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Effects of environmental carcinogen benzo(a)pyrene on canine adipose-derived mesenchymal stem cells

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ABSTRACT

Dogs and their owners share the same environment and are subjected to similar environmental risk factors for developing breast cancer. Adipose tissue-derived mesenchymal stem cells (ADMSCs) may affect development and progression of breast cancer. In this study, we evaluated the effects of environmental carcinogen benzo(a)pyrene (BaP) on proliferation and differentiation of ADMSCs isolated from dogs. We characterized eight canine ADMSC lines and studied the effects of BaP on cell proliferation and differentiation. BaP did not inhibit cell proliferation of ADMSCs; however, BaP significantly inhibited differentiation potential of ADMSCs into adipocytes. BaP down-regulated AhR protein levels; however, increased its translocation from the cytoplasm to nucleus and suppressed PPARγ expression during adipogenesis. BaP increased the expression of AhR signaling pathway protein, cytochrome P450 (CYP1A1) in ADMSCs. Our data suggest that canine ADMSCs are susceptible to the environmental carcinogen BaP through AhR and PPARγ signaling pathways and may contribute to canine mammary carcinogenesis.

1. Introduction

Breast cancer is the most common cancer in women, and will affect one in eight women in their lifetime (Breast Cancer Facts and Figure, 2013–2014). It is the second major cause of death of women in the Western world (Gangadhara et al., 2012; Macon and Fenton, 2013). Breast cancer's high morbidity correlates with its metastatic potential and the absence of effective treatments at late stages of metastasis (Coleman and Rubens, 1987; Gangadhara et al., 2012). The major risk factors for breast cancer development are heredity, lifestyle, behavior, and environmental factors (Macon and Fenton, 2013). Several studies show that almost 90% of breast cancers might originate from the cumulative effects of endogenous estrogens and environmental pollutants, such as organochlorine pesticides, polychlorinated biphenyls, dichlorodiphenyltrichloroethane, vinclozolin, phthalates, atrazine, bisphenol A (BPA), and BaP acting as endocrine-disrupting compounds (Bidgoli and Eftekhari, 2011; Macon and Fenton, 2013).

BaP is a polycyclic aromatic hydrocarbon found at significant levels in car exhaust (Ueng et al., 2004), cigarette smoke (Scherer et al., 2000), and fossil fuel emissions (Ciganek et al., 2004). This compound is one of the most widely studied air pollutants, and high levels of BaP have been detected not only in cigarette smoke, but also in various foods, e.g. potato chips (4 μg/kg), fried chicken (5 μg/kg), broiled/smoked meats (Lijinsky, 1991), smoked dried beef (5 μg/kg), cereals, and green leafy vegetables (Lee and Shim, 2007; Sinha et al., 2005). BaP activates the aryl hydrocarbon receptor (AhR), which subsequently induces cytochrome P-450 (CYP1A1, CYP1B1) expression (Whitlock, 1999). These enzymes convert BaP into BaP diolepoxide (BPDE) to form DNA adducts. The AhR interacts with several steroid hormone receptors, including estrogen, androgen, and progesterone receptors, and alters their functions (Kang and Lee, 2005). The downstream targets of AhR signaling pathways are cyclooxygenase-2, proteins of Wnt/β-catenin signaling pathway, and inflammatory cytokine receptors (Yoshioka et al., 2011). Like BPA, BaP has also been shown to predispose to certain types of tumors in the gastrointestinal tract and mammary gland in different animal models (Kang and Lee, 2005; Sinha et al., 2005).

The exposure to environmental contaminants from the air, water, and food suggests significant health risks for the human population. However, acquiring appropriate human epidemiological data of the influence of environmental pollutants in human breast cancer is difficult due to the prolonged latent period from exposure to development of cancer and possibility of multiple exposure routes (Backer et al., 2001). Because dogs share the same environment with their owners and are subjected to similar environmental risk factors for development

Abbreviations: ADMSC, adipose tissue-derived mesenchymal stem cells; AhR, aryl hydrocarbon receptor; BaP, benzo(a)pyrene; BPA, bisphenol A; CEBP, CCAAT/enhancer-binding protein; CYP1A1, cytochrome P450, family 1, subfamily A, polypeptide 1; Co, initial count; Ct, count after time t; dt, doubling time; FBS, fetal bovine serum; MSC, mesenchymal stem cells; PPARγ, peroxisome proliferator-activated receptors-gamma; ln, natural log; TGFβ3, Transforming growth factor beta 3; t, time between cell counts Ct and Co.

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of breast cancer as humans, dogs with naturally-occurring breast tumors are valuable models for human breast cancers (Backer et al., 2001). Dogs that developed mesotheliomas after asbestos exposure have been used as one of the predictive risk factors for development of the asbestos-related disease in people who shared a house with one of the dogs (Glickman et al., 1983). Additionally, the association between the applications of the herbicide 2,4-dichlorophenoxyacetic acid and diagnosed malignant lymphomas and non-Hodgkin's lymphoma in dogs and humans has been intensively studied (Hayes et al., 1981; Hoar et al., 1986). Increased risk of lung cancer in dogs has been suggested from exposure to second-hand-smoke in households in which owners smoke tobacco products (Reif et al., 1992). Dogs and people alike have also developed bladder cancers with confirmed similar geographical distribution when exposed to the same general environmental carcinogens (Hayes et al., 1981).

The effects of environmental carcinogens on mesenchymal stem cells are currently under intensive investigation. Mesenchymal stem cells (MSCs) are multipotent cells that can replicate and have the potential to differentiate into the bone, cartilage, fat, tendon, and muscle (Pittenger et al., 1999). MSCs, together with fibroblasts and adipocytes, form part of the stromal micro-environment surrounding breast epithelial cells (Swartz et al., 2012). MSCs within the breast tissue niche can promote breast cancer growth and metastasis through multiple molecular mechanisms (Grisendi et al., 2011; Karnoub et al., 2007). MSCs secrete cytokines and growth factors that lead to increased growth of carcinomas (Cekanova et al., 2006; Paunescu et al., 2011). Also, various studies have shown that hematopoietic stem cells and endothelial progenitor cells are preferential targets of environmental contaminants, causing the alteration of these cells' proliferation and differentiation (van Grevenynghe et al., 2005; van Grevenynghe et al., 2006). Therefore, it is important to understand the mechanisms of environmental carcinogens, such as BaP, during the breast MSC development and remodeling.

In this study, we evaluated the effects of BaP on proliferation and differentiation of canine ADMSCs in vitro. We identified mechanisms of BaP-induced inhibition of adipogenesis of canine ADMSCs through the AhR and PPARγ signaling pathways. We confirmed that the expression of adipogenesis-related marker PPARγ was reduced by BaP, thereby indicating a possible role of CYP1A1 as one of negative regulators of adipogenesis through the AhR signaling pathway in canine ADMSCs. In conclusion, our data suggests that canine ADMSCs, as part of breast stroma, are susceptible to the environmental carcinogen BaP through AhR and PPARγ signaling pathways.

2. Materials and methods

2.1. Antibodies and other reagents

Antibodies against CD90 (clone YKIX337.217) were purchased from the AbD Serotec (Raleigh, NC), CD29 (clone 18/CD29) were purchased from BD Biosciences (San Jose, CA), and CD19 (clone FMC63) were purchased from Millipore (Billerica, MA). Antibodies AhR (clone A-3, 122 kDa), CYP1A1 (clone H-70, 58 kDa), PPARγ (clone H-100, 55 kDa), tubulin (clone E-19, 50 kDa), lamin (clone H-110, 62/69 kDa), and actin (43 kDa) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals and reagents were purchased from Thermo Fisher Scientific (Pittsburgh, PA), unless otherwise specified.

2.2. Canine ADMSCs

Canine ADMSCs were isolated from non-utilized fat tissue from female dogs that underwent a routine spaying procedure at the University of Tennessee, College of Veterinary Medicine (UTCVM). The spaying procedures were conducted by board-certified veterinary internal medicine specialists in accordance with approval by the Institutional Animal Care and Use Committee at the University of Tennessee, College of Veterinary Medicine. The sample of fat tissues washed with PBS and digested by collagenase for 30 min at room temperature on an orbital shaker. The cell suspension was then filtered and washed with modified PBS, and the cellular pellet was collected and incubated with red blood cell (RBC) lysis solution for 5 min. The cells were then washed with modified PBS and cultured in DMEM/F12 media supplemented with 10% fetal bovine serum, 100 I.U. penicillin, and 100 μg/mL streptomycin in an atmosphere of 5% CO2 at 37 °C for 24–72 h. Isolated ADMSCs were characterized by doubling time and expression of the CD90 and CD29 markers.

2.3. Doubling time of canine ADMSCs

Canine ADMSCs were plated in triplicate in 6-well plates. Cells were trypsinized and counted using a hemocytometer 24, 48, and 72 h after plating. Doubling time was calculated using the following formula, $dt = t \times [\ln 2 / \ln(Ct / Co)$, wherein dt = doubling time, t = time (h) between cell counts Ct and Co, Co = initial count, Ct = count after time t, and ln = natural log. Doubling time by MTS proliferation assay (as described below) was calculated by culturing the cells in 96-well plates and performing an MTS assay at 0, 24, 48, and 72 h.

2.4. Cell proliferation assay (MTS)

Cell proliferation was determined using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega, Madison, WI). Canine ADMSCs (3×10^4 cells/well) were seeded in 96-well culture plates in four replicates and cultured for 24 h followed by treatment with 0, 0.5, 1, and 5 μM BaP for 24 h for short-term proliferation in four independent experiments. For long-term proliferation, the media with 0, 0.5, 1, and 5 μM BaP was changed twice a week for 3 weeks. After treatment, the cells were incubated with MTS reagent following the manufacturer's protocol. Absorbance at 490 nm using a plate reader (Bio-Tek Instruments, Inc., Winooski, VT) was normalized to the control group. BaP was dissolved in DMSO, and the same volume of DMSO was used in the control group as in treatment groups.

2.5. Flow cytometry analysis

Canine ADMSCs were trypsinized, rinsed with PBS, fixed with 1% formaldehyde, and washed twice with PBS. Cells were incubated with primary CD90 and CD19 antibodies at 4 °C for 1 h. Cells were then rinsed and re-suspended in PBS, and expression of CD90 and CD19 proteins was detected by flow cytometry (Coulter EPICS Elite Cytometer, Hialeah, FL) using Multicycle software (Phoenix, San Diego, CA).

2.6. Immunocytochemistry (ICC)

Canine ADMSCs were plated on 4-chamber slides (Lab-Tek II, Nalge Nunc, Naperville, IL) and cultured until they reached 80% to 90% confluency within 24–48 h, following the ICC protocol previously described (Cekanova et al., 2013). Briefly, after fixation and background blocking, ADMSCs were incubated with primary antibodies (CD90, CD29, and CD19) followed by secondary antibodies using a streptavidin–biotin detection system, and visualized with DAB-substrate. Nuclei of ADMSCs were counter-stained by DAPI and slides were mounted, cover-slipped, and evaluated under a Leitz DMRB microscope (Leica, Buffalo Grove, IL). The images were captured by a DP73 camera (Hunt Optics and Imaging, Pittsburgh, PA) attached to a microscope using cellSens software (Olympus, Center Valley, PA). The percentage of the CD29 or CD90 positive ADMSC cells was calculated (CD90 or CD29 positive cells / total cells × 100) in three fields at 20× objective magnification and data were presented as a mean ± S.D. as shown in Table 1.