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Cigarette Smoke Inhibits Recruitment of Bone-Marrow-Derived Stem cells to The Uterus

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Abstract

Cigarette smoking leads to female infertility and a decreased incidence of endometriosis. Bone marrow derived stem cells are recruited to uterine endometrium and endometriosis. The effect of cigarette smoking on stem cell recruitment to any organ is uncharacterized. We hypothesized that bone marrow-derived mesenchymal stem cell recruitment to the uterus and differentiation would be diminished by cigarette smoke. We used human mesenchymal stem cells (hMSC) *in vitro* and a mouse model of cigarette smoke exposure. After myeloablation female C57BL/6J received bone marrow cells from males. Mice were exposed to room air or smoke from unfiltered cigarettes. Immunofluorescence and Y-FISH was performed on uterine sections. *In vitro* hMSCs were treated with 8-Br-cAMP to induce endometrial cell differentiation with or without cigarette smoke extract (CSE) and decidualization assessed morphologically and by prolactin expression. After 4 weeks the total number of Y-chromosome cells in the uterus was reduced by 68% in the smoke exposed mice. Both leukocytes and bone marrow derived endometrial cells were reduced by 60% and 73%, respectively. Differentiation of bone marrow derived cell to endometrial epithelial cells was reduced by 84%. hMSC treated with CSE failed to show cytological characteristics of decidualization. mRNA levels of the decidualization marker prolactin were decreased by 90% in CSE treated cells. Smoking inhibits both recruitment of bone marrow derived stem cells to uterus and stem cell differentiation. Inhibition of stem cells recruitment may be a general mechanism by which smoking leads to long term organ damage through inability to repair or regenerate multiple tissues.

Keywords

smoking; tobacco; bone marrow-derived stem cells; endometrium; uterus

Introduction

Stem cells are progenitor cells that are capable of both self-renewal and differentiation into various cell types (Bongso *et al.* 2004; Gargett *et al.* 2007). Bone marrow-derived mesenchymal stem cells (BMDCs) are multipotent cells that can be induced into various cell

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types, including endothelial cells, hepatocytes, neurons, skin, cardiomyocytes, endometrial stromal fibroblasts (hESF) and gastrointestinal epithelium (Aghajanova *et al.* 2010; Blau *et al.* 2001; Alison *et al.* 2000; Theise *et al.* 2000; Mezey *et al.* 2003; Korblyng *et al.* 2002; Quaini *et al.* 2002; Lagasse *et al.* 2000). Bone marrow derived stem cells can specifically give rise to endometrial stromal and epithelial cells in both humans and mice. These bone marrow-derived stem cells likely contribute to both normal tissue homeostasis and repair. They however, are also responsible for the pathogenesis of endometriosis (Du *et al.* 2007). In human endometrium, a small population of clonogenic, self-regenerating and pluripotent mesenchymal stem cells (MSC) has been shown to be a potential stem/progenitor cell population (Chan *et al.* 2004; Gargett *et al.* 2009). Endometrial regeneration and repair is essential to the restore this tissue that must be replaced with each menstrual/estrus cycle in women/mice. The loss of BMDC recruitment to and differentiation in the uterus may reduce endometrial repair and reduce fertility as well as reduce the incidence of endometriosis.

Tobacco use is known to cause numerous diseases including lung cancer, heart and lung disease as well as oral cancers. Cigarette smoking can impair the regenerative functions of fibroblasts, epithelial cells, and mesenchymal cells in lung and oral cancer (Rennard *et al.* 2006). Cigarette smoking also has multiple effects on female fertility, including reduced uterine receptivity and increased risk of multiple pregnancies (Soares *et al.* 2007). However, smoking is “beneficial” to female smokers by decreasing incidence of endometrial cancer and endometriosis (Cramer *et al.* 1986; McGarry *et al.* 2002; Shiverick *et al.* 1999; 2005). While this effect has been ascribed to diminished ovarian function and resultant estrogen diminution, there are also direct uterine effects; one cigarette constituents, benzo(a)pyrene, can inhibit endometrial cell proliferation, attachment and invasion of basement membrane possibly by down-regulating epidermal growth factor receptor and E-cadherin (McGarry *et al.* 2002; Shiverick *et al.* 2005). Additionally, bone marrow-derived stem cells are also implicated in the pathogenesis of endometriosis (Du *et al.* 2007, Sasson and Taylor 2008). It is thus plausible that female smokers may have an increased risk of infertility and reduced risk of endometriosis because of impaired recruitment or differentiation of BMDCs. However, the effect of smoking on BMDC recruitment to the uterine has not been previously characterized. Here we present *in vivo* and *in vitro* data indicating that cigarette smoking has an adverse effect on BMDC recruitment to and differentiation in the uterus.

Results

Decreased bone marrow-derived cells in the uterine endometrium of cigarette-smoke exposed mice

Bone marrow-derive stem cells have been demonstrated to be a source of endometrial progenitor cells (Taylor 2004; Chan *et al.* 2004; Gargett *et al.* 2009; Aghajanova *et al.* 2010; Du *et al.* 2007). To examine the effects of cigarette smoking on the recruitment of bone-marrow-derive stem cells to the uterus, 1×10^7 unfractionated bone marrow cells from male donors were transplanted into female recipients through the tail vein. One month post transplantation, the animals were exposed to cigarette smoke (CS) or normal room air without smoke (NS) for one month. Y chromosome fluorescent in situ hybridization (FISH) was used to evaluate the total cells from male donor bone marrow, and CD45, a general leukocyte marker, was used to assess the total infiltrating self-and foreign leukocytes. As shown in Figure 1, the overall number of Y-chromosome positive (Y^+) cells observed in the uterus was decreased by 68% in the smoke exposed animals compared to those that were exposed to smoke free air ($p < 0.005$). The Y chromosome bearing cells in the uterus after bone marrow transplant consist of leukocytes and, as previously reported, stem cells with the capacity for endometrial regeneration (Du *et al.* 2007). Both populations of cells were reduced in the uteri of smoke exposed mice. Leukocytes were identified as Y^+ and $CD45^+$ cells and these cells were reduced by 60% in the smoke exposed compared to the non-

exposed animals ($p < 0.05$). Stem cells that differentiated from the transplanted bone marrow are Y^+ and also do not express leukocyte markers ($CD45^-$). Bone marrow stem cell derived endometrial cells were reduced by 73% in the uterus of smoke-exposed female mice compared to non-exposed female mice ($p < 0.005$).

Differentiation of bone marrow-derived stem cells into the endometrial epithelial cells is attenuated in the smoke-exposed mice

Previous studies have shown that smoking can adversely impact endometrial cell proliferation and differentiation (McGarry *et al.* 2002; Shiverick *et al.* 2005). In order to determine if smoking can also affect the differentiation of bone marrow derived stem cells into uterine endometrial epithelium cells, cytokeratin (an epithelial cell-specific marker) expression was assessed. As shown in Figure 2, in the epithelium, Y-chromosome positive and cytokeratin-expressing cells were decreased by 84% in the mice exposed to cigarette smoke compared to control ($p < 0.005$). This deficit is greater than the reduction in total cell recruitment ($p < 0.05$), indicating a reduction in differentiation of recruited stem cells to epithelial cells.

Differentiation of hMSCs is blocked by treatment of cigarette smoke extract in vitro

To determine if smoke extract affected differentiation of human mesenchymal stem cells (hMSC), we used a previously established in vitro differentiation protocol and a standard cigarette smoke extract (CSE). hMSC were treated with 1mM 8-Br-cAMP (cAMP) and either 0.05 μ g/ μ l CSE or vehicle control. At day 14 post treatment with cAMP only, the cell morphology changed dramatically and resembled typical endometrial stromal fibroblasts (Figure 3A). However, in the presence of CSE, the induction of this morphologic change was incomplete. Prolactin (PRL) is a well-known marker of endometrial stromal cells after differentiation (decidualization) induced by 8-Br-cAMP and recently has also been shown to be highly upregulated during hMSCs differentiation (Aghajanova *et al.* 2010). The expression of PRL by hMSCs was quantified by RT-PCR 14 days after treatment. PRL mRNA levels were significantly up-regulated by 200-fold in cAMP-treated cells compared to vehicle controls. However cells treated with cAMP and CSE together expressed 90% lower PRL than cells treated with cAMP alone (Figure 3B) ($p < 0.05$).

Discussion

Tobacco smoking has long been recognized as an addictive habit that leads to the development of numerous diseases, especially those of the respiratory and cardiovascular systems. Cigarette smoking has also a profound impact on fertility. Its effect on bone-marrow derived stem cell development, however, has not been characterized. Our results suggest that cigarette smoking can adversely affect bone marrow-derived stem cell recruitment to and differentiation in the uterus. The number of bone marrow-derived stem cells is greatly reduced in the uterus of female mice exposed to cigarette smoke compared to those exposed to normal room air. However, the effect of smoking on cell recruitment to uterus is not likely to be specific to stem cells, as leukocyte infiltration is also blocked; this suggests a common mechanism responsible for recruitment of both cell types. Cigarette smoking likely has a broad effect on many cell recruitment pathways. Indeed, in the setting of injury or infection, both leukocytes and stem cells are needed to foster tissue regeneration or clear invading pathogens; smokers are vulnerable to infections and exhibit diminished cellular immunity (Sopori *et al.* 2002). Our results, however, do not exclude the possibility that smoking may alternatively enhance inflammation (Kitamura *et al.* 2007) and autoimmunity (Costenbader *et al.* 2006; Klareskog *et al.* 2007; Vestergaard *et al.* 2002) in other organs. As opposed to most tissues, leukocyte recruitment is a normal physiologic event in the menstrual cycle where a flux of leukocytes is essential for pregnancy.

Bone marrow–derived stem cells contribute to both normal tissue homeostasis and repair as well as the pathogenesis of endometriosis (Du *et al.* 2007). Consistent with previous studies on woman smokers (Shiverick *et al.* 1999; Cramer *et al.* 1986), our results suggest that smoking could paradoxically reduce the incidence and progression of endometriosis, though the overall risks of smoking are far more profound. Reduction of endometrial stem cell recruitment caused by smoking may directly impact fertility. Diminished endometrial growth as indicated by a sonographically thin endometrium is closely correlated with human fertility (Bassil *et al.* 2001; Bromer *et al.* 2009; Dietterich *et al.* 2002; Weissman *et al.* 1999; Oliveira *et al.* 1993; Noyes *et al.* 1995; Kovacs *et al.* 2003; Zhang *et al.* 2005). The ability of the endometrium to provide a suitable environment for conception, implantation, early gestation and placentation is critical to pregnancy and fertility (Kathryn *et al.* 2009). This function likely relies on the ability to recruit stem cells to endometrium to maintain an adequate endometrial growth.

We acknowledge that irradiation followed by bone marrow transplant is an additional stress that may influence cell recruitment. As the control group was also irradiated and underwent bone marrow transplant, we believe that we have isolated the effect of smoking on bone marrow derived cell recruitment. However, the nature and magnitude of the effects may be influenced by the preceding treatment. It is possible that bone marrow transplanted animals have a propensity to the harmful effects of cigarette smoke on cell recruitment. This limitation is unavoidable as bone marrow transplant is required to introduce cells that can be identified experimentally.

In addition to altering recruitment, our data suggest that smoking may block stem cell differentiation. cAMP-induced differentiation of hMSCs is blocked when exposed to cigarette smoke extract. Previous studies have shown that cigarette-smoke conditioned medium reduced proliferation of endometrial cells (Shiverick *et al.* 1999; 2005; Khorram *et al.* 2010). Although not characterized in this study, we speculate that smoking may also affect the physiology of stem cells in many organs and this may be a novel mechanism underlying pathogenesis of multiple diseases associated with smoking. Future studies will investigate the molecular mechanism by which smoking blocks stem cell infiltration and differentiation.

Methods

Cell culture

Human mesenchymal stem cells were maintained in MSCBM (mesenchymal stem cell basic medium) medium containing 10% MCGS (mesenchymal stem cell growth supplement), 2% L-glutamine and 0.1% GA-1000. At 80% confluence, cells were trypsinized and plated at a density of 5×10^3 cells/cm² in DMED (high glucose) medium containing 10% fetal bovine serum (FBS), 1% 100× antibiotic-antimycotic. Nearly confluent cells were serum-starved overnight and treated for 14 days in the low-serum medium (2% FBS) with the following treatments: (a) 1mM 8-Br-cAMP (cAMP) (SigmaAldrich, St.Louis, MO); (b) 1mM 8-Br-cAMP (cAMP) and 0.05μg/μl CSE (cigarette smoking extract) (Arista Laboratories Inc. Richmond, VA); (c) DMSO vehicle control. Media were changed every third day.

Total RNA isolation, quantitative real-time RT-PCR

Total RNA from each cell culture was purified using the Qiagen RNeasy Plus Mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. For real-time RT-PCR analysis, 200ng of RNA was converted to cDNA using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). RT-PCR was performed using the LightCycler SYBR Green RT-PCR kit from Roche (Stockholm, Sweden). PCR for PRL was performed

for 40 cycles at 95°C for 15 seconds; 58.7°C for 20 seconds; and 72°C for 25 seconds, with the primers PRL (sense primer 5'-CATCAACAGCTGCCACACTT-3', antisense primer 5'-CGTTTGGTTTGTCTCCTCAAT-3'), B-actin (sense 5'-CGTACCACTGGCATCGTGAT-3', antisense 5'-GTGTTGGCGTACAGGTCTTTG-3').

Bone Marrow Cell Isolation and Transplantation

After irradiated with 1000 cGy, twenty 6-week old female C57BL/6J mice were transplanted with 3 million unfractionated bone marrow cells from age-matched male mice via tail vein injection. 4 weeks later, the mice were exposed to room air (non-smoke exposed or NS) or the smoke from unfiltered research cigarettes (cigarette smoke exposed or CS) using a smoking apparatus (Hautamaki *et al.* 1997) for 4 weeks at half cigarette per exposure, twice a day for the first week and 1 cigarette three times per day for the next three weeks. This level of treatment approximates low-moderate human exposure and is limited by lung capacity rather than weight. Animals were treated under an approved Yale University Institutional Animal Care and Use Committee protocol.

Y FISH and Immunofluorescence

Formalin-fixed paraffin-embedded uterine sections were deparaffinized and treated with BD Biosciences (San Diego) Retrieval solution. The Y-chromosome probe was generated following our previously published method (Du *et al.* 2007). Y-FISH was performed using a digoxigenin-labeled Y chromosome probe and anti-digoxigenin-rhodamine antibody (Roche Diagnostics, Basel, Switzerland). After Y-FISH, slides were incubated with 1:20 rat anti-mouse CD45 (BD Biosciences) at 4°C overnight followed by 1:500 anti-rat-Alexa 488 (Molecular Probes, Eugene,) for 1 hour at 37°C. Alternatively, slides were incubated with 1:100 anti-cytokeratin (ab9377, Abcam Inc. MA) for 1 hour at room temperature followed by 1:400 anti-rabbit-cy5 (Jackson ImmunoResearch laboratories, INC, PA) for 1 hour at 37°C. All slides were finished by using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Cells staining positive for Y chromosome, cytokeratin or CD45 were counted in 14 uterine sections per animal using 7 mice from 3 independent experiments. All cells in each uterine cross section were counted. Results were compared by Student *t*-Test.

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Figure 1.

Bone marrow-derived endometrial stromal cells are reduced in the cigarette smoke exposed female mouse uteri. Immunofluorescence staining of Y chromosome (red), CD45 (green) and nuclei (blue) of bone marrow-derived endometrial stromal cells in the transplanted, **A, B)** non-smoke (NS) or **C, D)** cigarette smoke (CS) exposed female mice uteri. **A, C)** Original magnification 100 ×, **B, D)** 400× magnification of part of **A, C)** respectively. Arrow heads indicates Y-chromosome and CD45 double positive cells. Arrows indicate Y-chromosome positive only cells. **E)** Cells counted from 14 uterine sections per animal. n=7 mice / group from 3 independent experiments. * $p<0.05$; ** $p<0.005$ by Student *t*-Test.

Figure 2.

Bone marrow-derived endometrial epithelial cells are reduced in the cigarette smoke exposed female mouse uteri. Immunofluorescence staining of Y chromosome (red), cytokeratin (yellow), CD45 (green) and nuclei (blue). Y chromosome-positive, cytokeratin-positive, and CD45-negative cells in the endometrium of **A**) nonsmoking (NS) and cigarette smoke-exposed (CS) group. **B**) Cells were counted from 14 uterine sections per animal. n=7 mice / group from 3 independent experiments. ** $p < 0.005$ by Student *t*-Test.

A **B** **Figure 3.**

Cigarette smoke extract (CSE) inhibits cAMP induced-differentiation of hMSC. hMSC cells were treated with vehicle control (Mock), or 1mM cAMP, or 1mM cAMP and 0.05mg/ml of CSE for 14 days. **A)** Morphological changes of hMSC. **B)** mRNA levels of PRL measured by Q-PCR and expressed as fold changes relative to vehicle control (mock) using delta delta Ct method. * $p < 0.05$ by Student *t*-Test.