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Low-dose mifepristone increased angiogenesis in a manner involving AQP1

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Abstract

Purpose To investigate the molecular mechanisms governing aquaporin-1 (AQP1)-mediated, mifepristone-induced angiogenesis and improve the understanding of low-dose mifepristone serving as an anti-implantation contraceptive drug.

Methods Human umbilical vein endothelial cells (HUVECs) were used to explore the effects of different concentrations of mifepristone (0, 65, and 200 nmol/L) on the activity of angiogenesis. Forty-five pregnant mice during the "window of implantation" were treated with different concentrations of mifepristone. HUVECs' proliferation was examined using a methyl thiazolyl tetrazolium (MTT) assay. The microvessel density (MVD) and the expression of AQP1 in endometrium were determined with immunohistochemical methods.

Results The MVD and the expression of AQP1 were significantly higher than controls. Mifepristone at 200 nmol/L significantly affected HUVECs' proliferation during culture over 12 h, and pretreatment with AQP1-specific siRNA significantly inhibited the mifepristone-enhanced cell proliferation.

Conclusions Low-dose mifepristone increased angiogenesis in a manner involving AQP1. This affords a new insight into the molecular mechanism underpinning the angiogenic effects of low-dose mifepristone.

Keywords Mifepristone · Endometrium · Implantation window · Angiogenesis · AQP1

Background

Endometrial receptivity toward embryo implantation is critical for pregnancy to occur. The window of implantation for the period of endometrial receptivity is short, occurs between days 20 and 24 of the menstrual cycle (5–9 days post-ovulation) [1]. In mouse, implantation occurs at a specific time, during a brief 24-h period 5 days after mating [2].

Endometrium is characterized by extensive vascular remodeling and expresses all the growth factors that are important for angiogenesis [3]. Angiogenesis is most intense in the secretory phase, and plays critical roles in endometrial growth and implantation [4–7]. Angiogenesis is a multistep process featuring degradation of matrix components by matrix metalloproteases, proliferation, and migration of endothelial cells to form tubular structures, and formation of matrix around the neovessels [8, 9].

Aquaporin-1 (AQP1), a water-selective member of the classical AQP family, primarily transports water across the plasma membrane, mediating rapid movement of bulky, osmotically driven fluid [10]. AQP1 plays a physiological role in the context of uterine receptivity. Moreover, reduced AQP1 expression in endometrial vessels or in the epithelium has been implicated in subjects with anovulatory uterine bleeding [11]. Study revealed that AQP1 promoted angiogenesis in the mouse hepatic vasculature [12]. Therefore, AQP1 expression in the uterus has recently become of considerable interest in the field of fertility research.

Mifepristone has high affinity for progesterone (P) and glucocorticoid receptors, which is effective and acceptable

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for the abortion within the first 9 weeks of pregnancy [13]. A study had shown that low-dose mifepristone did not affect ovulation, significantly affected endometrial receptivity and reduced pregnancy rates, which suggested that the dose required for effective contraception be lower than originally thought [14]. However, further study is required to determine the contraceptive mechanism of mifepristone.

Few studies have explored the effect of mifepristone on angiogenesis and AQP1 expression during the window of implantation. In the present study, we used immunohistochemistry and the methyl thiazolyl tetrazolium assay (MTT) to investigate how low-dose mifepristone acts as an anti-implantation contraceptive, and to analyze the effect of the drug on angiogenesis and AQP1 expression.

Materials and methods

Subjects

Forty-five female C57BL/6 mice (10–12 weeks of age) were used in this study. The procedures of this study were approved by the Animal Care and Use Committee of Zhejiang University (Hangzhou, China), and were in accordance with the university's guidelines for animal research. The mice were housed in a temperature- and humidity-controlled room with a 12/12 h light/dark cycle. Suitable mice were randomly allocated into one of three groups (control, 65 and 200 nmol/L mifepristone groups). The mice were caged overnight with fertile males of the same strain. The presence of a vaginal plug after mating was designated as day 1 of pregnancy. Mice on day 4 of pregnancy were treated with either vehicle (sesame oil) or RU486 (0, 0.155 and 0.755 mg/kg/day) and euthanized 24 h later on day 5, the day of implantation.

When mifepristone was administered at a concentration of 1 mg/day, the steady plasma level of mifepristone was found to be 65 nmol/L. When the serum mifepristone concentration reached 232.7 nmol/L, ovulation was inhibited [15]. Thus, 0, 65, and 200 nmol/L were selected for this experiment. The dose for mice was 0, 0.155, and 0.755 mg/kg/day, respectively.

Immunohistochemistry

The tissue sections were deparaffinized in xylene and rehydrated in a gradient series of alcohol baths. Antigen retrieval was performed by heating in a microwave at a medium-to-high temperature for 8 min, followed by heating at a low-to-high temperature for 5 min, and final cooling to room temperature over 20 min. Immunohistochemical staining featured the use of an anti-CD31 rabbit polyclonal antibody (Abcam) and an anti-AQP1 rabbit polyclonal antibody (Abcam) as the primary antibody. Binding was detected according to the manufacturer's instructions. Negative controls were incubated similarly, but with 1% (*w*/*v*) bovine serum albumin replacing the primary antibody. The microvessel density (MVD) was determined as described by Huang and Chen [16]. All sections were screened at lower magnification (×100) to identify 5 vascularized areas, and within these areas, microvessels were counted under high magnification (×400) by 2 investigators blinded to the study group and the results of the other observer. The MVD was the sum of microvessels in the five fields. Staining intensity was evaluated using a grading scale of 0–3 as follows: 0, no staining; 1, faint staining; 2, moderate staining and, 3, intense staining. Two investigators, each unaware of the identity of the slides, evaluated the staining intensity. The average value was calculated from the scores assigned for each slide by the two investigators [17].

Cells and cell culture

Human umbilical vein endothelial cells (HUVECs, female) were purchased from ScienCell (Carlsbad, California), and were cultured in endothelial cell medium (ECM; Scien Cell) supplemented with 5% (v/v) fetal bovine serum and an endothelial cell growth supplement (Scien Cell).

Cell proliferation assay

HUVECs (1×10^4/mL) were placed in 96-well plates and cultured for 24 h in phenol red-free ECM supplemented with 0.5% (v/v) charcoal-stripped FBS and various concentrations of mifepristone or siRNA. The 93-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma) was employed to measure cell proliferation, as described previously [18].

RNA interference experiments

Small interfering RNA (siRNA) duplexes were chemically synthesized by Ambion. The AQP1 siRNA sequences were: sense 5′-UAACCCUGCUCGGUCCUUUTT-3′ and antisense ′5-AAAGGACCGAGCAGGGGUUAat-3′. The 6-carboxyfluorescein (FAM)-labeled negative siRNA served as a control oligonucleotide. HUVECs were transfected with 5 nM siRNA with the aid of lipofectamine 2000 (Invitrogen).

Statistical analyses

Statistical comparison employed one-way analysis of variance followed by the Student–Newman–Keuls test. A P value < 0.05 was considered to reflect significance. All statistical analyses were performed using the Statistical Package for the Social Sciences version 19.0 (SPSS).

Results

Effect of low-dose mifepristone on the endometrial MVD

The CD31 was expressed principally on the surfaces of small endothelial cells that exhibited a distinct vascular structure (Fig. 1a). The MVD in two mifepristone-treated groups were significantly higher than controls ($P < 0.05$) (Fig. 1c). And there was no significant difference between two mifepristone-treated groups ($P > 0.05$) (Fig. 1c).

Effect of low-dose mifepristone on the AQP1 protein expression

The protein expression and localization of AQP1 in endometrium during the window of implantation were determined with immunohistochemical methods. AQP1 are lining at the interior surface of blood vessels (Fig. 1b). The staining intensity of AQP1 was found to be altered by 200 nmol/L mifepristone group ($P < 0.05$) but not 65 nmol/L group ($P > 0.05$) (Fig. 1d).

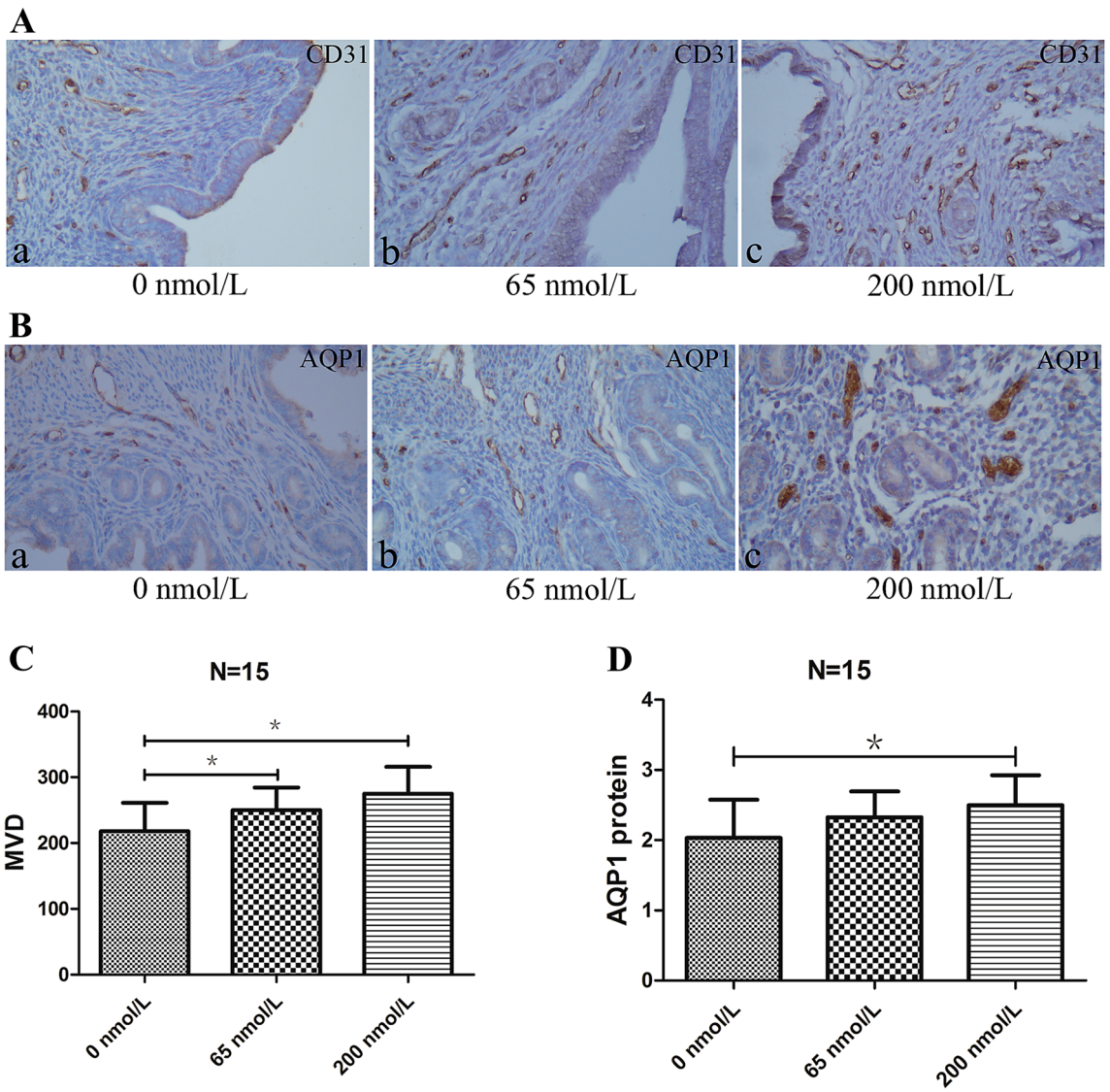

Fig. 1 **a**, **b** Expression of AQP1 (Aa–c) and CD31 (Ba–c) within the endometrium in 0, 65, and 200 nmol/L mifepristone group during the window of implantation. Magnification: ×400. **c** The MVD in two mifepristone-treated groups were significantly higher than controls ($P < 0.05$) and there was no significant difference between two mifepristone-treated groups ($P > 0.05$). **d** The staining intensity of AQP1 was found to be altered by 200 nmol/L mifepristone group but not 65 nmol/L group. Data are presented as mean values ± SDs. The number of repeat experiments is indicated by the *N* values. *$P < 0.05$ compared with the control

Effect of low-dose mifepristone on HUVECs' proliferation

The MTT assay was used to measure the effects of different concentrations of mifepristone (0, 65, and 200 nmol/L) on HUVECs proliferation over 6, 12, and 24 h. We found that HUVECs proliferation in the 200 nmol/L mifepristone group was significantly higher than control group after culture for 12 h ($P < 0.05$) (Fig. 2a).

AQP1 knockdown altered the HUVECs' proliferation triggered by mifepristone

To explore the role of AQP1 in angiogenesis triggered by mifepristone, HUVECs were transfected with AQP1-specific siRNA and grown with mifepristone at 200 nmol/L for 12 h. AQP1 siRNA suppressed the mifepristone-induced cell proliferation ($P < 0.05$, Fig. 2b).

Discussion

Implantation is a complex progressive process during which the blastocyst apposes, attaches to, and invades the underlying endometrial surface. Accumulating evidence suggests that AQP1 plays an important role in angiogenesis [19–21]. Angiogenesis is a crucial step in embryo implantation [22]. We postulated that mifepristone might affect angiogenesis by influencing AQP1 expression, and that inhibits human endometrial receptivity and embryo implantation in the endometrium.

Angiogenesis is weakest during menstruation, followed by a rapid increase during the early proliferative phase, a peak in mid-cycle, and a gradual decrease toward the end of the cycle [23]. MVD score assessed by immunomarking with CD31 is often used as an indirect estimate of angiogenesis [24]. CD31, a member of the immunoglobulin gene superfamily, is expressed principally on the surfaces of small endothelial cells that exhibited a distinct vascular structure [25]. In present study, there was no significant difference of MVD between the two mifepristone dosages, which suggested that 65 nmol/L mifepristone had the same effects on MVD as its triple dosage.

Histologically, tumors of AQP1-null mice consistently exhibit a markedly lower density of microvessels and islands of viable tumor cells surrounded by necrotic tissue [19]. We previously showed that low-dose mifepristone increased the expression levels of AQP1 mRNA and protein in human endometrium [26]. In present study, we found a similar result that 200 nmol/L mifepristone treatment enhanced the AQP1 expression in the mice endometrium, although the effect of 65 nmol/L mifepristone is not obvious. These results suggest that low-dose mifepristone might affect AQP1 regulation of angiogenesis during implantation.

Further study using HUVECs was required to explore the effects of low-dose mifepristone on angiogenesis. Mifepristone at 200 nmol/L group significantly increased HUVECs' proliferation over 12 h of culture, suggesting that mifepristone might affect angiogenesis by influencing HUVECs' proliferation, thus disturbing the endometrial microenvironment, in turn affecting whether pregnancy was maintained.

HUVECs exhibit stem cell potential and express AQP1 [27]. We used gene silencing to explore the effect of AQP1

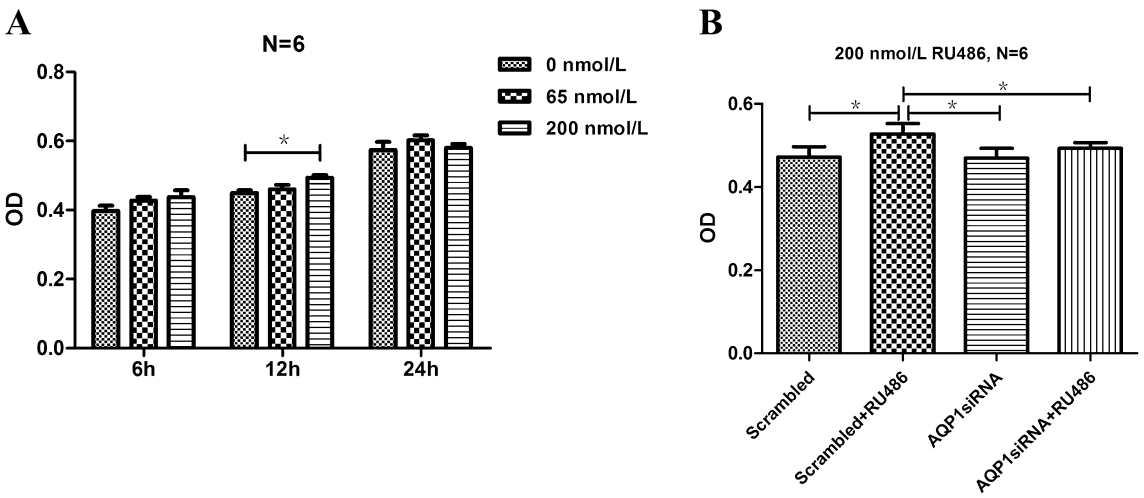

Fig. 2 **a** Effect of mifepristone (0, 65, and 200 nmol/L) on HUVECs' proliferation at different time points. Mifepristone (200 nmol/L) significantly increased HUVECs' proliferation after culture for 12 h ($P < 0.05$). **b** The role of AQP1 in angiogenesis triggered by mifepristone. Treatment with AQP1-specific siRNA suppressed the mifepristone-induced cell proliferation ($P < 0.05$). Data are presented as mean values ± SDs. The number of repeat experiments is indicated by the *N* values. *$P < 0.05$ compared with the control

on HUVECs' proliferation triggered by mifepristone, which, at 200 nmol/L, significantly increased HUVECs' proliferation over 12 h. Pretreatment with AQP1-specific siRNA significantly inhibited this mifepristone-enhanced cell proliferation. Thus, AQP1-mediated mifepristone-enhanced HUVECs' proliferation, suggesting that AQP1 is involved in mifepristone-induced angiogenesis.

The AQP1 promoter contains an estrogen response element [27]. In addition, estrogen (E2) increased HUVECs AQP1 expression, but this increase was not seen upon addition of an estrogen receptor (ER) inhibitor [27]. Mifepristone has been shown to act as an endometrial ER agonist [28]. Thus, we speculate that low-dose mifepristone may directly modulate AQP1 function via the ER, which may thus be an upstream regulatory factor of AQP1.

Conclusions

In preliminary work, we investigated how mifepristone might mediate ''endometrial contraception''. We found that AQP1 may modulate mifepristone-induced vascularization by enhancing cell proliferation. We conclude that AQP1 may play an important role in mifepristone-induced angiogenesis, triggering changes in the endometrial microenvironment, which in turn determine whether a pregnancy is maintained. However, further research is needed to define the exact mechanism by which mifepristone acts as a contraceptive.

Author contributions All authors made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; ZF and QZ involved in drafting the manuscript and revising it critically for important intellectual content; HL gave final approval to the version to be published. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest We declare that we have no conflict of interest.

Ethical approval The procedures of this study were approved by the Animal Care and Use Committee of Zhejiang University (Hangzhou, China) and were in accordance with the university's guidelines for animal research.

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