Effects of androgens on endothelial progenitor cells *in vitro* and *in vivo*

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A B S T R A C T

The beneficial or detrimental effects of androgens on the cardiovascular system are debated. Endothelial progenitor cells are bone-marrow-derived cells involved in endothelial healing and angiogenesis, which promote cardiovascular health. Oestrogens are potent stimulators of endothelial progenitor cells, and previous findings have indicated that androgens may improve the biology of these cells as well. In the present study, we show that testosterone and its active metabolite dihydrotestosterone exert no effects on the expansion and function of late endothelial progenitors isolated from the peripheral blood of healthy human adult males, whereas they positively modulate early 'monocytic' endothelial progenitor cells. In parallel, we show that castration in rats is followed by a decrease in circulating endothelial progenitor cells, but that testosterone and dihydrotestosterone replacement fails to restore endothelial progenitor cells towards normal levels. This is associated with persistently low oestrogen levels after androgen replacement in castrated rats. In a sample of 62 healthy middle-aged men, we show that circulating endothelial progenitor cell levels are more directly associated with oestradiol, rather than with testosterone, concentrations. In conclusion, our results collectively demonstrate that androgens exert no direct effects on endothelial progenitor cell biology *in vitro* and *in vivo*.

INTRODUCTION

In the last decade, our knowledge on vascular homoeostasis has significantly changed thanks to the discovery of EPCs (endothelial progenitor cells) in adult human blood [1]. EPCs originally reside in the bone marrow and other putative niches [2], they can be mobilized in the peripheral circulation in response to many stimuli and, once in the bloodstream, actively take part in endothelial repair and formation of new blood vessels [3]. Many experimental studies have substantiated this model, suggesting that vascular homoeostasis depends not only on resident cells, but also on the relevant contribution of circulating EPCs [4]. Reconstitution and maintenance of an intact endothelial layer is a crucial homoeostatic function of the cardiovascular system because endothelial damage is the earliest step in the atherogenetic process [5]. Angiogenesis is another fundamental event in health and disease, as it is involved in physiological conditions, such as body growth and the menstrual cycle, and in the setting of pathological conditions, such as ischaemic syndromes and wound healing. Being closely linked to

Key words: androgen, cardiovascular system, endothelial progenitor cell, endothelium, gender, oestrogen, stem cell.

Abbreviations: AcLDL, acetylated low-density lipoprotein; AR, androgen receptor; AUC, area under the curve; BMI, body mass index; BP, blood pressure; CVD, cardiovascular disease; DHT, dihydrotestosterone; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanime perchlorate; E2, 17β-oestradiol; EPC, endothelial progenitor cell; HDL, high-density lipoprotein; HUVEC, human umbilical vein endothelial cell; IF, immunofluorescence; KDR, kinase insert domain-containing receptor; LSD, least significance difference; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TA, tibialis anterior; vWf, von Willebrand factor; WB, Western blot.

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endothelial repair and angiogenesis, EPCs represent an integrated component of the cardiovascular system [6]. Consistent with this view, it has been shown that subjects with risk factors for or established CVD (cardiovascular disease) have a depletion in circulating EPCs [7]. Interestingly, patients with lower levels of EPCs in the bloodstream have a higher risk of cardiovascular events [8–10]. These findings have suggested that a depletion in EPCs is a pathogenic event that, through the inability to maintain an intact endothelium and to promote angiogenesis, translates risk factors into the development and progression of CVD [5]. Moreover, the comprehensive role of EPCs in CVD has suggested that EPC levels may be used as a surrogate marker of vascular damage and overall cardiovascular risk [11].

Cardiovascular risk gradients are primarily driven by gender: women of a reproductive age have a much lower incidence of cardiovascular events than age-matched men [12]. This is generally attributed to the vasculoprotective actions of female oestrogens, which are mediated by favourable effects on lipid profile, and inflammatory and haemostatic parameters [13]. Interestingly, experimental studies show that E2 (17β-oestradiol) mobilizes EPCs from the bone marrow and enhances EPC function [14,15]. We have demonstrated that women have higher levels and better functions of EPCs than age-matched men thanks to the cyclic EPC stimulation for endometrial regeneration [16]. Therefore sex-hormone-driven gender-specific modulation of EPCs may be one mechanism that accounts for the gender gradient in cardiovascular risk. There are some results suggesting that EPCs may be also regulated by androgens. In one study, hypogonadal men had a depletion in *ex-vivo*-determined circulating EPCs, which were restored by pharmacological treatment with testosterone [17]. In another study, potential favourable effects of androgens on EPCs were shown *in vitro* [18]; however, these findings were probably influenced by the methods used to enumerate and isolate EPCs [19,20], which have profound effects on study results [20,21]. In the present study, we have explored the role of androgens in EPC biology by showing: (i) the relationships among EPC levels, and testosterone and E2 concentrations in healthy men; (ii) the effects of testosterone and its active metabolite DHT (dihydrotestosterone) on early EPCs and late EPCs isolated *in vitro* using a standardized and extensively characterized protocol; and (iii) the effects of testosterone deficiency on circulating EPCs in a rat model of primary hypogonadism *in vivo*.

MATERIALS AND METHODS

Patients

Healthy men were recruited from a local community of middle-aged individuals who responded to an advertisement for health screening. Exclusion criteria were any recent (within 3 months) acute or chronic disease or infection, known CVD (ruled out by minimal criteria, including history and physical examination) and immunological disease. On the basis of these criteria, 62 healthy men were included. Age, smoking habit (of one or more cigarettes), BMI (body mass index; calculated from height and weight), waist circumference, and systolic and diastolic BP (blood pressure) were recorded. Blood samples were drawn for the determination of plasma fasting glucose, lipid profile [total cholesterol, HDL (high-density lipoprotein)-cholesterol and triacylglycerol (triglyceride) concentrations], sex hormones (E2 and total testosterone) and progenitor cells.

Informed consent was obtained from all subjects. The study protocol was in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and has been approved by the local Ethics Committee.

Cell culture

For the isolation of late EPCs, buffy coats of young ($\leqslant$40 year old) male healthy blood donors were used, and culture was performed as described previously [11,16]. Briefly, PBMCs (peripheral blood mononuclear cells) were separated using a Ficoll density gradient (Sigma). Cells were plated on to six-well fibronectin-coated plates (Becton Dickinson) at a density of 6×10^6 cells/well, and were grown in supplemented endothelial cell growth medium (Clonetics) for 15 days. Culture medium was changed first on day 4 and then every 2 days. Phenotypical characterization was performed at day 15. To study the effects of androgens on cultured EPCs, starting from day 1, growth medium was supplemented with 1, 10 or 100 nmol/l testosterone or DHT. As a positive control, E2 at 1, 10 or 100 nmol/l was used. With this culture system, attaching cells rapidly assume an endothelial-like shape and, starting from days 3–6 of culture, cells proliferate in clusters or small colonies made up of a central core of rounded cells surrounded by radiating spindle-shaped cells. Starting from days 10–12, cells also organize into large colonies of cobblestone confluent cells, which are considered endothelial colonies. Clusters were quantified every 3 days starting from day 6 until day 15 in ten randomly selected microscopic fields. It is currently agreed that cultures prolonged for 2 weeks allow positive selection of true EPCs that should represent the only surviving cell type [19,20]. The AUC (area under the curve) of the number of EPC clusters over days 6–15 was taken to represent the clonogenic expansion capacity.

Early EPCs were cultured from PBMCs of young male healthy blood donors, as described previously [22]. Briefly, cells were plated on to six-well fibronectin-coated plates at a density of 6×10^6 cells/well, and were grown in supplemented endothelial cell growth medium (Clonetics) without a medium change. After 4 days, cells were washed and stained with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanime

perchlorate)–AcLDL (acetylated low-density lipoprotein) and *Ulex* lectin (see below). Cells double-positive for DiI–AcLDL and lectin were considered as early EPCs and were counted in ten randomly selected high-power fields.

Characterization of cultured EPCs

After 15 days, late EPCs were characterized to confirm the endothelial phenotype. After washing with PBS, cells were incubated at 37 °C with 1 μg/ml DiI–AcLDL (Molecular Probes) for 1 h, followed by incubation in the dark with 15 mg/ml FITC-conjugated *Ulex* lectin (Sigma) for 2 h. Nuclei were stained in blue with Hoechst 33258 (Sigma). Dual-positivity for these markers has been suggested previously as a necessary, but not sufficient, criterion to identify EPCs [19]. For further characterization, we stained putative EPCs with other endothelial markers. Cells were fixed and incubated with anti-vWf (von Willebrand factor) (Dako Cytomation), anti-KDR (kinase insert domain-containing receptor) (Santa Cruz Biotechnology) and anti-CD31 (Chemicon International) antibodies and secondary Cy2-conjugated anti-(rabbit Ig) and anti-(mouse Ig) antibodies (Chemicon International). Positive cells were then visualized under a fluorescent microscope. For a further methodological confirmation of endothelial phenotype, cultured EPCs were detached using EDTA and analysed by flow cytometry for the expression of CD34 (a stem cell marker), KDR (R&D Systems) and CD31 (Becton Dickinson).

As an *in vivo* functional assay, we injected isolated EPCs into male Sprague–Dawley rat (Charles River) muscles subjected to 2 h of ischaemia/reperfusion injury, as described previously [16,23] The protocol was approved by the local ethics committee at the Institutions. Meanwhile, cultured EPCs were detatched with EDTA, washed with PBS and stained with 0.5 μg/ml of the orange dye CMTMR [5-(and-6)-(4-chloromethyl-benzoyl-amino)-tetramethylrhodamine] (Molecular Probes). Immediately after reperfusion, the TA (tibialis anterior) muscle was exposed and 2×10^6 of labelled EPCs/kg of body weight were injected intramuscularly. Animals were killed 2 weeks after EPC implantation and the TA muscles were harvested. Muscle capillaries were stained using an anti-(rat CD31) antibody (Chemicon International) on 5 μm muscle cryosections and were observed under a fluorescent microscope. Nuclei were counter-stained in blue with Hoescht 33358. Elongated cellular structures containing both rat CD31- and CMTMR-positive cells were considered chimaeric vessels bearing human EPCs.

Adhesion assay

The functional property of late EPCs to adhere to mature endothelium was evaluated *in vitro*, as described previously [11,16]. For this purpose, a monolayer of HUVECs (human umbilical vein endothelial cells; Clonetics) was prepared 48 h before the assay by plating 2×10^5 cells/cm^2 at early passages. EPCs were labelled with DiI–AcLDL as described above, and 1×10^5 cells were added to each well and incubated for 2 h at 37 °C. Non-attached cells were gently removed with PBS, and adherent EPCs were fixed with 4 % paraformaldehyde in PBS and were counted in ten random fields.

Expression of the AR (androgen receptor)

Late EPCs were assayed for the expression of the AR using IF (immunofluorescence) and WB (Western blot) analysis. IF was performed with cells stained with the mouse monoclonal anti-(human AR) antibody F39.4.1, which is directed against the N-terminal part of the AR protein (amino acids 301–320; Santa Cruz Biotechnology), and a secondary FITC-conjugated anti-(rabbit IgG) antibody. Nuclei were stained in blue with Hoechst 33358 (Sigma). For WB analysis, EPCs were detached with EDTA, and total protein extracts were obtained by cell lysis with a ice-cold buffer [20 mmol/l Tris/HCl (pH 7.4), 150 mmol/l NaCl, 5 mmol/l EDTA, 1.5 % Niaproof, 1.0 mmol/l Na_3PO_4, 0.1 % SDS and 0.5 mmol/l PMSF] with proteases inhibitors added. Protein concentrations were determined using the BCA assay (Pierce). Proteins were then separated by SDS/PAGE, transferred on to nitrocellulose membranes (Hybond ECL; Amersham Biosciences) and blocked overnight with 5 % (w/v) non-fat milk in PBS/0.05 % Tween 20. Membranes were probed with a primary anti-AR antibody (Santa Cruz Biotechnology). After incubation with the appropriate HRP (horseradish peroxidase)-conjugated secondary antibodies (Amersham Biosciences), immunoreactive proteins were visualized by chemiluminescence using the SuperSignal WestPico chemiluminescent substrate (Pierce). A positive control was provided by a prostate protein extract. Proteins were also extracted for WB analysis from HUVECs (Clonetics) at passage 4–5 and PBMCs immediately after isolation.

Aromatase activity

As an indirect assay of aromatase activity, confluent late EPCs were incubated with 0, 1, 10 or 100 nmol/l testosterone for 24 h and E2 concentrations were then measured (RIA oestradiol assay; Immunotech) in the culture medium supernatant. As a negative control, a cell-free system was prepared, consisting of plates filled with culture medium without cells, and incubated for 24 h with identical concentrations of testosterone.

Rat castration

To study *in vivo* the effects of androgen deprivation and restoration, we used a rat model of castration followed by androgen replacement. The protocol was approved by the local ethics committee of the Institutions. Briefly, adult male Sprague–Dawley rats underwent bilateral castration under ketamine/xylazine anaesthesia (50 and 20 mg/kg of body weight intraperitoneally respectively). A sham operation consisted of opening the scrotum,

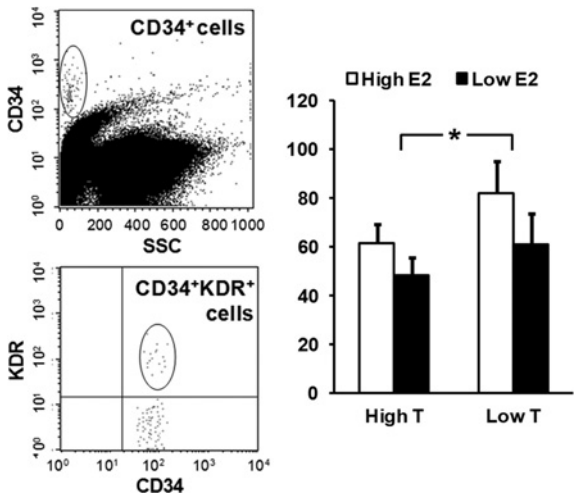

Figure 1 Progenitor cell count in healthy men

Left-hand panels, the gating strategy used to enumerate $CD34^+KDR^+$ cells in healthy men. First, $CD34^+$ cells were identified in the ungated white blood cell population. Then the expression of KDR was assayed in the $CD34^+$ cell gate in order to identify and quantify $CD34^+KDR^+$ cells (indicated by the oval). Right-hand panel, $CD34^+KDR^+$ cells were lower in men with high testosterone and low E2 compared with subjects with low testosterone and high E2. $^*P \leqslant 0.05$. Where not specified, the *x*- and *y*-axis represent arbitrary fluorescence units. T, testosterone.

manipulating the gonads and suturing the overlying skin. After a 4-week period of recovery, blood samples were drawn for determination of sex hormones (RIA oestradiol and total testosterone assays; Immunotech) and rat progenitor cell count. Then castrated rats were implanted subcutaneously with a silicone tube (internal diameter, 1.7 mm; external diameter, 3.4 mm; length, 10 mm; Dow-Corning) containing crystals of either testosterone ($n = 3$) or DHT ($n = 3$). At 2 weeks after the beginning of androgen treatment, blood was taken by heart puncture under deep urethane anaesthesia.

Progenitor cell counts

Circulating progenitor cells were analysed by flow cytometry on peripheral blood samples as described previously in detail [7,20]. Briefly, after red cell lysis, 10 μl of whole blood was incubated with FITC-conjugated anti-CD34 and PE (phycoerythrin)-conjugated anti-KDR. $CD34^+$ cells were gated and then this population was assessed for KDR expression in the mononuclear cell fraction (Figure 1). A total of 1×10^5 cells were always acquired, and human EPCs were defined as $CD34^+KDR^+$. Rat progenitor cells were defined as $Sca\text{-}1^+c\text{-}kit^+$ and $Sca\text{-}1^+CD31^+$ cells using PE-conjugated anti-(mouse Sca-1), FITC-conjugated anti-c-kit or FITC-conjugated anti-(rat CD31) monoclonal antibodies, and the same gating strategy used for human cells (see Figure 5). We have shown previously [23] that the rat $Sca\text{-}1^+/c\text{-}kit^+$ cell population is enriched with putative rat EPCs [23], and the $Sca\text{-}1^+CD31^+$ phenotype can be taken to represent the rat homologue of the human $CD34^+KDR^+$.

Statistical analysis

Values are expressed as means $\pm$ S.E.M., unless where specified. Progenitor cell count is expressed as cell number/10^6 cytometric events. Comparison between two or more groups was assessed using a Student's *t* test and ANOVA respectively. The LSD (least significance difference) test was used for multiple testing. The χ^2 test was used for frequencies and categorical variables. A multiple regression analysis was built with the EPC level as the dependent variable and sex hormone concentrations as explanatory variables to determine any independent associations. SPSS version 13.0 was used, and statistical significance was set at $P = 0.05$.

RESULTS

EPCs and sex hormones in humans

The 62 men enrolled in the present study were representative of a middle-aged (mean $\pm$ S.D., 46.8 $\pm$ 7.4 years) population with a low prevalence of cardiovascular risk factors. In the whole group, there was a non-significant correlation between $CD34^+KDR^+$ EPC levels and E2 ($r = 0.20$; $P = 0.10$) or testosterone ($r = -0.19$; $P = 0.12$) concentrations. A significant correlation was found between EPC levels and the testosterone/E2 ratio ($r = -0.33$; $P = 0.006$). Men were then divided into four groups according to their median levels of E2 and testosterone concentrations: group 1, high testosterone with high E2 ($n = 20$); group 2, high testosterone with low E2 ($n = 14$); group 3, low testosterone with high E2 ($n = 14$); group 4, low testosterone with low E2 ($n = 14$). Clinical characteristics of these four groups are shown in Table 1. Among the groups, there were minimal differences in BMI and lipid profile, whereas the other cardiovascular risk parameters were comparable. In terms of EPC levels, the only significant difference was between groups 2 and 3 (48.5 $\pm$ 13.2 compared with 82.0 $\pm$ 29.7; $P = 0.035$; Figure 1). A multiple regression analysis, including all of the variables listed in Table 1 as determinants of EPC levels, showed that E2 was positively associated with EPC levels, independently of testosterone (standardized $\beta = 0.28$; $P = 0.05$). Taken together, these results suggest that E2 is a stronger determinant of EPCs than testosterone.

Phenotype of late EPCs

To isolate late EPCs, we employed a culture method without pre-plating. This assay leads to purification of a population of blood-derived cells with a true endothelial phenotype and proliferative potential. During the first few days of culture, the cell population is heterogeneous. Subsequently, a change of medium and removal of

Table 1 Characteristics of the healthy middle-aged men divided into the four groups according to their median E2 and testosterone concentrations

Values are means ± S.D. Comparisons were performed with post-ANOVA LSD. *Significantly different compared with group 4 (Low E2/Low T); **significantly different compared with group 1 (High E2/High T); ***significantly different compared with group 2 (Low E2/High T). T, testosterone.

Characteristic	High E2/High T ($n = 20$)	Low E2/High T ($n = 14$)	High E2/Low T ($n = 14$)	Low E2/Low T ($n = 14$)
Age (years)	47.7 ± 6.8	45.9 ± 7.1	45.4 ± 7.0	48.0 ± 9.1
Smoking habit (%)	15	21	0	7
Family history (%)	55	43	36	57
BMI (kg/m^2)	26.4 ± 4.0	26.4 ± 3.6	28.8 ± 3.8*	25.9 ± 4.0
Waist circumference (cm)	97.0 ± 8.0	96.8 ± 10.4	100.6 ± 11.1	94.5 ± 11.0
Systolic BP (mmHg)	131.0 ± 10.3	129.3 ± 10.1	130.9 ± 16.6	127.1 ± 7.3
Diastolic BP (mmHg)	90.4 ± 9.3	86.9 ± 7.4	88.9 ± 10.4	85.7 ± 7.6
Plasma glucose (mg/dl)	94.3 ± 10.4	93.9 ± 10.4	98.2 ± 17.9	91.0 ± 15.0
Total cholesterol (mg/dl)	197.7 ± 28.4	201.3 ± 30.4	210.8 ± 23.7	214.5 ± 18.4
HDL-cholesterol (mg/dl)	49.4 ± 11.7	42.3 ± 6.8**	44.0 ± 6.5	43.9 ± 11.1
LDL-cholesterol (mg/dl)	128.1 ± 22.4	138.5 ± 28.5	143.7 ± 25.9	147.6 ± 22.3**
Triacylglycerols (mg/dl)	101.0 ± 48.3	102.5 ± 41.6	115.6 ± 50.5	114.8 ± 55.3
E2 (pg/ml)	19.2 ± 2.4	13.9 ± 2.4	18.6 ± 2.1	13.6 ± 2.3
Total testosterone (pg/ml)	20.7 ± 4.7	16.8 ± 3.3	11.5 ± 1.5	11.0 ± 2.8
CD34$^+$KDR$^+$ EPCs (per 10^6 events)	61.6 ± 22.1	48.5 ± 13.2	82.0 ± 29.7***	61.2 ± 22.8

non-adherent cells allows a progressive selection of cells with an endothelial-like morphology organizing in clusters and colonies (Figures 2A and 2B). After 15 days of culture, virtually all cells on the plate display an endothelial behaviour, as demonstrated by the uptake of AcLDL, binding of *Ulex* lectin and expression of typical endothelial markers, such as KDR [VEGFR-2 (vascular endothelial growth factor receptor-2)], CD31 and vWf (Figures 2C–2J). We confirmed the expression of CD31 and KDR using both IF and flow cytometry (Figures 2N–2R). These cells *in vitro* were also positive for CD34, revealing a very similar EPC phenotype to the one determined *ex vivo* with flow cytometry (CD34$^+$KDR$^+$). To have a final functional demonstration that isolated cells behave like EPCs *in vivo*, we injected them into rat muscles subjected to ischaemia/reperfusion injury. At 2 weeks after the ischaemic damage, injected cells were clearly incorporated into the endothelial layer of the host vasculature, suggesting that they take part in angiogenetic processes (Figure 2M).

Androgens have no effects on late EPC expansion *in vitro*

In a separate set of experiments ($n = 3$ each), the medium used to culture late EPCs was supplemented with 1, 10 or 100 nmol/l testosterone, DHT or E2. Cell clusters were counted every 3 days after plating as a measure of cell proliferation. Although the AUC of the number of clusters over time reflects the proliferative kinetics, cell clusters at day 15 is taken to represent the EPC yield and the end of the culture protocol. Both of these parameters were not significantly influenced by testosterone and DHT supplementation at any concentration (Figures 3A and 3B). Conversely, 10 nmol/l E2 was sufficient to increase the cluster numbers and AUC of EPC expansion at day 15 (Figures 3A and 3B).

Androgens have no effects on late EPC adhesion *in vitro*

As a functional assay, we studied the ability of EPCs to adhere to a mature endothelial layer. This is a critical property of EPCs in exerting their vasculoprotective action by means of both re-endothelialization and neoangiogenesis. Culture supplementation with testosterone or DHT had no effect on EPC adhesion at any concentration (Figure 3C). Conversely, 10 nmol/l E2 was sufficient to increase the adhesive property of EPCs to a mature layer of HUVECs (Figure 3C).

Androgens increase early EPCs

As a control experiment, we isolated early EPCs, which were defined as DiI–AcLDL- and lectin-double-positive cells 4 days after plating. Supplementation with 1 nmol/l testosterone, 1 and 10 nmol/l DHT, and 10 and 100 nmol/l E2 during the 4-day culture protocol significantly increased the generation of early EPCs from PBMCs, as compared with the unsupplemented experiments (Figure 3D).

AR and aromatase activity in late EPCs

Using IF, we have shown that cultured EPCs express the AR (Figure 2K), but WB analysis indicated that this expression was low compared with a prostate extract (Figure 4A). Aromatase activity was very low as well:

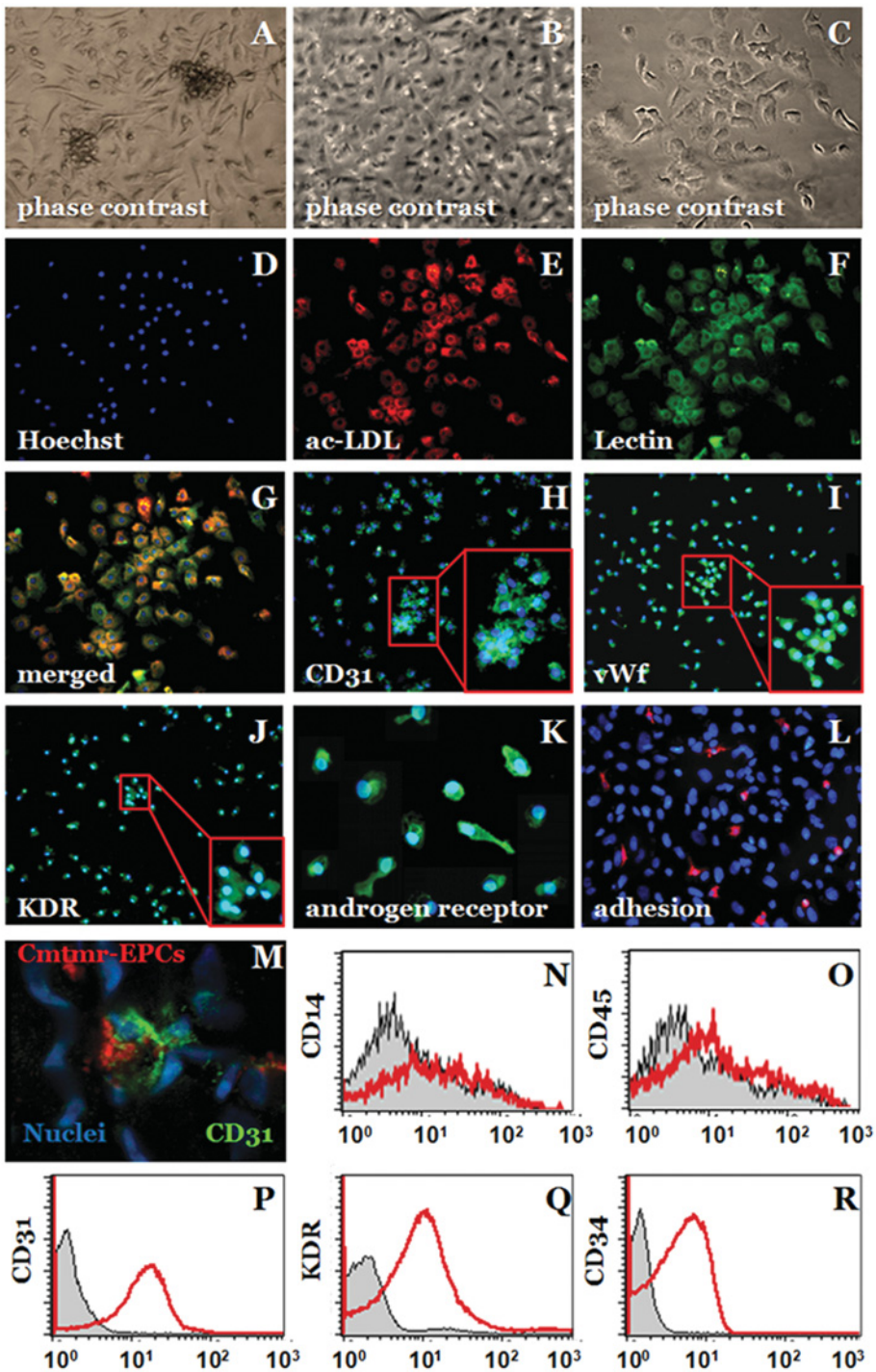

Figure 2 Characterization of EPCs cultured from healthy men

(A) Clusters/small colonies of EPCs at day 12. (B) Cobblestone monolayer of EPCs at day 15. (C–J) EPCs isolated from healthy male blood donors with a prolonged standardized protocol take up AcLDL, bind *Ulex* lectin, and stain positive for CD31, KDR and vWf. (K) Cultured EPCs at 15 days also stain positive for the AR. (L) A representative microphotograph of red (CMTMR)-labelled EPCs adhering on to the top of a HUVEC monolayer (nuclei are counterstained in blue). (M) A red (CMTMR)-labelled EPC incorporated into the host capillary network (FITC–CD31) after transplantation into an ischaemic rat muscle (nuclei are counterstained in blue). (N–R) The antigenic phenotype of EPCs was confirmed by flow cytometry as $CD45^{-}CD14^{-}CD31^{+}KDR^{+}CD34^{+}$. (A–L) Original magnification, $\times$ 20; (K) original magnification, $\times$ 40; (M) original magnification, $\times$ 40 (zoom, $\times$ 2.5).

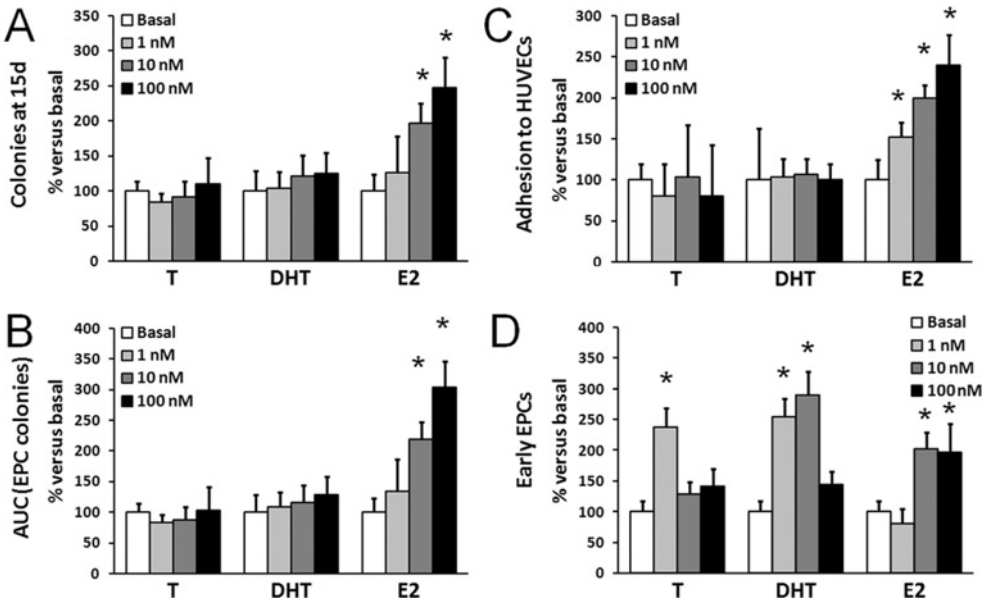

Figure 3 Effects of androgens on EPC expansion and adhesion

(A) Testosterone and DHT supplementation had no effects on the number of EPC colonies in culture. (B) A similar trend was evident for the AUC of EPC colonies over the 15 days of culture. (C) Testosterone and DHT had no effect on adhesion of EPCs to a HUVEC monolayer. E2 served as a positive control. (D) E2, testosterone and DHT increased the generation of early AcLDL- and lectin-double-positive EPCs. $^{*}P \leqslant 0.05$ compared with basal. T, testosterone.

incubation of EPCs with 10 nmol/l testosterone for 24 h induced approx. 0.03 nmol/l E2 in the culture medium (Figure 4B). However, it should be noted that, in the absence of a significant effect of androgens on cultured EPCs, the issue of aromatase activity is of limited value.

Effect of castration on circulating rat EPCs *in vivo*

It has been suggested that androgen deprivation in hypogonadal men is associated with EPC depletion [17]. To verify this observation, we have used an animal model of primary hypogonadism to simulate androgen deprivation. At 4 weeks after castration, circulating levels of Sca-1^{+}c-kit^{+} cells and Sca-1^{+}CD31^{+} cells fell markedly compared with baseline (Figure 5). As expected, there was also a decline in testosterone and E2 concentrations. Testosterone supplementation for 2 weeks was able to restore testosterone concentrations towards physiological levels, although it had modest and non-significant effects on E2 levels. Supplementation with either testosterone or DHT was unable to restore progenitor cell levels toward the baseline values (Figure 5). No differences were seen in EPC levels after sham-operation compared with baseline (results not shown).

DISCUSSION

The roles of androgens in the cardiovascular system are not clearly defined. In clinical studies, both androgen excess and deficiency have been reported to exert negative effects on cardiovascular parameters and endothelial function [24,25]. However, these studies may be confounded by the association between androgen deficiency, oestrogen deficiency and classic cardiovascular risk factors. EPCs represent an integrated part of the cardiovascular system, as they are involved in endothelial homoeostasis and angiogenesis [5]. Alterations in EPC biology are recognized as modifiable pathogenic biomarkers of cardiovascular risk and, at the same time, therapeutic targets of drugs with vascular effects. Ways to increase EPC levels are actively pursued through different strategies ranging from available medication to the frontiers of cell therapy. EPC assays have been used to test the 'endotheliotropic' activity of many drugs, including statins, ACEIs (angiotensin-converting enzyme inhibitors), glitazones and oestrogens [16,26–28].

In the present study, we have shown that androgens (testosterone and its active metabolite DHT) exert no direct effect on late EPCs *in vitro* and *in vivo*, as shown using standardized assays, including colony formation, adhesion and response to androgen replacement *in vivo*. These findings apparently contradict a previous report showing modest but significant increases in EPC proliferation, migration and colony formation after treatment with a synthetic androgenic agonist [18]. There may be several reasons for this discrepancy, including the use of a different AR agonist, but the most important issue appears to be the cell culture method. Foresta et al. [18] isolated putative EPCs according to the colony forming unit assay first described by Hill

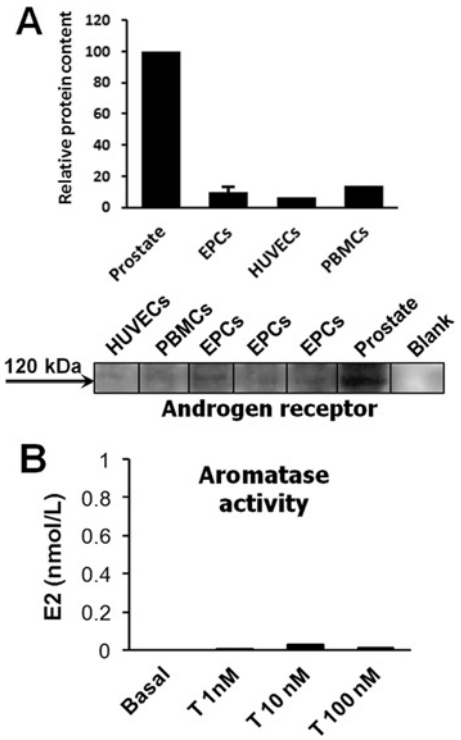

Figure 4 Expression of the AR and aromatase activity in EPCs

(A) WB analysis of the AR in cultured human EPCs (prostate extract was used as a positive control). EPCs express the AR (see Figure 2K), but protein quantification via WB analysis demonstrates a very low expression compared with the positive control. (B) Non-significant and non-linear conversion of testosterone into E2 by human EPCs in culture, suggesting low or absent aromatase activity. T, testosterone.

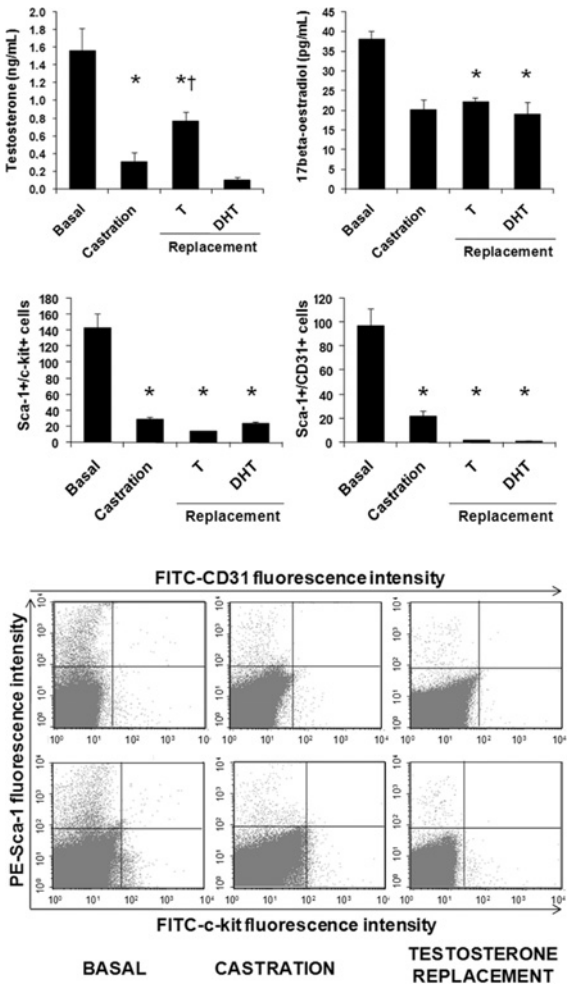

Figure 5 Effects of rat castration on sex hormones and EPCs

Upper panels, at 4 weeks after castration, testosterone concentrations were significantly reduced and were partly restored by testosterone replacement. Castration also lowered E2 levels, but testosterone and DHT replacement were unable to restore E2 levels. Castration was followed by a profound reduction of Sca-1$^+$c-kit$^+$ and Sca-1$^+$CD31$^+$ rat EPCs, whereas testosterone and DHT supplementation were unable to restore their circulating levels. $*P \leqslant 0.05$ compared with basal; $\dagger P \leqslant 0.05$ compared with castration. T, testosterone. Lower panels, the gating strategy used to enumerate rat EPCs according to the fluorescence intensity of PE–Sca-1, FITC–CD31 and FITC–c-kit antibodies. *x*- and *y*-axis of the flow cytometry plots show arbitrary fluorescence units.

et al. [29], which correspond to the so-called early 'monocytic' EPCs. Early EPCs are believed to reflect a population of immune cells mimicking endothelial cells [19,30,31]. These endothelial-like cells express CD45 and CD14, and have a limited proliferation capacity in culture, thus representing blood monocytes or macrophages. In the present study, we isolated late EPCs according to a highly standardized culture protocol and extensively characterized these cells *in vitro* and *in vivo*. These late EPCs are CD45$^-$CD14$^-$CD34$^+$KDR$^+$CD31$^+$ and AcLDL- and lectin-double-positive and, therefore, phenotypically different from monocytic cells. They also behave like EPCs *in vivo*, being able to incorporate in the host microvasculature after transplantation into ischaemic limbs. Although late EPCs isolated in the present study fulfil most criteria required to define true EPCs, including *in vivo* activity, the most recent methodological reviews suggest early EPCs have little in common with endothelial cells and should not be considered true EPCs [19,20,32]. Indeed, we confirm that androgens stimulate early EPCs, but show that androgens have no effects on late EPCs. Interestingly, as observed previously [21], different methods to study EPCs can yield different or even opposite results. On the contrary, E2 appears to stimulate the function of both early and late EPCs *in vitro* from male and female donors [14,15,33], and its effects on EPCs have also been substantiated *in vivo* [16,34]. Moreover, E2 regulates the EPC cycle for endometrial regeneration, providing fertile women with a larger EPC pool for cardiovascular homoeostasis [16]. These findings collectively suggest that oestrogens are more important than androgens in the regulation of endogenous EPCs in humans.

One point in favour of the effect of androgens on human EPCs *in vivo* is the observation that

hypogonadal men have low levels of circulating EPCs [17]. However, those hypogonadal men had low plasma concentrations of both E2 and testosterone. Therefore a reduction in EPC number could not be definitely attributed to testosterone deficiency. The observation that testosterone replacement in hypogonadal men was followed by restoration of a normal EPC pool is insufficient to support a direct effect of testosterone on EPCs, because testosterone treatment also increased E2 concentrations, probably through aromatase activity.

In the present study, we have developed an experimental model of primary hypogonadism by performing castration in adult male rats. As observed in humans, rat hypogonadism was associated with low testosterone and E2 levels, and a profound reduction in circulating Sca-1$^+$c-kit$^+$ and Sca-1$^+$CD31$^+$ progenitor cells, which represent rat EPCs. However, replacement therapy with an infusion of testosterone or DHT was unable to restore EPC levels. The most reasonable explanation for this negative result is that testosterone replacement was able to partially restore testosterone but not E2 concentrations, whereas DHT replacement could not generate E2, as DHT is not substrate of aromatase. The incomplete restoration of testosterone concentrations by exogenous testosterone would be unlikely to explain the sustained profound reduction in EPC levels, even if testosterone had a direct effect on EPCs because a partial effect on EPCs would be expected. The different gonadotropin levels in the experimental model as compared with the human condition are not likely to influence the effects of androgen deficiency on EPCs, as long as a direct effect of gonadotropins on EPCs is not assumed.

Taken together, the present experimental findings suggest that androgens have no direct effects on true human EPCs, and that E2 is likely to be the hormonal drive of EPC regulation in men as well as in women. To support this notion further in humans, we selected a sample of men from the general population. Although we found no significant correlation between EPCs and either testosterone or E2, there was a significant negative correlation between EPCs and the testosterone/E2 ratio, revealing a possible complex interplay between oestrogens and androgens in males, possibly influenced by aromatase activity. This negative correlation might actually reveal an even detrimental effect of testosterone on circulating EPCs. Moreover, when subjects were divided into four groups according to high or low levels of E2 and testosterone, it appeared that high E2 with low testosterone was associated with higher EPC levels than high testosterone with low E2. Finally, a multivariable analyses identified E2 as a determinant of EPC levels independently of testosterone. These results in healthy men support our experimental results indicating that androgens do not stimulate EPCs. It remains to be determined whether androgens eventually exert negative effects on EPCs. This is clearly not an argument against the desirable effects of androgen replacement in hypogonadal men, but does not support the use of androgens to stimulate EPCs.

In conclusion, in the present study, we found no evidence in support of a direct effect of androgens on EPC biology. As observed in other settings, the methods used to study EPCs are critically important and may largely affect study results [20,21]. Importantly, as already demonstrated in women, our results indicate that E2 rather than testosterone is likely to be the major hormonal drive of EPC kinetics in men.

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