RT-PCR of whole thymic RNA. Using total thymic RNA as template was necessitated by the observation that IL-7 production by dissociated whole thymic RNA. Using total thymic RNA as template was most consistent with a loss of IL-7–producing subset. It is possible that decreased intrathymic IL-7 production underlies the abnormal thymopoietic capacity observed during aging and that IL-7 administration can correct thymic defects. The mechanism for the loss of intrathymic IL-7 production during aging is unknown. Our results differ from those of a recent study of decreased IL-7 production by bone marrow stromal cells in aged mice that demonstrated a posttranscriptional mechanism rather than destruction of the IL-7–producing subset. It is possible that the mechanisms for the effects of aging and radiation on IL-7 production may be different.

Loss of the IL-7–producing thymic stromal cells may be a common mechanism that underlies many pathogenic processes, leading to thymic insufficiency, such as radiation therapy, high-dose chemotherapy, GVHD, aging, and human immunodeficiency virus infection. Several clinical results have suggested that thymopoietic capacity is damaged by chemotherapy, radiotherapy, and GVHD. Adolescent and adult recipients of high, but nonmyeloablative doses of chemotherapy do not regenerate naïve CD4<sup>+</sup> T lymphocytes as well as young children do. Similarly, adults who undergo BMT have impaired production of naïve CD4<sup>+</sup> T cells. The nature of the thymopoietic defect is difficult to ascertain in the clinical setting because thymic biopsy samples are not taken after chemotherapy or radiotherapy. The present experiments demonstrating decreased IL-7 transcripts and abnormal thymic maturation provide a model for how patients may develop treatment-related thymic insufficiency. Analyses of the post-BMT thymus indicate that there is a capacity for regeneration of the CD45<sup>−</sup> MHC class II<sup>+</sup> stromal cells after BMT. Further studies to determine the mechanism for the regeneration of CD45<sup>−</sup> MHC class II<sup>+</sup> stromal cells may allow the development of strategies either to protect these cells from radiation or to stimulate their recovery with epithelial cell–specific cytokines such as keratinocyte growth factor. Reconstitution of thymopoiesis in patients may require direct efforts to restore the function of the thymic epithelial cells.

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