Neuroendocrine regulation of human bone metabolism
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Chapter 5

The effects of beta-2 adrenergic agonist and antagonist on human circulating hematopoietic stem cells.

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Manuscript in preparation
Chapter 5

ABSTRACT

**Purpose:** Hematopoietic stem cells (HSC) reside in the bone marrow and migrate to the circulation to maintain hematopoiesis. In HSC transplantation, granulocyte-colony stimulating factor (G-CSF) is administered to force migration of HSC from the bone marrow into the circulation for harvest. Both the physiological and the G-CSF induced migration of HSC are reported to be under control of the sympathetic nervous system in mice. To study this mechanism in humans, we quantified peripheral blood HSC during beta-adrenergic agonist and antagonist treatment in healthy, postmenopausal women.

**Methods:** This study is an auxiliary study of a randomized controlled trial in which 32 healthy postmenopausal women were randomized to receive treatment with 17-β estradiol 2 mg/day; 17-β estradiol 2 mg/day and terbutaline 5 mg/day (selective beta-2 adrenergic agonist); propranolol 80 mg/day (non-selective beta-adrenergic antagonist); or no treatment during 12 weeks. Main outcome measure of the present study was the change in the percentage and absolute number of CD34+ HSC circulating in the peripheral blood after 12 weeks compared between the treatment and control groups. Data were analyzed with mixed model analysis.

**Results:** 12 weeks of beta-adrenergic agonist or antagonist treatment did not affect the percentage and absolute number of CD34+ HSC in the peripheral blood in healthy postmenopausal women.

**Conclusion:** Physiological migration of CD34+ HSC from the bone marrow into the blood is not affected by 12 weeks of beta-adrenergic agonist or antagonist treatment in healthy postmenopausal women.
INTRODUCTION

Hematopoietic stem cells (HSC) reside in the bone marrow. Under physiological conditions, HSC enter the circulation in low numbers which is known as HSC ‘migration’ [1]. The mechanisms regulating homeostatic HSC migration remain largely unknown, although recently circadian regulation of HSC migration was reported under the influence of the sympathetic nervous system [2]. Hematopoietic stem cell transplantation is an important treatment modality for hematologic malignant diseases such as leukemia. In most cases, donor HSC are harvested out of the blood. To increase the number of HSC in the blood, granulocyte-colony stimulating factor (G-CSF) is administered to the patient or donor to mobilize HSC from the bone marrow. The exact mechanism of G-CSF induced HSC mobilization is complex and not completely unraveled.

Over the last decade, several experiments showed that physiological HSC migration and G-CSF induced HSC mobilization depend critically on sympathetic nervous system signaling, both directly and indirectly. Katayama et al reported that the effect of G-CSF depends on changes in osteoblast morphology induced by noradrenergic signaling. These altered osteoblasts down regulate expression of CXCL12 and thereby release HSC from the bone marrow. Beta-blocker (propranolol) treatment for 3 weeks reduced HSC cell yield following G-CSF administration by >20% in C57/Bl6 mice while beta-agonist (clenbuterol) rescued HSC yield following G-CSF administration in dopamine beta-hydroxylase knockout mice, unable to synthesize norepinephrine [3]. Furthermore, G-CSF induced mobilization was impaired in beta-2 adrenergic receptor knockout mice [4]. Lucas et al subsequently elucidated the interaction by G-CSF and the sympathetic nervous system; G-CSF decreases reuptake of norepinephrine by the sympathetic nerve terminal in the bone marrow, thereby increasing the norepinephrine signal. Administration of desipramine, a norepinephrine reuptake inhibitor, together with G-CSF increased HSC mobilization in mice [5]. In addition, Spiegel et al demonstrated a direct effect of catecholamines on HSC migration. They demonstrated that HSC express the beta-2 adrenergic receptor and that in vitro, HSC migration is dose-dependently stimulated by norepinephrine. In vivo, epinephrine treatment enhanced migration of HSC to the peripheral blood [6]. These studies suggest that increased sympathetic nervous system activity enhances HSC migration and mobilization and that pharmacological interference is possible to increase HSC yields for transplantation. This would be a promising adjunctive treatment, however, whether this mechanism also exists in humans is not known.

In a previous study, we investigated the effect of beta-antagonist and beta-agonist treatment on bone turnover in healthy postmenopausal women [7]. In the present study
we investigated in the same trial the effect of beta-agonist and beta-agonist treatment on peripheral blood percentage and absolute number of CD34+ HSC in healthy, postmenopausal women. This auxiliary study was prespecified in the original protocol of the trial. Due to the design of the trial, the beta-agonist group received combined treatment with estradiol. We hypothesized that beta-agonist treatment would increase, and beta-agonist treatment would decrease HSC migration.

MATERIALS AND METHODS

Study Design and Setting
This study was a prespecified auxiliary study of a multi-arm parallel randomized controlled trial performed at the Endocrine Department of the Academic Medical Center of the University of Amsterdam (AMC/UvA) in The Netherlands from May 2010 until September 2012 [7]. Subjects were randomly allocated to treatment using a computer-generated (nQuery Advisor version 7.0, Statistical Solutions, Cork, Ireland) block randomization list with a block size of 4. The investigators were blinded to treatment allocation, but after randomization the investigators and subjects were not blinded to treatment. Laboratory personnel analyzing the samples was blinded to treatment. The study was carried out in accordance with the principles of the Declaration of Helsinki and the Institutional Review Board of the AMC/UvA approved the protocol. The trial was registered in the Netherlands Trial Register (TC 2874) before start of the study.

Subjects
32 healthy postmenopausal women who had their last menstrual cycle 12 to 60 months before inclusion were recruited from the general population via advertisements in local newspapers. Exclusion criteria were conditions or use of medication influencing bone metabolism and contraindications to treatment with estrogen, adrenergic beta-agonists and adrenergic beta-agonists. All subjects provided written informed consent before study inclusion.

Intervention
Subjects (n=8 per group) were randomized to receive treatment with 1] 17-β estradiol 2 mg daily (Zumenon, Abbott Products BV, Weesp, Netherlands), 2] 17-β estradiol 2 mg daily and terbutaline 5 mg daily (Bricanyl, AstraZeneca UK Ltd., Luton, UK), 3] propranolol slow release 80 mg daily (Propranolol retard, Pharmachemie BV, Haarlem, Netherlands) or 4] no treatment during 12 weeks.
Measurements
At baseline, the investigators took a complete history, measured weight and height, performed electrocardiography and drew venous blood samples after an overnight fast to determine peripheral blood CD34^+ HSC and leukocyte counts. After 4, 8 and 12 weeks subjects filled out questionnaires assessing study medication compliance and side-effects and provided venous blood samples after an overnight fast to determine peripheral blood CD34^+ HSC and leukocyte counts.

Main outcome
Change in peripheral blood percentage and absolute number of CD34^+ hematopoietic stem cells after 12 weeks was the main outcome measure.

CD34^+ hematopoietic stem cells
Peripheral blood was collected in the morning between 7.00 and 9.00 h after an overnight fast. Leukocyte counts were assessed by the hospital's routine diagnostic laboratory. Peripheral blood samples were incubated with allophycocyanin (APC)- conjugated CD45 and phycoerythrin (PE)- conjugated CD34 (BD Biosciences) for 20 minutes. After red cell lysis (FACS Lysing Solution, BD Biosciences) samples were analyzed by acquiring at least 250,000 to a maximum of 1 million events (to improve accuracy), on a FACSCanto II (BD Biosciences). The percentage of CD34^+ HSC was defined as the percentage of CD34-positive, CD45dim cells within the leukocyte fraction. Absolute numbers of CD34^+ HSC were calculated by multiplying the proportion of CD34-positive, CD45dim cells with the leukocyte number. These experiments were performed at the routine diagnostic laboratory of the department of Hematology at our institute, according to the routine CD34 analysis protocols used in stem cell transplantation procedures in The Netherlands [8, 9].

Statistical Analysis
Peripheral blood percentage and absolute numbers of CD34^+ HSC were ln-transformed prior to the statistical analysis and are reported as median and interquartile ranges (IQR). To assess the effect of the intervention including all time points, we performed a linear mixed model analysis with treatment and visit as categorical fixed effects, a random intercept and correction for heteroscedasticity (where appropriate). A two-sided P-value of 0.05 was considered significant. In case of a significant treatment and/or visit effects, pairwise t-test were carried out with Tukey’s correction for multiple testing. All statistical analyses were carried out using R statistical software for Windows (version 3.03, R Core Team. R: a language and environment for statistical computing 2014, package: nonlinear mixed effects (nlme)).
RESULTS

Subjects
Recruitment yielded 89 responses of which 29 subjects did not meet the inclusion criteria, 18 declined to participate and 4 could not be reached by telephone. 38 subjects were randomized of which 2 did not complete the study due to side-effects of treatment (one subject experienced headache from beta-blocker treatment and one had vaginal discharge from 17-β estradiol treatment) and 4 participants did not receive the allocated intervention or did not comply with the assessments (1 subject in the control group and 1 subject in the 17-β estradiol group withdrew for personal reasons, 1 in the 17-β estradiol group refused medication and 1 in the 17-β estradiol combined with beta-agonist group was excluded due to corticosteroid use which became known after randomization). Table I shows the baseline characteristics of the participants. At baseline, percentage of CD34+ HSC were significantly higher in the 17-β estradiol combined with beta-agonist group, despite the randomization. Compliance with study medication was 99% and there were no differences between treatment groups. No serious adverse events were recorded during the intervention.

CD34+ hematopoietic stem cells
Although the groups were randomized, baseline percentage CD34+ HSC was higher in the 17-β estradiol combined with terbutaline group (p=0.02). The distribution of percentage and absolute numbers of CD34+ HSC was right skewed and therefore we performed an ln-transformation prior to the analysis. Mixed model analysis showed that during the treatment period, the difference in percentage of CD34+ HSC between the treatment groups persisted, but there was no effect of treatment during the treatment period on percentage or absolute numbers of CD34+ HSC (Figure I).

TABLE 1 Baseline characteristics subjects

<table>
<thead>
<tr>
<th></th>
<th>17-B estradiol</th>
<th>17-B estradiol and terbutaline</th>
<th>Propranolol</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Age</td>
<td>52 (2.9)</td>
<td>52 (2.7)</td>
<td>53 (3.4)</td>
<td>53 (2.9)</td>
</tr>
<tr>
<td>Body Mass Index [kg/m2]</td>
<td>26 (3.8)</td>
<td>23 (3.2)</td>
<td>25 (2.5)</td>
<td>25 (2.7)</td>
</tr>
<tr>
<td>CD34+ HSC [10^9/L]</td>
<td>0.18 (0.06)</td>
<td>0.20 (0.10)</td>
<td>0.17 (0.07)</td>
<td>0.18 (0.15)</td>
</tr>
<tr>
<td>CD34+ HSC [%]</td>
<td>0.0337 (0.0197)</td>
<td>0.0454 (0.0282)*</td>
<td>0.0320 (0.0072)</td>
<td>0.0389 (0.024)</td>
</tr>
<tr>
<td>Leukocytes [10^9/L]</td>
<td>5.35 (3.3)</td>
<td>4.8 (3.3)</td>
<td>5.0 (3.6)</td>
<td>5.0 (1.5)</td>
</tr>
</tbody>
</table>

Age and BMI measurements are expressed as mean (standard deviation). CD34+ HSC and leukocyte are expressed as median (IQRrange). Units are provided in square brackets. HCS=hematopoietic stem cell. * significantly different (p=0.02)
The effects of beta-2 adrenergic agonist and antagonist on human circulating hematopoietic stem cells.

Figure I Percentage and absolute numbers of CD34⁺ HSC at baseline and after 12 weeks per treatment group.
DISCUSSION

In this study, we did not observe a change in percentage or absolute numbers of CD34+ hematopoietic stem cells in the peripheral blood after 12 weeks of beta-antagonist or beta-agonist treatment in healthy, postmenopausal women.

In animal studies, the presence of sympathetic nerve fibers connecting to the central nervous system and detectable catecholamine concentrations in the bone marrow have been established [8-10]. More recently, beta-2 adrenergic receptors on human hematopoietic stem cells were shown to be present and involved in HSC migration as noradrenaline dose-dependently increased HSC migration in vitro [6]. In mice, beta-agonist treatment increased and beta-blocker treatment decreased HSC yields in G-CSF induced mobilization in vivo [3]. Furthermore, physiological HSC migration follows a circadian pattern, under control of the sympathetic nervous system [2].

Fitch et al showed that the association of the SNS and HSC starts as early as embryogenesis by investigating GATA3 deficient mice. GATA3 is a transcription factor crucial for both SNS development and HSC emergence in the embryo. In GATA3 deficient mice the hematopoietic defect could be rescued by the addition of catecholamines, whereas the SNS defect could not be rescued [11]. The relation between SNS and HSC is important, in particular during states of injury. This was illustrated by a study showing that sympathetic denervation or neurotoxic chemotherapy-induced damage to the sympathetic nerves in the bone marrow compromised HSC mobilization and hematopoietic regeneration which was rescued by the administration of neuroprotective agents [12]. Furthermore, following hemorrhagic shock or ischemic injury, catecholamine concentrations in the bone marrow are increased, leading to increased HSC migration. Recalde et al showed that this postischemic HSC migration was enhanced by noradrenalin and clenbuterol administration [13]. This repair mechanism can also lead to prolonged bone marrow depression, Barabski et al showed that this can be prevented by the administration of beta-blockers before or shortly after the hemorrhage [14]. Finally, recent reports suggest bone marrow neuronal damage could be involved in the pathogenesis of myeloproliferative neoplasms, implicating potential treatment options by SNS manipulation [15].

In humans, the relation between the SNS and HSC migration and mobilization has less well been characterized. Iversen et al compared the HSC counts in peripheral blood in patients with spinal cord injury which includes SNS damage and control patients, but did not find any difference although the differentiation capacity of the HSC from spinal cord injury patients was less [16]. This supports our finding of no effect on HSC counts after pharmacological manipulation of sympathetic tone. Another study in humans
by Albiero et al investigated HSC mobilization in diabetic neuropathy and found that patients with neuropathy, had lower HSC counts in the peripheral blood [17]. However, the decrease in HSC counts was observed only in patients with more extensive neuropathy with two tests of autonomic function impaired, which might explain the incongruence with our finding.

So far, our study is the first to investigate the effect of beta-adrenergic agonist and antagonist treatment on HSC migration in healthy human subjects. We did not find any effect on HSC migration of twelve weeks of treatment with propranolol, a non-selective beta-blocker and terbutaline, a selective beta-2 agonist. A possible explanation for the lack of effect of terbutaline could be its beta-2 specificity. Although the osteoblast expresses the beta-2 AR and the effect of the SNS on HSC migration is considered to be, at least partially, mediated by the osteoblast, the HSC itself expresses the beta-3 adrenergic receptor, so any direct effect would have been missed with terbutaline treatment. In addition, the results can be masked by the combination of the terbutaline treatment with estradiol due to the design of the original trial. Effects of estradiol on HSC function have been described [18], but we included as a control group an estradiol monotherapy group and did not find any effect of estradiol alone on percentage or absolute numbers of CD34+ HSC. Therefore the combination of estradiol with terbutaline does not seem to explain the lack of effect. Furthermore, HSC migration is subject to circadian variation. During the resting time migration to the peripheral blood increases and during the active time of the organism, the migration is decreased [2]. We standardized the blood sampling in time, so circadian variation cannot have influenced our results. On the other hand, blood samples were taken early in the morning, when HSC migration is at its lowest [19], potentially masking the effect of our pharmacological treatment which could be more pronounced at other times of the day.

In conclusion, we did not find an effect on percentage or absolute numbers of CD34+ HSC in peripheral blood after 12 weeks of beta-adrenergic antagonist and agonist treatment in healthy postmenopausal women. From a clinical perspective, although this finding does not support the addition of beta-agonist treatment prior to HSC transplantation, it does not exclude the possible adjunctive role during treatment with G-CSF for HSC mobilization. This would be an interesting subject for further research.

ACKNOWLEDGEMENTS

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