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The Role of Estrogen Receptors and Androgen Receptors in Sex Steroid Regulation of B Lymphopoiesis

Glennda Smithson,* John F. Couse,† Dennis B. Lubahn,‡ Kenneth S. Korach,† and Paul W. Kincade2*†

Several observations suggest that sex steroids might participate in steady state regulation of B lymphopoiesis. B cell precursors decline dramatically in bone marrow of pregnant or estrogen-treated mice. Reciprocally, the same cell populations are increased in hypogonadal mice or male castrates. Estradiol treatment of hypogonadal mice reduced precursors to normal. However, questions remain about which hormones and receptors are the most important. Furthermore, these observations need to be reconciled with advances regarding new sex steroid receptors. We have now characterized B lymphopoiesis in androgen receptor-deficient testicular feminization (Tfm) mice. Testicular feminization mice had substantially elevated numbers of B cell precursors in the bone marrow and B cells in the spleen as compared with wild-type mice. Our previous studies indicated that hormone receptors in stromal cells may be important for estrogen-mediated suppression of B lymphopoiesis. We now show that estrogen-mediated inhibition of B cell precursor expansion in culture was blocked by a specific estrogen receptor antagonist (ICI 182,780). Stromal cells derived from ERα-targeted bone marrow were fully estrogen responsive. RT-PCR analyses of these stromal cells revealed splice-variant transcripts of ERα, as well as message for a recently discovered estrogen-binding receptor, ERβ. Thus, androgens may normally inhibit B lymphopoiesis through the androgen receptor, whereas estrogens might utilize one or more receptors to achieve the same physiologic response. The Journal of Immunology, 1998, 161: 27–34.

The number of B lymphocytes produced within marrow is tightly regulated by intrinsic processes that allow lineage-committed precursor cells to respond to external stimuli. Cells of the microenvironment regulate precursor survival and proliferation by providing critical and timely molecular signals. Several lines of evidence suggest that sex steroids may be physiologic regulators of lymphopoiesis (1–5). For example, increases in systemic levels of estrogen, or normal pregnancy, result in preferential reduction of lymphocytes (1, 2, 4). Treatment of mice with dihydrotestosterone (DHT)3, a form of testosterone that cannot be metabolized to estrogen, also reduced numbers of IL-7-responsive B cell precursors (4).

Conversely, reduced levels of circulating sex steroids resulted in up-regulation of B lymphopoiesis in the bone marrow. A mutation in the gonadotrophin-releasing hormone gene prevents appreciable production of sex steroids in hypogonadal (hpg) mice (6–8). These animals had substantially higher numbers of B cell precursors than littermate controls. A less dramatic increase in B cell precursor production was seen in male castrates (3). Female castration did not always result in an elevation in B cell precursors and may be both time and strain dependent (3, 9).

Estrogen appears to suppress B lymphopoiesis by altering cells in the marrow microenvironment. Direct treatment of B cell precursors with estrogen had no consequence on their survival, proliferation, or differentiation. However, the hormone reduced their expansion when stromal cells were also present in the cultures (2). Stromal cells and lymphocytes did not have to be in direct contact to observe the influence of estrogen. Moreover, estrogen-pulsed stromal cells produced some suppressive substance(s) (Ref. 2, and our unpublished observations). One estrogen receptor (ERα) was shown to be expressed by these stromal cells, and others have demonstrated functional receptors for both androgens and estrogens in marrow stromal cells (2, 10, 11).

Estrogen and testosterone commonly mediate their effects through well characterized nuclear receptors. When bound by ligand, the nuclear steroid receptors interact with hormone response elements in the DNA and trans-activate specific genes (12). The androgen receptor (AR) is encoded by a single gene on the X chromosome (13). Testicular feminization mice (Tfm) have a single point mutation in the AR gene causing a frame shift that results in unstable mRNA (14). However, low levels of a smaller, androgen binding AR protein that lacks the N-terminal domain have been detected in Tfm mice (14, 15). This domain of the AR is thought to be important for transcriptional activity (14). Thus, although Tfm mice retain residual androgen binding, they are androgen insensitive. The Tfm mutation is unique to male mice. Female Tfm homozygotes cannot be obtained by normal mating since male mice with the defective AR are sterile (14–17). Tfm are indistinguishable from normal female mice except for small internal testes (17). The lymphopoietic status of these mice has not been well characterized, but they have somewhat larger thymuses and spleens than littermate controls (18).

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‡Abbreviations used in this paper: DHT, dihydrotestosterone; hpg, hypogonadal; AR, androgen receptor; Tfm, testicular feminization; ER, estrogen receptor; ERKO, estrogen receptor knock out; sIg, surface Ig; PE, phycoerythrin.
Estrogen can interact with at least two distinct nuclear receptors designated estrogen receptor α (ERα) and estrogen receptor β (ERβ). ERα was cloned from mouse uterus and is required for most estrogen induced uterine functions (19, 20). ERβ was originally cloned from the rat prostate (21). The human (22) and mouse (23) ERβ homologues have recently been cloned. The ERβ is highly homologous to ERα with respect to the DNA and ligand binding domains in all three species. Interestingly, a variety of different size transcripts for ERβ are detected in mouse ovary, whereas only a single transcript for ERα is detected in that site (23). Like ERα, ERβ is a high affinity receptor that binds estrogen responsive elements in vitro and can induce transcription of estrogen regulated genes. A biologic function has not yet been attributed to ERβ, but it is hypothesized to mediate functions distinct from ERα based on its differential expression (24).

Recently, estrogen receptor knock out mice (ERKO) have been generated by inserting a neomycin gene into the second exon of the ERα gene (20, 25). These mutants have no detectable wild-type ERα mRNA. However, there is some residual estrogen binding in the uteruses of these animals that reacts with the ERα-specific Ab H222. Very low levels of mRNAs for two unique splice variants of ERα were detected in ERKO mice. One of these splice variants, potentially, could produce a protein that retains a high affinity for estrogen and could be responsible for residual estrogen binding in the uterus. However, in vitro studies suggest that this splice variant had a reduced transcriptional capacity, consistent with the relative unresponsiveness of the ERKO uterus to estrogen.

We have now exploited these deficient mice to determine the relative importance of sex steroids and their receptors for regulation of B lymphocyte formation. Loss of the androgen receptor resulted in an increase in B lineage cells, particularly those not yet expressing sIg. In vitro treatment with the antiestrogen drug ICI 182,780 suggested that estrogen suppressed B lymphopoiesis through an ER-specific mechanism. ERα−/− mice did not have abnormal response to B cell precursors. While this might be explained in part by redundancy with respect to other sex steroids, it is also possible that events in bone marrow are regulated by multiple estrogen receptors. Indeed, we show that stromal cells expressing sIg. In vitro treatment with the antiestrogen drug ICI 182,780 resulted in an increase in B lineage cells, particularly those not yet expressing sIg. In vitro treatment with the antiestrogen drug ICI 182,780 suggested that estrogen suppressed B lymphopoiesis through an ER-specific mechanism. ERα−/− mice did not have abnormal response to B cell precursors. While this might be explained in part by redundancy with respect to other sex steroids, it is also possible that events in bone marrow are regulated by multiple estrogen receptors. Indeed, we show that stromal cells from ERα gene-targeted mice were normally responsive to estrogen and that they express the recently described ERβ.

Materials and Methods

Animals

Male Tmf and wild-type control (T/a) mice (17), ranging in age from 5 to 17 wk, were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c mice (female 4–6 wk) were obtained either from the Oklahoma Medical Research Foundation Laboratory Animal Resources Center, or The Jackson Laboratory. ERKO mice have been previously described (25). ERKKO mice and wild-type controls ranged in age from 14 to 18 wk.

Flow cytometric analyses

Cells were isolated from the bone marrow and spleen of Tmf and ERKO mice and their respective controls, resuspended in staining buffer (PBS without Ca2+ and Mg2+ and with 3% heat-inactivated FCS) and stained as previously described (3). B lineage cells in the bone marrow and spleens were identified as follows: total B lineage cells are CD45R−/−, B cell precursors are CD45R+ IgM−/−, mature B cells are CD45R− IgM+ IgD− (27, 28). The Abs used to identify these antigens were PE rat anti-mouse CD45R (Mab 14.8; PharMingen, San Diego, CA) and FITC goat anti-mouse CD24, followed by goat anti-mouse IgG2a-FITC (Baxter, Mundelein, IL), PE rat anti-mouse CD45R, and biotinylated rat anti-mouse CD24, followed by streptavidin-Perdinin CP or Cy-Chrome streptavidin. Early pro B cells were identified as BP-1−/−, CD45−, CD24−/− cells and were gated as previously shown (1). Cells were sorted using the FACStar plus cell sorter.

Colony assays

Colony assays were done as previously described (26). Briefly, bone marrow cells were isolated and resuspended in assay medium containing 0.3% Bacto-agar (Difco Laboratories, Detroit, MI). For detecting IL-7-responding cells, 2 to 5 ng/ml of recombinant mouse IL-7 (R&D Systems, Minneapolis, MN) was used. LPS-responding B cells and myeloid precursors were detected in colony assays containing 250 ng/ml of endotoxin (Difco) or 25 µg/ml of 10% concentrated L cell or WEHI-3-conditioned medium, respectively. All cloning assays were performed in 35-mm dishes (Corning Glass, Corning, NY) and incubated at 37°C in 5% CO2. Colonies were counted on day 6.

Isolation of stromal cell clones

Stromal cells were isolated from the bone marrow of male ERKO and from wild-type mice. Single cell suspensions of bone marrow cells were plated at 5 × 105 cells/ml in stromal cell culture medium containing either DMEM or Opti-MEM I (Life Technologies, Gaithersburg, MD) with 20% FCS, 2 mM glutamine, 100 U penicillin/ml, 100 µg of streptomycin/ml, 5 × 10−4 M 2-ME and 10% BMS2 cell-conditioned medium (2, 29). ERKO stromal cells and their wild-type controls were cloned by limiting dilution. After cultures were confluent, the cells were detached with trypsin/EDTA (Life Technologies), washed, and resuspended in stromal cell cloning medium supplemented with 75 µg/ml endothelial cell growth supplement, 50 ng/ml fibroblast growth factor (both from Collaborative Biomedical Products, Bedford, MA), and 20 ng/ml insulin-like growth factor I (IGF-I) (HyClone). Plates were fed weekly and supplemented with cytokines.

OP42 stromal cells were derived from the spleen of a B6C3He-a/e osteopetrotic (op/op) mouse as previously described (2). OP42 stromal cells do not produce CSF-1 (30) but can support the growth of B cell precursors in the presence of exogenous IL-7 (2). OP42 stromal cells, ERKO stromal cell clones, and the corresponding wild-type clones were maintained in estrogen-low tissue culture medium (phenol-red free RPMI 1640, 5% charcoal dextran FCS (HyClone), 2 mM glutamine, 100 U penicillin/ml, 100 µg of streptomycin/ml, and 5 × 10−3 M 2-ME).

Cocultures

Early pro B cells (BP-1−, CD45−, CD24−) were cocultured with the indicated stromal cell lines. Stromal cells were plated at 80,000 cells/well in a 24-well plate with 1 ml of estrogen-low tissue culture medium for 24 to 48 h before the lymphocytes were added. B cell precursors and 2 ng/ml IL-7 (R & D Systems) were added to the wells and the cells cocultured for four days. Estrogen (Sigma, St. Louis, MO) and/or ICI 182,780 (a kind gift from Alan E. Wakeling and ICI Pharmaceuticals, Macclesfield, UK) were added to some cultures before addition of lymphocytes. B cell progenitor expansion was assessed as previously described (2). Briefly, each well was treated with 2 mM EDTA to remove all the cells. The detached cells were washed and resuspended in staining buffer and counted. Stromal cells were not included in cell counts. The cells were then stained with CD45R-PE, and the percentages of B lineage cells (CD45R+R) were assessed by flow cytometry.

Evaluation of ER status

The expression of ERα and ERβ transcripts in stromal cell clones derived from male ERKO or wild-type control mice was compared using RT-PCR. The Fast-Track 2.0 Kit from InVitrogen (San Diego, CA) was used to isolate mRNA. The RT reaction was done using 10 µl MgCl2, 5 µl of each 10 mM dNTP, 2.5 µl random hexamers, 2.5 µl (50 U) RNase inhibitor, 2.5 µl (250 U) murine leukemia virus (MuLV) reverse transcriptase, and 0.5 µg of cDNA template. The expression of ERα and ERβ mRNAs in the stromal cell clones was compared to the corresponding wild-type and ERα−/− controls using the Fast-Track Kit (InVitrogen). The primers used for ERα and ERβ were designed to amplify specific fragments of each transcript. The primers used were 5′-ATCTAAGCCGCTCCTATGG (ERα forward) and 5′-TCCGTGCGTTCTTTCTCG (ERα reverse) and 5′-CGGAATGCAGGTCTGTTCG (ERβ forward) and 5′-GGTGAGGTCCCTCTCGGTC (ERβ reverse), respectively. The PCR conditions were as follows: 1 cycle of 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. The amplified products were excised from the gel, purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and cloned into the pGEM-T Easy Vector (Promega, Madison, WI). The cloned cDNAs were sequenced using the ABI Prism 310 automated sequencer (Applied Biosystems, Foster City, CA). The sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Kit Version 2.1 (Applied Biosystems). The sequences were analyzed using the Lasergene software package (DNASTAR, Madison, WI).
poly(A) RNA (all reagents from Perkin-Elmer, Foster City, CA) in a total volume of 50 µl. The RT reactions were incubated at 24°C for 12 min, 42°C for 30 min, and 95°C for 5 min, and then held at 4°C in a Perkin-Elmer GeneAmp 9600. The PCR reactions were then conducted in the following mix per 25-µl reaction: 5.0 µl 5× InVitrogen buffer (Buffer I for ERα and ERβ and Buffer I for β actin), 50 pM of each primer, 2.0 µl (10 mM) dNTP mix (InVitrogen), 0.25 µl (1.25 U) AmpliTaq, and 0.5 µl of RT reaction. The PCR reactions were cycled 35 times at 95°C for 1 min, 58°C for 45 s, and 72°C for 30 s, and then extended at 72°C for 7 min and held at 4°C. The ERα, actin, and ERβ primers have been previously described (20, 21).

Results

Tfm mice have increased numbers of B cell precursors

We used Tfm mice to assess the importance of the AR for normal B cell production in the bone marrow. These animals have a point mutation in the X-linked AR gene and are insensitive to androgens (14, 15, 31). Flow cytometric analyses comparing bone marrow from wild-type and Tfm mice showed markedly increased percentages of B cell precursors (CD45R⁺, IgM⁻) in the mutant animals (illustrated in Fig. 1 and summarized in Fig. 2). A similar increase was seen in their absolute numbers (wild-type 10⁶ vs Tfm 4.7 ± 1.9 × 10⁶). IL-7-responsive cells, which give rise to pre-B cells, were enumerated with a semisolid agar-cloning procedure (28) and also were found to be increased (Fig. 3). In comparison, LPS-responsive B cells and myeloid progenitors assessed in similar semisolid agar assays were not affected by the Tfm mutation (Fig. 3). The number of nucleated cells in Tfm bone marrow was slightly increased as compared with controls (Tfm 21.9 ± 7.3 × 10⁶ vs wild-type 17.0 ± 5.3 × 10⁶/bone) but did not reach significance (p = 0.08). The incidence of MAC-1⁺ cells in the bone marrow, which are primarily myeloid cells, was significantly decreased (Fig. 2). We also examined the production of erythroid cells because sex steroids (32, 33) negatively regulate GATA-1, an essential transcription factor for erythroid production. Despite the lack of androgen responsiveness, the percentage of TER119⁺ cells was reduced in Tfm bone marrow (Fig. 2). Numbers of MAC-1⁺ (CD116) and TER119⁺ cells were not significantly decreased in the AR-deficient animals, perhaps reflecting the slight increase in total nucleated cells. Together, these data show a clear increase in B cell precursors in Tfm bone marrow as assessed by flow cytometry and in a functional assay, indicating the AR may be important in determining the size of the B cell precursor compartment.

The increase in B cell precursor production correlated with an increase in numbers of more mature B lineage cells in the bone marrow (Fig. 2) and periphery. Tfm mice had significantly more splenic IgM⁺ B cells than wild-type controls (54.5 ± 10.8 × 10⁶ wild-type vs 89.4 ± 17.5 × 10⁶ for Tfm; p = 0.001), which was reflected in a 1.5-fold greater number of nucleated spleen cells (p = 0.001). This suggests that increased production of B cells within bone marrow is not compensated for by a decreased rate of survival in peripheral tissues. Thus, these findings document augmented and selective expansion of B cell production in the bone marrow of Tfm mice and a corresponding increase in the level of B cells in the peripheral lymphoid organs such as spleen.

B lymphopoiesis in bone marrow is preferentially altered by the Tfm mutation. B lineage cells and their subsets were discriminated by flow cytometric analyses as follows: “B cell precursors” were identified as CD45R⁺, and IgM⁻ cells and B cells were identified as IgM⁺, MAC-1⁻, and TER119⁻ staining detected myeloid and erythroid lineage cells respectively. Statistical significance was determined by paired Student’s t test. Significant differences are indicated by asterisks (*, p ≤ 0.05; **, p ≤ 0.002). All of the data are averaged from three independent experiments using a total of six control (open bars) and nine Tfm mice (shaded bars).
The increase of B cell precursors in Tfm mice could result from down-regulated estrogen levels, since the AR regulates the enzymes required for estrogen synthesis in male mice (34–36). However, DHT, a nonmetabolizable form of testosterone, was able to alter stromal cell-dependent B cell precursor growth in vitro. Early pro-B cells (CD24low, CD45R- , BP-1-) from normal mice (BALB/c) were cocultured with OP42 stromal cells (2) in the presence of DHT (10⁻⁶ M). Under these defined conditions, the androgen inhibited precursor expansion by 51 ± 14% (n = 6; p = 0.002). In the same group of experiments, expansion of B cell precursors was decreased 49 ± 10% (n = 5; p = 0.004) by estradiol (10⁻⁶ M). Furthermore, like estrogen (2), DHT at this concentration had no direct effect on IL-7-responding cells in semi-solid agar-cloning assays. In an average of four independent experiments, the number of colonies in cultures treated with 10⁻⁶ M DHT was 105 ± 30% of diluent treated controls. Therefore, while androgens and their receptors might be functionally linked to other endocrine networks in vivo, testosterone alone was sufficient to negatively affect B lymphopoiesis in stromal cell-containing cultures.

The pattern of selectively increased B lymphopoiesis shown here for Tfm mice is very similar to that previously observed in castrated male or hpg mice (3). Moreover, the same populations of B cell progenitors were depleted after estrogen treatment and during pregnancy of normal animals (1, 4). Together, these findings suggest that both androgens and estrogens contribute to steady state regulation of B lymphocyte formation.

**ERKO mice have normal levels of B cell precursors**

In contrast to the results obtained with AR-deficient castrated, and hpg mice, no significant increases in B cell precursors were found in ERKO (25) mice. In fact, the percentages of CD45R⁻ , sIgM⁻ cells were slightly lower, and CD45R⁺ , sIgM⁻ cells were decreased by 49 ± 10% (n = 5; p = 0.04) by estradiol (10⁻⁶ M). Furthermore, like estrogen (2), DHT at this concentration had no direct effect on IL-7-responding cells in semi-solid agar-cloning assays. In an average of four independent experiments, the number of colonies in cultures treated with 10⁻⁶ M DHT was 105 ± 30% of diluent treated controls. Therefore, while androgens and their receptors might be functionally linked to other endocrine networks in vivo, testosterone alone was sufficient to negatively affect B lymphopoiesis in stromal cell-containing cultures.

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**ERKO stromal cells can respond to estrogen in vitro**

Estrogen levels are elevated 10-fold in female ERKO mice (20), and, although the uteri of these animals are virtually unresponsive to this hormone, the bone marrow might retain functional estrogen receptors. To test this possibility, we isolated stromal cells from the bone marrow of ERKO and wild-type mice and compared their ability to support the growth of a subset of normal B cell precursors in the presence of estrogen. Previous studies indicated that estrogen may induce stromal cell production of a factor(s) that reduced the expansion of cells with undifferentiated characteristics (CD24low, CD45R⁻ , BP-1⁻) (Ref. 2; and our unpublished observations). Estrogen suppressed B cell precursor expansion in cocultures containing ERKO-derived stromal cells to a level comparable with cultures containing either wild-type-derived stromal cells or the OP42 stromal cell clone (2) (Fig. 6). These results were reproducible in four experiments done with both cloned and bulk cultured stromal cells.
FIGURE 5. ICI 182,780 blocks the effects of estrogen in B cell precursor/stromal cell cocultures. OP42 stromal cells (2) were cocultured with B cell precursors (CD45R<sup>+</sup>, CD43<sup>+</sup>, CD24<sup>low</sup>) in the presence of exogenous IL-7 (2 ng/ml). The darkened circles represent the fold increase in B cell precursors after coculture with OP42 stromal cells in the presence of estrogen. Open circles represent the fold increase in B cell precursors after coculture with OP42 stromal cells in the presence of estrogen and 10<sup>-7</sup> M ICI 182,780. The increase of diluent (ethanol) controls was 7.2-fold. The increase in diluent controls treated with 10<sup>-7</sup> M ICI 182,780 was 5.7.

demonstrating this was not a phenomenon associated with a single ERKO stromal cell line. Thus, the loss of ERα in ERKO stromal cells did not prevent them from responding to estrogen and depressing B cell precursor expansion.

FIGURE 6. Stromal cells derived from ERKO mice are responsive to estrogen in culture and suppress the expansion of B cell precursors. Stromal cells were cocultured with B cell precursors (CD45R<sup>+</sup>, CD43<sup>+</sup>, CD24<sup>low</sup>) in the presence of exogenous IL-7 (2 ng/ml) with either estradiol (10<sup>-6</sup> M) or ethanol diluent (1:100). Three experiments were done with cloned cell lines from either ERKO or wild-type mice, and one experiment was done with stromal cells from bulk cultures. Similar levels of inhibition were seen with both, and the results were pooled. OP42 is a previously described estrogen-responsive stromal clone, and it was used as a positive control for estrogen inhibition (2). The data were normalized and represent numbers of CD45R<sup>+</sup> cells recovered from estradiol-containing cocultures as compared with those present in diluent-treated wells. There was a statistically significant decrease in B cell precursors with estrogen addition to each stromal cell type as compared with controls (*, p ≤ 0.03).

FIGURE 7. Stromal cells derived from normal and ERKO mice express transcripts for ERα and ERβ. RT-PCR analysis was done with mRNA isolated from wild-type and ERKO ovaries, a stromal cell line cloned from wild-type bone marrow, and ERKO bone marrow as described in Materials and Methods. The expected m.w. of the ERα amplimers was 685 bp from full length ERα transcripts and 514 bp for unique ERKO E1 and 354 bp for ERKO E2 (mutant ERα transcripts). Transcripts for the ERβ (262 bp) and β actin (540 bp) are also shown.

ERKO-derived stromal cells express mRNA for multiple ERs

Estrogen might mediate its effects on B lymphopoiesis in ERKO mice through a receptor other than the classical ERα. The ability of ERKO stromal cells to respond to estrogen and inhibit B lymphopoiesis demonstrates they have a functional estrogen response mechanism. The ERα is encoded by a single mRNA in normal mice (19), but not in gene-targeted animals. Two smaller splice variants of ERα (ERKO-E1 and ERKO-E2) are present in these animals. ERKO-E2 does not code for a functional protein. The ERKO-E1 splice variant can produce a protein with similar affinity for estradiol as the full length ERα, but with decreased transcriptional activity (20). A primer set that binds sequences 5’ and 3’ of the disrupted portion of the ERα gene amplified a single 685-bp product from wild-type ovary tissue and a wild-type stromal cell clone (Fig. 7). A 514-bp product (corresponding to ERKO-E1) and a 354-bp product (corresponding to ERKO-E2) were found in ERKO ovary tissue and in a cloned stromal cell line derived from ERKO bone marrow. Thus, ERKO stromal cells have detectable levels of ERKO-E1 and ERKO-E2 mRNA.

Estrogen can also be bound by a recently discovered receptor designated ERβ (21–23). A 262-bp ERβ product was detected in stromal cells and ovaries of normal and knockout mice using previously described primers (21). While ERβ is highly homologous to ERα in both ligand and DNA binding sites, a physiologic function has not previously been demonstrated for this receptor. Thus, estrogen could regulate lymphocyte precursor expansion in ERKO mice by interacting with either ERβ or the functionally compromised ERKO-E1 variant.

Discussion

This study extends numerous observations that suggest sex steroids participate in normal steady state regulation of B lymphocyte production (1, 4, 3, 39). Evidence for an increased rate of lymphopoiesis in AR-deficient mice now implicates androgens in this process. Evaluation of ERα-targeted mice indicates this receptor is dispensable for some responses to estrogen and points to a possible role for the recently discovered ERβ within bone marrow. Marrow stromal cells regulate both hemopoiesis and bone morphogenesis and are potentially influenced by both androgens and estrogens.
(40–43). Thus, male and female sex steroid levels may have consequences on bone structure and, as shown here, events that take place within bone marrow.

A missense mutation in the only known AR renders Tfm mice unable to respond to androgens (14, 15, 31). One previous study noted that Tfm mice have increased splenic weight and cellularity as well as alterations in the thymus (18). Another group suggested that B lymphocyte formation might be augmented by this mutation (44), and our observations are consistent with this hypothesis. B lymphocyte lineage cells were selectively expanded in marrow of Tfm mice (Fig. 2). This included cells responsive to IL-7 (Fig. 3) and one defined by the absence of slgM and presence of CD45R (Figs. 1 and 2). It is interesting that IL-7-responsive B cell precursors are depressed to the greatest degree by pregnancy or estrogen treatment (1, 4). Furthermore, these precursor subsets were expanded in sex steroid-deficient hpg or castrated male mice (3). We conclude that the availability of these hormones, as well as the ability to respond to them, is a critical parameter for determining how many B lymphocytes are formed.

Numbers of splenic B cells are substantially elevated in three circumstances of reduced sex steroid influence: the inability to make all sex steroids (hpg), deficiency of androgens (castrated male), or hormone unresponsiveness (Tfm mice). This interesting correlation may indicate that the average lifespan of mature lymphocytes in the periphery cannot decline sufficiently to compensate for the increased production of cells in bone marrow. While it is also possible that sex steroids have a direct influence on mature B cells (45), we observed no deficiency either during pregnancy or following estrogen treatment (1, 4). The only splenic B cells to be affected in those circumstances had characteristics of newly formed lymphocytes (1, 4). Animals treated with insulin-like growth factor-1 (IGF-1) also have abnormally high numbers of splenic lymphocytes, and a case has recently been made for a role of thyroxine in promoting B lymphocyte formation (46, 47). It will be important to learn if these hormones counterbalance the negative regulatory role of sex steroids and if the target cells are similar.

It has long been known that the development of humoral immunity in birds is sensitive to androgens, and the epithelium of the bursa of Fabricius represents the most likely target (48, 49). Our previous studies suggest that one mechanism estrogen might utilize to suppress B lymphopoiesis is by inducing stromal cell production of a negative regulator(s) (Ref. 2; and unpublished observations). Stromal cell lines have been shown to express receptors for androgens as well as estrogens (42, 43), and we now report that DHT suppresses the expansion of B lineage precursors when added to short term cocultures of lymphocytes and stromal cells. This finding demonstrates that androgens can directly influence lymphopoiesis and do not have to work by altering estrogen levels.

Tfm mice have intact estrogen receptors, but there are at least two possible reasons why estrogen does not compensate for the loss of AR and maintain normal levels of B cell production. The initial step of estrogen production in males involves the conversion of pregnenolone to either progesterone or 17-hydroxyprogrenenolone, catalyzed by the steroidogenic enzyme 17α-hydroxylase. This activity is confined to Leydig cells in male rodents, and synthesis of the enzyme appears to be regulated by the AR (34–36). Since Tfm mice lack an AR, estrogen production may be reduced and levels of the hormone too low to compensate for decreased androgen responsiveness. A second possibility is that, while androgens play the dominant role in males, estrogens are the primary sex steroid regulating B lymphopoiesis in females.

ERKO mice provide an important model to identify estrogen-mediated responses in which the ERα is important (50) and where, potentially, other receptors may play a predominant role (51). Male mice targeted for ERα (ERKO mice) had no perturbations of B lymphocyte formation, and this might be taken as evidence that androgens were sufficient for normal control of lymphopoiesis. However, we predicted that B lymphopoiesis would be abnormally increased in female ERKO mice. This hypothesis derived in part from the finding that estrogen replacement of hpg mice was sufficient to bring numbers of B cell precursors into the normal range (3). Therefore, it was surprising to find that precursor numbers were normal or reduced in female ERα-targeted animals. This might result from the expression of an alternative estrogen receptor(s) in bone marrow stromal cells and/or from greatly elevated estrogen levels in the bloodstream. Alternatively, as in males, normal or lower levels of precursors in female mice might be due to high circulating levels of androgens.

Stromal cells isolated from ERKO mice responded to estrogen and suppressed the expansion of B cell precursors in culture. Furthermore, a cloned stromal cell line derived from ERKO bone marrow possessed mRNA for ERα variants unique to ERKO mice (ERKO-E1 and ERKO-E2 (20)). The ERKO-E1 splice variant of ERα can produce a protein that has decreased transcriptional activity in vitro (20). However, no protein of this kind was detectable in the uteri of ERKO mice by Western blot. Transcription of genes for the progesterone receptor, lactoferrin, and glucose-6-phosphate dehydrogenase were all up-regulated in response to estrogen treatment in the uteri of wild-type, but not ERKO mice (20). Although the ERKO-E1 transcript was detected in stromal cells isolated from ERKO mice, it would be difficult to account for normal lymphopoiesis in females in terms of this functionally compromised receptor.

We also detected transcripts for a second type of estrogen receptor in stromal cells derived from ERKO mice. This receptor was originally cloned from a cDNA library isolated from rat prostate and is designated ERβ (21). Recently, clones for this receptor have been isolated in humans (22) and mice (23). Protein for this receptor has not been identified in normal tissues. However, ERβ synthesized in vitro had an affinity for estrogen similar to ERα. Transient transfection of Chinese hamster ovary (CHO) cells with ERβ cDNA and a reporter plasmid containing an estrogen response element suggested that ERβ is transcriptionally active (21). Furthermore, the action of ERβ can be blocked by ICI 164,384 (ICI 182,780 is a more potent derivative of this drug (38)) and numerous other anti-estrogen drugs (22, 24). In the rat, RT-PCR experiments revealed differential expression of ERβ and ERα (24). Recent studies support the idea that ligand-mediated activation of these receptors can have different consequences (52). Our observations raise the interesting possibility that ERβ mediates some responses of bone marrow cells to estrogen.

Systemic estrogen levels are normally limited in females by a pituitary feedback that inhibits further release of gonadotrophs (53). Targeting of the ERα may disrupt this pathway because female ERKO mice have 10 times the normal level of estrogen (20). This fact, together with the presence of one or more active estrogen receptors in bone marrow, might account for the slightly reduced B lymphopoiesis in female gene-targeted mice. In addition, female ERKO mice had somewhat elevated levels of testosterone, which could potentially diminish lymphocyte formation via the AR.

Studies of sex steroids in relationship to bone density provide precedent for our findings involving lymphocyte formation and indicate that similar biologic responses can be achieved by different classes of hormones. There is a delicate balance between bone
formation by osteoblastic stromal cells and bone resorption by heterotypically derived osteoclasts. IL-6 is important in the recruitment and activation of osteoclasts, and it has been proposed that sex steroids suppress IL-6 induction in osteoblasts. Furthermore, functional AR and ER have been detected in osteoblast-like cells (10, 42, 43, 54). If circulating levels of either estrogen or testosterone are reduced, bone loss occurs (43, 55). Reduction of estrogen or androgen levels in IL-6-deficient mice does not reduce bone mass (43, 55). Thus, androgens and estrogens may have overlapping effects on two functions of bone marrow cells, bone remodeling and lymphopoiesis.

These studies extend our understanding of the complex interrelationships between sex steroids, their receptors, and B cell production. Functional ARs are required for normal numbers of B cells to be made in male mice. One potential mechanism involves ligation of ARs in marrow stroma and subsequent alteration of their ability to support lymphocyte growth. The situation is potentially more complex in the case of estrogen, which similarly inhibits precursor expansion in culture and can be blocked by ER-specific drugs. It remains to be seen if ERα and ERβ are redundant with respect to responses of bone marrow cells to this hormone, or if their differential expression corresponds to tissue specific differences. ER gene-targeted mice and stromal cells derived from them should provide important tools for further investigation of these issues.

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References


