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Enhanced cyclooxygenase-2 expression levels and metalloproteinase 2 and 9 activation by Hexachlorobenzene in human endometrial stromal cells

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ABSTRACT

Hexachlorobenzene (HCB) is an organochlorine pesticide that induces toxic reproductive effects in laboratory animals. It is a dioxin-like compound and a weak ligand of the aryl hydrocarbon receptor (AhR). Endometriosis is characterized by the presence of functional endometrial tissues outside the uterine cavity. Experimental studies indicate that exposure to organochlorines can interfere with both hormonal regulation and immune function to promote endometriosis. Altered expression of metalloproteinases (MMPs) in patients with endometriosis, suggests that MMPs may play a critical role. In the endometriotic lesions, prostaglandin E_2 (PGE_2) produced by cyclooxygenase-2 (COX-2), binds to its EP4 receptor (EP4), and via c-Src kinase induces MMPs activation, promoting endometriosis. We examined the HCB action on MMP-2 and MMP-9 activities and expression, COX-2 levels, PGE_2 signaling, and the AhR involvement in HCB-induced effects. We have used different *in vitro* models: (1) human endometrial stromal cell line T-HESC, (2) primary cultures of Human Uterine Fibroblast (HUF), and (3) primary cultures of endometrial stromal cells from eutopic endometrium of control (CESC) and subjects with endometriosis (EESC). Our results show that HCB enhances MMP-2 and MMP-9 activities in T-HESC, HUF and ESC cells. The MMP-9 levels were elevated in all models, while the MMP-2 expression only increased in ESC cells. HCB enhanced COX-2 and EP4 expression, PGE_2 secretion and the c-Src kinase activation in T-HESC. Besides, we observed that AhR is implicated in these HCB-induced effects. In conclusion, our results show that HCB exposure could contribute to endometriosis development, affecting inflammation and invasion parameters of human endometrial cells.

1. Introduction

Endometriosis is an estrogen-dependent inflammatory disease of reproductive-age women characterized by the presence and growth of endometrial tissue in ectopic sites [1]. The prevalence of the disease is 10–20% in childbearing-age women, increased to 20–30% in women with subfertility, and to 40–60% in women with dysmenorrhea [1,2]. Organochlorine pollutants may play a role in the development of this disease, however, the results of clinical trials are discordant, and it is not clear how the effect of exposure to these compounds is linked to endometriosis. Dioxins and dioxin-like compounds have in particular been associated with the disease, mainly on the basis of their effects on cytokines, immune system, hormones, and growth factors which are thought to increase the risk of endometriosis [3]. Given their capacity to persist in the environment, these compounds tend to accumulate

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in the food chain and the body, being found in adipose tissue, maternal milk and blood, and umbilical blood [4]. Several studies that focused on dioxins or dioxin-like compounds and endometriosis have observed significantly higher concentrations in serum of women with endometriosis than in those without the condition [5].

Hexachlorobenzene (HCB) is a widely distributed organochlorine pesticide and has been classified as a probable human carcinogen [6]. It has been reported that HCB induces toxic reproductive effects [7] and is an endocrine disruptor in laboratory animals [8]. Regional studies demonstrate the presence of this pollutant in mother's milk [9] as well as in samples of bovine milk for human consumption [10]. HCB is a "dioxin-like" compound that binds to the aromatic hydrocarbon receptor (AhR), accumulates in lipid tissue and induces the expression of xenobiotic metabolic enzymes [11]. Some of the biological effects of these compounds are mediated by activation of cytosolic AhR complex (AhR-dioxin-c-Src), triggering: (a) membrane actions, where c-Src activates growth factor receptors, and (b) nuclear actions, where AhR regulates gene transcription including cyclooxygenase-2 (COX-2) [12,13].

COX is the rate-limiting enzyme in the metabolic conversion of arachidonic acid to prostaglandins. Two isoforms of COX have been described: COX-1 is constitutively expressed in many tissues, while COX-2 is inducible by mitogen, growth factors [14] and cytokines [15]. A positive feedback cycle indicates that aromatase, an enzyme involved in estrogen biosynthesis, and COX-2, are responsible for the continuous local formation of estrogen and prostaglandin E_2 (PGE_2) in endometriotic stromal cells [2]. PGE_2 is a major mediator of the pain resulting from the pathophysiology of endometriosis [16]. Multifaceted actions of PGE_2 are mediated through G-protein coupled receptors designated EP1, EP2, EP3 and EP4 [17]. Selective inhibition of PGE_2 receptors EP2 and EP4 suppresses expression and/or activity of matrix metalloproteinases (MMPs) and thereby decreases migration and invasion of human immortalized endometriotic epithelial and stromal cells [17]. In this investigation, the authors found that interactions between EP2/EP4 and MMPs are mediated through c-Src kinase [17]. Takenaka et al. [18] ascertained that EP4 was higher than other EP1–3 gene expression in endometriotic stromal cells, suggesting that EP4 would be the crucial factor for PGE_2 signaling.

Involvement of MMPs in the development of endometriosis is being increasingly confirmed by several research groups, with studies indicating that the pattern of MMPs expression in the endometrium of these women significantly differs from that of healthy women [19]. Studies have shown that the expression of MMPs is increased in the ectopic endometrium, indicating that MMPs may participate in the displacement of the endometrium. Among the MMP family, MMP-2 and MMP-9 are closely associated with the formation of endometriosis [20,21]. In previous studies, we have observed that HCB induces MMP-2 expression and MMP-9 activation in human breast cancer cell line MDA-MB-231, and stimulates tumor growth and metastasis in breast cancer animal models [22].

The objective of the present study was to investigate if HCB exposition may induce alterations that promote endometriosis. We have used three *in vitro* models: (1) human endometrial stromal cell line T-HESC, (2) primary culture of Human Uterine Fibroblast (HUF), and (3) primary cultures of endometrial stromal cells from eutopic endometrium of control patients (CESC) and patients with endometriosis (EESC). We examined the HCB mechanism of action on cell viability, MMP-2 and MMP-9 expressions and activities, COX-2 expression levels and PGE_2 signaling. In addition, we evaluated the AhR role in HCB-induced MMPs activities, COX-2 expression and c-Src activation.

2. Materials and methods

2.1. Chemicals

HCB (>99% purity, commercial grade) was obtained from Aldrich-Chemie GmbH & Co. (Steinheim, Germany). Anti-c-Src and anti-phospho-Y416-c-Src antibodies were purchased from Cell Signalling Technology Inc. (MA). Anti-MMP-2 antibody was obtained from Santa Cruz Biotechnology (CA, USA); and anti-MMP-9 antibody was purchased from Millipore (Temecula, California, USA). Anti-COX-2, anti-AhR and anti-cytokeratin antibodies were obtained from Abcam Ltd. (Cambridge, UK). Monoclonal antibody anti-β-Actin, antibiotic–antimycotic, trypsin, glutamine, α-naphthoflavone (ANF) and 4,7-orthophenanthroline (4,7 PHE) specific inhibitors for AhR, as well as gelatin of zymography assay were obtained from Sigma Chemical Co. (St. Louis, MO). Polyclonal anti-EP4 antibody was purchased from Santa Cruz Biotechnology, INC (Santa Cruz, CA). For PGE_2 determination, [5,6,8,9,11,12,14,15 (n)-3H]-PGE_2 (130 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, IL, USA). PGE_2 antiserum was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-vimentin antibody was obtained from DAKO Cytomation, Glostrup, Denmark. The secondary antibodies peroxidase-accomplished goat, rabbit and mouse anti-immunoglobulin, and polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad Laboratories (CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Promega (Southampton, UK). The enhanced chemiluminescence kit (ECL) and protein electrophoresis molecular markers were purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). Phenol red-free DMEM: F-12 and RPMI culture media were obtained from Beckton and Dikinson. Fetal bovine serum (FBS) was purchased from Invitrogen Life Technology (Carlsbad, CA). All other reagents used were of analytical grade.

2.2. Collection of human biopsies

A total of 19 women in reproductive age who underwent diagnostic laparoscopy for infertility participated in this study were: 12 with untreated endometriosis (Stages I, II, III and IV according to the Revised American Society for Reproductive Medicine Classification 1997) [23] and 7 controls. Endometriosis was confirmed by laparoscopy and histological documentation. Control subjects were infertile women with tubal factor or unexplained infertility undergoing diagnostic laparoscopy. To avoid false negatives, only patients who did not complain of pelvic pain were considered for the control group. All patients were infertile, showed regular menstrual cycles and had not received any hormonal medical treatment for the last 6 months. All subjects signed informed consent prior to evaluation. Biopsies of eutopic endometrium were obtained from all subjects as described previously [24]. Biopsies were taken with a metal Novak curette from the posterior uterine wall. This study was approved by the Ethics and Research Committee from the Biology and Experimental Medicine Institute (IBYME-CONICET) of Buenos Aires, Argentina.

2.3. Isolation and culture of human endometrial stromal cells (ESCs)

The eutopic tissue biopsies were collected under sterile conditions, placed in 1:1 formula of DMEM:F-12 culture medium and transported to the laboratory. Endometrial stromal cells (ESCs) were isolated from endometrial biopsies as was previously described [25] with minor modifications. The eutopic endometrium was minced with scissors and digested with collagenase type IA in culture medium for 2 h at 37 °C. Then, the samples were