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ARTICLE

Anti-platelet therapy is efficacious in treating endometriosis induced in mouse

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Abstract In light of recent findings showing that platelets play important roles in the development of endometriosis in general and in fibrogenesis in particular, this study investigated the efficacy of Ozagrel, a TXA_2 synthase inhibitor, in a murine model of endometriosis. In addition, another mouse experiment was conducted to evaluate the effect of timing of platelet depletion and of sequential depletion of platelets and macrophages on the development of endometriosis. It was found that both the Ozagrel treatment and different platelet depletion schemes resulted in significant reduction in lesion growth (all *P*-values <0.01) along with improved hyperalgesia in mice with induced endometriosis. They also significantly reduced the expression of markers of proliferation, angiogenesis, inflammation and fibrosis as well as decreased macrophage infiltration in endometriotic lesions (all *P*-values <0.05). Compared with untreated mice, pre-emptive depletion of platelets as well as platelet depletion after induction resulted in significant reduction in lesion weight (both *P*-values <0.001), while sequential depletion of platelets and macrophages yielded similar reduction. These results, in conjunction with other roles that platelets play in the development of endometriosis, strongly argue for the potential of anti-platelet therapy in treating endometriosis.

KEYWORDS: endometriosis, fibrosis, hyperalgesia, macrophage, Ozagrel, platelet

Endometriosis is a common gynaecological disorder caused by the deposition and growth of endometrial-like tissues outside of the uterine cavity. It is an oestrogen-dependent disease, affecting roughly 6–10% of women of reproductive age (Giudice and Kao, 2004). It is a leading cause of disability in women of reproductive age and a major contributing cause for dysmenorrhoea, pelvic pain and subfertility (Farquhar, 2000), impacting negatively on their quality of life (Nnoaham et al., 2011). Currently, its pathogenesis and pathophysiology are poorly understood (Bulun, 2009). Therefore, there is

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a palpable disappointment over the frustratingly slow pace in drug development for endometriosis (Vercellini et al., 2011).

As with their eutopic counterpart, endometriotic lesions undergo cyclic and repeated bleedings that signify tissue injury and subsequent repair (Brosens, 1997), suggesting that platelets should be involved in the development of endometriosis. Indeed, it has recently been shown that platelets can promote angiogenesis and cellular proliferation (Ding et al., 2015), and platelet depletion and the treatment with a recombinant P-selectin can effectively reduce lesion area in murine models of endometriosis (Ding et al., 2015; Guo et al., 2015a). Platelets also induce the expression of oestrogen receptor β (ERβ) in endometriotic stromal cells (Zhang et al., 2015), which has shown recently to modulate apoptosis complexes and the inflammasome to drive the pathogenesis of endometriosis (Han et al., 2015). Consistent with the role of platelets in endometriosis, it has recently been reported that women with endometriosis are in a hypercoagulable state, featuring elevated platelet activation in their peripheral blood (Wu et al., 2015).

Endometriotic lesions are not passively affected by activated platelets, however. It has been recently reported that endometriotic stromal cells also secrete platelet-inducing factors such as thrombin and thromboxane A_2 (TXA_2) (Guo et al., 2016), effectively forming a two-way communication with platelets. Once activated and aggregated in the endometriotic lesions, platelets promote angiogenesis and cellular proliferation, and facilitate epithelial-mesenchymal transition, fibroblast-to-myofibroblast transdifferentiation, resulting in smooth muscle metaplasia and ultimately fibrosis (Zhang et al., 2016a, 2016b). Consequently, endometriotic lesions can be viewed as wounds that undergo repeated tissue injury and repair, ultimately leading to fibrosis (Guo et al., 2015a, 2015b). This may explain, at least in part, why endometriosis is very difficult to be managed by medication.

However, aside from the demonstrated effect of platelet depletion and of treatment with a recombinant P-selectin, no other anti-platelet treatment has been attempted in the preclinical setting. In addition, it is unclear as whether a preemptive depletion of platelets would be effective to forestall the induction of endometriosis. Nor is it clear whether later depletion of platelets would be just as effective as early platelet depletion.

In wound healing, platelets are among the first that are recruited to the wound site, followed by neutrophils, macrophages and then other immune cells (Witte and Barbul, 1997). One study published a decade ago indicates that endometriotic lesions are uncannily similar to a wound site, recruiting neutrophils and macrophages to the lesions in an orderly and sequential fashion (Lin et al., 2006). Since activated platelets recently been found to play a critical role in initiating inflammation (Sreeramkumar et al., 2014), depleting platelets first and then macrophages may appear to be an appealing strategy to disrupt the normal healing (promotional) process, hindering the development of endometriosis.

This study investigated the efficacy of Ozagrel, a TXA_2 synthase inhibitor (Loo et al., 1987), in a murine model of endometriosis. In addition, an additional mouse experiment was conducted to see whether the timing of platelet depletion has any effect on endometriosis development. Moreover, the study evaluated the effect of sequential depletion of platelets first and then of macrophages in the development of endometriosis.

Materials and methods

Animals and chemicals

All mice used in this study were purchased from Bikai Experimental Animal Centre (Shanghai, China). All mice were maintained under controlled conditions with a light/dark cycle of 12/12 h and had access to food 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 3 March 2014.

Ozagrel Sodium was purchased from YaoDa Pharmacology Industry Company (Shenyang, China). All other chemicals were purchased from Sigma unless stated otherwise.

Mouse experiment 1: effect of Ozagrel treatment on mice with induced endometriosis

Thirty-one ~6-week old female C57BL/6 mice, about 16–18 g in weight, were used for this study. After two weeks of acclimatization, endometriosis was surgically induced as reported previously (Lu et al., 2010). Briefly, surgery was performed under aseptic conditions to auto-transplant small pieces of endometrial tissue fragments to the peritoneum in lower parts of the abdominal and pelvic cavity. One day prior to the surgery and also on the day of surgery, but before the start of surgery, a hotplate test, described below, was administered to all mice and the latency, along with bodyweight, was measured. The two hotplate tests were performed within a 24-hour interval and the resultant latency readings were averaged to yield a single measurement, which represented a more accurate measurement of the mouse's sensitivity to noxious thermal stimuli. The mice were anaesthetized with 100 mg/kg ketamine hydrochloride. A laparotomy was performed and the right uterine horn was removed. The excised horns were immersed in a sterile saline solution and opened longitudinally. Each uterine segment was cut into four smaller fragments of roughly equal size. A total of four uterine tissue fragments were sutured to the peritoneal wall of the lower part of the lateral abdominal and pelvic cavity with a 7/0 braided silk suture, with the endometrium facing the abdominal wall. Then the midline incision was closed with a 3/0 braided silk suture. After surgery, all mice were administrated i.m. with penicillin of 40,000 IU to prevent possible infection.

Two weeks after the surgical induction of endometriosis, the second hotplate test was administrated along with bodyweight measurement. Again, the test was administered twice, one on day 13 and the other on day 14, within a 24-hour interval, and the two latency readings were averaged to yield a single measurement. Then the mice were randomly divided into three groups: Group HO (high-dosage Ozagrel, $n = 10$) that received intraperitoneal (i.p.) injections of Ozagrel 30 μg/g bodyweight in 300 μl sterile saline every two days for the next two weeks; Group LO (low-dose Ozagrel, $n = 10$) that received i.p. injections of Ozagrel 15 μg/g bodyweight in similar fashion and duration; and Group UT (control, or untreated group, $n = 11$) that received i.p.

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