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Undifferentiated Cell Markers in the Endometrium of Women with Endometriosis

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Abstract

Endometriosis is a benign chronic disease characterized by the presence of tissue resembling the endometrium outside the uterine cavity. The etiology of endometriosis appears to be polygenic and multifactorial, but the exact pathogenic mechanisms are still unclear. In recent years, adult stem cells have been identified in various human tissues including the endometrium, and these cells may be associated with the pathogenesis of proliferative gynecological diseases such as endometriosis. The identification of adult stem/progenitor cells (SPCs) in tissues is highly complex and can be inferred from the identification of undifferentiated cell markers. This study investigated the expression of undifferentiated markers Musashi-1, Oct-4, and c-kit in eutopic endometria of patients with and without endometriosis and endometriotic lesions using immunohistochemistry (IHC) on a non-biotin polymer detection system. Four tissue specimens of normal endometrium and six samples of eutopic and ectopic endometria from patients with endometriosis were evaluated. Categorical variables were analyzed by the chi-square test and immunoreactivity scores (IRS) were tested using the Kruskal-Wallis test. Musashi-1 protein expression presented as cytoplasmic or nuclear staining, Oct-4 staining was predominantly nuclear, and c-kit protein expression presented as transmembrane and cytoplasmic staining. The three markers were positively immunostained in all endometrial compartments evaluated (glandular epithelium, stroma, and endothelium). The percentage of Musashi-1 expressing cells was significantly higher in the endothelium of patients with endometriosis ($p < 0.05$). Musashi-1 and c-kit demonstrated staining of cell groups, which may correspond to stem cell clusters. Overall, the three markers demonstrated high staining intensity and percentage. c-kit expression was significantly higher in glandular epithelium than in the stromal compartment ($p < 0.05$), and stromal IRS was significantly higher in eutopic endometrial samples from patients with endometriosis ($p < 0.05$). In conclusion, putative endometrial SPCs may play a role in the physiopathology of endometriosis, not only in the origin of ectopic implants, but also through involvement in neoangiogenesis.

Keywords: *Surface antigens. Adult stem cells. Endometrium. Endometriosis. Physiopathology.*

Introduction

Endometriosis is a benign chronic disease that is characterized by the presence of glandular and/or stromal tissue resembling the endometrium outside the uterine cavity. The incidence of endometriosis varies widely according to the study population, affecting 5–15% of women of reproductive age and approximately 35–50% of infertile patients and/or patients with chronic pelvic pain (GAZVANI and TEMPLETON, 2002; JENSEN et al., 2002; VINATIER et al., 2001).

Even though it has been extensively studied, the pathogenesis of endometriosis is still poorly understood, and a combination of several aberrant biological processes may be involved (BULUN, 2009). Evidence for the existence of adult stem/progenitor cells (SPCs) in the human endometrium is now emerging (GARGETT, 2007), suggesting that these cells may be involved in the physiopathology of endometrial proliferative disorders such as endometriosis (DU and TAYLOR, 2007; GARGETT and MASUDA, 2010; MARUYAMA et al., 2010; Oliveira et al., 2012). Studies

with SPCs have generated great interest over the last decade due to their potential therapeutic implications (MIMEAULT and BATRA, 2008), and somatic SPCs have been identified in various tissues and organs including bone marrow, breast, prostate, brain, liver (LI and CLEVERS, 2002), and, more recently, endometrium (CHAN, SCHWAB, and GARGETT, 2004).

Despite the large number of studies on stem cells, cell markers that are specific for adult SPCs have not yet been identified. Thus, the identification of a panel of molecular markers would be of great importance for the characterization of adult quiescent SPCs. However, to date, SPCs have been characterized phenotypically by the expression of markers typical of undifferentiated progenitor cell lineages and the absence of markers of more differentiated cell types (GRITTI, VESCOVI, and GALLI, 2002; SOUZA et al., 2003). The nuclear transcription factor Oct-4 (PESCE and SCHÖLER, 2001) and the stem cell factor receptor c-kit (SOGO et al., 1997) are two cell markers widely used in stem cell studies. Expression of the RNA-binding protein Musashi-1, which is associated with self-renewal of progenitor cells, has been detected in neural SPCs, and this molecule may be a promising marker of endometrial SPCs (GÖTTE et al., 2008).

This study aimed to correlate the presence of endometrial SPCs with the pathogenesis of endometriosis by analyzing the expression of Oct-4, c-kit, and Musashi-1 markers in ectopic endometria of women with endometriosis and eutopic endometria of women with and without endometriosis.

Methods

This is a controlled cross-sectional study that included eutopic endometrial samples from premenopausal women who underwent hysterectomy for various medical indications (n = 4), endometriotic tissue samples (n = 6), and eutopic endometrial samples from patients who underwent surgery for treatment of deep infiltrating endometriosis with intestinal involvement (n = 6) confirmed in the histopathological study. The study was approved by the Research Ethics Committee at Federal University of Minas Gerais (COEP–UFMG), Belo Horizonte, Brazil, under protocol number ETIC/0628.0.203.000-09. Patients with polyps/endometrial hyperplasia and/or patients who were under treatment for endometriosis or receiving hormone suppression treatment in the previous six months were not included in the study.

Immunohistochemistry

Tissue samples were fixed in 10% formalin and embedded in paraffin blocks. Sections were incubated at 56 °C for 24 h, deparaffinized in xylene, and rehydrated in a decreasing ethanol series. Heat-induced antigen retrieval was performed in a steamer (98–100 °C) for 45 min to achieve optimal exposure of the epitopes of interest in ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) for Oct-4 and c-kit and citrate buffer (pH 6.0) for Musashi-1.

All immunohistochemistry steps were performed using the Novolink™ nonbiotin polymer detection system kit (Novocastra®, Newcastle Upon Tyne, UK).

Endogenous peroxidase activity was blocked with peroxidase block for 5 min.

Sections were incubated for 5 min with protein block to reduce background staining. Next, sections were incubated with rabbit polyclonal primary antibody (Abcam, Cambridge, UK) diluted 1:50 (Oct-4 and c-kit) and 1:100 (Musashi-1). Following incubation with the primary antibodies for 60 min at room temperature in a moisture chamber, a post-primary block was added for 5 min to enhance penetration of the polymer reagent. Immunohistochemical reactions were developed using 3,3'-diaminobenzidine (DAB) and sections were counterstained with Harris's hematoxylin.

Human intestinal tissue sections were used as positive controls for Musashi-1, Oct-4, and c-kit antibodies. Two sections of eutopic endometrium from a healthy patient were used as negative controls, which included the omission of the primary antibody in one section and incubation with rabbit normal serum (normal rabbit IgG –0.1 mg/mL; Calbiochem, Darmstadt, Germany) diluted 1:100 in the other section.

The slides were examined under an Olympus CX31 light microscope (Olympus, Tokyo, Japan) by two independent observers and the median scores between the two observers were considered. The percentage of positive cells was estimated semiquantitatively at 40x magnification. The stained sections were scored for each marker evaluated according to the immunoreactive score (IRS) (Remmele and Stegner, 1987), which has been validated in human endometrium for the Oct-4 antibody (BENTZ et al., 2010). The predominant cell staining pattern (nuclear, transmembrane, and/or cytoplasmic), endometrial

location (glandular epithelium, stroma, and vascular endothelium), and immunostaining of cell clusters were also evaluated.

Statistical analysis

Categorical variables were analyzed by the chi-square test and corrected by the likelihood ratio test. The immunoreactivity scores were tested using the Kruskal-Wallis test, whereas nonparametric paired samples were analyzed using the Wilcoxon test. The significance level was set at $p < 0.05$.

Results

Positive staining of Musashi-1 was found in the cytoplasm and nucleus of 93.7% of samples, but not in the cell membrane of any of the samples (Fig. I-A). No staining of Oct-4 was found in the cell membrane, little staining was found in the cytoplasm (6.7% of samples), and the predominant staining pattern of Oct-4 was nuclear (96.9%) (Fig. I-B). c-kit staining was found in the membrane (43.0%) and cytoplasm (75.0%), but not in the nucleus.

Musashi-1 demonstrated glandular and stromal staining in a high percentage of samples (93.8%) and endothelium staining in 50.0% of samples. Oct-4 staining signal was strong in glandular epithelium (93.8%), moderate in stroma (75.0%), and intermediate in vascular endothelium (46.2%). c-kit staining signal was strong in glandular epithelium (81.3%), moderate in stroma (43.8%), and only 6.7% of samples were stained in vascular endothelium. Musashi-1 staining was significantly higher in vascular endothelium samples from the patients with endometriosis than in normal and eutopic samples ($p = 0.021$) (Table 1). Conversely, no significant differences in Oct-4 and c-kit expression were observed across groups ($p > 0.05$, Table 1).

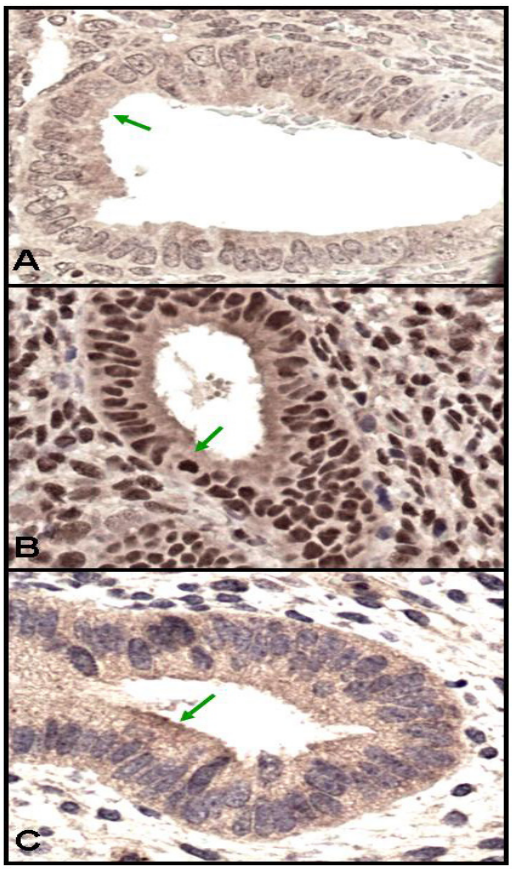

Figure I. *Eutopic endometrial sample from a healthy patient (200x) showing the cell staining pattern of Musashi-1, Oct-4, and c-kit antibodies. The arrow indicates the predominant immunostaining pattern for each antibody. (A) Cytoplasmic and nuclear expression of Musashi-1 protein. (B) Predominantly nuclear expression of Oct-4. (C) Cytoplasmic and transmembrane expression of c-kit protein.*

Table1. *Expression of Musashi-1, Oct-4, and c-kit proteins according to endometrial location and tissue type (number of stained samples/total number of samples).*

Protein/location	Normal tissue	Eutopic with endometriosis	Lesion
Musashi-1			
Glandular epithelium	4/4	5/6	6/6
Stroma	4/4	5/6	6/6
Vascular endothelium*	0/4	4/5	3/5
Oct-4			
Glandular epithelium	4/4	6/6	5/6
Stroma	4/4	4/6	4/6
Vascular endothelium	3/4	2/4	1/5
c-kit			
Glandular epithelium	4/4	4/6	5/6
Stroma	1/4	3/6	3/6
Vascular endothelium	0/4	1/5	0/6

*p < 0.05, Chi-square test.

Analysis of the pooled samples for Musashi-1 staining showed a moderate percentage of stained cells and staining intensity, and no significant difference in staining intensity was observed between glandular and stromal compartments. Additionally, there were no significant differences in immunostaining percentage, intensity, and IRS for Musashi-1 across sampling groups/tissue types.

Analysis of the pooled samples for Oct-4 expression showed a high proportion of stained cells (> 80%) and high staining intensity (moderate and strong) (Fig. II), resulting in high IRS for all samples. In addition, no significant difference in IRS between glandular and stromal compartments was observed for Oct-4 (pooled samples). Similarly, there were no significant differences in immunostaining percentage, intensity, and IRS for Oct-4 across sampling groups/tissue types.

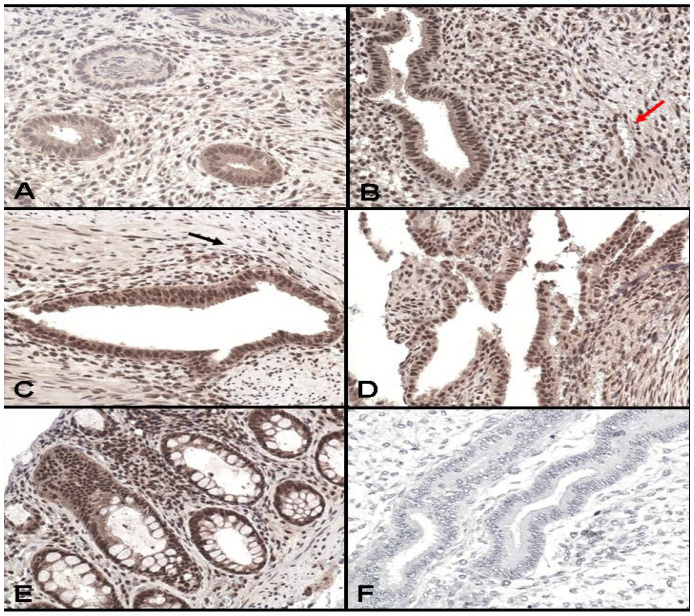

Figure II. *(A) Endometrial sample from a healthy patient showing strong glandular and stromal staining. (B) Eutopic endometrial sample from a patient with endometriosis showing strong staining. Stained endothelial cells. (C and D) Endometriotic lesions with strong and moderate immunostaining. Transition zone between the endometriotic lesion and normal tissue. (E) Positive control. (F) Negative control. (200x – Oct-4).*

Analysis of the pooled samples for c-kit staining showed heterogeneity in the percentage of stained cells (10–50%) and intensity ranging from weak to moderate staining. The comparison between glandular and stromal compartments for c-kit staining (immunoreactive scores) showed that protein expression was significantly higher in the epithelial compartment than in the stromal compartment (Fig III).

Additionally, stromal IRS was significantly higher in eutopic endometrial samples than in normal and lesion samples ($p < 0.05$). Conversely, no significant differences in total and glandular IRS were observed for c-kit staining across sampling groups/tissue types (Fig. IV).

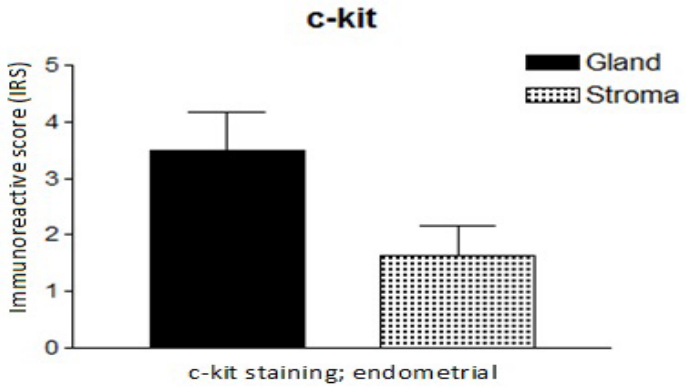

Figure III. *Glandular and stromal immunoreactive scores (IRS) for c-kit staining (pooled samples, $p < 0.05$).*

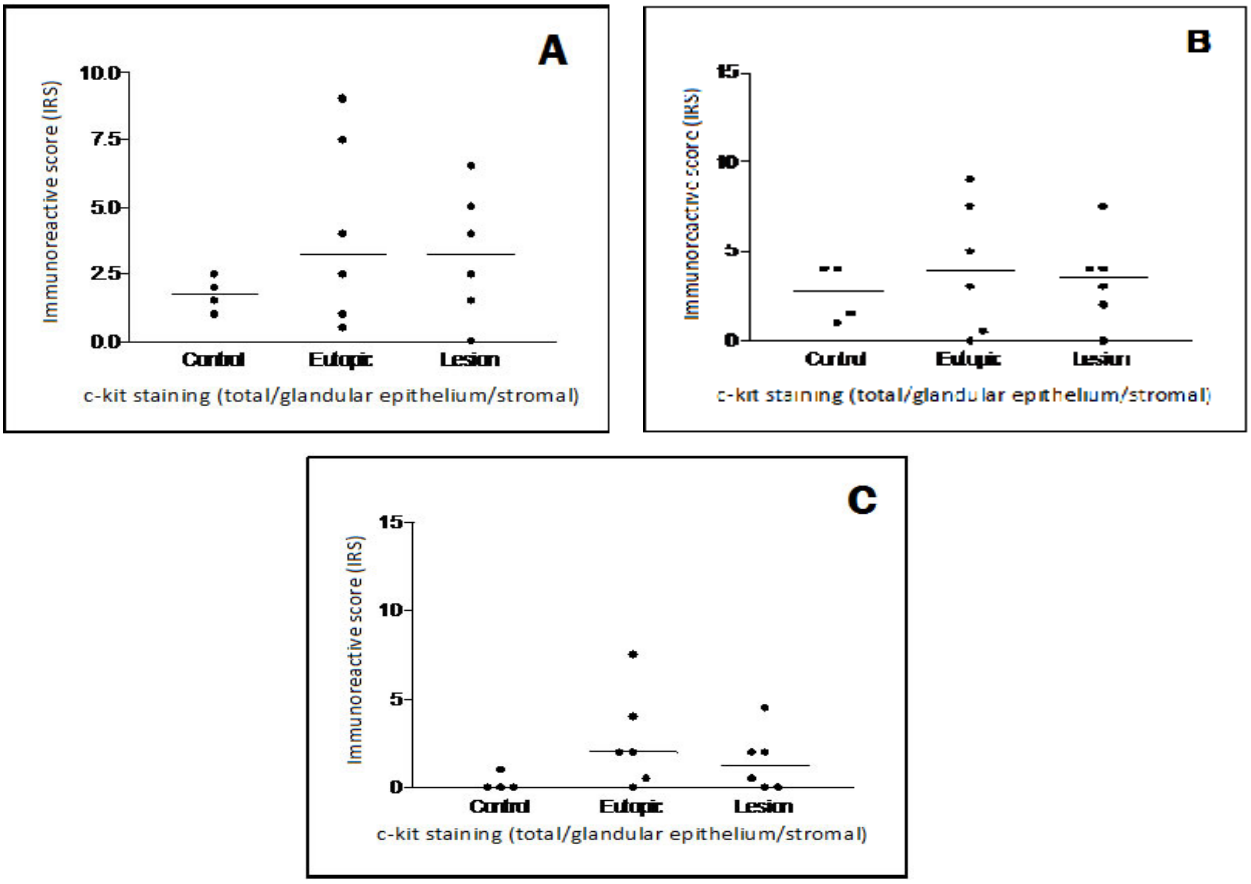

Figure IV. *Immunoreactive scores (IRS) for c-kit staining between sampling groups. (A) Total IRS, (B) glandular epithelium IRS, and (C) stromal IRS.*

Clustered staining was observed only for Musashi-1 and c-kit.

Discussion

In this study, we show that the pattern of cellular expression of the three markers evaluated (Musashi-1, Oct-4, and c-kit) was partially consistent with their known cellular locations. Musashi-1 protein expression presented as cytoplasmic or nuclear staining, a similar pattern to the one described by Götte et al. (2008). Because Oct-4 is a transcription factor, its pattern of cellular expression was, as expected, predominantly nuclear (96.7% of samples). Conversely, c-kit protein expression presented as transmembrane and cytoplasmic staining, but no nuclear staining was observed, and these results are consistent with the known function of c-kit as a transmembrane tyrosine-kinase receptor (SOGO et al., 1997).

The three markers were positively immunostained in all endometrial compartments evaluated (glandular epithelium, stroma, and endothelium). A similar staining proportion was observed between glandular epithelium and endometrial stroma for Musashi-1 and Oct-4, but staining was predominantly epithelial for c-kit.

Staining positivity for Musashi-1 was higher in samples of endometriotic lesions than in samples of eutopic endometrium with and without endometriosis, suggesting a possible correlation between SPCs and neoangiogenesis. Even though Götte et al. (2008) did not examine the vascular compartment, Musashi-1 expression was significantly higher in the stroma of endometriotic lesions than in normal endometrium. Similarly to our findings, the authors of that study also found strong staining in cell groups, which may correspond to SPC clusters.

Oct-4 was not differentially expressed in endometrial compartments across sampling groups/sample types. Similar results were reported by Matthai et al. (2006), who detected Oct-4 expression in all endometrium samples using reverse transcriptase polymerase chain reaction (RT-PCR), but a variable pattern of expression using immunohistochemistry (IHC). Moreover, Oct-4 demonstrated expression in epithelial cells of eutopic endometrium and ectopic epithelial cells of stage IV endometriotic lesions, and protein expression was significantly higher in the latter (Sbracia et al., 2007). Bentz et al. (2010) investigated Oct-4 expression using RT-PCR and IHC in the different phases of the menstrual cycle and found that Oct-4 was not differentially expressed in follicular and lutheal phase endometrium.

The expression of c-kit was predominantly glandular, but the protein was also expressed in the stromal compartment, and, rarely, in the vascular endothelium.

Additionally, in endometrial stroma, c-kit expression was significantly higher in samples of eutopic endometrium from patients with endometriosis than in samples from normal endometrium and endometriotic lesions. Nevertheless, evidence of c-kit expression in the endometrium is conflicting. For instance, Lammie et al. (1994) found no evidence of c-kit expression in the endometrium. Conversely, Elmore et al. (2001) reported that 93% of benign proliferative glandular endometrial samples and 79% of secretory endometria immunostained positively for c-kit. Later, Cho et al. (2004) reported that c-kit was not detected in the fetal endometrium, but it was prominently expressed in the stroma and basalis glands in all other lifetime endometrium samples.

Osuga et al. (2000) investigated the expression of c-kit in peritoneal fluid of women with and without endometriosis and suggested that SPCs may play a role in the pathogenesis of endometriosis. Moreover, Uzan et al. (2005) suggested that the higher expression of c-kit in deep endometriosis indicates that SPCs are involved in the pathogenesis of endometriosis, and similar findings were also reported by Sbracia et al. (2007).

The three markers evaluated in our study showed high staining intensity and percentage for candidate SPC markers. Based on the results of other assays for SPCs such as clonogenic assays (CHAN, SCHWAB, and GARGETT, 2004), the expected expression for Musashi-1 should be lower than was observed in our study, whereas our findings for Oct-4 and c-kit expression are consistent with other studies (Sbracia et al., 2007; Bentz, 2010; Elmore et al., 2001). Taken together, these results suggest that Musashi-1, Oct-4, and c-kit have low specificity for the identification of SPCs and may be expressed in cells other than SPCs. Thus, novel molecules that have greater specificity for undifferentiated cells and are validated by other trials must be identified in future studies.

In conclusion, our results suggest a possible role for putative endometrial SPCs in the physiopathology of endometriosis, because the proportion of stained cells

with undifferentiated markers was equal to or greater than in specimens from patients with endometriosis. The higher staining intensity for Musashi-1 in the vascular endothelium compartment of endometriotic lesions may be related to the angiogenesis of endometriosis, and the high percentage of c-kit-expressing cells in eutopic endometrial samples from patients with endometriosis may represent further evidence for the participation of reflowed SPCs in the origin of ectopic endometrial implants.

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