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ARTICLE

Progressive development of endometriosis and its hindrance by anti-platelet treatment in mice with induced endometriosis

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Abstract We have recently shown that platelets drive smooth muscle metaplasia (SMM) and fibrogenesis in endometriosis through epithelial-mesenchymal transition (EMT) and fibroblast-to-myofibroblast transdifferentiation (FMT). To see whether this is true *in vivo*, this prospective, randomized, and serially evaluated mouse investigation was conducted. Endometriosis was induced in female Balb/C mice, which were then randomly divided into two groups: Tanshinone IIA (TAN) and control (CTL) groups. TAN mice were treated with TAN but CTL mice received none. Every week until the 6th week after induction, five mice from each group were killed. Lesion weight was measured and lesion samples were subjected to immunohistochemistry and histochemistry analysis of platelet aggregation (CD41), E-cadherin, TGF-β1, phosphorylated Smad3, α-SMA, collagen I, CCN2, LOX, desmin and SM-MHC, and the extent of fibrosis was evaluated by Masson trichrome staining. It was found that endometriotic lesions exhibited progressive cellular changes consistent with the progressive EMT, FMT, SMM, and fibrogenesis. TAN treatment resulted in significant hindrance of EMT, FMT, SMM and fibrogenesis, and reduced lesion weight (all *P*-values <0.05). These data corroborate the notion that endometriotic lesions undergo progressive EMT and FMT, giving rise to SMM and ultimately fibrosis. This understanding sheds new light onto the natural history of endometriosis.

KEYWORDS: endometriosis, epithelial-mesenchymal transition, fibroblast-to-myofibroblast transdifferentiation, fibrosis, platelet, smooth muscle metaplasia

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Introduction

Characterized by the deposition and growth of endometrial-like tissues outside the uterine cavity, endometriosis is an oestrogen-dependent disorder and a major contributor to pelvic pain and subfertility affecting 6–10% of women of reproductive age (Giudice and Kao, 2004). Despite exponential growth in the number of publications on endometriosis in the last four decades (Guo, 2014), its pathophysiology is incompletely understood (Giudice and Kao, 2004). As a result, the development of effective, targeted therapy or preventative measures for this debilitating disease, especially the development of non-hormonal drugs, has been painfully slow (Guo, 2014), over which there is a palpable disappointment (Vercellini et al., 2011).

One seemingly insurmountable roadblock to the elucidation of the pathophysiology of endometriosis is our rudimentary knowledge of the natural history of endometriosis, even though the consensus is that over time endometriosis is a progressive and dynamic disease in spontaneous and induced endometriosis (D'Hooghe et al., 1996a, 1996b; Harirchian et al., 2012). Despite thousands of published studies documenting various histologic, cellular and molecular aberrations in endometriotic lesions, the lesions appear to refuse to part with their secrets of natural history. Consequently, the currently widely used rAFS/rASRM staging system does not correlate well with either the severity of symptoms, progression or prognosis (Koninckx et al., 2011). It is also difficult to piece together most, if not all, published studies.

Taking the cue from the observation that ectopic endometrium experiences cyclic and thus repeated bleeding (Brosens, 1997), a cardinal sign of tissue injury, we have recently shown that platelets play important roles in the development of endometriosis (Ding et al., 2015; Guo et al., 2015a, 2015b, 2015c; Zhang et al., 2015) and proposed that endometriotic lesions are essentially wounds that undergo repeated tissue injury and repair (ReTIAR) (Ding et al., 2015; Guo et al., 2015a, 2015b, 2015c). Our recent investigation indicates that, as the result of this ReTIAR, the endometriotic lesions, stimulated by platelet-derived transforming growth factor-β1 (TGF-β1), activate the TGF-β1/Smad3 signalling pathway and undergo epithelial-mesenchymal transition (EMT) and fibroblast-to-myofibroblast transdifferentiation (FMT), resulting in increased cellular contractility and collagen production, leading ultimately to fibrosis (Zhang et al., 2016a, 2016b). Prolonged exposure to activated platelets also leads to increased expression of α-smooth muscle actin (α-SMA) as well as markers of differentiated smooth muscle cells (SMC) in endometriotic stromal cells, which may be responsible for what is termed smooth muscle metaplasia (SMM) that is nearly universally seen in endometriotic lesions (Itoga et al., 2003; Khare et al., 1996; Matsuzaki and Darcha, 2013; Mechsner et al., 2005).

In light of these findings, we postulated that during the progression of endometriosis, we should see indications of the activated TGF-β1/Smad3 signalling pathway, progressive EMT and FMT, as shown by increased vimentin expression but decreased E-cadherin expression in the epithelial component of the endometriotic lesions. In addition, we should see increased expression of α-SMA, a marker for myofibroblasts and SMC (Hasegawa et al., 2003; Hinz et al., 2001), in the stromal component of the lesions. Concomitant with the increased α-SMA expression and other markers of SMC, we should see progressive SMM as well as increased fibrotic tissue content in endometriotic lesions. Our recent serial evaluation of endometriotic tissue samples harvested from time points after induction of endometriosis in female baboons are consistent with our hypothesis (Zhang et al., 2016a, 2016b).

In this study, this hypothesis was further tested in a mouse model of endometriosis. Our goal was two-fold. First, to test this hypothesis using serially harvested endometriotic tissue samples and immunohistochemistry (IHC) and histochemistry analyses. Second, to test the hypothesis that anti-platelet treatment should be effective in impeding the EMT, FMT, SMM and fibrogenesis.

Materials and methods

Mouse experiment protocol

Ninety virgin female Balb/C mice, 6 weeks old and about 16–18 g in bodyweight, were purchased from Shanghai BiKai Laboratory Animal Centre (Shanghai, China) and used for this study. They were maintained under controlled conditions with a light/dark cycle of 12/12 h, and had access to chows and water *ad libitum*. All experiments were performed under the guidelines of the National Research Council's Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and approved by the institutional experimental animals review board of Shanghai OB/GYN Hospital, Fudan University on 14 May 2014.

Among the 90 mice, 30 were randomly selected as donors that contributed endometrial tissue fragments, and the remaining 60 were recipients who received endometrial tissues from donor mice. After 2 weeks of acclimatization and before the endometriosis-inducing procedure (see below), all recipient mice were administrated a baseline hotplate test as reported previously (Ding et al., 2015). The donor mice were initially treated i.m. with oestradiol benzoate (0.2 μg/g bodyweight, Xinyi Chemistry, Shanghai, China) after 1 week of acclimation. One week later they were killed and their uteri were removed and harvested as in (Bacci et al., 2009). The uterine tissues were seeded in a Petri dish containing warm sterile saline, and split longitudinally with a pair of scissors, as in (Somigliana et al., 1999, 2001). The maximal diameter of the processed fragments was consistently smaller than 1 mm. Approximately 40–50 fragments per mouse were then injected i.p. into recipient mice. The recipient mice were divided randomly into two groups in equal size: Tanshinone IIA (TAN) and control (CTL) group. Mice in the TAN group were injected i.p. with TAN 12.5 μg/g dissolved in 300 μl sterile saline (shielded from light) every other day (i.e. days 2, 4, 6, . . ., 38 and 40), starting from 2 days after the induction of endometriosis (day 0, the day when induction was performed). Mice in the CTL group received no treatment at all. This 'intact' mode was chosen mainly because the study aimed to see the natural development history of endometriosis at the price of getting more conservative treatment effect due to the promotional effect of stress resulting from repeated injections (Cuevas et al., 2012; Long et al., 2016). To minimize possible individual variation, endometrial tissue

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