Clinical significance of M2 macrophages expressing heme oxygenase-1 in malignant transformation of ovarian endometrioma

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Phenotype
Heme oxygenase-1

Abstract
Malignant transformation of endometriosis is a rare and still poorly understood event, but is associated with the distortion of the pro-oxidant and anti-oxidant balance. The aim of the present study was to quantify the numbers of macrophages polarized as M1 or M2 phenotypes and the expression of heme oxygenase (HO)-1 in tissue sections from patients with benign ovarian endometrioma (OE) and its malignant transformation (endometriosis-associated ovarian cancer, EAOC). We performed a retrospective study at the Department of Gynecology, Nara Medical University hospital from December 2012 to March 2015. This study included 53 patients with OE (n = 33) and EAOC (n = 20), and we evaluated polarized functional status of macrophages by immunohistochemical staining of CD68, CD11c, CD163 and HO-1. The number of the M1 phenotype (CD11c⁺, p = 0.001) and the M2 phenotype (CD163⁺, p = 0.009) was significantly lower in EAOC patients than in OE patients. Analyzing the correlations between the studied markers, the expression of CD68, CD11c, and CD163 proteins significantly correlated with each other (p < 0.001). The number of M2 phenotypes expressing HO-1 was significantly decreased in the EAOC group, compared with the OE group (P < 0.001), demonstrating sustained downregulation of an antioxidant marker, HO-1, in EAOC. In conclusion, reduced number of M2 macrophages expressing HO-1 may have an important role in promoting malignant transformation of OE.

1. Introduction

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterus, most often in the pelvic peritoneum and ovaries. This disorder affects an estimated 10% of women in the reproductive age group and is basically an estrogen-dependent benign gynecological disease. Repeated episodes of retrograde menstruation or ovarian hemorrhage occur in the peritoneal cavity or ovarian endometrioma (OE), respectively [1]. Endometriosis results in a local accumulation of hemoglobin, heme and iron species, which causes severe oxidative stress and antioxidants depletion, leading to distortion in the redox balance [2]. Altered homeostatic redox balance of the environment may support chronic inflammation, uncontrolled proliferation and then malignant transformation [2]. Actually, endometriosis increases the subsequent risk of developing endometriosis-associated ovarian cancer (EOAC) [3,4]. Endometriotic lesions are highly infiltrated with various leukocytes, including macrophages that secrete antioxidants and immunosuppressive factors [1,5–7]. Endometriosis infiltrating macrophages might adapt to these stressful environmental conditions by secreting antioxidants that control excess oxidative stress [1].

Oxidative stress and inflammation in the surrounding environment contribute to several aspects of macrophage functions including recruitment, activation and the shift in cell polarity. Macrophage polarization may have a distinct role in the inflammatory, immune and neoplastic diseases [8,9]. Macrophages are classified into the pro-inflammatory, classically activated M1 macrophages that support tumor progression and malignancy [8,9]. For the phenotypical characterization of infiltrating macrophages, immunohistochemistry employing selected literature-based prototype-antibodies against CD11c, CD163 and CD68 was evaluated in this study. Although CD11c is a specific marker in macrophages and dendritic cells [10], CD11c-based immunohistochemistry is used as a M1 phenotype specific marker [11,12].

CD11c, a member of integrin family, induces tissue injury and the
inflammatory response [13]. The protein expression of M2 phenotype specific marker CD163 is tested via immunohistochemistry. CD163 is a hemoglobin/haptoglobin scavenger receptor and acts to protect tissues from oxidative damage [5]. CD68 is specifically expressed by tissue macrophages and used as a pan-macrophages marker [14].

When the microenvironment is altered in endometriosis by an excess oxidative stress and inflammatory insult, knowledge on how macrophages respond to its changes is limited. An antioxidant enzyme, heme oxygenase-1 (HO-1), is a key mediator that allows the resolution of inflammatory processes [15,16]. HO-1 is responsible for the cata-

bolism of heme to carbon monoxide (CO), biliverdin, and iron. HO-1 characteristics were collected from a database containing medical re-

2.1. Tissue samples

sections from patients with OE and EAOC.

3. Results

3.1. Study population

The major clinical and pathological characteristics of the two groups of patients are listed in Table 1. There was a significant difference between the OE and EAOC group in median values of various factors such as age (p < 0.001), tumor size (p < 0.001), menopausal status (p < 0.001) and CA125 levels (p < 0.001). There were no statistical differences for parity status between the two study groups (P > 0.05).

3.2. The number and phenotypes of macrophages in the OE and EAOC group

We identify and quantify the amounts of accumulated cells in tissue samples by immunostaining. Sample immunohistochemical images are shown in Fig. 1. CD68-, CD11c- and CD163-positive cell staining is mainly seen in the cytoplasm and/or membrane of macrophages. Generally, CD163+ cells and CD68+ cells were ampler than CD11c+ cells in both tissues. CD marker-positive cells revealed a randomly dispersed distribution, but 'hot-spot' areas were identified in the OE and EAOC tissue specimens. A greater number of CD68+ cells, CD11c+ and


table 1

demographic and clinical characteristics of the study population.

<table>
<thead>
<tr>
<th>Baseline characteristics of the two groups</th>
<th>OE group</th>
<th>EAOC group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>182</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Benign ovarian endometrioma</td>
<td>35</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Clear cell (n=13)</td>
<td>51.1 ± 11.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrioid (n=3)</td>
<td>38 (26-65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous (n=1)</td>
<td>50 (36-69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucinous (n=1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other (n=2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIGO stage</td>
<td>IA(n=5)</td>
<td>IIC(n=13)</td>
<td></td>
</tr>
<tr>
<td>IC(n=13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II(n=2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis, mean ± SD</td>
<td>37.2 ± 8.5</td>
<td>51.1 ± 11.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>median (range)</td>
<td>38 (26-65)</td>
<td>50 (36-69)</td>
<td></td>
</tr>
<tr>
<td>Nulliparous n (%)</td>
<td>10 (30.3%)</td>
<td>10 (50.0%)</td>
<td>0.152</td>
</tr>
<tr>
<td>Premenopause n (%)</td>
<td>32 (97.0%)</td>
<td>11 (55.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A maximum diameter of the cyst, mean ± SD</td>
<td>72.0 ± 24.4</td>
<td>131.6 ± 47.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>median (range)</td>
<td>70 (25-137)</td>
<td>120 (38-230)</td>
<td></td>
</tr>
<tr>
<td>CA125, mean ± SD</td>
<td>73.9 ± 47.1</td>
<td>687.7 ± 1428.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>median (range)</td>
<td>61 (17-235)</td>
<td>110 (6-6272)</td>
<td></td>
</tr>
</tbody>
</table>

OE ovariana endometrioma, EAOC endometriosis-associated ovarian cancer, FIGO The International Federation of Gynecology and Obstetrics.
cells, or CD163⁺ cells was observed in the stroma of both tissues. The number of CD68⁺ cells (Fig. 1B and G), CD11c⁺ cells (Fig. 1C and H) and CD163⁺ cells (Fig. 1D and I) were lower in the EAOC group, compared with the OE group. Our immunohistochemical results also showed that CD68, CD11c and CD163 expression was downregulated in the majority of EAOC samples compared with corresponding adjacent noncancerous endometriotic tissues (Fig. 2).

The number of macrophage infiltration according to CD68, CD11c and CD163 counts are summarized in Table 2. The mean CD68⁺, CD11c⁺ and CD163⁺ counts in the EAOC group were significantly lower than those in the OE group. We further analyzed M1-like cells/ M2-like cell ratio (M1/M2) based on cell staining with CD11c⁺ cells and CD163⁺ cells in the consecutive sections. The M1/M2 ratio was significantly lower in the EAOC group, compared with the OE group (p < 0.001).

Table 3 shows comparison of the correlations between the studied markers, including CD68, CD11c, and CD163. Analyzing the correlations between the studied markers, the expression of CD68, CD11c, and CD163 proteins significantly correlated with each other (p < 0.001).

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>The OE group</th>
<th>The EAOC group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c expression</td>
<td>34.0 (0.6-73.8)</td>
<td>4.1 (0.8-21.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>CD68 expression</td>
<td>100.9 ± 40.0</td>
<td>68.6 ± 18.3</td>
<td>0.007</td>
</tr>
<tr>
<td>CD163 expression</td>
<td>94.5 ± 28.6</td>
<td>72.8 ± 27.4</td>
<td>0.009</td>
</tr>
<tr>
<td>HO-1 expression</td>
<td>0.321 (0.007-1.0)</td>
<td>0.065 (0.011-0.213)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

OE ovariana endometrioma, EAOC endometriosis-associated ovarian cancer, HO-1 heme oxygenase-1.
ectopic endometrial cells and epithelial cancer cells were generally
4. Discussion

tissues, compared with the OE tissues (P
3.3. The HO-1 expression in OE and EAOC tissues

Next, the HO-1 expression was evaluated using im-
munohistochemistry in the OE and EAOC group (Fig. 1E and J). The
ectopic endometrial cells and epithelial cancer cells were generally
unstained. HO-1 staining was strongly observed in the stroma.
The number of HO-1-positive cells were significantly lower in the EAO

tissues, compared with the OE tissues (P < 0.001) (Table 2). Since HO-1
acts as a target for M2 macrophages, we further investigated the co-
expression pattern of HO-1 and CD163 in OE and EAOC tissues. Indeed,

Comparison of the association between CD163 and HO-1. The Pearson
and Spearman's rank correlation coefficient.

<table>
<thead>
<tr>
<th></th>
<th>CD11c</th>
<th>CD68</th>
<th>CD163</th>
</tr>
</thead>
<tbody>
<tr>
<td>y</td>
<td>0.484</td>
<td>0.422</td>
<td>0.270</td>
</tr>
<tr>
<td>rs</td>
<td>0.524</td>
<td>0.552</td>
<td>0.270</td>
</tr>
<tr>
<td>p</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 3

Comparison of the correlations between the studied markers. Correlations be-
tween the distribution of CD68+, CD11c+, and CD163+ cells of each case are shown.

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First, we found that CD163+ M2-like cells were outnumbered by
CD11c+ M1-like cells in the two groups. M1 macrophages secrete in-
flammatory cytokines and contribute to the adaptive immune response
through Th1 responses [8,18]. In contrast, anti-inflammatory M2
macrophages induce Th 2 responses and tissue repair [8,18]. Environ-
mental stimuli shape macrophage plasticity in the OE and EAOC
groups, and modify macrophages phenotypes from an anti-tumor M1
type to a pro-tumor M2 type [8,18–20]. It has been established that
CD163 is classified into the marker of alternatively activated M2 phe-
notype and acts as a hemoglobin scavenger receptor on macrophage
[5]. The possible role of macrophages in carcinogenesis has not been
directly examined between the OE and its malignant transformation.
However, our data support the finding that endometriosis environment
itself has a propensity to develop into ovarian cancer.

Second, although HO-1 expression in the stromal macrophages has
been seen in the other cancer tissues [21], the impact of HO-1 ex-
pression on EAOC progression has not been explored. We have found
that the CD163+ M2-like cells were HO-1+ in OE, but malignant
transformation may be associated with, and at least partly due to,
reduced number of M2 phenotypes expressing HO-1. Endometriotic cyst
fluid contains much higher levels of iron-related compounds, such as
hemoglobin species, heme and free iron, compared with EAO sample
[22]. Repeated episodes of hemorrhage in OE induce excess oxidative
stress and trigger DNA damage, mutations and genome instability,
demonstrating the dichotomy between cytototoxicity and proliferation
in endometriotic cells [1,2,19,23]. Thus, stimuli in environment create
increased cellular susceptibility to oxidant-mediated cell killing or

carcinogenesis. In benign OE, autooxidation and Fenton reaction of ho-
moglobin from the ferrous Fe2+ (oxyhemoglobin) state to the ferric
Fe3+ (methemoglobin) leads to the increase of ROS and DNA damage in cells, which
produces HO-1 in the EAOC microenvironment. EAOC macrophages
produce HO-1 in the EAOC microenvironment. EAOC macrophages
demonstrate sustained downregulation of an antioxidant marker, HO-1,
possibly due to decreased oxidative stress. Taken together, the pattern
of redox balance supports that reduced oxidative stress may be involved
in the pathogenesis of malignant transformation.

Third, the expression of HO-1 in macrophages was decreased in
some cancers such as lung cancer [26]. Theoretically, HO-1 down-
regulation leads to the increase of ROS and DNA damage in cells, which
may promote the initiation of carcinogenesis [27]. Conversely, HO-1
has been detected in tumor-infiltrating macrophages and shows the
impact on cancer progression, aggressiveness, invasion, metastasis, and
poor prognosis [27,28]. At late phase of tumorigenesis, HO-1 over-
expression may promote cancer progression through inducing the ex-
pression of angiogenic factors, such as vascular endothelial growth
factor (VEGF) [29]. Several lines of evidence have highlighted the role
of HO-1 in cancer progression through modulating tumor micro-
environment [27]. Thus, the function of HO-1 in the pathogenesis of
cancer progression remains controversial.

In conclusion, the aberrant microenvironment in endometriotic
milieu can induce alterations in macrophage recruitment and its po-
larization phenotype, which significantly induces the shift to M2 phe-
notype expressing HO-1. Reduced number of M2 macrophages expres-
sing HO-1 may have an important role in promoting malignant
transformation of endometriosis. Although the exact reasons for EAOC
carcinogenesis are unclear at the present time, this study supports the
homeostatic redox balance hypothesis that there are at least two phases

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of EAOC tumorigenesis: the initial wave of the reduced infiltration of M2 macrophages would be followed by the second wave of subsequent increase of ROS and DNA damage in endometriotic cells, and then the final big wave of EAOC carcinogenesis. The specific molecular mechanisms by which reduced HO-1 expression favors the promotion of malignant transformation of endometriosis still require further investigation.

Funding

This study was not funded.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional review committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The protocols were approved by the ethics committee of Nara Medical University (Approved number 1570/2017). This study is a retrospective observational study, carried out by the opt-out method of our hospital website.

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