Original Article

The Late-Stage Foetal Liver Microenvironment Is Essential for Later Sensitivity of B-Lymphopoiesis to Suppression by Oestrogens

(stem cells / foetal liver haematopoiesis / B-lymphopoiesis / oestrogen)

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Abstract. B-lymphopoiesis in FL differs notably from that of adult B-lymphopoiesis in being resistant to suppression by oestrogens due to the lack of expression of oestrogen receptors in B-cell progenitors and precursors. We have transplanted middle-stage FL cells (E14.5) to adult male mice and demonstrated that B-lymphopoiesis derived from FL cells remained resistant to suppression by exogenous oestrogen for several months compared to adult BM cells. This significant difference strongly suggests that the late-stage FL environment exerts an inductive action on the haematopoietic stem cells and is mandatory for later sensitivity of B-lymphopoiesis to suppression by oestrogens. The results also provide the first *in vivo* functional confirmation of a differential responsiveness of FL- and adult BM-derived B-lymphopoiesis to suppression by oestrogens.

Introduction

B-lymphopoiesis is suppressed in the bone marrow (BM) of pregnant mice due to the action of increased levels of sex steroid hormones (Medina et al., 1993; Medina and Kincade, 1994) and is stimulated by oestrogen deficiency (Masuzawa et al., 1994; Smithson et al., 1998; Onoe et al., 2000; Shim et al., 2004). These studies on suppression of B-lymphopoiesis by sex steroid hormones have been based on demonstration that early pro-B cells are directly supressed by oestradiol in culture (Kouro et al., 2001) and by demonstration that B-cell progenitors and precursors from adult BM express both oestrogen receptors (ER), ER α and β, as well as those for androgens and glucocorticoids (Igarashi et al., 2001). *In vivo*, very early lymphoid progenitors present in BM were shown to be significantly suppressed by oestrogens (Medina et al., 2001). A paradoxical situation exists in the middle- and late-stage foetal liver (FL), where an intensive B-lymphopoiesis occurs (Strasser et al., 1989; Chang et al., 2005) in the presence of high levels of oestrogens. This has been explained by the absence of ERs on the B-cell precursors and progenitors from FL (Igarashi et al., 2001), making them insensitive to oestrogens. Igarashi et al. (2001) also demonstrated that FL-derived haematopoietic stem cells/progenitor cells (HSC/P) still lacked the receptors for sex steroid hormones four weeks after transplantation of middle-stage FL HSC/P to irradiated RAG-1-deficient mice, and that the B-lymphopoiesis derived from these cells was less sensitive to inhibition by oestrogens compared to that derived from adult BM HSC/P.

There are several features in which FL and BM haematopoietic cells differ between each other (Hardy and Hayakawa, 1991; Medina and Kincade, 1994). However, since BM haematopoiesis is directly derived from FL haematopoiesis by migration of late-stage FL HSC/P to newly formed BM (Christensen et al., 2004), it is evident that the BM microenvironment or an intrinsic programme in HSCs eventually converts the FL phenotype of haematopoietic cells into the adult BM phenotype. Actually, such a conversion was demonstrated to occur during the first two postnatal weeks regarding expression of IL-7 receptor that is absent on FL B-cells (Kikuchi et al., 2005, Kikuchi and Kondo, 2006).

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Abbreviations: BM – bone marrow, ER – oestrogen receptor, FL – foetal liver, HSC/Ps – haematopoietic stem cells/progenitor cells, WBCs – white blood cells.

In the present study, we investigated whether FL14.5 HSC/Ps are able to gain sensitivity to oestrogens without passing through the late-stage FL phase. Our results strongly suggest that exposure of HSC/P to the late-stage FL microenvironment is mandatory for later sensitivity of B-lymphopoiesis to suppression by oestrogens.

Materials and Methods

Animals

C57BL/6 mice (B6-Ly5.2 and B6-Ly5.1, Charles River Laboratories) 8- to 12-weeks-old were maintained in a clean conventional animal facility with a light-dark cycle of 12 hours and fed *ad libitum*. To set up mating, females were examined in the afternoon, and those in oestrus were placed in cages with males (two females with one male). The morning after mating, the females were checked for the presence of a copulation plug in the vagina, and this day was designated as embryonic day 0.5 (E0.5).

Cells for transplantation

Foetal livers from (E14.5) embryos and BM from both femurs of normal adult mice were collected. A single-cell suspension was prepared in a phosphate-buffered saline (PBS) solution containing 0.5% albumin by repeated flushing the tissues through needles of 18 and 27 gauges. Finally, cells were passed through a nylon mesh with a pore size of 70 μm (Falcon 2350, Becton, Dickinson Labware, Franklin Lake, NJ), counted and appropriately diluted.

Transplantation to primary recipients

Eight to twelve-week-old B6-Ly5.1 or Ly5.2 host male mice were lethally irradiated with a dose of 11 Gy (5.5 + 5.5) from a ^{60}Co source. Two hours after irradiation the mice were intravenously injected with a mixture of 5 x 10^6 B6-Ly5.2 BM cells and 5 x 10^6 B6-Ly5.1 FL cells resuspended in 0.5 ml of PBS with 0.5% albumin. The presence of FL-derived cells was followed in the peripheral blood for up to 13 weeks.

Retransplantation to secondary recipients

Host male mice 8 to 12 weeks old were lethally irradiated with a dose of 11 Gy (5.5 + 5.5) from a ^{60}Co source. Two hours after irradiation they were intravenously injected with 10^7 BM cells obtained from mice that had been sublethally irradiated (4 Gy) and transplanted with 10^7 FL (E14.5) cells 20 weeks ago. Half of these animals were treated with oestrogens as described. After 20 weeks there was 22% FL chimerism in the BM of control mice and 28% chimerism in BM of mice treated with oestrogens. The transplanted cells were suspended in 0.5 ml of PBS with 0.5 % albumin. The presence of FL-derived cells was followed in the peripheral blood for up to 17 weeks.

Oestrogen treatment

Oestrogens (estradiolum hemihydricum; Estrofem, Novo Nordisk A/S, Bagsvaerd, Denmark) were added to food starting one day before transplantation in an amount corresponding to delivery of approximately 100 μg of 17β oestradiol/mouse/day. Additionally, oestrogen-treated mice received 100 μg of estradioli dipropionas (Agofollin, Biotika, Slovak Republic) i.m. one day before transplantation to substitute a decreased food intake following preconditioning with irradiation. The oestrogen treatment resulted in diminished weight of the testes and seminal vesicles and by partial obliteration of the cavity of long bones harbouring BM. These are well-known effects of oestrogen administration (Samuels et al., 1999; Onoe et al., 2000; Perry et al., 2000; Erlandsson et al., 2003) and the examinations were used to confirm oestrogen exposure.

Analysis of recipients

At 2, 4, 8, 13, and 17 weeks after transplantation, peripheral blood from recipients was obtained from the retro-bulbar plexus. Aliquots (~50 μl) from each blood sample were added to separate tubes filled with 3 ml lysis buffer (0.15 M NH_4Cl, 0.035 M NaCl, and 0.1 mM EDTA), and red blood cells were lysed for 10 min. Cells were washed twice, resuspended in PBS, and stained with phycoerythrin (PE)-conjugated anti-Ly5.1 and fluorescence isothiocyanate (FITC)-conjugated anti-Ly5.2 antibodies. They were simultaneously stained with biotinylated anti-B220 antibody. This was followed by addition of streptavidin-PE-Cy-5. All antibodies and reagents were purchased from Pharmingen (San Diego, CA). Multicolour analysis was performed in FACS Calibur (Becton, Dickinson, San Jose, CA). Gating for Ly5.1$^+$ and Ly5.2$^+$ cells was performed, and Ly5.1$^+$Ly5.2$^+$ artificial doublets were omitted from the analysis.

Statistical analysis

Groups of eight recipient mice given the same treatment were used to calculate the mean values and standard deviations of the percentage of FL-origin and adult BM-origin cells. These values were used to calculate P values using the two-tailed Student's *t*-test.

Results

Sensitivity of B220$^+$ cells of FL origin or adult BM origin to oestrogens after transplantation into lethally irradiated recipients

Lethally irradiated male Ly 5.1 or Ly 5.2 mice were transplanted with a mixture of 5 x 10^6 Ly 5.1 FL (E14.5) cells and 5 x 10^6 Ly 5.2 BM cells. Fig. 1 presents the percentage of all FL-origin white blood cells (WBCs), as well as the percentages of FL-origin B220$^+$ cells until day 90 after transplantation. FL cells were more efficient in restoring haematopoiesis in lethally irradiated

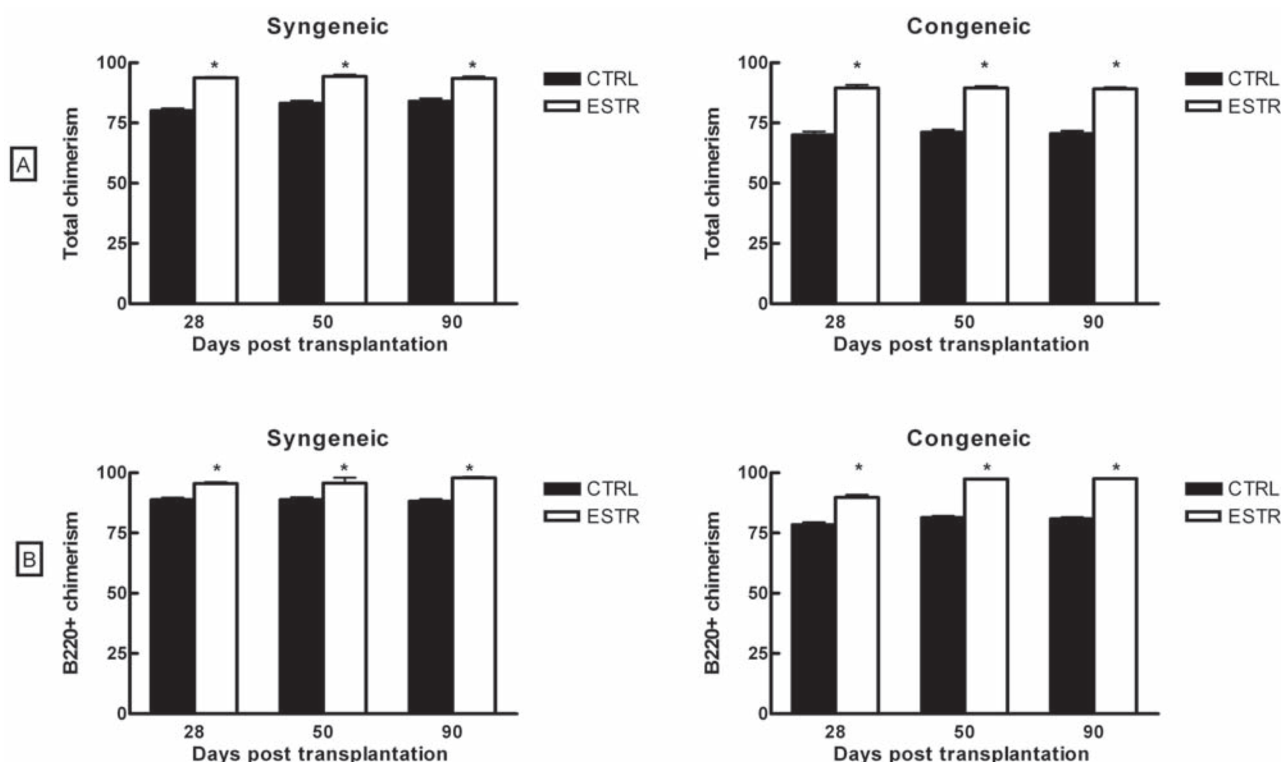

Fig. 1. Cells of FL origin in peripheral blood: total chimerism (A) and B220$^+$ chimerism (B) after transplantation to syngeneic (Ly5.1) and congeneic (Ly5.2) mice. (*) P values < 0.05.

recipients as compared to the same number of BM cells. Oestrogen administration further enhanced the percentage of FL-origin WBCs in the blood, as well as B220$^+$ FL-origin cells, over those of competing BM-origin cells (Fig. 1). On the other hand, the ratio between CD3$^+$ cells and Gr-1/Mac$^+$ cells of FL and BM origin was not changed by oestrogens (results not shown).

Sensitivity of B220$^+$ cells of FL origin and adult BM origin to oestrogens after retransplantation into lethally irradiated recipients

Sublethally (4 Gy) irradiated Ly5.2 mice transplanted with 5 x 10^6 Ly5.1 FL cells (E14.5) presented with FL-origin chimerism as shown in Fig. 2A. The chimerism was significantly higher in the group treated with oestrogens. There was 22% chimerism in control mice and 28% in oestrogen-treated mice in blood at the time of sacrifice and BM transplantation to secondary recipients. Oestrogens increased the proportion of FL-origin blood cells 28 days and 50 days after the secondary transplantation (Fig. 2B), and the proportion of B220$^+$ cells of FL origin from 28 days until 90 days (Fig. 2B).

Discussion

Common lymphoid progenitors from adult BM possess characteristic phenotypic features that include lack of expression of lineage-specific markers (Lin$^-$ cells), low levels of expression of c-Kit and Sca-1 antigens, high expression of Flt3 and IL-7R receptors, and expression of TdT and RAG-1 enzymes (Medina et al., 2001; Igarashi et al., 2002). The progenitors are slowly proliferating in adult BM (Pelayo et al., 2006), and oestrogens are potent inhibitors of B-lymphopoiesis acting on early developmental stages of B-cell progenitors and early pro-B cells (Kouro et al., 2001). The B-cell progenitors from FL significantly differ from their adult BM counterparts. They are actively proliferating (Pelayo et al., 2006), and their growth and maturation are independent of IL-7 stimulation (Hardy and Hayakawa, 1991; Carvalho et al., 2001). Furthemore, they are resistant to suppression by oestrogens (Kincade et al., 2002). In mice, the conversion of the FL phenotype of B-cell lymphopoiesis into the adult BM phenotype was shown to occur during the first two postnatal weeks in the case of IL-7 sensitivity, but has not been precisely established for resistance to oestrogens. Nevertheless, Igarashi et al. (2001) demonstrated that after transplantation of middle-stage FL (E15) haematopoietic cells to adult irradiated recipients, the B-lymphopoiesis derived from FL was still significantly less sensitive to suppression by oestrogens as compared to that of adult BM after four weeks.

We have transplanted adult male mice with a mixture of FL and adult BM cells and examined sensitivity of the haematopoiesis derived from the two sources to oestrogens utilizing the Ly5.1/Ly5.2 transplantation model. The model is widely used under the assumption of full histocompatibility. Since minor immune reactions were reported in this experimental settings by van Os et al. (2001), we have used transplantation of a mixture of

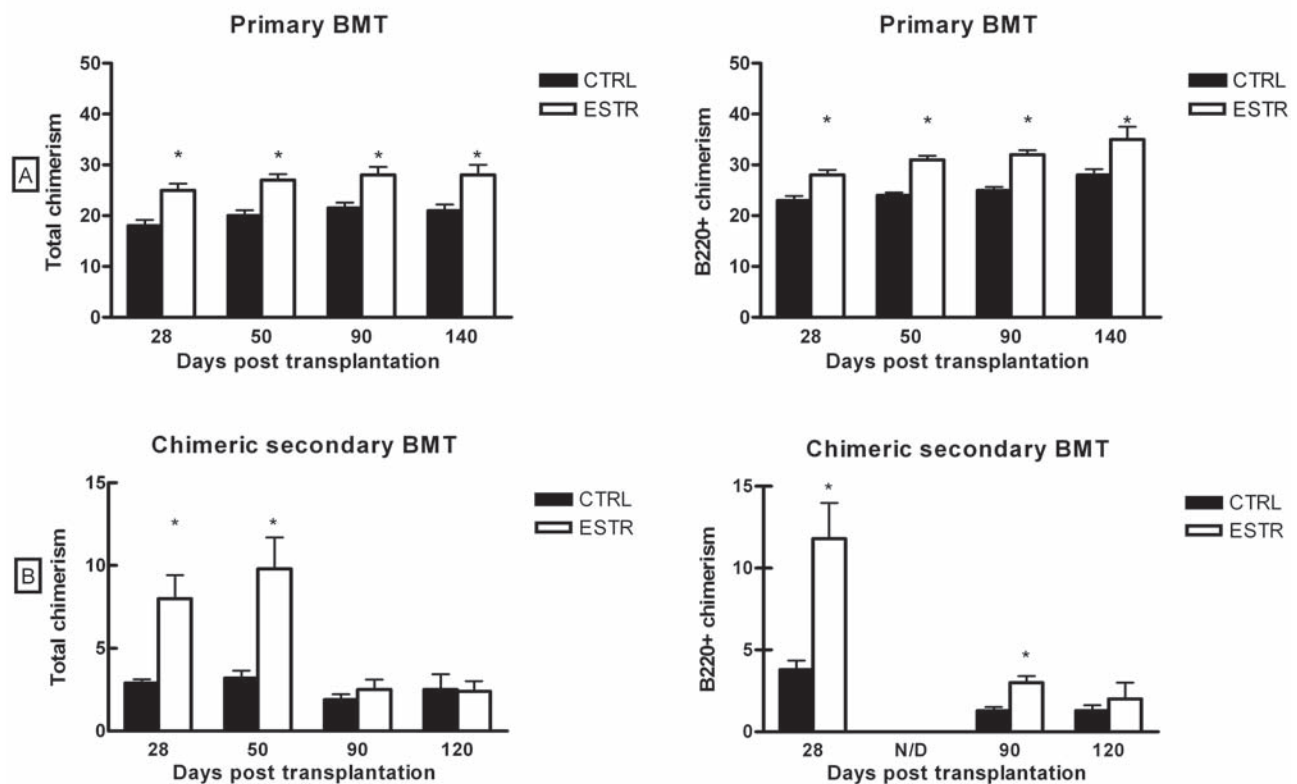

Fig. 2. Cells of FL origin in peripheral blood after primary and secondary transplantation. (*) P values < 0.05.

foetal and adult BM cells into lethally irradiated syngeneic and congeneic recipients with respect to the FL Ly5.1 cells (for details see Fig. 1). Although a slightly higher engraftment occurred in the syngeneic recipients (Ly5.1), there was no significant difference in the sensitivity of B-lymphopoiesis to oestrogens in both experiments (Fig. 1). The higher levels of FL-origin B-cells in oestrogen-treated mice demonstrated their higher resistance to suppression by oestrogens as compared to BM-origin cells. The phenomenon was present for the entire 90-day lasting examination period.

To examine whether the insensitivity to oestrogens is a permanent feature of B-lymphopoesis derived from E14.5 FL cells, FL/BM chimeric haematopoiesis was transplanted to secondary recipients in another experiment. This allowed following oestrogen effects on FL-derived B-lymphopoiesis up to 260 days (Fig. 2). The difference in sensitivity to oestrogens between FL- and BM-origin B-lymphopoiesis was also expressed in secondary recipients for at least 50 days (Fig. 2B).

Our previous results demonstrated that HSCs/progenitors from different stages of FL development were ready to home and function in the environment of adult haematopoiesis (Chang et al., 2005). The different phenotype of FL and adult BM B-cell progenitors reported by Igarashi et al. (2001) and Medina et al. (2001) thus presented a challenge that we approached by examining the timing of conversion of the oestrogen-resistant phenotype of FL B-lymphopoesis into the sensitive phenotype of the adult BM B-lymphopoesis. The results provided the first *in vivo* functional confirmation of a differential responsiveness of FL- and adult BM-derived B-lymphopoiesis to suppression by oestrogens. Surprisingly, the B-lymphopoiesis derived from FL cells, obtained from embryonic day 14.5, remained significantly less sensitive to inhibition by oestrogens compared to that of adult BM origin for several months and even after transplantation into secondary recipients.

Hence, although HSC/P cells from different stages of FL development can home and engraft to damaged BM microenvironment successfully (Chang et al., 2005), an exposure of HSCs to the late-stage FL microenvironment seems to be critical and mandatory for the proper timing and a full conversion of the FL phenotype of the B-lymphopoiesis into that of the adult BM phenotype.

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