Dioxin stimulates RANTES expression in an in-vitro model of endometriosis

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The industrial contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is associated with inflammatory disorders in women and other mammals. The current studies were performed to investigate the effect of TCDD on RANTES expression in an in-vitro model of endometriosis. The biochemical effects of dioxins are mediated by binding to aryl hydrocarbon receptors (AhR). This study showed that both normal and endometriotic endometrial stromal cells express AhR protein, which was observed to be down-regulated by 40–60% after exposure to TCDD. Treatment with TCDD for 24 h increased the luciferase activity of the RANTES promoter by 2.5 ± 1.0-fold in stromal cells derived from normal endometrium and endometriotic implants. When AhR were over-expressed in these cells, luciferase activity increased 6.1 ± 1.4-fold, and RANTES protein secretion increased from undetectable to 31 ± 10 pg/100 000 cells. TCDD failed to activate a RANTES construct with a mutated dioxin response element. Other AhR ligands had similar effects to TCDD on RANTES transcription and secretion. Control transfections using tumour necrosis factor (TNF)-α and nuclear factor (NF)-κB response element reporters indicated that these pathways are not activated by TCDD in endometrial stromal cells. This study has demonstrated that functional AhR are present in endometrial and endometriotic stromal cells and that TCDD up-regulates the expression of RANTES, providing a possible mechanistic link between dioxin exposure and chemokine expression in endometriosis.

Key words: AhR/dioxin/endometrial stromal cells/endometriosis/RANTES

Introduction

Endometriosis is a common gynaecological disorder, affecting 5–15% of women of reproductive age (Goldman and Cramer, 1990; Haney, 1990). Among theories proposed to explain this anomaly, Sampson’s hypothesis of retrograde menstruation is most widely accepted (Sampson, 1927). This theory, however, falls short in completely explaining the pathogenesis of this disease. Retrograde menstruation occurs in 90% of women (Halme et al., 1984), and the vast majority manifest no pathology. Therefore, other factors must be involved in the development of endometriosis.

The aryl hydrocarbon receptor (AhR) is a nuclear receptor whose natural ligand has not yet been identified. Similar to steroid receptors, AhR acts as a ligand-dependent transcription factor that mediates a broad range of responses induced by xenobiotic toxicants including arylhydrocarbons, dioxins,furans and coplanar polychlorobiphenyls (Fernandez-Salguero, 1996).

The AhR has multiple domains including a basic helix–loop–helix (HLH) domain near the N terminus; the basic region contributes to DNA binding and the HLH region to protein–protein dimerization. The C terminal segment contains a complex transactivation domain, consisting of multiple stimulatory and inhibitory subdomains (Whitlock, 1999). The toxic biochemical effects of dioxins are mediated by AhR binding. The liganded AhR associates with its arylhydrocarbon receptor nuclear translocator (ARNT) and moves from the cytoplasm to the nucleus. The heteromeric complex acts as a signal transducer and transcription factor for target genes, including cytochromes P450 (1A1, 1A2, 1B1), detoxifying phase II enzymes, and growth regulatory genes involved in cell proliferation, differentiation and inflammation (Whitlock, 1990; Sutter et al., 1991). AhR/ARNT complexes function as transcription factors by binding to dioxin response elements (DRE) within target genes (Whitlock, 1993). The most potent AhR ligand is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a well known environmental pollutant. TCDD is an established carcinogen in experimental animals (Chahoud et al., 1989; Huff et al., 1991), but its effects on humans are less well studied (Johnson, 1993; Bertazzi et al., 2001).

In monkeys, exposure to dioxin correlates directly with an increased prevalence and severity of endometriosis (Rier et al., 1993; Yang et al., 2000). However, the true effect of TCDD on human endometriosis remains a matter of debate (Koninckx et al., 1994; Mayani et al., 1997; Scialli, 2001).

Endometriosis is associated with abnormal immune responses in women affected by this disease (Steele et al., 1984; Braun and Dmowski, 1998). The peritoneal environment in these women reveals a predominant monocytic infiltrate (Halme et al., 1987; Oosterlynck et al., 1994). RANTES is a key chemokine expressed by normal endometrium (NE) and endometriotic implant (EI)
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stromal cells (Hornung et al., 1997). The concentration (Khorram et al., 1993) and bioactivity (Hornung et al., 2001a) of RANTES are elevated in the peritoneal fluid of women with endometriosis. We postulated that xenobiotic agents, such as TCDD, might influence the immune response in women with endometriosis. An in-vitro model of endometriosis was therefore used to investigate the effect of TCDD on RANTES gene expression.

Materials and methods

Materials

A total of 10 nmol/l TCDD (Radian Corp., CA, USA) was added in 0.1% ethanol carrier directly to the cells. A total of 1 µmol/l 9,10-dimethyl-1,2-benzanthracene (DMBA) and benzo(a)pyrene (BaP) (Sigma Chemicals, St Louis, MO, USA) were suspended in 0.1% ethanol and mixed before treatment (Casper et al., 1999). Vehicle controls (0.1% ethanol) were used as a comparison.

Subjects

Healthy women with ovulatory menstrual cycles who had not received hormones or GnRH agonist therapy for at least 6 months before surgery were enrolled in this study. Subjects undergoing elective surgery for leiomyomata uteri or other benign uterine conditions were recruited for NE biopsies. Women with laparoscopically confirmed endometriomas were recruited for EI biopsies. Biopsies were obtained after the patients provided written informed consent, under a study protocol approved by the Committee on Human Research at the University of California, San Francisco (UCSF). Endometrial tissue was obtained by Pipelle® (Cooper Surgical, Shelton, CT, USA) aspiration biopsies. All samples were obtained in the proliferative phase of the cycle and this was confirmed histologically according to established criteria (Noyes et al., 1950).

Cell culture

In brief, dispersed stromal cells were separated from glandular cells and debris by filtration through narrow gauge sieves with apertures of 40 µm. Stromal cells were plated and subcultured to eliminate contamination by macrophages or other leukocytes in Modified Eagle's Medium (MEM)-α supplemented with 10% fetal calf serum (FCS), antibiotics and nucleosides. All experiments were initiated after 24 h incubation in 2.5% charcoal-stripped FCS. Extensive characterization of cell cultures prepared using this protocol previously confirmed >95% purity with cells retaining functional steroid receptors and cytoskeletal markers of their endometrial stromal origin (Ryan et al., 1994).

Immunocytochemistry

NE and EI stromal cells were plated onto Lab-Tek (Miles Laboratories, Naperville, IL, USA) 8-chamber slides and stained using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). Immunoperoxidase staining was performed using mouse monoclonal IgG antibodies against human vimentin (1:100 dilution; Sigma), rabbit polyclonal IgG antibodies against human cytokeratin (1:200 dilution; Sigma) and AhR (2 µg/ml; Santa Cruz Biotechnology, CA, USA). Non-immune, species-specific antibodies at the same concentrations were used as controls.

Western blots

Media were removed by aspiration, the cells were washed with 10 ml cold Tris-buffered saline (TBS), and scraped into 500 µl of extraction buffer (20 mMol/l Tris, pH 8.0, 137 mMol/l NaCl, 10% glycerol, 1% Triton X-100, 2 mMol/l EDTA, 1 mMol/l Pefablock, 2 µmol/l leupeptin, 0.14 µmol/ml aprotinin, 1 mMol/l vanadate). Cells were lysed by repeated freeze-thaw cycles and centrifuged at 15 000 g for 15 min at 4°C. Supernatants of extracts were denatured in sample buffer and 100 µg total protein sample was electrophoresed on 8% polyacrylamide sodium dodecyl sulphate (SDS) gels at 30 mA for 2 h. Proteins were transferred to Immobilon PVDF membranes (Millipore) for 2 h in a semi-dry transfer system (Hoefer Scientific Instruments, San Francisco, CA, USA). Membranes were blocked for 1 h in TBS-Tween plus 5% instant non-fat dry milk, then probed with 1.0 µg/ml anti-AhR polyclonal antibody H-211 (Santa Cruz Biotechnology) at 4°C overnight. The membranes were washed in the same solution before addition of 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG, and specific bands were visualized by the ECL (Amersham) method on Kodak X-omat film. Bands were quantified by transmission scanning using NIH Image 1.60 software.

RT–PCR

Total RNA was extracted from stromal cell cultures (NE and EI) using the TRizol reagent kit (Gibco BRL, Gaithersburg, MD, USA). The conditions for preparing the internal standards, the RT–PCR reaction and the primers used for semi-quantitative RT–PCR have been described previously (Hornung et al., 1997). Briefly, specific oligonucleotide primers were designed to amplify sequences from human RANTES mRNA (193 bp) and human GAPDH (240 bp) as a positive control (Ercolani et al., 1988) and reaction products were separated on 2% agarose gels.

ELISA

A specific sandwich ELISA was used to quantify RANTES in conditioned media (Quantikine; R&D Systems, MN, USA). Aliquots of culture supernatants were each tested in duplicate at several dilutions to ensure linearity and compared with reference standards of recombinant human RANTES. In our laboratory, the assay is sensitive to 8 pg/ml, with intra- and inter-assay coefficients of variation of 3 and 7% respectively. The assay is specific for human RANTES, with no known cross-reactivity with other cytokines or chemokines (R&D Systems Catalogue, 2001).

Gene promoters and luciferase assays

A full length (961 bp) fragment of the human RANTES promoter (Nelson et al., 1993) was subcloned into the pGL2 vector (Promega, Madison, WI, USA). The DRE of the wild-type RANTES promoter CACCGG at nucleotide positions −821/−816 was mutated to CTCGAG with QuickChange® site-directed mutagenesis kits (Stratagene) using oligonucleotides containing the desired mutation. Nuclear factor (NF)-κB and tumour necrosis factor (TNF)-α response element reporter constructs were gifts from Dr Dale Leitman (UCSF). The recombinant human (r)AhR vector [psG5-(hu)AhR] was engineered in the laboratory of Dr Jean-François Savouret (INSERM Unité 135, Bicêtre, France) from a sequence generously provided by Dr ChrisBradfield (Mc Ardle Cancer Research Center, University of Wisconsin, USA). All constructs and mutants were sequenced by the UCSF Biomolecular Resource Center to verify that the correct sequences and mutations were present.

Transient transfections were performed in human NE and EI stromal cells grown in MEM-α with 2.5% FCS and antibiotics in 12-well plates at ~50% confluence. A total of 0.45 µg of pGL2-RANTES promoter (firefly luciferase, experimental reporter) was added to each well using QIAGEN Effectene® reagent (Valencia, CA, USA). The RANTES promoter transfection efficiencies were normalized to an independent control plasmid (0.1 µg renilla luciferase reporter) co-transfected simultaneously. In some experiments, AhR was over-expressed by co-transfection of an expression vector (0.25 µg/well). Salmon sperm DNA was added to normalize total transfected DNA in each well. The results are presented as a percentage of luciferase activity between treated and untreated cells after correcting for transfection efficiency. Each reporter vector was assayed in at least three independent cultures. The same amount of empty pGL2 vector was analysed in pilot experiments as a control and revealed low basal activity.

Data are presented as mean ± SD. Comparisons between treatments were evaluated using Wilcoxon non-parametric tests with StatView software. Two-tailed tests with P < 0.05 were considered significant.

Results

Immunocytochemistry revealed that NE and EI stromal cells express AhR. The receptor protein was localized in both the cytoplasm and nuclei of endometrial stromal cells (Figure 1). Vimentin and cytokeratin served as positive and negative controls respectively for the immunocytochemistry analyses.

The specificity of the AhR immunolocalization was confirmed
AhR activation induces RANTES expression

Figure 1. Immunocytochemistry of endometrial stromal cells. (A,B) Normal endometrium (NE) and endometriotic implant (EI) stromal cells stained with anti-vimentin antibody as a positive control. (C,D) NE and EI stromal cells stained with anti-cytokeratin antibody as a negative control. (E,F) NE and EI stromal cells stained with anti-AhR antibody; AhR is present in both the cytoplasm and nucleus.

Figure 2. TCDD exposure decreases the amount of AhR protein. Western blot of normal endometrium (NE) and endometriotic implant (EI) stromal cells lysates, probed for human AhR (110 kDa). Cells were treated with or without TCDD (10 nmol/l) for 2 days. Stromal cells transfected with rAhR vectors were included as positive controls.

Figure 3. TCDD up-regulates RANTES mRNA. Semi-quantitative RT-PCR of normal endometrium (NE) and endometriotic implant (EI) stromal cells treated with or without TCDD. TCDD treatment increased RANTES mRNA over the basal amount.

We used semi-quantitative RT-PCR assays. TCDD treatment increased the expression of RANTES mRNA in NE and EI stromal cells by ~2-fold (Figure 3). Introns-spanning primers used to amplify transcripts of a constitutive gene, GAPDH, indicated that the RNA...
Figure 4. TCDD treatment increases RANTES protein secretion. Supernatants of normal endometrium (NE) and endometriotic implant (EI) stromal cells treated with TCDD were analysed for RANTES production by ELISA. TCDD treatment increased the secretion of RANTES protein by stromal cells transfected with rAhR, but not in non-transfected cells. *P < 0.05 compared with basal RANTES secretion.

Figure 5. TCDD activates RANTES gene expression. Luciferase reporter gene assay in endometrial stromal cells using the wild-type RANTES promoter reporter construct (–961 bp, 0.3 µg/well). Transfection efficiency was normalized by renilla luciferase. In the absence of rAhR vectors, TCDD increased luciferase activity by 2.5 ± 1.0-fold, *P < 0.05 compared with control RANTES expression. In the presence of rAhR vectors, TCDD treatment increased luciferase activity by 6.1 ± 1.4-fold, *P < 0.05 compared with controls. Even in the presence of rAhR, TCDD did not increase luciferase activity from a RANTES construct with a mutated DRE site.

preparations were of good quality and not contaminated by genomic DNA.

With over-expression of AhR (Figure 2, lanes 3 and 6), enhanced production of RANTES protein was detected in endometrial stromal cells treated with TCDD for 48 h; RANTES secretion by NE and EI stromal cells was increased from undetectable to 31 ± 10 pg/100 000 cells (n = 3, P < 0.01, Figure 4).

To explore the role of specific domains within the RANTES gene promoter, endometrial stromal cells were transiently transfected with wild-type RANTES promoter constructs cloned upstream of the firefly luciferase reporter gene.

For the full length RANTES construct (961 bp of 5’ flanking DNA), containing a single DRE consensus motif, 24 h treatment with TCDD increased luciferase activity by 2.5 ± 1.0-fold (P < 0.05) in stromal cells from both NE (n = 3) and EI (n = 3) sources. When rAhR was over-expressed in these cells, the luciferase activity increased 6.1 ± 1.4-fold (P < 0.05, Figure 5).

To further investigate the role of this DRE site, we used site-directed mutagenesis to disrupt DRE in the RANTES promoter construct (Figure 5). With the mutated DRE construct (Figure 6), TCDD failed to increase luciferase activity even when rAhR was over-expressed (n = 3, Figure 5).

TCDD exposure had no effect on endometrial stromal cells transiently transfected with NF-κB or TNF-α response element constructs in the presence of rAhR. The relative luciferase activities were 1.0 ± 0.2-fold (n = 5, Figure 7A) and 1.2 ± 0.1-fold (n = 5, Figure 7B) respectively. Neither of the results were statistically significant. By contrast, TNF-α stimulated the TNF-α response element luciferase construct >3-fold (data not shown).

Cells treated with a combination of DMBA/BaP (alternative AhR ligands) showed similar effects on RANTES as determined by both luciferase activity and protein secretion (data not shown).

Discussion

Dioxins are a family of halogenated heterocyclic hydrocarbon by-products of industrial processes such as paper bleaching and herbicide synthesis. Dioxins directly alter the expression of target
genes (Whitlock, 1990) or may act as anti-estrogens or weak estrogens through interference with the estrogen receptor (Safe et al., 1991). The increased incidence of endometriosis in populations from industrialized countries has suggested a possible link with exposure to dioxins and other AhR ligands (Rier et al., 1993; Yang et al., 2000). However, social as well as environmental factors could be responsible for this association. Later age at first pregnancy, shorter duration of breast-feeding and smaller family size may contribute to the risk of endometriosis. While a study by Pauwels et al. showed an increased odds ratio of 4.3 for the association of serum dioxin equivalents and endometriosis, this failed to reach statistical significance (Pauwels et al., 2001). Whether endometriosis is associated with high exposure to dioxin-like compounds remains to be proven.

Our study examined the presence and activation of AhR in cultures of human NE and EI stromal cells. We found AhR protein to be localized in both cytoplasm and nuclei of endometrial stromal cells. Western blotting indicated that AhR protein concentrations were decreased by TCDD treatment, reportedly through the ubiquitin–proteasome pathway (Pollenz, 1996; Ma and Baldwin, 2000). Our findings are of interest with respect to a recent study, which reported high levels of AhR mRNA in endometriotic ovarian cysts (Khorram et al., 2002). From these and our results, we suggest that the relationship between dioxin exposure and endometriosis is not direct. The co-expression of other factors, such as cellular AhR and ARNT are necessary to activate the transcription of target genes. The potential AhR modulator 7-ketocholesterol (Savouret et al., 2001) might also influence the effect of dioxin exposure in this pathway.

At least two phenomena appear to limit RANTES production in our in-vitro model. One is the down-regulation of AhR by TCDD (Ma and Baldwin, 2000) and the other is the spontaneous loss of nuclear receptors in cultured cells (Sadovsky et al., 1992). To restore TCDD responsiveness in our cells, rAhR expression vectors were constructed and transfected. Under this condition, TCDD treatment for 48 h dramatically increased RANTES secretion. A combination of DMBA and BaP, also ligands for AhR, caused a similar stimulation in RANTES secretion. This finding supports a generalized effect of AhR ligands on this inflammatory pathway.

To investigate whether the induction of RANTES secretion is transcriptionally regulated, we established a model system of transiently transfected human RANTES promoter-luciferase reporter constructs in primary human NE and EI stromal cells. Treatment with TCDD increased luciferase activity up to 6-fold. A similar stimulation was noted using DMBA and BaP. However, when we mutated the DRE site, TCDD treatment no longer increased RANTES expression. These data indicate that TCDD up-regulates RANTES gene expression, and that this effect is both AhR- and DRE-dependent.

A previous study from our group showed that NF-κB activation is responsible for interleukin-1β stimulation of RANTES gene expression in endometriotic stromal cells (Lebovic et al., 2001). It was also reported that TNF-α treatment can increase RANTES mRNA and protein production in endometrial cells (Altman et al., 1999; Hornung et al., 2001b). To test the hypothesis that TCDD could directly activate NF-κB or TNF-α and thereby stimulate RANTES gene expression, we transiently transfected NF-κB or TNF-α response element luciferase constructs into endometrial stromal cells. Exposure to TCDD failed to activate NF-κB or TNF-α response elements in the absence or presence of over-expressed AhR. These findings suggest that the increase in RANTES expression by AhR complexes is not mediated through NF-κB or TNF-α pathways.

In conclusion, the combination of TCDD and AhR up-regulates the expression of RANTES via a pathway that appears to be DRE-dependent and independent of TNF-α and NF-κB activation. Other AhR ligands have similar effects. These findings provide a mechanistic link between xenobiotic exposure, macrophage recruitment and inflammation in endometriosis.

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References


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