Angiostatic agents prevent the development of endometriosis-like lesions in the chicken chorioallantoic membrane

A prospective study was performed to determine the effects of the angiostatic compounds anti-hVEGF antibody, TNP-470, endostatin, and anginex on the vascularization and on endometriosis-like lesion formation in the chicken chorioallantoic membrane model. Endometriosis-like lesion formation was significantly impaired after treatment with angiostatic agents, which was associated with decreased vessel densities in the surrounding chorioallantoic membrane and more necrosis in the endometriosis-like lesions. (Fertil Steril® 2005;83:793–5. ©2005 by American Society for Reproductive Medicine.)

Direct and indirect evidence in literature suggests that angiogenesis is required for the development of endometriosis: Endometrium is angiogenic (1), increased angiogenesis is found around peritoneal implants (2), and increased endothelial cell proliferation has been observed in eutopic endometrium of endometriotic patients (3). In addition, the soluble VEGF receptor, sFlt, and the angiogenesis inhibitor, angiostatin, were shown to be effective in the prevention of ectopic lesion formation in mouse models of endometriosis (4, 5). We evaluated the vascularization and endometriosis-like lesion formation after transplantation of human endometrium together with angiostatic agents on the chicken chorioallantoic membrane (CAM). The CAM model has the advantage that all lesions can be retracted and studied and the process of lesion formation can be followed closely. The angiostatic agents tested were: [1] a humanized anti-human VEGF-A antibody, because it was demonstrated that VEGF-A is an important angiogenic factor in human endometrium (6), [2] TNP-470, an analogue of the fungus-derived antibiotic fumagillin (7), and the specific angiogenesis inhibitors [3] endostatin (8) and [4] anginex (9).

Proliferative endometrium was collected from 15 women (25–42 years of age) who underwent laparoscopy for benign indications. The women had regular menstrual cycles and no visible or symptomatic endometriosis. The endometrium was collected by transvaginal biopsy using a sampling device (Gynotec, Malden, The Netherlands). The tissue was minced in small fragments of 1- to 2-mm³ and transplanted on CAMs. From each endometrial biopsy, tissue was embedded in paraffin for histologic evaluation. Institutional Ethical Review Board approval was obtained, and the women signed an informed consent form. The women had regular menstrual cycles (25–42 years of age) who underwent laparoscopy for benign indications. The women had regular menstrual cycles and no visible or symptomatic endometriosis. The endometrium was collected by transvaginal biopsy using a sampling device (Gynotec, Malden, The Netherlands). The tissue was minced in small fragments of 1- to 2-mm³ and transplanted on CAMs. From each endometrial biopsy, tissue was embedded in paraffin for histologic evaluation. Institutional Ethical Review Board approval was obtained, and the women signed an informed consent form.

Fertilized eggs of Lohman-selected White Leghorn chickens were prepared as described previously (10). On day 7 of incubation, a 10-mm silicon ring was placed on the CAM to allow drug administration (65 μl). On day 10 of incubation, an endometrium fragment of 1- to 2-mm³ was transplanted onto the CAM and within the ring. Two hours after transplantation, anti-human VEGF antibody (HuMV833; Protein Design Labs, Fremont, CA; 3 mg/kg/d, n = 22), TNP-470 (Takeda Chemical Industries, Osaka, Japan; 20 mg/kg, once every 2 days, n = 22), endostatin (Entremed Inc., Rockville, MD; 2 mg/kg/d, n = 10), anginex (provided by Prof K. H. Mayo, Minneapolis, MN; 8 mg/kg/d, n = 24), or vehicle (65 μl saline/d, n = 10) were administered. Doses were based on the average weight of a 10-day-old chicken embryo. For each reagent, the same treatment regimen was applied to 10 CAMs without endometrium fragment to study the effect of the reagent on CAM morphology and on vascular development.

Seventy-two hours after transplantation of the endometrium fragment onto the CAM, color micrographs of the area within the ring were made. Calculation of the vascularization index was performed as described previously (1). An increase in vascularization index in CAMs without an endometrium fragment was defined as developmental angiogenesis. Enhanced angiogenesis due to the transplantation of endometrium onto the CAM was defined as endometrium-induced angiogenesis.

At the end of the experiments, CAMs were fixed in 3.7% buffered formaldehyde and embedded in paraffin. Paraffin sections (4 μm) were cut from the entire specimen (150–200 sections); every fifth section was stained with hematoxylin/eosin to locate the lesions. The size of the necrotic areas in the endometriosis-like lesions was quantified using a microscope coupled to a computerized morphometry system (Quantimet 570, Leica, The Netherlands). Sections were evaluated twice under identical circumstances, and the average of the evaluations was taken.

Comparisons were made using the nonparametric Mann-Whitney U test. Dichotomous variables were compared using χ² tests. Correlations were calculated using Spearman correlation tests. Vascular density indices were counted blindly,
twice under identical circumstances, by two observers. The average of the counts was taken. Intraobserver and interobserver variabilities were between 5% and 10%.

Treatment with TNP-470, endostatin, and anginex significantly inhibited the development of vessels in CAMs without an endometrium fragment (developmental angiogenesis) by 45% (P<.05), 51% (P<.03), and 48% (P<.01), respectively (Fig. 1A). Only the anti-human VEGF antibody did not decrease the vascularization of CAMs without an endometrium fragment (Fig. 1A). Because the VEGF antibody only recognizes VEGF of human origin, this suggests that this antibody does not neutralize VEGF of chicken origin. Alternatively, VEGF may not be involved in the vascular development of the CAM at this stage.

Transplantation of human endometrium onto the CAM led to a strong angiogenic response in the chicken tissue (Fig. 1A), and to the formation of endometriosis-like lesions (Fig. 1B). The angiostatic agents TNP470, endostatin, and anginex significantly inhibited this angiogenic response to the presence of human endometrium by 43% (P<.05), 43% (P<.03), and 38% (P<.01), respectively (Fig. 1A). Administration of the anti-human VEGF antibody also significantly (P<.05) antagonized the endometrium-induced angiogenic response. These findings suggest that the endometrium induces an angiogenic response while implanting in the ectopic environment and that VEGF is an important mediator.

Transplantation of a human endometrium fragment onto the CAM resulted in the formation of endometriosis-like lesions in a majority of CAMs (Fig. 1B). In vehicle-treated control CAMs, this was observed in 20 of 24 CAMs (83%). Administration of the angiostatic agents significantly reduced endometriosis-like lesion formation. Anti-human VEGF antibody reduced lesion formation by 59% (P<.005), TNP470 by 95% (P<.0001), endostatin by 75% (P<.001), and anginex by 43% (P<.05). The vascularization index in the CAMs was significantly (P<.05) associated with formation of endometriosis-like lesions. The CAMs with a lesion had a higher vascularization index than CAMs in which no lesion was formed. This suggests that the angiogenic response induced by the endometrium is important for lesion development. This is supported by the fact that endometriosis-like lesions in the CAMs treated with angiostatic agents showed significantly more necrosis. Treatment with anti-human VEGF antibody, endostatin, and anginex resulted in a necrotic area of 9.4% (P<.05), 32% (P<.01), and 16% (P<.01) of the area of the endometriosis-like lesion, respectively, compared to 4% of the endometriosis-like lesion in control CAMs. The percentage of necrosis was strongly and inversely correlated with the vascularization index (R = −0.63, P<.0001), indicating that a disturbed vascularization as a consequence of the presence of the inhibitors is responsible for the necrosis present in the CAM lesions.

Vessel density index (A) and the number of lesions formed (B) in CAMs after administration of vehicle and angiostatic agents. The photographs in (A) show examples of a control CAM, a CAM after transplantation of human endometrium, and after treatment with an angiogenesis inhibitor. The photographs in (B) show examples of a control lesion and a lesion in a CAM treated with an angiogenesis inhibitor. (αVEGF = anti-human VEGF antibody.)
This in vivo study shows that a proper endometrium-induced angiogenic response in the ectopic environment is critical for implantation and subsequent survival of the tissue. Angiostatic therapy may therefore provide a suitable alternative to prevent endometriosis to recur after surgical or hormonal therapy.

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