Microarray analysis provides insight into the early steps of pathophysiology of mouse endometriosis model induced by autotransplantation of endometrium

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Aims: To characterize the biochemical alterations that occur in the peritoneal tissue of the mouse endometriosis model during early development of the lesion using microarray analysis.

Main methods: The endometriosis model was induced by autotransplantation of endometrium in 8-week-old female ICR mice. Peritoneum only (excluding the transplant) was obtained 24, 48, and 96 h after the autotransplantation and subjected to microarray analysis. To interpret the large amounts of data generated and to enable a functional analysis, genes were classified using Gene Ontology (GO) and Medical Subject Heading (MeSH) terms, and the results were compared with previous reports on endometriosis.

Key findings: Of the upregulated genes, those involved in the inflammatory response, cell adhesion, extracellular matrix, wound healing, hormones, and leukocytes were significantly enriched 24 and 48 h after autotransplantation. Those of cytokines, antibody-producing cells, dendritic cells, inflammation, and infertility were enriched after 96 h. Analysis using GO and MeSH provided different information. Particularly, MeSH showed a link between an anatomical and diseased phenotype with common genes found to be upregulated.

Significance: The factors occurring during early development of endometriosis induced by autotransplantation are increase in adhesion molecules and inflammatory responses rather than angiogenesis. Data presented herein may reveal a novel therapeutic gene targets and will contribute to knowledge for the treatment of this currently incurable disease.

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Introduction

Endometriosis, the incidence of which is on the rise, is a common gynecological disorder, prevalent in 6–10% of women of reproductive age (Giudice and Kao 2004). Despite this high prevalence, its pathogenesis has not been clarified and treatment options are limited. There have been some reports on gene expression and protein production patterns that are shared by experimental and human disease, but these are mostly single gene and single protein studies. To identify gene expression profiles associated with endometriosis, microarrays are being applied to analysis of human endometriosis (Arimoto et al. 2003; Byster et al. 2002, 2007; Kao et al. 2003; Matsuzaki et al. 2004, 2005, 2006; Sha et al. 2007). However, human studies are limited by ethical and practical considerations including: [1] the need for repeated surgical procedures to monitor disease progression, [2] difficulties in control of variables, and [3] difficulties in studying the early steps of disease development (Flores et al. 2007). To identify the pathogenic factors of this disorder, animal models of endometriosis by autotransplantation of endometrium were created (Uchiide et al. 2002). Uchiide et al. (2002) found that proliferation of stromal cells and an infiltration of cells related to allergic inflammation was induced in the peritoneal tissue to which the endometrium was autotransplanted. This observation was consistent with that from human endometriosis (Sugamata et al. 2005). It has been shown that proliferative lesions with angiogenesis are induced by autotransplantation of endometrium (Fainaru et al. 2008; Lin et al. 2006), and that expression of cytokines and chemokines in this rat model, including peritoneal stromal tissue and the uterus transplant, is similar to that that occurs in human endometriosis (Umezawa et al. 2008a). Microarrays have also been applied for analysis of the rat endometriosis model (Flores et al. 2007; Konno et al. 2007). Flores et al. (2007) showed that the gene expression profile of the rat endometriosis was consistent with human endometriosis; however, there has been no observation of the biochemical changes that occur, specifically in the peritoneal tissue adjacent to the endometrial transplant, excluding the factors occurring in this transplant. Although the hypothesis that
retrograde seeding of endometrial cells during menstruation induces endometriosis is widely accepted (Sampson 1927), this disorder occurs rarely in sites with no retrograde menstruation, such as pericardium, pleura, and brain (Giudice and Kao, 2004) and even in males (Oliker and Harris 1971; Pinkert et al., 1979; Schrodt et al. 1980; Martin and Hauck 1985). Considering these reports, analyzing the pathology in the peritoneal site of the endometriosis model may reveal key factors associated with its etiology.

**Materials and methods**

**Animals and treatments**

Female ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed in a room under controlled temperature (23 ± 1 °C), humidity (55 ± 5 °C) and light (12:12 light:dark cycle with lights on at 8:00 a.m.) with ad libitum access to chow and water. All experimental animals were handled in accordance with institutional and national guidelines for the care and use of laboratory animals.

Endometriosis was induced in the mice at 8 weeks of age (27.1–37.1 g), when a 5 mm × 5 mm piece of uterine tissue was attached to each side of the peritoneum by surgical autotransplantation. Peritoneal tissues adjacent to the endometrial transplant were obtained as endometriotic lesions (n = 2 per mouse) at 24 (n = 4 mice), 48 (n = 4 mice), and 96 h (n = 4 mice) after the transplantation. As a control, a piece of abdominal white adipose tissue was autotransplanted to each side of the peritoneum. Peritoneal tissue adjacent to adipose tissue (n = 2 per mouse) were obtained at 24 (n = 3 mice), 48 (n = 4 mice), and 96 h (n = 4 mice) after the transplantation. Each tissue sample was divided into specimens; one for microscopic analysis and the other for gene expression analysis.

**Light microscope analysis**

Tissue samples for microscopic analysis were fixed and treated as previously described (Umezawa et al. 2008b) and embedded in paraffin. Paraffin-embedded tissue sections were stained with hematoxylin-and-eosin (HE) for histological analysis. To identify mast cells in each specimen, the paraffin sections were stained with toluidine blue as previously described (Ihara et al. 2004).

**Total RNA extraction**

Tissue samples for gene expression analysis of the uterine transplant and the peritoneal tissue only was immediately frozen in liquid nitrogen. Frozen tissues were homogenized with a Digital Homogenizer (As One Corp., Osaka, Japan) in Isogen (Nippon Gene Co., Ltd., Tokyo, Japan), and total RNA was isolated with chloroform. RNA was precipitated in isopropanol, washed with ethanol, and resuspended in RNase-free water. The RNA quantity was determined by spectrophotometry at OD260 in a Smart Spec 3000 (Bio-Rad Laboratories Inc., Tokyo, Japan). 2 mg of RNA from each sample was provided for quantitative real-time PCR and the rest was used for microarray analysis.

**Antisense RNA microarray using the Mouse FANTOM Array**

RNAs for microarray analysis were pooled for each group. Each pooled RNA was purified and concentrated using the RNeasy Micro Kit (Qiagen, Hilden, Germany). Amino allyl-modified antisense RNA (aRNA) was synthesized from concentrated RNA and then purified and labeled with Cy3 and Cy5 using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion Inc., TX, USA). Cy3- and Cy5-labeled samples were purified using the Amino Allyl MessageAmp II aRNA kit.
Amplification Kit. The generated targets were mixed and hybridized to the Mouse FANTOM Array ver. 1 (AGC Techno Glass Co. Ltd., Chiba, Japan) consisting of 13,728 gene probes. Microarrays were scanned with two different photomultiplier sensitivities by a ScanArray (Packard BioChip Technologies, MA, USA). Scanner output images were normalized and quantified using ScanArray Express (Perkin Elmer, MA, USA) and SilicoCyte (CytoGenomics, MO, USA). Normalization was performed so that the overall intensity ratio of Cy3 and Cy5 was equal to one. Statistical analysis was performed with analysis of variance. The expression threshold levels were more than 1.5-fold and less than 0.67-fold with a P value of less than 0.5.

**Quantitative real-time PCR**

Total RNA solutions for quantitative real-time PCR were reverse-transcribed into cDNA as previously described (Umezawa et al. 2008a). Quantitative PCR was performed in duplicate with specific oligo-primer pairs and SYBR Green Realtime PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan) using an Mx3000P Real-Time PCR System (Stratagene, CA, USA) according to the manufacturers’ instructions. Relative expression levels were calculated for each sample after normalization against the expression of six selected genes was conducted: Ahr, Irak1bp1, Pycard, 1700112J16Rik, and DOH4S114 was upregulated in peritoneal tissue at 24, 48, and 96 h after autotransplantation of endometrial tissue, compared to those on which adipose tissues had been transplanted (control tissues). 110, 266, and 331 were found to be downregulated at 24, 48, and 96 h. Ninety-six hours after autotransplantation of endometrium, the infiltration of mast cells seen in the rat endometriosis model was also observed in the lesions (Fig. 1H).

**Table 1**

<table>
<thead>
<tr>
<th>GO term</th>
<th>24-h enrichment factor</th>
<th>P value</th>
<th>48-h enrichment factor</th>
<th>P value</th>
<th>96-h enrichment factor</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extracted categories of upregulated genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>1.49</td>
<td>0.08</td>
<td>1.75</td>
<td>0.03</td>
<td>1.28</td>
<td>0.09</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>2.03</td>
<td>0.04</td>
<td>1.05</td>
<td>0.24</td>
<td>1.27</td>
<td>0.15</td>
</tr>
<tr>
<td>Oscillation</td>
<td>1.41</td>
<td>0.17</td>
<td>1.11</td>
<td>0.24</td>
<td>1.77</td>
<td>0.04</td>
</tr>
<tr>
<td>Wound healing</td>
<td>2.35</td>
<td>0.04</td>
<td>0.49</td>
<td>–</td>
<td>1.32</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Extracted categories of downregulated genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Response to oxidative stress</td>
<td>1.13</td>
<td>0.25</td>
<td>1.20</td>
<td>0.23</td>
<td>1.24</td>
<td>0.04</td>
</tr>
<tr>
<td>Ion binding</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>–</td>
<td>4.76</td>
<td>0.01</td>
</tr>
<tr>
<td>Metal ion binding</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>–</td>
<td>6.14</td>
<td>0.003</td>
</tr>
</tbody>
</table>

**Results**

**Histopathology of mouse endometriosis model**

Ninety-six hours after autotransplantation of endometrium, the transplant had induced the endometriosis model, with proliferation of peritoneal stromal cells similar to the model induced in rats (Uchide et al. 2002) (Fig. 1G). Infiltration of mast cells seen in the rat endometriosis model was also observed in the lesions (Fig. 1H).

**Analysis of mRNA microarrays**

From the 13,728 genes printed on the Mouse FANTOM Arrays, 192, 182, and 223 were found to be upregulated in peritoneal tissue at 24, 48, and 96 h after autotransplantation of endometrial tissue, compared to those on which adipose tissues had been transplanted (control tissues). 110, 266, and 331 were found to be downregulated at 24, 48, and 96 h. Ninety-six hours after autotransplantation of endometrium, the infiltration of mast cells seen in the rat endometriosis model was also observed in the lesions (Fig. 1H).

**Table 2**

<table>
<thead>
<tr>
<th>MeSH term</th>
<th>24 h enrichment factor</th>
<th>P value</th>
<th>48 h enrichment factor</th>
<th>P value</th>
<th>96 h enrichment factor</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extracted categories of upregulated genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokines</td>
<td>0.89</td>
<td>–</td>
<td>1.24</td>
<td>0.13</td>
<td>1.53</td>
<td>0.04</td>
</tr>
<tr>
<td>Hormones</td>
<td>2.76</td>
<td>0.02</td>
<td>1.15</td>
<td>0.28</td>
<td>0.95</td>
<td>–</td>
</tr>
<tr>
<td>Antibody-producing cells</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>–</td>
<td>4.60</td>
<td>0.02</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>1.84</td>
<td>0.06</td>
<td>0.96</td>
<td>–</td>
<td>1.84</td>
<td>0.05</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>3.10</td>
<td>0.002</td>
<td>1.62</td>
<td>0.14</td>
<td>1.66</td>
<td>0.10</td>
</tr>
<tr>
<td>Inflammation</td>
<td>1.06</td>
<td>0.20</td>
<td>1.11</td>
<td>0.19</td>
<td>1.82</td>
<td>0.02</td>
</tr>
<tr>
<td>Infertility</td>
<td>1.34</td>
<td>0.36</td>
<td>1.39</td>
<td>0.36</td>
<td>3.44</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Extracted categories of downregulated genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphokines</td>
<td>0.77</td>
<td>–</td>
<td>1.12</td>
<td>0.20</td>
<td>1.89</td>
<td>0.03</td>
</tr>
<tr>
<td>Receptors, chemokine</td>
<td>0</td>
<td>–</td>
<td>0</td>
<td>–</td>
<td>2.72</td>
<td>0.01</td>
</tr>
<tr>
<td>Vascular endothelial growth factors</td>
<td>0.74</td>
<td>–</td>
<td>1.44</td>
<td>0.20</td>
<td>2.52</td>
<td>0.01</td>
</tr>
<tr>
<td>Phagocytes</td>
<td>0</td>
<td>–</td>
<td>0.73</td>
<td>–</td>
<td>2.75</td>
<td>0.02</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0</td>
<td>–</td>
<td>1.41</td>
<td>0.36</td>
<td>2.10</td>
<td>0.04</td>
</tr>
<tr>
<td>Nerve degeneration</td>
<td>0.88</td>
<td>–</td>
<td>0.64</td>
<td>–</td>
<td>1.93</td>
<td>0.03</td>
</tr>
</tbody>
</table>

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detected at 24 h and upregulation of Ahr, Pycard, and Sfa3 was detected at 96 h. Of these seven points, the results of six were confirmed, (all except, DOH4S114) with no discrepancy. For Irak1bp1 and Pycard at 24 h and Ahr at 24 and 96 h, the difference in mean between experimental and control groups reached statistical significance.

Functional categorization of microarray data

From 52 GO terms reported to have some relation to the development and pathology of endometriosis, 9 were significantly extracted (Table 1). Of the genes upregulated, those linked to inflammatory response, wound healing, and extracellular matrix at 24 h, those linked to cell adhesion at 48 h, and ossification at 96 h were extracted. Of the genes downregulated, those involved in the response to oxidative stress, signal transduction, ion binding, and metal ion binding were extracted at 96 h. 13 MeSH terms were also significantly extracted from 76 terms (Table 2). Of the upregulated genes, those linked to hormones and leukocytes at 24 h, those with cytokines, antibody-producing cells, dendritic cells, inflammation, and infertility at 96 h were extracted. Of the downregulated genes, those linked to lymphokines, chemokine receptors, vascular endothelial growth factors, phagocytes, monokines, and nerve degeneration were extracted at 96 h.

Discussion

Several studies have shown the usefulness of the autotransplantation model as an appropriate tool for studying the natural history of endometriosis in rats (Uchiide et al. 2002; Flore et al. 2007; Umezawa et al. 2008a) and mice (Rossi et al. 2000). In the present study, gene expression changes that occur in stromal proliferative lesions of peritoneum onto which endometrium had been autotransplanted were focused upon, in order to identify the response induced by autotransplantation of endometrium, associated with the early stages of development of endometriosis. The mouse model was used in the present study because murine genomic information has been much more elucidated than for the rat. Sham-operated control was prepared through autotransplantation of white adipose tissue to observe the specific reactions induced by autotransplantation of endometrium.

Histopathological characteristics of the mouse model, i.e., stromal hyperplasia and infiltration of mast cells in peritoneal tissues, were similar to the rat model (Uchiide et al. 2002). This observation supported the validity of the mouse model for analyzing the molecular events induced in the endometriosis model.

The results of the microarray analysis showed changes in expression of hundreds of genes at 24, 48, and 96 h after autotransplantation. To validate the microarray results, real-time PCR analysis of the expression level of selected genes was conducted. Six genes were selected and real-time PCR was conducted for the microarray data validation. Although only a subset of upregulated and downregulated genes was validated with this method, the results for these from the microarray analysis were comparable to those of the quantitative PCR, in accordance with results from previous studies that have confirmed the correlation between microarray and real-time RT-PCR data (Flore et al. 2007).

Interestingly, Ahr was upregulated in the early stages of development of the lesion induced by autotransplantation. Khorram et al. (2002) reported that Ahr mRNA expression is increased in endometriotic ovarian cysts but not in ovarian cancer, and that this suggested a potential role of Ahr in the pathogenesis of endometriosis. Ahr agonists, i.e. dioxins and dioxin-like toxicants such as polychlorinated biphenyl are well known to be associated with the incidence of endometriosis (Rier and Foster 2002). It was previously reported that diesel exhaust, which has aromatic hydrocarbons that stimulate Ahr (Mason 1994; Takeda et al. 2004), enhances the pathology of the rat model of endometriosis (Umezawa et al. 2008b). These effects of environmental toxicants on progression of endometriosis suggest an important role of Ahr in the pathogenesis.

From the comprehensive microarray analysis of gene expression, the phenotype of stromal proliferative lesions was found in the mouse endometriosis model induced by autotransplantation of endometrium. Analysis with gene annotation using GO and MeSH terms was conducted to provide biological and functional meaning to the large amounts of data generated by the microarray analysis. Although most researchers uses GO for providing annotation to genes (Flore et al. 2007), MeSH terms are also used to complement the interpretation of microarray data from the biological viewpoint (Nakazato et al. 2007). As a result, six categories of Biological Process, one of Cellular Component, and two of Molecular Function in GO terms; and five in the Chemicals and Drugs Category, five in the Anatomy Category, and three in the Diseases Category of MeSH terms were significantly extracted. This result shows that the use of GO terms provide more information associated with biological processes, although MeSH has a similar category (Biological Sciences Category), while the use of MeSH has the advantage of producing anatomical and disease information with respect to the genes of interest. Application of MeSH in annotation with GO is helpful for interpretation of groups of genes in a biological context.

Several microarray studies of endometriosis have been reported previously. Human studies have shown gene expression differs in eutopic and ectopic endometrium in patients, and differences in eutopic endometrium in women with and without endometriosis. They have also found dysregulation of genes associated with cell adhesion, extracellular matrix and its remodeling (Eyster et al. 2007; Sha et al. 2007), apoptosis, aromatase, progesterone receptors, angiogenic factors (Kao et al. 2003), immune system, inflammatory pathways, and signal transduction pathway components (Eyster et al. 2007; Kao et al. 2003), especially the RAS/RAF/MAPK signaling pathway (Matsuzaki et al. 2004, 2005, 2006). Similarly, a recent study using the rat model showed upregulation of genes involved in cell adhesion, integrin binding, extracellular matrix, metalloendopeptidase activity, collagen catabolism, response to wounding, angiogenesis, and immune and inflammatory response in rat endometriosis (Flore et al. 2007). In this current study that focused on the peritoneal side of the lesion in the mouse endometriosis model, aside from normalization of the lesion after 96 h of the autotransplantation, it was observed that an upregulation of genes associated with inflammation and infertility is involved in the pathology of endometriosis. Interestingly, in the earlier stage of the lesion from 24 h after the autotransplantation, genes associated with cell adhesion, extracellular matrix, and inflammatory response were upregulated. Since these factors in stromal lesions of endometriosis patients are upregulated in treatment of human chorionic gonadotropin (Huber et al. 2007), further investigation is needed to clarify the meaning of altered expression of genes associated with the factors.

Many reports have suggested that immunoinflammatory processes contribute to the pathogenesis of endometriosis (Kyama et al. 2003). The results from the current study also showed an enhanced inflammatory response including activation of antibody-producing cells, dendritic cells, and leukocytes with upregulated expression of cytokines and downregulated expression of genes associated with phagocytes and monocytes. While an increased number of macrophages, helper T lymphocytes, and natural-killer (NK) cells in peritoneal fluid (PF) (Hill et al. 1988) and accentuated activation of peritoneal macrophages (Halme et al. 1984, 1988) were observed in patients with endometriosis, decrease of natural killer activity of PF has also been reported (Oosterlynck et al. 1994); however, it was reported that the number of activated T cells (CD25+ CD3+) that can produce lymphokine activating NK cells was decreased in women with endometriosis (Ho et al. 1995). Endometrial cells can be eliminated
by NK cells because human leukocyte antigen (HLA)-G, which is a ligand of KIR2DL4, one of the receptors on NK cells, is expressed by the glandular epithelium of peritoneal endometriosis (Barrier et al. 2006) and eutopic endometrium in the menstrual phase (Kawashima et al. in press). These findings suggest that immunotolerance with decreased NK activity against endometrial cells in the peritoneal cavity may lead to development of endometriosis. The results from the current study show that a downregulation of genes associated with lymphokines, chemokine receptors, phagocytes and monocytes is induced in the lesion. This observation may explain the localized immunotolerance against ectopic endometrial cells in the peritoneal cavity.

It has also been reported that angiogenesis plays an important role in the pathogenesis of endometriosis (Ferrara et al. 2003; Kao et al. 2003; Koninckx et al. 1998; Nap et al. 2004). Unexpectedly, this current study indicates that downregulation of genes associated with vascular endothelial growth factors occurs in the lesion 96 h after autotransplantation. This apparent discrepancy could be caused by the difference in the time and subject of analysis between the studies, i.e., the lesions from 24–96 h after autotransplantation and not including the transplant, were analyzed in this study.

**Conclusion**

From the results presented in this study, reactions induced by autotransplantation of endometrium in peritoneal tissue that elucidate the early stages of pathogenesis in endometriosis are summarized as follows (Fig. 2): ectopic endometrial cells provided by retrograde menstruation induce an upregulation of genes associated with inflammatory response, adhesion molecules and extracellular matrix, followed by inflammation and cytokine expression, activation of antibody-producing cells and dendritic cells, and local immunosuppression. The factors occurring during early development of endometriosis induced by autotransplantation of endometrium are: differential expression of genes associated with adhesion molecules and inflammatory responses rather than angiogenesis and growth factors. Data presented here contributes important information on the early stages of development of endometriosis, and will greatly contribute to development of novel therapies that inhibit the responses induced by ectopic endometrium.

**Acknowledgments**

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lfs.2009.03.015.

**References**


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**Fig. 2.** A model of the development of endometriosis based on data from this study.
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