NEW RESEARCH HORIZON

Review

Endothelial progenitor cells as a new cardiovascular risk factor in Klinefelter’s syndrome

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Abstract: Klinefelter syndrome (KS) is associated with a significant reduced life expectancy (2.1 years) including greater mortality from cardiovascular diseases. Underlying causes that may involve low levels of testosterone as well as the extra X chromosome are not fully understood. Low testosterone may have a direct affect on vascular tissue or act indirectly via metabolic effects. Testosterone levels may act genomically on cardiac function via the androgen receptor (AR) or non-genomically. Recently, it has been demonstrated that a reduced number of circulating endothelial progenitor cells (EPCs) is an independent predictor of morbidity and mortality from cardiovascular diseases. Because EPCs have never been studied in KS, we evaluated the number of circulating EPCs in 68 adult 47,XXY Klinefelter men and 46 healthy males. Patients and controls were divided into two groups, according to the absence or presence of cardiovascular risk factors (CRFs). Controls without CRFs had significantly higher levels of EPCs than controls with CRFs; on the contrary, KS patients without CRFs had EPCs levels similar to KS men with risk factors and significantly lower with respect to controls without CRFs. The number of EPCs in patients with hypogonadism was not different from that of those with normal testosterone levels. Twenty-two hypogonadal patients were re-evaluated after 6 months of androgen therapy, but we did not observe any modification in the number of EPCs. These primary hypothesis-generating data suggest that factors involved in KS, whether hypogonadism, CRFs or other genetically determined factors related to the supernumerary X chromosome might contribute to a reduction in EPCs number and that this could be considered another CRF contributing to the increased mortality of these subjects.

Key words: Klinefelter / cardiovascular diseases / endothelial progenitor cells

Introduction

Until few years ago, knowledge of long-term health outcomes for Klinefelter subjects was limited. Recently, three registry-based studies from Denmark (Bojesen, 2004; Bojesen et al., 2006a) and UK (Swedlow et al., 2005) provided comprehensive reports on morbidity and mortality among Klinefelter subjects. These studies showed that men with Klinefelter syndrome (KS) have an increased morbidity, with more frequent hospitalization than controls and a significant reduced life expectancy (2.1 years). In particular, morbidity was increased from congenital malformations of the heart and urinary tract, from cancers, with more admissions for breast cancer and mediatinal tumours, for endocrine and metabolic disorders, especially hypogonadism, diabetes and hypothyroidism, and for psychiatric diseases (Bojesen et al., 2006a). Mortality was particularly raised from infectious, diabetes, pulmonary embolism and peripheral vascular disease (Swedlow et al., 2005; Bojesen et al., 2006a), but it was significantly reduced from ischaemic heart diseases (Swedlow et al., 2005), maybe related to the reduced prevalence of hypertension in these subjects and to their increased amount of adiponectin (Bojesen et al., 2006b). Hypogonadism, in fact, has been shown to increase the levels of adiponectin that may counteract the other risk factors seen in Klinefelter patients (Bojesen et al., 2006b).

To date, the cause of the elevated morbidity and mortality from vascular diseases is unknown, but it could be related to the chromosome aberration itself, to the hypogonadal status and to environmental factors (socioeconomic status, smoking) (Bojesen et al., 2006a). In particular, a gene-dose effect of non-inactivated genes on the supernumerary X chromosome could explain the increased incidence of congenital heart malformations. It has been previously reported that in KS the frequency of mitral valve prolapse is higher than in healthy male population (Fricke et al., 1984), and this could justify an increased risk of cardiac arrhythmias and endocarditis with sudden death in these subjects. Anyway, a more recent
related to coagulation system, leading to deep vein thrombosis, leg relationship between plasminogen activator inhibitor-1 and with an increased thromboembolic risk, because of the reverse (Andersen et al., 2008).

The low levels of testosterone, instead, could cause an increased risk of vascular diseases and diabetes, independently from extra X chromosomes (Bojesen et al., 2006a). In fact, although the effect of androgens on vascular health is a matter of debate, recent evidence supports a protective role of these steroids on the cardiovascular system. A direct effect of testosterone on the vessel wall has been suggested by studies showing that a reduction in plasma testosterone contributes to increased arterial stiffness (Dockery et al., 2003), whereas testosterone supplementation in animal models inhibits atheroma formation (Alexandersen et al., 1999). In men with coronary artery disease, testosterone administration increases angina threshold, induces coronary vasodilatation (Rosano et al., 1999) and produces endothelium-dependent and -independent vasorelaxation. Furthermore, high endogenous testosterone concentrations appear to be associated with reduced mortality, probably reducing inflammatory markers, insulin and haemostatic factors concentrations and/or through coronary vasodilatation and improved endothelial function (Khaw et al., 2007). Finally, it has been demonstrated that low testosterone levels are associated with abdominal adiposity, type 2 diabetes and metabolic syndrome (Oh et al., 2000; Bhasin et al., 2001; Laaksoenen et al., 2004; Bojesen et al., 2006b; Zitzmann et al., 2006; Kapoor et al., 2007; Rodriguez et al., 2007; Selvin et al., 2007) which are characterized by endothelial dysfunction and which are associated with an increased risk of cardiovascular diseases.

In subjects affected by KS, it has been reported an increased prevalence of truncal obesity and metabolic syndrome and it seems plausible that a vicious cycle might ensue in these subjects: low testosterone levels (caused by a primary testicular failure) cause an increase in body fat (especially intra-abdominal fat), and subsequently carbohydrate metabolism deteriorates causing insulin resistance (Bojesen et al., 2006b), which further worsens the hypogonadism with a direct inhibitory effect on testosterone production by the Leydig cells (Pitteloud et al., 2005). This cycle might expose Klinefelter patients to an increased risk of cardiovascular diseases.

Furthermore, a recent study analysing the increased prevalence of cardiovascular diseases in Klinefelter patients found a reduced systolic left ventricular long-axis function, involving both the displacement rate and the velocity of the left ventricle (Andersen et al., 2008). The authors suggested that testosterone influences cardiac function through genomic effects via the nuclear AR (that is present in most organs including the heart and vascular smooth muscle) and/or by non-genomic effects via a membrane G protein-coupled receptor (Andersen et al., 2008). Ventricular dysfunction was significantly related to the metabolic status of the subjects (insulin resistance, abdominal adiposity) and to their levels of free-testosterone and was absent in Klinefelter subjects without metabolic syndrome, excluding an affection of the left ventricular function related to chromosome aberration itself (Andersen et al., 2008).

Finally, Klinefelter subjects have been reported to have problems related to coagulation system, leading to deep vein thrombosis, leg ulcers and thromboembolic events probably related to hypogonadism (Campbell and Price, 1981). In fact, androgen deficiency is associated with an increased thromboembolic risk, because of the reverse relationship between plasminogen activator inhibitor-1 and testosterone levels (Winkler, 1996; De Pergola et al., 1997) and because of the prothrombotic effect of estrogens (Rosendaal et al., 2001) that are increased in KS. This hypercoagulability state is worsened by obesity and diabetes (Ageno et al., 2008), which are often present in Klinefelter patients.

**New developments**

Recently, it has been demonstrated that a reduced number of circulating endothelial progenitor cells (EPCs) is an independent predictor of morbidity and mortality from cardiovascular diseases (Werner et al., 2005) and of atherosclerotic disease progression (Schmidt-Lucke et al., 2005). Although there is an ongoing debate regarding the true molecular phenotype of EPCs, they are generally defined as CD34, CD133 and vascular endothelial growth factor 2/kinase insert domain-receptor (KDR) positive cells (Asahara et al., 1997; Fadini et al., 2008). The majority of these cells originates from haematopoietic stem cells of the bone marrow and under specific signals from a damaged endothelium differentiates and shifts into the systemic circulation, contributing to the neoangiogenic process and to the repair of the damaged endothelial monolayer. Because of their role in endogenous maintenance of functional endothelium, EPCs are currently considered an integrated component of the cardiovascular system (Fadini et al., 2007).

Many studies have showed that classic risk factors for atherosclerosis directly reduce the mobilization and survival of EPCs by impairing nitric oxide (NO) bioavailability (Dimmel et al., 2001; Vasa et al., 2001; Aicher et al., 2003; Assmus et al., 2003; Hill et al., 2003; Laufs et al., 2004; Dimmeler and Zeiher, 2004) and that a reduced number of circulating EPCs is detected in many pathological conditions, such as diabetes, coronary, cerebral and peripheral artery diseases (Loomans et al., 2004; Forastera et al., 2006a), which are characterized by endothelial dysfunction.

There are some data suggesting that androgens may influence EPCs mobilization from bone marrow. A direct effect of testosterone on EPCs was suggested by the evidence that hypogonalad men have a low number of circulating EPCs that increase significantly after testosterone replacement therapy (Forastera et al., 2006a). These results were also confirmed in vitro by showing an increase in EPCs proliferation, migration and colony formation activity induced by testosterone in an AR-mediated pathway (Forastera et al., 2008). Even if the exact mechanism by which androgens act on EPCs is still unknown, it seems that they act on the NO/cyclic guanosine monophosphate (cGMP) system in the bone marrow. NO acts primarily in a paracrine manner to increase EPCs number: it enhances the production of cGMP that, in turn, causes metalloproteinase MMP-9 activation and therefore the mobilization of EPCs from the bone marrow (Forastera et al., 2006b) (Fig. 1). Many studies have shown that androgens positively modulate NO synthesis in human corpora cavernosa (Charnness et al., 1995; Lugg et al., 1995; Reilly et al., 1997; Baba et al., 2000; Zhang et al., 2005) and that phosphodiesterase (PDE)5 (the enzyme that degrades cGMP) is expressed in the rat corpora cavernosa (Zhang et al., 2005). Recent reports have also demonstrated that human bone-marrow expresses PDE5 (Forastera et al., 2007) and have hypothesized that it is up-regulated by testosterone (Forastera et al., 2009), explaining the low levels of circulating EPCs in hypogonadal subjects: reduced levels of androgens might cause a lowered NO

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Figure 1 (A) Role of testosterone on endothelial progenitor cells (EPCs) mobilization from the bone marrow. Testosterone positively modulates NO synthetase (NOS) and PDE-5 expression in the human bone marrow, in an androgen receptor (AR)-mediated pathway. NO acts primarily in a paracrine manner to increase EPCs number, enhancing the production of cGMP that, in turn, causes MMP-9 activation and therefore the mobilization of EPCs from the bone marrow. (B) Klinefelter subjects have reduced levels of testosterone that might cause a lowered NOS activity and therefore a lowered NO production. This could cause a reduced cGMP production in the bone marrow that, in turn, leads to a lowered MMP9 activation and mobilization of EPCs towards the peripheral circulation. However, testosterone therapy does not normalize EPC number, suggesting also possible reduced androgen sensitivity due to a different CAG length and/or inactivation in the AR gene. NO, nitric oxide; sGC, guanilate cyclase; cGMP, cyclic guanosine monophosphate; GTP, guanosine triphosphate; ROS, reactive oxygen species; PDE, phosphodiesterase.
Hypothesis generating primary data

Although subjects with KS have a higher prevalence of CRFs and are often hypogonadal, EPCs in KS have never been studied. We evaluated circulating EPCs number in 68 consecutive subjects affected by non-mosaic 47,XXY KS (mean age 30 ± 8 years), referred to our centre for infertility evaluation and in 46 controls (mean age 33 ± 9 years), matched by age and body mass index, selected from 50 blood donors volunteers. Exclusion criteria for patients and controls were previous major cardiovascular events and previous or ongoing therapy with gonadotropin, testosterone (T), statin or PDE type 5 inhibitor, all known factors able to modify the number of circulating EPCs.

As hypogonadism and metabolic syndrome reduce the number of EPCs, patients and controls were divided into two groups, according to the absence or presence of cardiovascular risk factors (CRFs) and underwent clinical evaluation, biochemical and hormonal evaluation and EPC measurement.

CRFs included waist circumference > 102 cm, hypertension (blood pressure > 130/85 mmHg in three measurements), HDL cholesterol < 40 mg/dl, fasting glycemia > 110 mg/dl, triglycerides > 150 mg/dl, LDL cholesterol > 160 mg/dl, smoking, homeostasis model assessment (HOMA) > 2.4, hyperhomocysteinemia (> 15 mg/dl). We include among CRFs also hypogonadism (defined as calculated free-testosterone < 0.250 nmol/l), because testosterone seems to play a protective role on the cardiovascular system, acting directly on the vessel wall (Alexander et al., 1999; Rosano et al., 2002; Kang et al., 2002; Dockery et al., 2003; Khaw et al., 2007) besides of its role on EPC mobilization. Furthermore, many studies have shown a close association between low testosterone levels and abdominal obesity, type 2 diabetes and metabolic syndrome (Bhasin et al., 2001; Laaksonen et al., 2004; Bojesen et al., 2006; Zitzmann et al., 2006; Kapoor et al., 2007; Rodríguez et al., 2007; Selvin et al., 2007), which are associated with endothelial dysfunction and increased risk of cardiovascular diseases.

Clinical evaluation included waist and BMI measurement and blood pressure. Hormonal parameters (evaluated by commercial electrochemiluminescence immunoassay methods, Elecsys 2010, Roche Diagnostics, Mannheim, Germany) and fasting glucose, insulinemia, LDL cholesterol, HDL cholesterol and triglycerides were evaluated at 8.00–10:00 am. Free testosterone was calculated from total T and SHBG concentrations, using the method of Vermeulen (Vermeulen et al., 1999). HOMA was calculated as (fasting glucose × 0.055) × (insulinemia/22.5).

Blood samples for circulating EPC counts were evaluated by flow cytometry, as previously described (Foresta et al., 2005). Briefly, analysis was performed on 150 µl peripheral blood incubated with fluorescein isothiocyanate-labelled monoclonal antibodies against human CD34 (Becton Dickinson, Milano, Italy), allophycocyanin-labelled monoclonal antibodies against human AC133 (Miltenyi Biotec, Bergisch Gladbach, Germany) and monoclonal antibodies against human VEGFR2 (Sigma-Aldrich, Milano, Italy). EPCs are defined by CD34, AC133 and VEGFR2 positivity.

Table I Hormonal, biochemical parameters and endothelial progenitor cells (EPCs) serum concentrations in KS subjects and in controls.

<table>
<thead>
<tr>
<th></th>
<th>Klinefelter (n: 68)</th>
<th>Controls (n: 46)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (IU/l)</td>
<td>31.0 ± 11.5</td>
<td>4.8 ± 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>19.3 ± 6.6</td>
<td>4.1 ± 2.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T (nmol/l)</td>
<td>11 ± 4</td>
<td>19.4 ± 4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>27 ± 11</td>
<td>30.4 ± 4.7</td>
<td>NS</td>
</tr>
<tr>
<td>Free-T calculated (nmol/l)</td>
<td>0.223 ± 0.09</td>
<td>0.432 ± 0.102</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Estradiol (pnmol/l)</td>
<td>113 ± 33</td>
<td>91 ± 40</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>EPCs (cells/ml)</td>
<td>42 ± 34</td>
<td>67 ± 44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>47 ± 100</td>
<td>46 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>146 ± 100</td>
<td>143 ± 108</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (BMI)</td>
<td>26 ± 5</td>
<td>23.2 ± 1.5</td>
<td>NS</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>98 ± 16</td>
<td>96 ± 19</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>82 ± 16</td>
<td>83 ± 11</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting insulinemia (mU/l)</td>
<td>11.7 ± 8</td>
<td>10.2 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>HOMA</td>
<td>2.4 ± 1.8</td>
<td>2.3 ± 1.4</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>127 ± 15</td>
<td>120 ± 18</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80 ± 15</td>
<td>76 ± 12</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD of the mean. NS, not significantly different.
In order to highlight the role of low testosterone levels (free testosterone <0.250 nmol/l) in the reduction of EPCs in the most numerous group of KS patients (with risk factors), we compared EPCs levels between patients with low testosterone levels (36 subjects; T: 8.2 ± 2.9 nmol/l; calculated free-T: 0.162 ± 0.06 nmol/l) and those with normal testosterone concentrations (24 subjects; T: 14.2 ± 3.7 nmol/l; calculated free-T: 0.316 ± 0.06 nmol/l). This analysis showed that the number of circulating EPCs was similar in these two groups (50 ± 40 and 34 ± 27 cells/ml). Therefore, it seems that KS subjects have low EPCs number independently from testosterone plasma levels. Furthermore, 22 hypogonadal patients were re-evaluated after 6 months of testosterone replacement therapy.

Although we observed, as expected, higher levels of total testosterone, calculated free-testosterone and estradiol with respect to baseline, and a reduction in LH levels, we did not obtain the normalization of LH values. In Klinefelter subjects, in fact, is very difficult to obtain the normalization of LH without causing an excessive increase of testosterone concentrations and the goal of therapy is the normalization of testosterone levels. The number of EPCs did not increase after therapy (Table III) (42 ± 34 versus 44 ± 51 cells/ml) and the number of EPCs post-treatment remained similar to EPCs of controls with CRFs (33 ± 25 cells/ml). These data further suggested that in KS subjects the reduction of EPCs number is not related to low testosterone levels (free testosterone <0.250 nmol/l).

**Table II** EPCs serum concentration in KS patients and controls, with and without cardiovascular risk factors and with metabolic syndrome.

<table>
<thead>
<tr>
<th>All subjects</th>
<th>Without risk factors</th>
<th>With risk factors</th>
<th>With metabolic syndromea</th>
</tr>
</thead>
<tbody>
<tr>
<td>KS patients</td>
<td>n=68</td>
<td>n=8</td>
<td>n=60</td>
</tr>
<tr>
<td>EPCs (cells/ml)</td>
<td>42±34</td>
<td>33±21</td>
<td>43±36</td>
</tr>
<tr>
<td>Controls</td>
<td>n=46</td>
<td>n=25</td>
<td>n=21</td>
</tr>
<tr>
<td>EPCs (cells/ml)</td>
<td>67±44b</td>
<td>100±23</td>
<td>33±25b</td>
</tr>
</tbody>
</table>

*P < 0.001 versus all groups of KS patients.

**Table III** Hormonal and EPCs data in 22 hypogonadal KS subjects (free testosterone <0.250 nmol/l) before and after 6 months of testosterone therapy (testogel 50 mg/day).

<table>
<thead>
<tr>
<th></th>
<th>Before treatment</th>
<th>After treatment</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (IU/l)</td>
<td>32 ± 13</td>
<td>27 ± 17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LH (IU/l)</td>
<td>19 ± 6</td>
<td>16 ± 8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>T (nmol/l)</td>
<td>9 ± 3</td>
<td>23 ± 11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Free-T calculated (nmol/l)</td>
<td>0.186 ± 0.07</td>
<td>0.602 ± 0.409</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Estradiol (pmol/l)</td>
<td>109 ± 31</td>
<td>153 ± 53</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>EPCs (cells/ml)</td>
<td>42 ± 34</td>
<td>44 ± 51</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD of the mean.

**Implications**

We showed that KS subjects, other than a high prevalence of hypogonadism (36/68, 53%) as expected, have a high frequency of smoking (34/68, 60%), truncal obesity (26/68, 38%) and hyperinsulinemia (25/68, 37%). Furthermore, all KS patients, independently from testosterone levels and from the presence or absence of CRFs, had low levels of circulating EPCs that further increase their cardiovascular risk. The number of EPCs is not only significantly lower with respect to controls but also remain similar to controls with CRFs even when testosterone was increased to normal ranges after 6 months of androgen replacement therapy.

In the light of our data, it seems that in KS subjects androgens have no effect on EPC number and that CRFs might contribute, but are not the only cause of the low levels of EPCs. At present, we do not have an explanation for these findings, but we could hypothesize reduced androgen sensitivity, maybe related to a different length of CAG repeats of the AR, which modulates androgen sensitivity. In fact, it has been reported that CAG length influences some clinical features of Klinefelter subjects and that a non-random inactivation of the X chromosome (where the AR map) might exist (Zitzmann et al., 2004). Furthermore, it is possible that other genetically determined factors (related to the supernumerary X chromosome) could be involved. The extra X chromosome might be responsible for an impaired ability of bone marrow to produce and/or release EPCs. Constitutional chromosome abnormalities, in fact, are associated with an increased risk of haematological malignancies and, although at present there is no agreement about an association between KS and haematological neoplastic diseases, cases of leukaemia, lymphoma, myelodysplastic syndrome, aplastic anaemia and erythropoetin-resistant anaemia have been described in KS (Yamauchi, 1993; Bakshi et al., 2004; Shimizu et al., 2005). The molecular mechanism is unknown, but it has been supposed that sex chromosome abnormality causes myelodysplastic changes in the bone marrow that predispose to haematological malignancies. These same mechanisms could explain also a possible damage on proliferation and/or maturation and/or migration of EPCs from the bone marrow in KS subjects.

New in vitro studies on EPCs from Klinefelter subjects will clarify the role of androgens on EPC system and the bone marrow’s functionality in these patients.

Anyway, because KS subjects have a low number of EPCs, which is itself an independent risk factor for cardiovascular diseases, in these subjects the first recommendation remains the lifestyle modification with the correction of all modifiable factors that affect EPCs number. In the future, an EPC therapy could also be hypothesized. Initial studies have shown optimism for the potential of EPCs as a therapeutic area in vascular regenerative medicine, but to date only few aspects have been elucidated with respect to their physiology and further studies are necessary to discover and understand all mechanisms involved in EPCs mobilization, homing and survival. These findings will provide the basis for their real therapeutic applications in ischaemic diseases and maybe in preventive medicine.
Summary

KS is associated with a significant morbidity related to vascular diseases. This increased risk is probably mediated by several mechanisms, some of which directly dependent from the chromosome aberration itself and others depending from hypogonadism. In KS subjects, the low number of EPCs could be considered a new CRF that in conjunction with the hypercoagulability state and the increased prevalence of cardiac valve malformations, visceral obesity and insulin resistance, might contribute to the increased mortality of these subjects.

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References

Klinefelter syndrome and cardiovascular diseases


