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A dioxin-like compound induces hyperplasia and branching morphogenesis in mouse mammary gland, through alterations in TGF-β1 and aryl hydrocarbon receptor signaling

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ABSTRACT

Hexachlorobenzene (HCB) is a widespread environmental pollutant and a dioxin-like compound that binds weakly to the aryl hydrocarbon receptor (AhR). Because AhR and transforming growth factor β1 (TGF-β1) converge to regulate common signaling pathways, alterations in this crosstalk might contribute to developing preneoplastic lesions. The aim of this study was to evaluate HCB action on TGF-β1 and AhR signaling in mouse mammary gland, through AhR +/+ and AhR −/− models. Results showed a differential effect in mouse mammary epithelial cells (NMuMG), depending on the dose: 0.05 μM HCB induced cell migration and TGF-β1 signaling, whereas 5 μM HCB reduced cell migration, promoted cell cycle arrest and stimulated the dioxin response element (DRE) -dependent pathway. HCB (5 μM) enhanced α-smooth muscle actin expression and decreased TGF-β receptor II mRNA levels in immortalized mouse mammary fibroblasts AhR +/+, resembling the phenotype of transformed cells. Accordingly, their conditioned medium was able to enhance NMuMG cell migration. Assays in C57/Bl6 mice showed HCB (3 mg/kg body weight) to enhance ductal hyperplasia, cell proliferation, estrogen receptor α nuclear localization, branch density, and the number of terminal end buds in mammary gland from AhR +/+ mice. Primary culture of mammary epithelial cells from AhR +/+ mice showed reduced AhR mRNA levels after HCB exposure (0.05 and 5 μM). Interestingly, AhR −/− mice exhibited an increase in ductal hyperplasia and mammary growth in the absence of HCB treatment, thus revealing the importance of AhR in mammary development. Our findings show that environmental HCB concentrations modulate AhR and TGF-β1 signaling, which could contribute to altered mammary branching morphogenesis, likely leading to preneoplastic lesions and retaining terminal end buds.

1. Introduction

Environmental exposure can alter mammary gland (MG) development, impair lactation and increase susceptibility to cancer (Rudel et al., 2011). Hexachlorobenzene (HCB) is an environmental pollutant which was used as a fungicide in the past and is still released into the environment as a byproduct from several industrial processes. The International Agency for Research on Cancer has classified HCB as a possible human carcinogen (ATSDR, 2015) and several studies demonstrate the presence of this pollutant in human milk, baby formula, and cow milk for human consumption (Chen et al., 2014; Der Parsehian, 2008). We have previously reported that HCB activates estrogen receptor α (ERα) and induces estrogen-like responses in human breast cancer cells acting as an endocrine disruptor (ED) (García et al., 2010), while other authors have revealed that some EDs affect MG development (Fenton, 2006).

HCB is a dioxin-like compound and a weak ligand of the aryl hydrocarbon receptor (AhR) (Hahn et al., 1989). The AhR is a ligand-dependent transcription factor that binds to its DNA consensus sequence known as the dioxin response element (DRE) and regulates the expression of many detoxification genes such as cytochrome P450 1A1 (CYP1A1). Furthermore, after dioxin activated-AhR, c-Src is released

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from its cytosolic complex and stimulates growth factor receptors, including the transforming growth factor -β (TGF-β) receptor II (TβRII) (Galliher and Schiemann, 2006) and human epidermal growth factor receptor (HER1) (Park et al., 2007). AhR has been studied because of its role in the toxic effects caused by environmental pollutants, including dioxins, organochlorine compounds, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons. Evidence has shown that this receptor plays an important role in normal physiologic functions such as development, cell cycle regulation, and immune response (Murray et al., 2014; Safe et al., 2013). Moreover, Hushka et al. (1998) have demonstrated that the inactivation of AhR results in impaired mammary development and lactation. However, the role of the AhR in cancer is complex, with clear discrepancies between pro- and anti-tumorigenic activities (Bekki et al., 2015; Jin et al., 2012; Pontillo et al., 2013).

TGF-β1 is a pluripotent cytokine critically important in mammary morphogenesis and significantly activated during breast cancer development (Moses and Barcellos-Hoff, 2011). It has been reported that TGF-β1 plays a dual role: it limits proliferation in epithelial cells but induces epithelial-mesenchymal transition (EMT) in carcinoma cells, accelerating cancer progression and metastasis (Lebrun, 2012). In the canonical pathway, TGF-β1 engagement of TβRII leads to the phosphorylation and activation of TGF-β receptor I (TβRI), which subsequently phosphorylates Smad2/3. Once activated, Smad2/3 rapidly interacts with Smad4 and this complex translocates to the nucleus to regulate the expression of TGF-β-responsive genes. In addition, TGF-β1 also stimulates an ever expanding array of molecules, including ERK1/2, p38, JNK, and PI3K/Akt (Parvani et al., 2011). Recently, we have reported that HCB activates TGF-β1 and enhances Smad3, p38, and JNK phosphorylation levels in the ERα-negative MDA-MB-231 breast cancer cell line (Miret et al., 2016). TGF-β1 and AhR signaling interact both in reciprocal regulation and toward common targets such as cell cycle control (Haarmann-Stemmann et al., 2009). Wolff et al. (2001) have demonstrated that TGF-β1 triggers cell type-specific effects on AhR by inhibiting receptor expression and activation in lung cancer cells, while it enhances receptor function in hepatoma cells. Furthermore, analyses in cell systems and mouse models lacking AhR expression have demonstrated that AhR is relevant to maintain TGF-β1 activity. The upregulation of this cytokine could have a role in producing some of the phenotypes identified in AhR-depleted mice (Gómez-Duran et al., 2009).

Understanding the mechanisms regulating normal MG development may contribute to explaining how tumors promote their own growth and invasion, changing these normal mechanisms. Given our previous observations in mammary cells and animal models, and considering epidemiological data available, we hypothesize that HCB could act as a risk factor in breast cancer. Unlike its well characterized mechanisms of action in breast cancer (García et al., 2010; Miret et al., 2016; Peña et al., 2012; Pontillo et al., 2011, 2013; Randi et al., 2006), little is known about HCB effects on normal MG. Therefore, the aim of the present study was to investigate HCB effects on mouse normal MG by means of in vitro and in vivo assays. To examine the role of AhR, we worked with wild-type (AhR +/+) and AhR-null (AhR −/−) mice, as well as AhR +/+ and AhR −/− mammary epithelial and fibroblast cells. HCB action was analyzed in terms of cell proliferation, cell cycle, cell migration, and AhR and TGF-β1 signaling pathways, as well as MG structure and histology. Because stromal cells dictate the different mammary side-branching patterns (Sternlicht, 2006), conditioned medium (CM) assays were conducted to determine whether different factors secreted by HCB-treated fibroblasts have impact on epithelial cells.

2. Materials and methods

2.1. Chemicals

HCB (> 99% purity, commercial grade) was obtained from Aldrich-Chemie GmbH & Co. (Steinheim, Germany). Anti-Smad3, anti-phospho-Smad3, anti-phospho T202/Y204 ERK1/2, anti-p38, and anti-phospho-p38 antibodies were purchased from Cell Signaling Technology, Inc. (MA, USA). Anti-ERα antibody was obtained from Chemicon International Inc. (CA, USA). Anti-progesterone receptor (PR) antibody was purchased from Santa Cruz Biotechnology, Inc. (TX, USA). Anti-AhR antibody was obtained from Abcam Ltd. (Cambridge, UK). Anti-α-smooth muscle Actin (α-SMA), anti-β-Actin and anti-Vimentin (V9) antibodies, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), diaminobenzidine tetrahydrochloride (DAB) tablets, dimethyl sulfoxide (DMSO), phenylmethylsulfonyl fluoride (PMSF), epidermal growth factor (EGF), insulin, bovine serum albumin (BSA), α-naphthoflavone (ANF) inhibitor, SB431542 inhibitor and carmine alum were obtained from Sigma-Aldrich Chemical Co. (MO, USA). Anti-ERK1/2 antibody was purchased from Upstate (NY, USA). Anti-Keratin 14 (AF64) antibody was obtained from Covance (NJ, USA). Anti-proliferative cell nuclear antigen (PCNA) antibody was from Dako Laboratories (CA, USA). The enhanced chemiluminescence kit (ECL) was purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). The precision plus protein dual color standard and the Iscript reverse transcription supermix were obtained from Bio-Rad Laboratories (CA, USA). The high pure RNA isolation kit and collagen were purchased from Roche (IN, USA). The collagenase III was obtained from Gibco, Invitrogen Life Technology (MA, USA). The specific oligonucleotides for mouse AhR, CYP1A1, HER1, insulin-like growth factor-1 (IGF-I), TGF-β1, TβRI, and TβRII were purchased from Integrated DNA Technologies Inc. (IA, USA). The UltraTek HRP (Anti-Polyvalent) was obtained from Scytek Laboratories (UT, USA). The anti-E-cadherin antibody was graciously provided by Dr. Ibarra (Universidad de Buenos Aires), and anti-P-cadherin antibody by Dr. Vazquez-Levin (IBYME, CONICET). All other reagents used were of analytical grade.

2.2. Mice and treatment

Wild-type (AhR +/+) and AhR-null (AhR −/−) C57BL/6 N mice were produced by homologous recombination in embryonic stem cells as previously described (Fernandez-Salguero et al., 1995). Mice were maintained in a controlled environment: 24 ± 2 °C, 50 ± 10% relative humidity, and a 12–12 h light/dark cycle. Five-week-old virgin female AhR +/+ and AhR −/− mice were randomly separated in four groups (control AhR +/+, HCB-treated AhR +/+, control AhR −/− and HCB-treated AhR −/−) with 5 animals each one. Animals were treated humanely and with regard for alleviation of suffering. All the experiments involving animals were performed following the guidelines established by the Animal Care and Use Committee of the University of Extremadura. HCB (3 mg/kg body weight) was dissolved in corn oil and mice were treated by intraperitoneal injection (i.p.) (0.1 ml) four times a week for 21 days. Control animals were injected i.p. corn oil (0.1 ml) as vehicle. Treatment with HCB had no effect on the general health of the animals, as shown by the healthy appearance of mice and no effect on body weight or water and food consumption. The stages of the estrous cycle were determined using vaginal smears and the animals were euthanized on the morning of the first estrous phase. The fourth MG both right and left were removed under sterile conditions and processed for whole mounts and immunohistochemical studies. The HCB doses used in our experiments were chosen after reviewing the relevant literature. HCB (3 mg/kg b.w.) was assayed previously in other toxicological studies and had an androgenic effect in mice (Ralph et al., 2003). Besides, HCB (3 mg/kg b.w.) was assayed previously in our laboratory and found to promote tumor growth and metastatic focus in mice lungs (Pontillo et al., 2013). Although in this study, the HCB