Gender differences in vascular thromboses are well known, and there is evidence that platelets may be involved in these differences and that sex hormones affect platelet function. We characterized the expression of the estrogen receptor α (ER α), estrogen receptor β (ER β), progesterone receptor (PR), and androgen receptor (AR) in the megakaryocyte lineage. Megakaryocytes generated ex vivo from normal human CD34+ stem cells contained RNA for ER β and AR, which increased with cell differentiation. Platelets and human erythroleukemia (HEL) cells also contained ER β and AR transcripts. No ER α or PR messenger RNA or protein was detected in the megakaryocyte lineage. Immunofluorescence microscopy showed that ER β protein was present in glycoprotein (GP) IIb+ megakaryocytes and the HEL megakaryocytic cell line in a predominantly cytoplasmic location. AR showed a cytoplasmic and nuclear distribution in GPIIb+ and GPIIb− cells derived from CD34+ cells and in HEL cells. Western immunoblotting confirmed the presence of ER β and AR in platelets. Megakaryocyte and HEL AR expression was up-regulated by 1, 5, and 10 nmol/L testosterone, but down-regulated by 100 nmol/L testosterone. These findings indicate a regulated ability of megakaryocytes to respond to testosterone and suggest a potential mechanism through which sex hormones may mediate gender differences in platelet function and thrombotic diseases. (Blood. 2000;95:2289-2296)

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and the androgen receptor (AR), and that AR expression is regulated by testosterone. Thus, genomic effects in megakaryocytes and/or signaling properties in platelets could contribute to the known gender differences in platelet function.

**Methods and materials**

**Reagents**

Appyrase was the generous gift of Dennis Perry (McMaster University, Hamilton, Ontario, Canada). Bovine serum albumin (BSA), prostaglandin E1 (PGE1), prostaglandin I2 (PGI2), normal goat serum, charcoal, and dextran were obtained from Sigma (St Louis, MO). RPMI 1640, Ham’s F12K medium, Stem Pro culture media, 10 × HEPES buffer, and bovine insulin were from GibcoBRL (Gaithersburg, MD). Heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin solution were from Gemini Bio-Product (Calabasas, CA). Pegylated recombinant human megakaryocytic growth and differentiation factor (PEG-rhMGDF) was a gift of Amgen (Thousand Oaks, CA). Mouse and rabbit immunoglobulin (IgG)–G were purchased from Pierce (Rockford, IL). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). General laboratory reagents were obtained from either Sigma or Baker Scientific (Bridgeport, NJ).

**Human subjects**

Informed consent was obtained from all volunteers and patients. All studies were approved by the Johns Hopkins University School of Medicine Institutional Review Board and were conducted according to the principles of the Helsinki Declaration.

**Enrichment of CD34+ cells from leukopheresis units or bone marrow**

To obtain stem cells from leukopheresis units, low-density mononuclear cells from normal donors were first separated by centrifugation over HISTOPAQUE 1077 (Sigma) according to the manufacturer’s suggested protocol. Mononuclear cells were washed twice in 1% BSA in phosphate-buffered saline (PBS) (pH 7.4, 0.137 mol/L NaCl, 4.3 mmol/L Na2HPO4, 1.4 mmol/L KH2PO4, 2.7 mmol/L/L KCl) without Ca2+ and Mg2+. Cells were incubated with biotinylated anti-CD34+ antibody (gift from the Johns Hopkins Oncology Center, Baltimore, MD) for 30 minutes and washed, and then CD34+ cells were isolated from the mononuclear cell fraction by passage over an avidin column (CellPro, Bothell, WA). In a second protocol, donor bone marrow for allogeneic transplantation was processed by our clinical Graif Engineering Laboratory (Johns Hopkins University, Baltimore, MD). Small mononuclear cells were separated from marrow by clinical elutriation centrifugation. This lymphoid-rich fraction was labeled with biotinylated anti-CD34 antibody and passed over a CPRATE column (CellPro). The CD34-depleted fraction was washed, relabeled with biotinylated anti-CD34 antibody, washed again, and passed over an avidin column as previously described for leukopheresis products. Yields from the 2 preparation techniques were as follows: leukopheresis, 0.8 to 1.2 × 106 CD34+ cells from 1 × 109 mononuclear cells; CD34-depleted bone marrow fraction, 3 to 7 × 109 CD34+ cells from 1 × 109 mononuclear cells. CD34+ cells were resuspended in Stem Pro culture media without phenol red and without serum, cultured with 1 ng/mL IL-3 for 2 days, and immediately harvested (termed day-0 cells) or treated with 50 ng/mL PEG-rhMGDF for 7 days (termed day-7 cells). Approximately 25% of the day-7 cells were megakaryocytes as assessed by flow cytometry with the use of the megakaryocyte-specific marker GPIb or GPIbα.

**Cell lines and cell culture**

The human erythroleukemia (HEL) and Dami megakaryocytic cell lines were obtained from the American Type Culture Collections (ATCC; Rockville, MD). The Dami cells were obtained from the ATCC in 1989 and demonstrate greater GPIHβ (integrin β3) expression than do our HEL cells, despite their likelihood of being a subclone of HEL. PC3 and LNCap, both human prostate cancer cell lines, were gifts from Dr John Isaacs, and T47D, a human breast cancer cell line19 was a gift from Dr Saraswati Sukumar (both from Johns Hopkins University). These cell lines were used as controls for ERα, ERβ, the progesterone receptor (PR), and AR. HEL and Dami cells were grown in RPMI 1640 containing 10% serum and 1% penicillin/streptomycin. T47D cells were grown in RPMI 1640 containing 10% serum, 1% penicillin/streptomycin, and 0.2 IU/mL insulin. PC3 cells were cultured in Ham’s F12K media containing 10% FBS and 1% penicillin/streptomycin with 2 mmol/L L-glutamine, and 1.5 g/L sodium bicarbonate. Depending on the experiment, cells were cultured in FBS (GibcoBRL) or charcoal-stripped FBS. The FBS was stripped with the use of a standard protocol (Sigma) containing 0.25% charcoal (vol/vol) and 0.0025% dextran (vol/vol) in 10 mmol/L HEPES (pH 7.4).

**Platelet preparation**

Whole blood was obtained into acid-citrate-dextrose (0.1 mol/L trisodium citrate, 0.11 mol/L dextrose, and 71 mmol/L citric acid monohydrate) anticoagulant with the use of a 19-gauge needle and platelet-rich plasma (PRP), prepared as previously described.20 The top, middle, and bottom one third (by volume) of the PRP were designated PRP-upper, PRP-middle, and PRP-lower, respectively. Total red blood cells, white blood cells, and platelets were counted. Platelets were obtained by centrifugation of PRP at 800g for 20 minutes. Gel-filtered platelets were also prepared from PRP obtained as described previously. Then, 7.5 µmol/L PGE1 in 1.50 dilution and freshly prepared aprotase (20 µL/mL) were added, and the PRP was centrifuged at 800g for 20 minutes. The platelet pellet was resuspended in 1 mL buffer (138 mmol/L NaCl, 12 mmol/L NaHCO3, 10 mmol/L KCl, 5.5 mmol/L glucose, 0.36 mmol/L NaHPO4, 0.35% BSA, and 10 mmol/L HEPES, pH 7.4) containing aprotase and PGE1. Platelets were purified over a Sepharose CL-2B (Pharmacia, NJ) column. The eluate possessed normal adenosine-diphosphate–inducible aggregation in the presence of fibrinogen.

**Reverse transcription–polymerase chain reaction**

Total RNA was prepared from different cell samples with the use of a single-step guanidine thiocyanate/phenol kit (RNA STAT-60; Tel-Test, Friendswood, TX). One microgram of total RNA was reverse-transcribed in a 20-µL reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 4 mmol/L MgCl2, 100 pmol random hexanucleotides (Pharmacia), 15 units of ribonuclease inhibitor (Gibco BRL), and 220 units M-MLV reverse transcriptase (Gibco BRL) at 37°C for 1 hour. Polymerase chain reaction (PCR) conditions varied slightly according to primers, but the ranges were as follows: 30 to 35 cycles of 1 minute at 94°C, 30 to 60 seconds at 52°C to 60°C, and 30 to 60 minutes at 72°C. Then, 1× PCR buffer (20 mmol/L Tris-HCl, pH 8.0, 50 mmol/L KCl) was used in the presence of 200 mmol/L deoxynucleotide-triphosphates (dNTPs), and 2.0 mmol/L MgCl2, 10 pmol of each primer per reaction, and 2.5 units of Gibco Taq polymerase. Then, 10 µL of the PCR reactions was separated on 2% agarose gels and visualized by ethidium bromide staining. All reverse transcription (RT) – PCR analyses were confirmed at least twice in separate experiments.

The PCR primers for the ER α were as follows: 5′-CAG GGG TGA AGT AGG GTC TGC TG-3′ (sense) (priming site in exon 4, nucleotides 1060 to 1083 as numbered by Green et al17); 5′-ATG CGG AAC CGA GAT GAT GTA GC-3′ (antisense) (priming site in exon 6, nucleotides 1520 to 1543). These primers yield a product of 247 base pairs (bp). The primers for the ER β were as follows: 5′-ATC TTT GAC ATT GTG CTC TG-3′ (antisense) (priming site in exon 6, nucleotides 1520 to 1543). These primers yield a product of 247 bp. An optimized PCR buffer was used for these primers (1×: 80 mmol/L Tris-HCl, pH 9.0, 20 mmol/L [NH4]2SO4). The primers for the PR were as follows: 5′-AAG GAG GCC CTG CCG CAG GTC TAC-3′ (sense) (priming site in exon 1; nucleotides +1591 to +1614 with respect to starting A TG codon as numbered by Misrahi et al23); 5′-CGG CCA CTG GCT CAG GAG GCA-3′ (antisense) (priming site in exon 4). These primers yield a product of 515 bp. An optimized PCR buffer was used for these primers (1×: 80 mmol/L Tris-HCl, pH 9.0, 20 mmol/L [NH4]2SO4). The primers for the AR were as follows: 5′-CAG ATG GTC ATT CAG TAC TC-3′ (sense) (priming site at nucleotides +2559 to +2581 as numbered by

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Lubahn et al24); 5'-TGC TGA AGA GTA GCA GTG CTT TC-3' (antisense). These primers yield a product of 247 bp. The primers for the human β actin were as follows: 5'-TAC CTC ATG AAT ATG TTC A-3' (sense) (priming site at nucleotides 2227 to 2245 as numbered by Nakajima-Lijima et al23); 5'-TTC GTG GAT GCC ACA GGA C-3' (antisense). These primers yield a product of 247 bp. There was no sequence similarity among these primers and they are specific for their corresponding complementary DNA (cDNA).

**Immunostaining and fluorescence microscopy**

Cells were washed once in PBS containing 0.05% sodium azide (Sigma), cytofuged onto glass slides, fixed in 4% paraformaldehyde for 10 minutes at 4°C, washed 3 times in PBS, and washed twice in PBS with 50 mmol/L NH4Cl. Cells were permeabilized with 0.05% saponin in PBS containing 10% normal goat serum for 30 minutes at 22°C. The first primary antibody incubation was performed in PBS containing 10% normal goat serum and 0.05% saponin for 1 hour at 37°C followed by washing in PBS containing 0.05% saponin for 3 times for 5 minutes each at 22°C. Cells were then incubated with the first fluorochrome-conjugated secondary antibody for 30 minutes at 37°C followed by washing 3 times in PBS containing 0.05% saponin for 5 minutes each at 22°C. The nucleic acid staining dye Dapi (10 mg/mL) was diluted in the preparatory solution A of Slowfade Antifade kit (Molecular Probes, Eugene, OR) in 1:100 dilution. After an initial primary and secondary antibody staining, the procedure was repeated for the second (Molecular Probes, Eugene, OR) in 1:100 dilution. After an initial primary and secondary antibody staining, the procedure was repeated for the second primary and secondary antibody staining, and the slides were then mounted in the preparatory solution A containing Dapi. Each fluorochrome was analyzed individually by means of an inverted confocal laser scanning Fluorescence Microscope (LSM; Zeiss, Germany).

We used 3 primary antibodies in dual color immunofluorescence confocal microscopy: (1) S222, a mouse monoclonal antibody specific for human platelet GPIb (integrin \(\alpha I_{IIb}\beta 3\) (Immunotech, Westbrook, ME), was used at 20 µg/mL; (2) C-19, a rabbit polyclonal IgG specific for a peptide of the human AR (Santa Cruz Biotechnology, Santa Cruz, CA), was used at 4 µg/mL; and (3) purified rabbit antisera to human ER β (Alexis Corporation, San Diego, CA) was used at 20 µg/mL. Secondary antibodies were a 1:125 dilution of fluorescein-isothiocyanate–conjugated goat antigoat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and a 1:100 dilution of Rhodamine Red X-conjugated goat antirabbit (Jackson ImmunoResearch Laboratories).

**Western immunoblotting**

Immunoblotting was performed as previously described.26 Cells were lysed in 15 mmol/L Hepes, pH 7.0, 145 mmol/L NaCl, 0.1 mmol/L MgCl2, 10 mmol/L ethylene glycol-bis-N,N,N′,N′-tetraacetic acid (EGTA), 1% Triton X-100, 1 mmol/L NaVO3, 250 µg/mL 4,2-aminoethyl-benzene sulfonylfluoride, 15 µg/mL of protease inhibitors (chymostatin, antipain, and pepstatin), and 55 µg/mL of the protease inhibitor leupeptin. Protein concentration was determined by the Bradford technique. For AR analysis, the lysing detergent was 2% sodium dodecyl sulfate (SDS). Lysates were separated by 8% SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Amersham, Buckinghamshire, England). A series of polyclonal antiseras were used to probe for ER β: L-20 and N-19 (Santa Cruz Biotechnology, Inc), anti–ER β (Upstate Biotechnology, Lake Placid, NY), and Ab-1 (Oncogene Research Products, Cambridge, MA). A series of polyclonal antisera were used to probe for AR: PG-21 (Upstate Biotechnology), C19 and N-20 (Santa Cruz Biotechnology), and NCL-ARp (Vector Laboratories, Burlingame, CA). Numerous experiments indicated that the anti–ER β from Upstate Biotechnology, and PG21 yielded the best results. Blocking peptides from the amino termini of ER β and AR were purchased from Upstate Biotechnology, and Santa Cruz Biotechnology, respectively. The specificity of fragments detected by immunoblot was assessed by preincubating the primary antibody with the various peptides. For 10 µg/mL of antibody, 35 to 70 µmol/L peptide was used.

**Radioimmunoassay**

Total testosterone was measured with the use of the Coat-A-Count Kit (Diagnostic Products, Los Angeles, CA) according to the manufacturer’s protocol. Testosterone levels in charcoal-stripped FBS were undetectable (lower limit of detection is 0.14 nmol/L) and in FBS was 3.5 nmol/L.

**Results**

**Identification of RNA transcripts for sex hormone receptors**

Specific nuclear and cytoplasmic receptors mediate the biologic effects of sex hormones. We surveyed the megakaryocyte/platelet lineage for the expression of ER α, ER β, PR, and AR. Using total RNA from various cell lines and oligonucleotide primers specific for each receptor cDNA, we probed for the respective transcripts by RT-PCR. T47D, a breast cancer cell line, served as positive control for all 4 receptors (Figure 1, lane 7) and demonstrated the ability of our primers to amplify the expected targets. ER β and AR transcripts were identified in day-0 CD34+ cells not treated with PEG-rhMGDF (lane 1) and appeared to increase after CD34+ cells were treated with PEG-rhMGDF for 7 days (lane 2). The ER β and AR RT-PCR products from the day-7 cells were cloned, and nucleotide sequencing confirmed them to be authentic ER β and AR transcripts (data not shown). Approximately 25% of the day-7 cells expressed the megakaryocyte-specific marker GPIIb, and many showed an increased cell size and a nucleus with several lobes, morphologic features typical of megakaryocytes (Figures 2 and 3). ER α and PR messenger RNAs (mRNAs) were not observed in the day-0 or day-7 cells (Figure 1, lanes 1 and 2), or after longer exposure to PEG-rhMGDF (not shown). The absence of total testosterone in day-0 and day-7 cells supported the above results.
of ERα and PR transcripts in CD34+ or CD34+ derived cells was not due to poor quality RNA, since β actin (Figure 1, bottom panel), ERβ, and AR transcripts were readily amplified in all RNA preparations.

We studied mRNA obtained from platelets prepared by density centrifugation and by gel filtration. The former was contaminated by leukocytes, while the latter had no leukocytes (Table 1). Like the cells of bone-marrow origin, gel-filtered platelets also contained ERβ and AR, but not ERα or PR transcripts (Figure 1, lane 3). The upper two thirds of platelet-rich plasma corroborated these results, but the lower third reproducibly did not show ERβ or AR transcripts (data not shown). Whether this discrepancy was due to substantial leukocyte contamination and a relative lack of platelet mRNA in the lower third of the PRP or to a true difference in platelets of different density is not clear. The megakaryocytic cell lines Dami and HEL also contained transcripts for ERβ and AR, but not ERα or PR (Figure 1, lanes 5 and 6). The PC3 prostate cancer cell line is known to lack the AR, 27 but there are no prior reports as to which ER it contains (Figure 1, lane 4). These data support the presence of ERβ and AR transcripts, but not ERα or PR transcripts, in the megakaryocytic lineage.

Localization and distribution of the estrogen receptor β and androgen receptor in the cultured CD34+ cells

Because the day-7 CD34+ derived cells contained a heterogeneous population of cells (ie, both megakaryocytes and nonmegakaryocytic hematopoietic cells), we could not be certain of the cell type that gave rise to the transcripts observed in Figure 1. We used dual color immunofluorescence confocal microscopy with antibodies specific for ERβ, AR, and the megakaryocyte-specific marker GPIIb to assess protein expression in cells of CD34+ origin that had been treated with PEG-rhMGDF for 7 days. ERβ was present in GPIIb+ cells in a predominantly cytoplasmic location (Figures 2D and 2E). Similarly, intense staining for AR was seen in GPIIb+ day-7 cells (Figures 3C, 3E, and 3G). The identity of the GPIIb-negative, AR-positive cells is unknown, but such cells were also observed in the day-0 CD34+ cells (not shown), suggesting AR expression occurs early in hematopoiesis. AR showed primarily a nuclear distribution under these conditions. In both Figures 2 and 3, the same microscopic field is shown in each panel to demonstrate co-localization of the megakaryocyte-specific marker and the ERβ and AR. Specificity of staining is demonstrated by the

Figure 2. Localization of ERβ in human megakaryocytes using dual color immunofluorescence microscopy. CD34+ bone marrow cells, grown in serum-free and phenol-red-free medium, were treated for 7 days with PEG-rhMGDF and harvested for microscopy. (A) Negative control using mouse and rabbit IgGs as irrelevant primary antibodies. The same field is shown in panels B through E. (B) Dapi staining of nucleic acid. The polyploid nucleus is easily appreciated. (C) GPIIb detected by monoclonal antibody SZ22 (green). (D) ERβ detected by specific rabbit polyclonal antisera (red). (E) The co-localization of GPIIb and ERβ shown by summing the channels used in panels C and D. Although both GPIIb and ERβ localize predominantly to the cytoplasm, the intensity of the GPIIb stain dominates in panel E to the extent that less yellow color is appreciated. Original magnification of panels B through E: ×1300.

Figure 3. Localization of AR in human megakaryocytes using dual color immunofluorescence microscopy. Similar to Figure 2 except that antisera specific for AR were used. (A) Negative control using mouse and rabbit IgGs as irrelevant primary antibodies. The same field of cells is shown in panels B through E. (B) Dapi staining of nucleic acid. (C) AR detected by specific rabbit polyclonal antisera C-19 (red). (D) GPIIb (green). (E) The co-localization of GPIIb and AR shown by summing the channels used in panels C and D. Panels F and G contain the same cell to emphasize the polyploid features of the megakaryocyte. (F) Dapi staining of nucleic acid. (G) Co-localization of GPIIb and the AR. Original magnification for panels B through E: ×1000. Original magnification for panels F and G: ×600.
Table 1. Cell counts on different platelet preparations*

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Platelet count per mm³</th>
<th>White blood cell count per mm³</th>
<th>Red blood cell count per mm³</th>
<th>Volume used to make total RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRP-upper</td>
<td>500 000</td>
<td>550</td>
<td>0</td>
<td>6 mL</td>
</tr>
<tr>
<td>PRP-middle</td>
<td>398 000</td>
<td>330</td>
<td>0</td>
<td>3 mL</td>
</tr>
<tr>
<td>PRP-lower</td>
<td>17 500</td>
<td>18 800</td>
<td>2 950</td>
<td>000</td>
</tr>
<tr>
<td>Gel filtered</td>
<td>235 500</td>
<td>0</td>
<td>0</td>
<td>3 mL</td>
</tr>
</tbody>
</table>

*This procedure was repeated on several occasions to confirm the absence of red blood cells in the upper and middle platelet-rich plasma (PRP) fractions.

lack of fluorescence with the use of control mouse and rabbit IgG (Figures 2A and 3A). We also observed ER β and AR in GPIIb-expressing HEL cells, a transformed cell line with megakaryocytic properties (data not shown). Thus, the expression of ER β and AR protein correlated with the RNA data.

Expression of the estrogen receptor β and androgen receptor in human platelets

Members of the family of steroid receptors have been shown to possess nongenomic activity,28,29 and we sought to determine whether the corresponding protein was present in platelets that are anucleate. Immunofluorescence microscopy identified the AR in normal human platelets from men and women, but we were not able to observe ER β in platelets of either sex by this technique (not shown). The anti–ER β antisera used in the immunofluorescence analyses do not work on Western blotting (according to the manufacturer), and perhaps they were less efficient in detecting their target under the conditions of our platelet immunofluorescence experiments. However, Western blot analysis showed both ER β and AR protein in human platelets (Figure 4). Specificity for both platelet ER β and AR was demonstrated by the ability of the appropriate peptide to block antibody binding (Figure 4, lanes 5-8), while an irrelevant peptide did not block antibody binding (Figure 4, lanes 9-12). LNCap and HEL cells contained the expected approximately 65-kd ER β and approximately 110-kd AR proteins. Figure 4A suggests that ER β in platelets may be slightly larger than in HEL and LNCap cells. A second preparation of anti–ER β antisera also suggested platelet ER β was larger in platelets (not shown). To some extent, these studies were limited by reagents (see “Discussion”), but perhaps platelet ER β has alternately spliced mRNAs or posttranslation modifications. In data not shown, a different anti–AR antibody bound to the same 110-kd band on Western blot, providing further evidence that this polypeptide in platelet and HEL cells is the authentic AR. We suspect the signal at approximately 62 kd may represent a proteolytic fragment of AR, which has been repeatedly observed with AR from prostatic cells,30-32 since we observed almost exclusively the approximately 62-kd fragment until lysing cells in an SDS buffer.

Ex vivo effect of sex hormones

Sex hormones can autoregulate their corresponding receptors,33,34 often in a complex fashion. In these next studies, we wanted to determine whether sex hormones affected receptor expression in HEL cells. Using estradiol, we observed no changes in ER β expression (data not shown). However, as shown in Figure 5, HEL-cell AR expression varied according to the testosterone exposure, with an increase in AR at 1, 5, and 10 nmol/L testosterone (compare Figure 5B-5D with Figure 5A, the no-testosterone control). However, AR expression was reduced when cells were treated with 100 nmol/L testosterone (Figure 5E). The lack of AR induction in FBS containing 100 nmol/L testosterone (Figure 5E) was a consistent finding, suggesting that regulation of AR expression is not a linear function of testosterone concentration. A similar dose-response was seen when cells were cultured in charcoal-stripped FBS (data not shown). The reason for the punctate appearance of the AR seen in HEL cell nuclei is unknown, but in light of the numerous alternately spliced forms of the AR mRNA that have been reported, perhaps this represents the “speckle” or “coiled body” nuclear structures known to be rich in splicing factors.35,36 We further pursued these hormonal effects on AR expression using ex vivo–generated megakaryocytes. As with HEL cells, we observed prominent AR expression in megakaryocytes treated with 10 nmol/L testosterone (Figure 6G) and a reduction in AR expression with 100 nmol/L testosterone (Figure 6K compared with Figures 6C and 6G). The difference in AR expression between HEL cells and megakaryocytes in response to no testosterone (Figure 5A versus Figure 6C) is most likely due to the persistent inhibitory effects of ethanol on AR expression37 at the 48-hour time point (Figure 5) versus loss of ethanol via evaporation by 13 days (Figure 6).

Discussion

Although sex hormones were first reported to affect platelet function more than 25 years ago, little mechanistic data exist and no previous information has been available on either ER β or AR in the megakaryocyte lineage. The major findings in this study are the following: (1) ER β and AR can be identified in normal human megakaryocytes and platelets; (2) both ER β and AR transcripts are up-regulated during megakaryocyte differentiation; and (3)
megakaryocytic AR expression is regulated by hormonal manipulation. Although the downstream effects of ERβ and AR in these cells are unknown, potential genomic effects in megakaryocytes and/or signaling properties in platelets may contribute to the known gender differences in platelet function and vascular disease.

A survey of the megakaryocyte/platelet lineage for receptors for estrogen, progesterone, and testosterone was performed, and transcripts for ERβ and AR were identified. No transcripts were detected for ERα or PR in the megakaryocytic lineage, although we cannot exclude the possibility of an alternately spliced ERα or PR mRNA not detected by our PCR primers. However, for ERα at least, no platelet protein was detected by Western immunoblotting (data not shown). Prior to the identification of ERβ, Tarantino et al 11 used monoclonal antibody H222 to show the presence of an ER in the S01 megakaryocytic cell line and in megakaryocytes, although no other markers of the megakaryocyte lineage were used. This antibody was raised against an ER purified from the MCF-7 breast cancer cell line38 that is now known to contain both ERα and ERβ.39 Perhaps there is some cross-reactivity of H222 with ERβ and the signal detected in megakaryocytes by Tarantino et al 11 was ERβ.

CD34+ stem cells are pluripotent and give rise to a heterogeneous population, even when induced to differentiate with PEG-rhMGDF. Immunofluorescence staining of this population demonstrated both ERβ and AR proteins in GPIIb-positive megakaryocytes (Figures 2 and 3). Several isoforms of both ERβ and AR have been described in other tissues, 40-42 and our immunoblotting studies raised the possibility of a platelet ERβ of greater-than-expected size (Figure 4 and data not shown). However, for unclear reasons, these size differences were not consistently observed with all 4 ERβ antibodies used, prohibiting firm conclusions about a possible difference in platelet ERβ size.

Steroid receptors bind steroid response elements within and adjacent to target genes, although the mechanism by which steroid receptors regulate transcriptional activity is not well understood.43,44 ERβ may affect transcription differently than ERα, and presumably it is ERβ that mediates the known estrogenic effects in megakaryocyte genes. Megakaryocytic AR most likely mediates the androgenic regulation of platelet thromboxane A2 receptor expression,15 and a similar mechanism could regulate other
androgen-responsive genes involved in platelet function. Androgen therapy has been used for decades in the treatment of bone-marrow failure states, such as aplastic anemia and paroxysmal nocturnal hemoglobinuria. Our findings support a hypothesis that in certain hormonal milieus, sex hormones may affect hematopoiesis. Along these lines, an AR has been identified in erythroid precursors that may contribute to the higher hemoglobin concentrations in men than in women.

An increasing number of nongenomic effects of sex hormones have been reported, including effects in platelets. The cytoplasmic distribution of megakaryocyte and platelet ER and AR raises the possibility that some hormonal effects on the megakaryocyte lineage may be nongenomic. In vitro testosterone appears to enhance platelet aggregation, and our own studies suggest that estradiol inhibits aggregation of platelets from men (unpublished observations). Since these nongenomic effects of sex hormones may not always be mediated through the corresponding hormone receptor, further studies are needed to assess the role of platelet ER and AR in these immediate, nontranscriptional effects.

The effects of hormones on a given tissue is complex, in part because receptor levels vary among tissues and even in the same tissue at different developmental or pathologic states. Tissue-specific effects are manifest further by the ability of testosterone to both up-regulate and down-regulate AR mRNA. Using a range of testosterone concentrations, we found that low concentrations of testosterone (1 to 10 nmol/L) up-regulate AR expression, while 100 nmol/L down-regulates AR expression (Figure 5). Similar findings were seen with megakaryocytes (Figure 6). Such nonlinear dose-response to testosterone concentration has also been seen in LNCap cells, in which a low concentration of testosterone has been shown to increase AR protein (by decreasing AR turnover rate without changing mRNA levels), while higher concentrations cause AR down-regulation. Posttranslational destabilization of AR mRNA appears to be the predominant mechanism resulting in down-regulation of AR mRNA by androgen in some cell types, and perhaps this is the effect of higher testosterone concentrations in the megakaryocyte lineage. A thorough characterization of this complex aspect of megakaryocyte biology was not the intention of these studies. Rather, our goal was to assess whether the megakaryocytic AR was functional, as assessed by some phenotypic change in response to its ligand, and our data support the transcriptional functionality of the AR in megakaryocytes.

Cardiovascular disease is a complex, multifactorial process in which the gender of an individual and the individual’s unique platelet physiology affect the propensity to develop or resist thrombosis. The interaction of these 2 traits may be linked to the expression of sex hormones and their corresponding receptors. The identification of ER and AR in the megakaryocyte lineage may enable new strategies for investigating the prothrombotic effects of estrogens and androgens suggested previously by HERS and other studies. It is important to note that we found comparable levels of ER and AR in the platelets of both men and women, suggesting that receptor levels per se could not account for any gender difference in platelet function. However, our data do not exclude the possibility that variations over time in either the ligands (estriol or testosterone) or in the expression of the receptors themselves could alter events of downstream AR engagement and, hence, megakaryocyte and/or platelet physiology.

References


Human megakaryocytes and platelets contain the estrogen receptor β and androgen receptor (AR): testosterone regulates AR expression


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