

Original Paper

Increased expression of IgE-dependent histamine-releasing factor in endometriotic implants

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Abstract

A complex network of cytokines mediates the immunoregulatory responses leading to endometriosis. Recent intensive studies suggest that monocyte and T cell chemoattractants contribute to the inflammatory environment of endometriotic implants. The relationship between the inflammation present during endometriosis and the development of endometriotic implants in the peritoneal cavity remains unclear. On the other hand, the association between endometriosis and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) exposure has been discussed in recent years, and our previous results revealed that IgE-dependent histamine-releasing factor (HRF) is inducible by TCDD. The present study aimed to clarify the expression, localization, and function of HRF in endometriosis. Northern blot analysis demonstrated that HRF is overexpressed in endometriotic implants. RT-PCR with Southern blot analysis, however, showed that HRF overexpression was not always accompanied by CYP1A1 induction in endometriotic implants, suggesting that HRF is inducible in endometriosis without exposure to TCDD. HRF is also inducible by macrophage colony-stimulating factor (M-CSF). Immunohistochemistry showed CD68-positive macrophages in the stroma of endometriotic implants, adjacent to regions with prominent HRF accumulation. HRF-overexpressing cells exhibited high implantation efficiency in comparison to control cells when the cells were injected into the peritoneal cavities of nude mice. These results suggest that the accumulation of macrophages in endometriotic implants induces HRF; the overexpression of HRF accelerates the growth of endometriotic implants. Copyright © 2003 John Wiley & Sons, Ltd.

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Introduction

Endometriosis is a common gynaecological disorder, affecting 10% of all women of reproductive age [1]. Endometriotic tissue undergoes cyclic proliferation and breakdown similar to that of the eutopic endometrium and can cause, for example, cyclical dysmenorrhoea, dyspareunia, pelvic pain, and catamenial haematuria. In addition, 30–40% of infertile patients have this disease [2]. Although the mechanism whereby endometrial cells implant and proliferate ectopically in certain patients remains unknown, the deregulation of inflammatory cytokines may contribute to the development of endometriosis [3,4]. In accordance with this idea, monocyte activation and recruitment into the peritoneal cavity is one of the most consistently reported immunological abnormalities of endometriosis [5–8].

Dioxins, which are classified as endocrine disruptors, are ubiquitous in the environment. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) is the

most toxic compound in this group and causes a variety of toxic effects, including immunotoxicity, hepatotoxicity, teratogenicity, and tumour promotion [9,10]. Changes in gene expression induced by TCDD and related chemicals are initiated when the toxins bind to the aryl hydrocarbon receptor (AhR), which then dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT), to form a complex that interacts with gene regulatory elements that contain an XRE (xenobiotic response element) motif [11,12]. Following chronic exposure to TCDD, monkeys developed moderate to severe endometriosis in a dose-dependent manner [13], prompting several studies examining the association of dioxin with endometriosis [14–18]. The conclusion that TCDD exposure did not correlate with endometriosis, however, was reported recently [19,20]. Thus, the relationship between dioxin exposure and endometriosis has remained elusive. We have previously identified TCDD target genes [21–23] including IgE-dependent histamine-releasing factor (HRF). HRF detectable in

T cells, B cells, mononuclear cells, and fibroblasts [22,24,25] is thought to be secreted upon allergic stimulation [25]. In mice and humans, HRF induces the recruitment of eosinophils [25] and the release of histamine, IL-4, and IL-13 from basophils [24,26]. In addition, HRF is secreted from macrophages that have been stimulated by macrophage colony-stimulating factor (M-CSF) [25].

In the present study, we examined the expression of TCDD target genes, including HRF and CYP1A1, in endometrial tissues and endometriotic implants. Immunohistochemistry identified the cells responsible for HRF expression and the localization of specific cell types within the implants. Furthermore, we analysed the physiological effects of high HRF expression using an *in vivo* mouse model. These results suggest that the local cytokine environment induces HRF expression, thereby promoting the development of endometriosis.

Materials and methods

Tissue samples

For RNA preparation, we obtained the following samples from 18 patients: (1) endometriotic implants ($n = 21$), (2) eutopic endometrial tissue from patients with endometriosis (by curettage; $n = 4$), (3) normal endometrial tissue from patients with no endometriosis ($n = 6$). Some samples were obtained from different sites from the same individual. Samples were frozen in liquid nitrogen and stored at -80°C until RNA preparation. Endometriotic implants were obtained from the ovary. To exclude normal ovarian tissue, only the surface of endometriotic cysts was taken. Normal endometrial tissue samples for RNA preparation and formalin-fixed, paraffin-embedded samples of normal proliferative and secretory endometrial tissue were obtained from patients undergoing biopsy for leiomyoma or uterine prolapse. Pathology samples were staged by histological examination, varying from endometriosis stage III and IV (r-ASRM: revised American Society for Reproductive Medicine classification of endometriosis, 1996). The women included in this study did not exhibit endometrial hyperplasia or neoplasia, and had not received any anti-inflammatory or hormonal medications before surgery. Written consent was obtained prior to surgery, according to the protocol approved by the Institutional Review Board for Human Research of the Tokyo Medical University Hospital.

Northern blot analysis

Northern blotting was performed as previously described [21]. The HRF probe was prepared as previously described [22]. A human β -actin cDNA control probe (CLONTECH Laboratories, Inc., CA, USA) was used as a standard.

RT-PCR with Southern blot analysis

First strand cDNAs were synthesized from total RNAs using an oligo dT primer as previously described [27]. Next, $2\text{ }\mu\text{l}$ ($1\times$) and $10\text{ }\mu\text{l}$ ($5\times$) of the resultant single-strand cDNA solutions were used as templates for PCR. After adding four primers (5'-CCACAACCACCAAGAACTGCTTAG-3' and 5'-TGAAGGGGACGAAGGAAGAGTG-3' for CYP1A1, 5'-GGGAAATCGTGCGTGACATTAAG-3' and 5'-TGTGTTGGCGTACAGGTCCTTG-3' for β -actin, respectively), we amplified the CYP1A1 and β -actin cDNA fragments by PCR cycling with an initial denaturation at 95°C for 2 min; 22 cycles of 95°C for 0.5 min, 65°C for 0.5 min, and 72°C for 1 min. Amplification products were fractionated by electrophoresis on agarose gels, and then blotting and hybridization were performed. The CYP1A1 cDNA probe was obtained by reverse transcription PCR using the primer pair described above. A human β -actin cDNA probe (CLONTECH) was used as a control. These cDNA probes were labelled with ^{32}P using the Rediprime II random prime labelling system (Amersham Pharmacia Biotech UK, Ltd).

Antibody preparation and Immunohistochemistry

A peptide antibody against polypeptide 101 to 116 (GKLEEQRPERVKPFMT), derived from human HRF, was raised in rabbits using standard methods. This antibody was named HRF-GKL. For immunohistochemical analysis, deparaffinized sections were incubated overnight with a mixture of anti-HRF antibodies, HRF-TPY [22] and HRF-GKL (dilution of 1:100), or anti-human CD68 (dilution 1:100; Dako Japan, Kyoto, Japan). For anti-HRF staining, deparaffinized sections were subjected to heat-induced antigen retrieval with a pressure sterilizer. LSABC (Dako Japan, Kyoto, Japan) was used for detection, using 3,3'-diaminobenzidine as a chromogen. Haematoxylin was used for counterstaining.

Western blot analysis

Western blot analysis was performed as previously described [22]. Membranes were probed with anti-HRF antibodies (HRF-GKL and HRF-TPY) at a dilution of 1:2000, respectively. Signals were detected using the ECL plus western blotting detection system (Amersham Pharmacia Biotech UK Ltd).

Cell culture and retroviral infection

NIH3T3 cells were obtained from the American Type Culture Collection (ATCC). The cells were grown in DMEM (GIBCO BRL, Life Technologies, Inc., MD, USA), supplemented with 10% FBS at 37°C in a 5% CO_2 environment. For retroviral transduction, the mouse HRF cDNA containing the full-length ORF was amplified by PCR using the primers 5'-TTGGATCCATGATCATCTACCGGGACCTC-3' and

5'-TTGAATTCTTAACATTTCTCCATCTCTAA-3'.

The resultant cDNA fragment was digested with *Bam*HI and *Eco*RI, and then cloned into the *Bgl*II-*Eco*RI site of the retroviral expression vector MSCV-puro (CLONTECH). Preparation of recombinant retroviruses and infection protocols was performed as described [28]. Twenty-four hours after infection, cells were selected in 1 µg/ml puromycin (Clontech) for 2 weeks.

Animals and treatment

For the implantation assay, aliquots of 5×10^5 cells were injected into the abdominal cavities of 6-week-old female BALB/C nude mice. Two weeks later, animals were sacrificed, allowing measurement of the number of implantation colonies.

Results

The expression patterns of the TCDD-inducible genes HRF and CYP1A1 are not concordant in endometriosis

To determine the expression patterns of HRF during endometriosis, we performed northern blot analysis. We discovered high HRF expression levels in endometriotic implant tissues from three of five patients (Figures 1A and 1B). Certain members of the human cytochrome p-450 gene superfamily (e.g., CYP1A1, CYP1A2, and CYP1B1) are induced by dioxin; the induction of CYP1A1 serves as a prototype target for the dioxin-dependent regulation of gene expression. Therefore, to determine the relationship of dioxin exposure to HRF expression, we examined CYP1A1 expression using RT-PCR with Southern blot analysis [29,30]. CYP1A1 was not induced in all cases that showed high HRF expression (Figures 1A and 1B). Thus, although HRF expression may have been induced by TCDD in some cases, these observations suggest that HRF was induced in some endometriotic implants independently of TCDD exposure.

HRF is highly expressed in endometriotic implants

We next confirmed the overexpression of HRF in endometriotic implants in additional endometriosis patients. We examined seven more cases by northern blot analysis (Figure 2A). We observed high HRF expression levels in endometriotic implants in comparison with normal endometrial tissues and eutopic endometrium from patients with endometriosis (Figure 2B). There was no significant correlation between clinical stage and HRF expression in endometriotic implants.

Immunohistochemistry of HRF in normal endometrium and endometriotic implant

Next, we determined the endometrial cell types expressing HRF by immunohistochemistry. Polyclonal

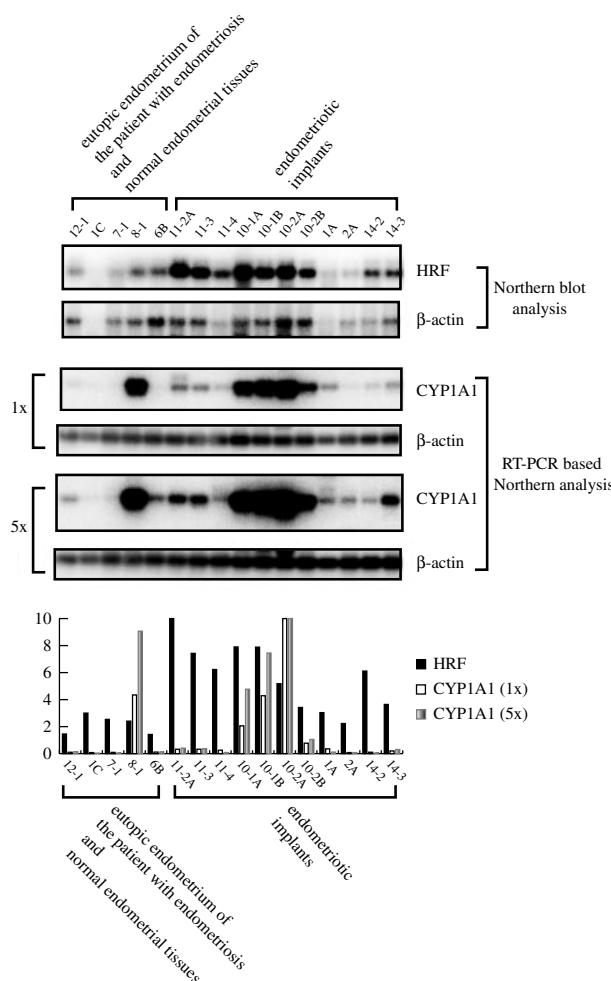


Figure 1. HRF and CYP1A1 expression in normal endometrial tissues, eutopic endometrial tissues from patients with endometriosis and endometriotic implants. (A) HRF mRNA levels were examined by northern blot analysis. Blots were reprobed with a human β-actin probe to determine total RNA levels. The CYP1A1 mRNA levels within the samples examined by northern blot were also determined by quantitative RT-PCR with Southern blot analysis. To confirm the accuracy of the quantitation, fivefold different concentrations of the cDNA samples (1× and 5×) were used as PCR templates. β-Actin was used as an internal control for mRNA quantity. (B) Graphical representation of the HRF and CYP1A1 mRNA levels is also shown. mRNA levels were normalized to β-actin signals using densitometry (Molecular Imager, Nippon Bio-Rad, Tokyo, Japan). The mRNA levels present in samples 11-2A for HRF and 10-2A for CYP1A1 were arbitrarily set to 10, respectively. When multiple samples are from a single individual, an average value from the multiple samples is calculated and shown. Error bars represent maximum values in the multiple samples. Cases 12-1, 7-1, 8-1, and 6B are normal endometrial tissues and Case 1C marked with an asterisk is eutopic endometrium from the patient with endometriosis

antibodies against HRF identified this protein in both the endometrial glands and stromal cells of normal tissues. Endometrial glands exhibited stronger expression than stromal cells (Figures 3A and 3B). No significant changes in expression patterns were noted between secretory and proliferative phases.

We also examined HRF expression in endometriotic implants. HRF was present in both the stromal

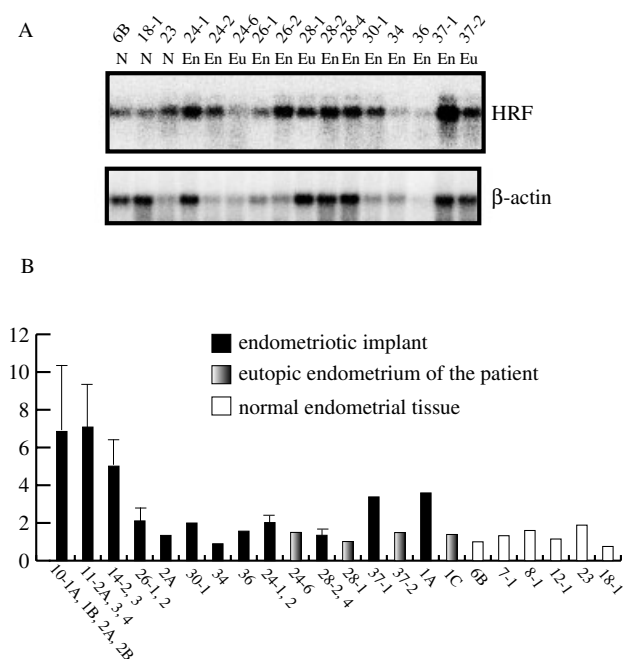


Figure 2. HRF expression in endometriotic implants. (A) Northern blot analysis of HRF expression in normal endometrial tissues, eutopic endometrial tissues from patients with endometriosis, and endometriotic implants. The blots were reprobed with a human β -actin probe to determine total RNA levels. N, Eu and En above the columns represent the normal endometrial tissues, eutopic endometrial tissues from patients with endometriosis, and endometriotic implants, respectively. (B) Graphical representation of HRF mRNA levels determined by northern blot analysis for the samples examined in Figures 1A and 2A. HRF mRNA levels were normalized to the β -actin signals by densitometry (Molecular Imager, Nippon Bio-Rad, Tokyo, Japan). The mRNA level of sample 6B was arbitrarily set to 1. When multiple samples are from a single individual, an average value is calculated and shown. Error bars represent maximum values of the multiple samples

and epithelial components of the ovarian endometriotic implants (Figures 3C and 3E). In contrast to the weak HRF expression of normal endometrial stromal cells, both the endometrial glands and the stromal cells showed similar intensities of HRF expression in the endometriotic implants of the ovary. These specific signals for HRF were not observed when pre-immune serum was used as a control (data not shown).

The mechanism of HRF induction in endometriotic implants, however, remains a mystery. In agreement with reports demonstrating that macrophages induce HRF during activation by M-CSF [25], recruitment of CD68-positive macrophages is observed in endometriotic implants [7]. Thus, we utilized CD68 staining of implant serial sections to identify CD68-positive macrophages within the HRF-overexpressing regions (Figure 3F). A control section stained with haematoxylin and eosin demonstrates the general morphology of the endometriotic implant. From these results, we consider that macrophages might contribute to HRF production in endometriotic implants.

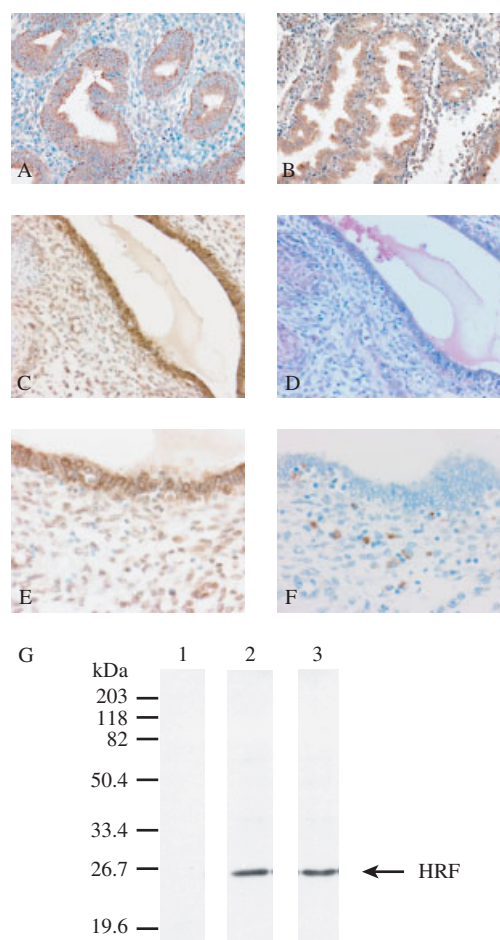


Figure 3. Immunohistochemical analysis of HRF and CD68 expression. Positivity is visualized as a brown stain. Haematoxylin was used for counterstaining. (A, B) Detection of HRF protein in normal endometrial tissue. (A, proliferative phase; B, secretory phase; original magnification $\times 200$). (C) Detection of HRF protein within an ovarian endometriotic implant (original magnification $\times 200$). (D) H&E staining of serial sections demonstrating the morphology of the endometriotic implant (original magnification $\times 200$). (E) Detection of HRF protein from the same field as (C) at a higher magnification (original magnification $\times 400$). (F) Immunohistochemical localization of CD68-positive macrophages in serial sections of endometrial implants (original magnification $\times 400$). (G) Western blot analysis with the total extract from a human-derived cell line, Jurkat, to confirm the specificity of the antibodies against HRF that were used for immunohistochemical analysis. Lane 1, pre-immune serum; lane 2, HRF-GKL antibody; lane 3, HRF-TPY antibody

HRF accelerated implantation of NIH 3T3 cells in the peritoneal cavity

We also determined the physiological effects of elevated HRF expression. The pathogenesis of endometriosis remains unclear [31,32]. According to the predominant hypothesis, endometriosis arises from the implantation and proliferation of endometrial tissue that reaches the peritoneal cavity by tubal reflux (retrograde menstruation). We examined the influence of HRF on this implantation. First, we created stable transfectants of NIH 3T3 cells overexpressing HRF. We confirmed high HRF expression following infection with an HRF-containing retroviral

vector (pMSCVHRF) (Figure 4A). We then injected these cells (pMSCVHRF-3T3 cells) into the peritoneal cavities of nude mice. pMSCV-HRF-3T3 cells possessed a high transplantation ability, compared with cells transfected with the vector control (pMSCV-3T3) (Figure 4B). These data suggest that HRF plays an important role not only in immunological dysfunction but also in the initial development of endometriotic implants.

Discussion

HRF plays an important role in late-phase allergic inflammatory responses by triggering histamine release from basophils in the absence of antigen. HRF induces immune cell activation, and stimulates histamine release from basophils and mast cells when IgE is present on the cell surface [24]. In the present study, we have demonstrated that HRF is highly expressed in endometriotic implants. However, we did not observe allergic symptoms in the patients examined in this study. The increased expression of HRF in endometriotic implants may not be sufficient to induce allergic symptoms. If the endometriosis patients have allergic disease, HRF overexpression in endometriotic implants might exacerbate allergic symptoms. On the other hand, HRF has other functions such as IL-4, IL-8, and IL-13 production [26,33]. Thus, we consider that HRF may influence the local inflammatory environment in endometriotic implants.

What is the function of HRF upregulation in endometriotic implants? In recent years, studies have

indicated that the concentration and activation status of macrophages are increased in endometriosis [3,4]. It has been suggested that TNF- α or IL-1 β produced by endometriotic implants can induce expression of RANTES mRNA in a subset of endometriotic stromal cells [6,7]. These cells synthesize both RANTES protein and/or monocyte chemotactic protein-1 (MCP-1), which recruit macrophages into the endometriotic lesion [5]. Macrophages by themselves synthesize and release M-CSF [34,35]. M-CSF aids the survival, proliferation, and differentiation of macrophages [36] and induces HRF secretion from these cells [25]. Taken together, our results suggest that the induction of CD68-positive macrophage accumulation by proinflammatory cytokines induces increased levels of HRF in endometriotic implants (Figure 3). HRF expression, acting in cooperation with other cytokines, may cause chronic pain through immune cell activation.

For TCDD, our results suggest that HRF induction is independent of TCDD exposure in endometriotic implants (Figure 1). However, HRF is a TCDD target gene [22]; and, although there are diverse conclusions about the relationship between TCDD exposure and promotion of endometriosis [14–20], it is still possible that TCDD or such compounds cause endometriosis in some patients. In addition, the overexpression of HRF may play an important role in the initial development of endometriotic implants as shown in this study (Figure 4). Thus, we consider that HRF expression is inducible in endometriotic implants without TCDD exposure, and that some endometriotic implants are induced by TCDD exposure as a result of HRF expression.

In summary, this study confirms that HRF is highly expressed in endometriotic implants. In addition, our results suggest that HRF is induced by accumulation of CD68-positive macrophages in endometriotic implants. The overexpression of HRF in endometriotic implants of epithelial and/or stromal cell origin may be sufficient for the acquisition of a growth advantage. We postulate that the inflammatory environment created by this cascade contributes to both the clinical symptoms characteristic of endometriosis and the development of endometriotic implants.

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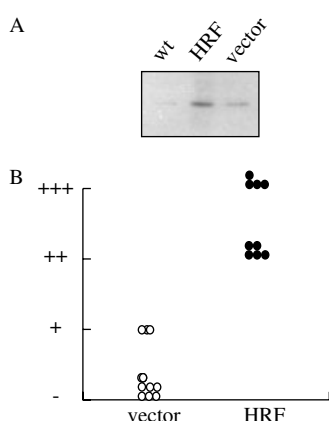


Figure 4. Implantation assay. (A) Western blot analysis of HRF protein in NIH 3T3 cells with an anti-HRF antibody (HRF-TRY). wt, parental NIH3T3 cells; HRF, cell line (pMSCVHRF-3T3) stably expressing HRF following infection with an HRF containing retroviral vector; vector, control cells infected with empty vector (pMSCV-3T3). (B) High implantation efficiency of HRF-overexpressing cells in nude mice. The symbols on the vertical axis indicate the following conditions. +++, innumerable implantation colonies were observed; ++, tens of implantation colonies were observed; +, several implantation colonies were observed; –, no implantation colonies were observed. Individual mice injected with either control cells or HRF-overexpressing cells are represented by open circles or closed circles, respectively

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