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The cholinergic system is involved in regulation of the development of the hematopoietic system

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Abstract

Gene expression profiling demonstrated that components of the cholinergic system, including choline acetyltransferase, acetylcholinesterase and nicotinic acetylcholine receptors (nAChRs), are expressed in embryonic stem cells and differentiating embryoid bodies (EBs). Triggering of nAChRs expressed in EBs by nicotine resulted in activation of MAPK and shifts of spontaneous differentiation toward hemangioblast. In vivo, non-neural nAChRs are detected early during development in fetal sites of hematopoiesis. Similarly, in vivo exposure of the developing embryo to nicotine resulted in higher numbers of hematopoietic progenitors in fetal liver. However postpartum, the number of hematopoietic stem/progenitor cells (HSPC) was decreased, suggesting an impaired colonization of the fetal bone marrow with HSPCs. This correlated with increased number of circulating HSPC and decreased expression of CXCR4 that mediates migration of circulating cells into the bone marrow regulatory niche. In addition, protein microarrays demonstrated that nicotine changed the profile of cytokines produced in the niche. While the levels of IL1 α , IL1 β , IL2, IL9 and IL10 were not changed, the production of hematopoiesis-supportive cytokines including G-CSF, GM-CSF, IL3, IL6 and IGFBP-3 was decreased. This correlated with the decreased repopulating ability of HSPC in vivo and diminished hematopoietic activity in bone marrow cultures treated with nicotine. Interestingly, nicotine stimulated the production of IL4 and IL5, implying a possible role of the cholinergic system in pathogenesis of allergic diseases. Our data provide evidence that the nicotine-induced imbalance of the cholinergic system during gestation interferes with normal development and provides the basis for negative health outcomes postpartum in active and passive smokers.

Keywords

Stem cells; regulatory niche; nAChR; cytokines

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Introduction

Under in vitro conditions, embryonic stem cell (ESCs) lines are capable of developing into a variety of tissues, including hematopoietic. Thus, ESCs provide a model system that allows us to better understand the cellular and molecular mechanisms regulating development of human hematopoietic stem cells (HSCs). Different strategies have been applied to generate HSCs from ESCs. One approach involved using ESC-derived embryoid bodies (EBs) and generated HSCs with similar developmental kinetics to cells developing in vivo (Keller et al., 1993).

In vivo, development of the hematopoietic system begins early during embryogenesis and includes the generation of hematopoietic cells in the yolk sac and aorta-gonad-mesonephros and their subsequent migration into the organs that are the sites of fetal hematopoiesis. At day 12 of embryonic development (E12) in the mouse, the fetal liver is colonized by multipotent and self-renewing HSCs, which subsequently migrate to the bone marrow, where they remain throughout life. In the bone marrow, self-renewal, proliferation and differentiation of HSCs are regulated by the microenvironmental niche (Schofield, 1983). The cellular compartment of the niche is heterogeneous and is represented by cells of hematopoietic (macrophages, lymphocytes, osteoclasts, etc) and mesenchymal (stromal cells, osteoblasts, adipocytes, etc.) origin. Extracellular matrix (ECM) molecules and cell surface associated and soluble factors are produced by the cells that compose the hematopoietic niche and contribute to the highly complex structure of the hematopoietic niche (Chabannon and Torok-Storb, 1992). Soluble factors produced by the niche include positive (G-CSF, GM-CSF, M-CSF, IL-6, IL-3, IL-12, SCF, Flt-3L, etc.) and negative (TGF- β , TNF- α , MIP-1 α and INF γ) regulators of HSC proliferation. It is vital to maintain the correct balance between positive and negative regulators in order to maintain the optimal ratio of proliferating and quiescent cells in the bone marrow.

Despite recent advances in understanding of the structure and function of the hematopoietic niche, the role of the cholinergic system in regulation of hematopoietic homeostasis has not been well investigated. A role of the cholinergic system is however anticipated due to the fact that elements of the non-neural cholinergic system, including acetyltransferase (ChAT), acetylcholinesterase (AChE) and acetylcholine receptors (AChRs), are expressed by cells of hematopoietic origin (Deutsch et al., 2002; Kawashima and Fujii, 2004; Wessler et al., 1998). Nicotinic AChRs (nAChRs) belong to a superfamily of ionotropic receptors and are expressed on a variety of non-neural cells, including hematopoietic cells and cells composing the hematopoietic niche. Nicotine-mediated activation of nAChRs affects DNA synthesis, interferes with cell proliferation, and influences cytokine and ECM production and adhesion molecule expression (Carty et al., 1996; Tipton and Dabbous, 1995; Tomek et al., 1994).

In the current study we demonstrate that nicotine-mediated stimulation of nAChRs expressed by the developing tissue, results in activation of MAPK signaling and increased differentiation towards hemangioblast. In vivo, both the engraftment of circulating HSCs into the bone marrow and their lymphoid differentiation are inhibited by nicotine. The production of cytokines that support proliferation of HSCs, including IL-3, IL-6, G-CSF, GM-CSF and IGFBP-3, is decreased by nicotine contributing to the impaired hematopoiesis-supportive function of the niche. Also, we observed decreased levels of chemokines produced by the niche, decreased CXCR4 expression on hematopoietic cells and increased mobilization of HSPC implying that the recruitment of circulating HSPC and their retention in the niche is negatively influenced by nicotine.

Materials and Methods

Mice

All experiments were conducted in agreement with the institutional policy on animal use and approved by the Institutional Animal Care and Use Committee (IACUC). Eight- to 12-week-old female mice BALB/c were obtained from Harlan Inc. (Indianapolis, US) or bred in-house. The animals were kept under standard pathogen-free conditions. Where indicated, nicotine (Sigma; 10^{-8} M/mouse) was administered intravenously.

Cell Lines

ESCs were cultured on a feeder layer established from HS27 human fibroblasts grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with non-essential amino acids (AA), L-Glutamax and 10% fetal bovine serum (FBS). Prior to use for ESC cultures, the feeder layers were irradiated with 40Gy (4000 rads). Human ESCs (WA09 line) were grown on the feeder layers in DMEM/F12 supplemented with 20% knockout serum replacement (SR), 10mM non-essential AA, 1mM L-Glutamax, 10ng/ml h-FGF and 0.1mM 2-mercaptoethanol (ME) (Invitrogen, Carlsbad, CA). The cultures were fed every two days and passaged by the manual dissection method every 6 days. To induce ESC differentiation, the ESC colonies were removed from the feeder layers and cultured in DMEM/F12 supplemented with 20% FBS, 10mM non-essential AA, 2 mM L-Glutamax and 0.1 mM 2-ME in suspension as clustered spheroids of cells referred to as embryoid bodies (EBs).

Bone marrow cultures

Short-term bone marrow cultures (STBMC) were established from freshly isolated bone marrow cells (10^6 cells/ml) and cultured in DMEM supplemented with 20% horse serum (StemCell Tech) and 10^{-6} M hydrocortisone (Sigma, St Louis, MO) in 6 well plates at 37°C in a humid atmosphere containing 5% CO₂. Cultures were fed weekly by changing half of the culture medium. Non-adherent cells were collected at week 3 from the culture medium, counted, and assayed for colony-forming units (CFU).

Clonogenic assays

For colony forming unit (CFU) assays, cell suspension (1×10^4 /ml of plating mixture) was mixed with semisolid methylcellulose medium supplemented with cytokines (StemCell Technologies, Canada). The cultures were incubated in a humidified incubator with 5% CO₂ in air at 37°C for 7–14 days. For long-term culture initiating cell (LTC-IC) assays, cells were plated in a limiting dilution on 96-well plates onto a layer of S17 stromal cells in RPMI medium supplemented with 5% FCS, 5×10^{-4} M 2-mercaptoethanol and 2 mM L-glutamine. The cultures were fed weekly for 5 weeks. Wells containing colonies of small round hematopoietic cells were counted under the microscope. For spleen colony-forming unit (CFUs-8) assay, cell suspension was injected into the lethally irradiated recipients (4×10^4 /mouse), spleens were isolated after 8 days, fixed in Tellesnicky's solution and colonies were counted after several hours of fixation.

Fluorescent activated cell sorter (FACS) analysis

For cell surface immunostaining, 5×10^5 cells were stained according to standard procedures (Serobyan et al., 2005). Rat anti-mouse CD3, CD4, CD19 and CXCR4 specific monoclonal antibodies, control isotype-matched IgG and secondary goat anti-rat IgG-FITC were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). FITC-conjugated CD16, PE-conjugated CD11b, CD45 and CD31 specific monoclonal antibodies and control isotype-matched PE- or FITC-conjugated IgG were purchased from PharMingen

(PharMingen, San Diego, CA). Fluorescence analysis was performed on FACScan (Becton Dickinson) and analyzed using CellQuest program.

Immunohistochemistry

EBs were seeded on collagen-coated glass-slides, 1 hour at 37°C, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X100 and used for the intracellular detection of nAChRs proteins. Rabbit anti-rat $\alpha 4$, $\alpha 7$, $\beta 2$ and $\beta 4$ -specific antibody and mouse anti-rat $\alpha 3$ -specific antibody staining was visualized with goat anti-rabbit Alexa-Fluor 594-conjugated (Invitrogen, Carlsbad, CA) and goat anti-mouse Alexa-Fluor 488-conjugated (Invitrogen) secondary antibodies (1:1000). Rabbit anti-human OCT4 and mouse anti-human SSEA1-specific Abs were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Images were captured with an Olympus FLUOVIEW FV 1000 ($\times 60$ objective) and analyzed using Open Lab software.

Gene expression in hESCs

Triplicate cultures of hESCs were cultured as described above, harvested and total RNA was isolated using a Qiagen RNA isolation kit. Probe preparation and chip hybridization was performed according to the manufacturer's recommendations (Illumina, San Diego, CA). Genes of interest were defined as detectable if their hybridization signal intensities in all three samples were detected with at least 99% confidence.

RT-PCR

Expression of the nAChRs was detected by RT-PCR. RNA was isolated from ESCs and EBs using a kit from Qiagen Corp. (Valencia, CA). A Qiagen kit was also used to transcribe cDNA initiated by oligo-dT. A segment of the cDNA was detected using RT-PCR for nAChRs using previously described primers (Kuo et al., 2002).

Protein microarray

Production of cytokines and chemokines in murine bone marrow cultures was examined by using the RayBio Mouse Cytokine Antibody Array III&3.1 according to the manufacturer's recommendations.

Statistical analysis

Statistical analysis was carried out by Student's *t*-test.

Results

Expression of cholinergic system components by ESCs

Pluripotent ESCs possess the ability to differentiate into multipotent "specialized" stem cells, including HSCs, which subsequently generate mature blood cells. In Figure 1A, a human ESC WA09 colony grown on the feeder layer is shown. The pluripotent nature of cultured ESC colonies was confirmed by the expression of OCT4 (Figure 1B). To induce ESC differentiation, the colonies were removed from the feeder layers and cultured in suspension as clustered spheroids of cells referred to as embryoid bodies (EBs) (Figure 1C).

To address the question of whether nicotine might influence spontaneous differentiation of ESCs, we first screened for genes encoding components of the cholinergic system. Using gene arrays, we found that choline acetyltransferase (ChAT), acetylcholinesterase (AChE) and nicotinic acetylcholine receptors (nAChRs), are expressed in both ESCs and EBs (Figure 1D). Interestingly, there was no statistically significant difference in the expression of these genes in ESCs versus EBs. The RT-PCR analysis revealed that mRNA for nAChRs subunits alpha

1, 2, 3, 4, 5, 6, 7, 9 and 10 subunits, and beta 1, 2, 3 and 4 is present in both ESCs (not shown) and EBs (Figure 1 E). Protein expression was confirmed for $\alpha 3$ and $\alpha 7$ by immunohistochemistry. In line with the RT-PCR results, we found that very small clusters within the developing EB express low levels of $\alpha 4$ and $\beta 4$ subunits of nAChR (Figure 1 F). This is in agreement with our previous finding that nAChRs are expressed on the hematopoietic tissue during development (Serobyan et al., 2005).

The effect of nicotine on the development of hematopoietic system

Since activation of nAChRs by nicotine has been shown to cause phosphorylation of MAPK cascades (Heeschen et al., 2002), we next asked whether nicotine would stimulate activation of ERK1/2 (p42,p44) in EBs. Exposure of 10-days-old EBs to nicotine resulted in rapid, time-dependent phosphorylation of ERK kinase (Figure 2 A), which was detectable at 10 minutes of exposure to nicotine.

To investigate the role of the cholinergic system in ESC, differentiating EBs were cultured with or without nicotine. At day 10, EBs were dissociated and the number of CD45+ and CD31 cells was evaluated by FACS. We found that nicotine increased the number of both CD31+ endothelial cells, and hematopoietic CD45+ cells (Figure 2 B), suggesting a stimulation of the hemangioblast formation.

To investigate whether nicotine interferes with fetal development of the hematopoietic system *in vivo*, BALB/c mice were administered intravenous injections of nicotine daily throughout the entire pregnancy. On the day 12 of embryonic development (E12), the fetal livers from control and nicotine-exposed fetuses were isolated and the number of hematopoietic progenitors was investigated using the CFUs-8 assay. The number of CFUs-8 in nicotine-exposed fetal livers was a 1.8-fold higher as compared to control (28.1 ± 8.6 in control versus 49.5 ± 8.3 nicotine-exposed embryos, $p < 0.01$).

After birth, the injections of nicotine were discontinued and the offspring was analyzed 1 month later. Morphological staining of peripheral blood cells revealed that mice exposed to nicotine during gestation have decreased counts of lymphocytes (62.8 ± 6.2 in control versus 22.3 ± 2.5 in mice). This was confirmed by FACS analysis (Figure 3 A). FACS analysis of spleen and bone marrow cells showed that the number of CD11b+ and CD16+ cells (myeloid lineage) in the bone marrow and liver was increased. We found that the number of CD3+, CD4+ and CD19+ cells (lymphoid lineage) in bone marrow and spleen was 2-fold lower, as compared to control. In addition, we detected a 2.4-fold decrease in the number of CD31+ cells in spleen of mice exposed to nicotine during gestation compared to controls, whereas in bone marrow and liver the number of CD31+ cells was not significantly changed (Figure 3 A).

The changed ratio between myeloid and lymphoid cells in different organs could be due to the effect of nicotine on cell compartmentalization or on cell generation. Thus, we next investigated whether the decreased numbers of lymphocytes in mice exposed to nicotine during gestation may have been caused by lower numbers of lymphoid progenitors. The number of committed progenitors in the bone marrow of these mice was investigated using a methylcellulose CFU assay. The offspring from control (PBS) and nicotine administered mothers was analyzed before birth (E15), on the day of delivery and one, two, three and four weeks after birth. On E15 and during the first two weeks, a significant decrease in the number of both myeloid and lymphoid progenitors was detected in the bone marrow of nicotine-exposed mice as compared to control. Although the number of myeloid progenitors measured by CFU-GM and CFU-B reached normal levels at week three and four, the total number of lymphoid progenitors (CFU-B) in nicotine-exposed mice remained lower than in the control group. The significant decrease in the number of committed progenitors found in bone marrow of nicotine-exposed mice at the early stages of postnatal life is likely to be due to the low number of multipotent HSC in the

bone marrow of these mice. Thus, we evaluated the number of HSCs, as measured by long-term culture-initiating cells (LTC-IC), using the limiting dilution assay. Before birth (E15) and at day one of postnatal life, we found a 10-fold decrease in the number of LTC-IC in bone marrow of nicotine-exposed versus control newborns (Figure 3 B, C). At week one and two a trend of slow recovery in LTC-IC numbers in the bone marrow of nicotine-exposed mice was monitored.

Effect of nicotine on the hematopoiesis-supportive function of the niche

Since cytokines and chemokines are important regulatory factors within the niche network, we next examined whether nicotine affects expression of these soluble factors that regulate HSC proliferation. The adherent layers of bone marrow cell cultures were incubated with nicotine and the production of soluble factors was evaluated by protein microarray technology. We observed that the production of hematopoiesis-supportive factors including G-CSF, GM-CSF, IGFBP-3, IL-3 and IL-6 was decreased in these cultures after treatment with nicotine (Figure 4 A). To confirm the physiological relevance of the nicotine-induced decrease in the production of hematopoiesis-supportive cytokines, we next cultured short-term bone marrow cultures (STBMC) with or without nicotine (10^{-8} M). At week 3, non-adherent cells in STBMC were harvested and assayed for the number of committed progenitors. In line with our cytokine array results, the treatment with nicotine resulted in a significant ($p=0.03$) reduction in the numbers of progenitors (Figure 4B) generated in STBMC.

Interestingly, we observed increased production of M-CSF and the Th2 cytokines IL4 and IL5 in cultures treated with nicotine (Figure 4 A). While M-CSF drives differentiation of monocytic lineage, IL-5 stimulates proliferation of eosinophil progenitors and generation of mature eosinophils, cells that contribute to the pathogenesis of allergy and parasite infection. The expression levels of other important cytokines including IL1, IL2 and IL9, was unchanged by the nicotine treatment.

In addition to the panel of cytokines, the expression of MIP-1 α , a chemokine that acts synergistically with SDF-1 and contributes to the recruitment of hematopoietic cells into the niche, was significantly decreased following nicotine treatment (Figure 4 A). We have also monitored that the expression of SDF-1, a chemokine mediating transendothelial migration of HSC was not significantly changed by nicotine treatment, whereas the expression of CXCR4, the receptor for SDF-1, on hematopoietic cells was decreased (Figure 4 E). To investigate whether the function of the SDF-1/CXCR4 pathway in mediating migration of HSC and progenitors is affected by nicotine, we performed HSC transplantation. HSCs were collected from bone marrow or peripheral blood of mice that received nicotine treatment during 18 days and injected into lethally irradiated recipients. We found that the number of both leukocytes and platelets was lower in mice that were reconstituted with HSCs from nicotine-treated donors (Figure 4 C, D). Since CXCR4/SDF-1 pathway is involved in the retention of HSCs within the niche, we also investigated the effect of nicotine on HSCs mobilization. Peripheral blood from mice treated with nicotine was collected and examined for the number of progenitor cells using CFUs-8 assay. Nicotine significantly increased the number of circulating CFUs-8 as compared to control (Figure 4 F). Thus, the effect of nicotine on the expression of chemokines and chemokine receptors might interfere with the recruitment of circulating HSCs and their retention in the niche.

Discussion

ESCs have the potential for generation of unlimited numbers of tissue-specific multipotent stem cells. In addition, ESCs represent a unique experimental model for studying the role of specific molecular pathways on tissue development *in vitro*. The aim of this study was to investigate whether the cholinergic system is involved in regulation of the development of non-

neural tissues, in particular hematopoietic. Using gene expression arrays, we could demonstrate that ESC and EB express elements of the cholinergic system, including ChAT, AChE, BUTE and AChRs. Nicotinic AChRs expressed on ESCs and EB can be stimulated by both endogenous factors (e.g. acetylcholine), and exogenous stimuli (e.g. nicotine), implying that the function of the cholinergic system in the developing embryo can be influenced by maternal smoking. Indeed, the placenta does not pose a metabolic barrier to nicotine transfer from the mother (Pastrakuljic et al., 1998), and therefore nicotine can readily cross through the placenta and accumulate in utero (Luck et al., 1985). In addition, the expression of nicotine-metabolizing enzymes appears either near birth (CYP2B) or 2 week postpartum (CYP2A) (Nagata et al., 2003; Rich and Boobies, 1997). Together, in agreement with a phenomenon referred to as the 'fetal basis of adult disease' (Barker, 1998), this provides a basis for pathological shifts in the developmental patterns of nicotine-exposed embryos leading to postpartum health consequences.

It has been suggested that during EB formation, the tissues undergo developmental patterns similar to those during in vivo growth of the embryo. Protein analysis performed by immunohistochemistry or FACS demonstrated that nAChRs are expressed on both EB and fetal liver. To understand whether these receptors are functionally active, we stimulated EBs with nicotine at concentrations similar to those seen in smokers. While concentration of nicotine in the serum of smokers can be up to $3 \times 10^{-7} \text{M}$ (Hukkanen), our cultures were exposed to 10^{-8}M nicotine, thus reflecting the in vivo situation. Similarly to our previously published observation with endothelial cells (Serobyan et al., 2005), we found that nicotine stimulated phosphorylation of MAPK in EBs at concentrations as low as 10^{-8}M .

During EB formation, ECS spontaneously differentiate into various lineages including hematopoietic. We asked whether exposure to nicotine might interfere with the hematopoietic lineage development. To address this question, control and nicotine-treated EBs were dissociated and the number of differentiated cells was evaluated using lineage-specific antibodies. FACS analysis showed that nicotine supported differentiation of cells toward hematopoietic and endothelial lineages as measured by the CD45 and CD31 markers. Similar findings were observed in vivo, when the developing embryos were exposed to nicotine in utero. We found that in fetuses exposed to nicotine, the number of CD45+ and CD31+ cells was increased in fetal livers. In addition, the number of progenitors in fetal liver was higher in nicotine-exposed fetuses compared to control. Both in vivo and in vitro results suggest a promoting effect of nicotine on the formation of hemangioblast during early stages of development.

The switch from the fetal liver to the bone marrow site of hematopoiesis occurs at later stages of ontogenesis and correlates with the maturation of newly formed hematopoietic niches (Tavassoli, 1991). We found that the number of HSCs colonized fetal bone marrow (E15) was lower in nicotine-exposed fetuses compared to control. This correlated with the lower number of committed progenitors in bone marrow before birth and early postpartum. While the number of lymphocytes was decreased, we detected a relative increase in the number of monocytes in nicotine exposed mice, which could be explained by nicotine-induced expression of M-CSF, a cytokine that drives monocytic differentiation of myeloid progenitors and their maturation. Together, our results suggest that at this later stage of development nicotine has a negative effect on hematopoiesis.

Under the postpartum conditions of gradually recovering nicotine-metabolizing enzymes, the quantity of LTC-ICs was slowly recovered: by week three the total number of LTC-ICs in the bone marrow of nicotine-exposed mice was not significantly different from that of control. A tendency similar to LTC-IC was observed for the myeloid, but not for the lymphoid lineage. While the number of myeloid progenitors recovered at week three postpartum, the number of

lymphoid progenitors remained low throughout the monitoring period. At this point there remains the question of whether lymphoid lineage in offspring exposed to nicotine during gestation will recover at later times, or whether these changes are irreversible and reflect nicotine-induced alteration of HSC differentiation.

Both adult and fetal HSCs are recruited to the bone marrow niches by the SDF-1 gradient (Nagasawa et al., 1996). We have previously demonstrated that the spontaneous migration of HSPCs in control Transwell cultures might be facilitated by nicotine (Seroby et al., 2005). However, the physiological relevance of this finding is not clear at this point, since in vivo the migration of cells is governed by chemokines. In contrast to control cultures, in cultures supplemented with SDF-1 we monitored the inhibitory effect of nicotine on chemokine-mediated migration of HSPC. While this phenomenon may be partially mediated by decreased activation of MMP-9 (Seroby et al., 2005), another explanation is a nicotine-mediated decrease in expression of CXCR4, the receptor for SDF-1 on hematopoietic cells. The decreased expression of CXCR4 can also account for the diminished homing and engraftment of HSCs isolated from the nicotine-treated donors.

The CXCR4/SDF-1 pathway is involved not only in the recruitment of HSPC into the niche, but also - along with other adhesion molecules - in their retention (Ma et al., 1999). Among other adhesion molecules, CD44 is responsible for HSC – niche adhesive interactions (Khalidoyanidi et al., 1996), and its cell surface expression can be decreased by nicotine (Khalidoyanidi et al., 2001). Therefore, the cross-talk between HSCs and the niche might be affected by nicotine-induced changes of the profile of adhesion molecules expressed by HSCs and the niche. In line with this, we monitored the increased number of hematopoietic progenitors circulating in blood of mice treated with nicotine, suggesting the effect of nicotine on HSC mobilization.

In addition to adhesive interactions, the hematopoietic microenvironment provides HSC with factors that support the proliferation and maturation of HSC on various stages of their differentiation. IL-6 is one of the factors which play an important role in the regulation of HSC cell proliferation (Miura et al., 1993). We have previously reported a negative effect of nicotine on IL-6 production in newborns exposed to nicotine during gestation (Seroby et al., 2005). In this study we examined the effect of nicotine on cytokine production by the niche in vitro. Adherent layers of bone marrow cultures represent an in vitro model for the niche, since these cultures consist of various cell types reflecting the complexity of the niche. We found that in addition to IL-6, nicotine decreased the production of other cytokines including G-CSF, GM-CSF, IGFBP-3 and IL-3. These cytokines are important for proliferation of both HSCs and committed progenitors. Low concentrations of cytokines produced by the niche are expected to contribute to the nicotine-induced changes in the niche and are reflected by decreased hematopoietic activity in vitro and in vivo. In vitro, we observed that treatment with nicotine resulted in a significant decrease in the progenitor cell production in STBMC. In line with this observation, the recovery of hematopoiesis following bone marrow transplantation is delayed in mice treated with nicotine.

Interestingly, we observed an increased production of IL4 and IL5 – Th2 cytokines – in the niche treated with nicotine. IL-5 stimulates proliferation of eosinophil progenitors and their maturation into mature eosinophils. While the number of eosinophils in peripheral blood is low in healthy individuals (less than 1%), eosinophilia is one of the symptoms of allergic diseases. Our findings suggest that the increased levels of IL-5 seen in the presence of nicotine may contribute to the enhanced risk of allergic disease in susceptible cohort of patients, a correlation which has been observed in some, but all studies.

The response to nicotine is likely to vary depending on the cell type, cell surface or intracellular binding, tissue of origin and the length of exposure. Similarly to endothelial cells (Serobyán et al., 2005; Heeschen et al., 2002), short-term exposure to nicotine resulted in MAPK phosphorylation in EBs. Phosphorylation of MAP kinases can result in increased chemokine production due to transcriptional activation. Since this is, however, only seen following prolonged activation of ERK1/2 (Cowley et al., 1994), we do not expect that the effect of nicotine on MAP kinases influenced chemokine expression. It is, however possible that p38 activation was involved in the up-regulation of IL-4 and IL-5, as has been previously described for T-lymphocytes (Mori et al., 1999).

Conclusion

Overall, we have demonstrated that the cholinergic system is involved in regulation of hematopoiesis during both embryonic development and postpartum. Stimulation of nAChRs expressed in the niche by endogenous or exogenous (e.g. nicotine) ligands interferes with cytokine production and the expression of adhesion molecules, thus affecting the hematopoiesis-supportive function of the stem cell niche.

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Figure 1. The expression of cholinergic system in ESCs

The hESC WH09 line was cultured on human skin fibroblasts (A). The cultured hESCs expressed OCT4, a marker for undifferentiated cells as shown in red; SSEA-1, a differentiation marker, is not expressed on these cells (green). Nuclei are stained by DAPI (blue) (B). EBs were grown from hESC colonies by culturing in differentiating media in ultra low attachment plates (C). Microarray analysis of differential gene expression in hESCs versus EBs is shown (D). In the log transformed graph the hybridization signals for over 10,000 genes are plotted. Hybridization signals obtained from EB samples (X-axis) are compared to those from ESC samples (Y-axis). Genes that were statistically significantly detected in the samples are shown. Non-detected genes are not shown. Each sample was averaged from four biological replicates. The expression of nAChR on EB was examined by RT-PCR (E). The expression of nAChRs in differentiated EBs was detected by immunohistochemistry (F). The right panel shows the overlay (OL) of the nuclei staining (DAPI, shown in blue) and nAChR $\alpha 3$ (green), nAChR $\alpha 4$, nAChR $\alpha 7$ and nAChR $\beta 4$ (all in red).

Figure 2. The effect of nicotine on ESC differentiation

Nicotine-induced activation of Erk kinase (A). EBs were serum-free starved, exposed to 10^{-8} M nicotine for the indicated periods of time, lysed and then protein samples were analyzed by Western blotting. Erk1/2 phosphorylation was detected by phospho-Erk specific antibodies (upper panel). Antibodies directed against total Erk was used as the loading control (lower panel). B: EBs were cultured with or without nicotine, and the expression of CD45 and CD31 was evaluated on day 14 by FACS. The isotype match IgG was used as negative control (not shown). The results of one out of two similar experiments are shown.

Figure 3. The effect of nicotine on HSC differentiation

Control mice and mice exposed to nicotine during gestation (n=3 per group) were sacrificed, and their peripheral blood, liver, bone marrow and spleen cells were isolated and used for FACS analysis. The cell surface expression of CD19, CD3, CD4, CD45, CD31, CD16 and CD11b is shown on representative counterplots selected out of three similar immunostainings. Isotype matched IgG was used as a negative control (A). Bone marrow cells harvested from femurs and tibias of fetuses (E15), newborns (at day of birth, DB), and postpartum were examined for the presence of granulocyte-macrophage progenitors (CFU-GM) (B) and B-cell progenitors (CFU-B) (C) in a methylcellulose assay. Data are shown as a percent decrease, where the numbers from the control group are taken as 100%. D: Bone marrow cells were examined for the presence of long-term culture-initiating cells (LTC-IC) using the limiting dilution assay. Data shown are means $\pm$ SD of the LTC-IC content in 10^6 bone marrow cells.

Figure 4. Effect of nicotine on the bone-marrow niche

A: The adherent layers of bone marrow cultures (non-starved) were incubated with 10^{-8} M nicotine. The conditioned media was harvested after 72 hours and assayed for the presence of cytokines and chemokines using RayBio Mouse Cytokine Antibody Array. One representative experiment is shown. Duplicate spots are represented in the following sequence as indicated in the figure (A). Bone marrow cells were cultured with 10^{-8} M nicotine during 3 weeks. The non-adherent cells were harvested from the control and nicotine-treated cultures and assayed for the number of colony forming units (CFU) using methylcellulose assay (B, $p < 0.05$). Peripheral blood and bone marrow cells were collected from control and nicotine-treated donors, and injected into the lethally irradiated recipients. The recovery of white blood cells (WBC) and platelets (PLT) in the recipients on day 16 is shown in C and D. The expression of CXCR4 on the peripheral blood and spleen cells from control mice and mice exposed to nicotine was evaluated by FACS (E). Peripheral blood from control and nicotine-treated mice (10^{-8} M during 18 days) was collected and assayed for the number of spleen colony-forming units (CFUs-8). The data shown are means $\pm$ SD the number of CFUs-8 in one representative experiment (F, $p < 0.01$).