Epigallocatechin-3-gallate inhibits estrogen-induced activation of endometrial cells in vitro and causes regression of endometriotic lesions in vivo

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BACKGROUND: Epigallocatechin-3-gallate (EGCG), the major component of green tea, is a pleiotropic substance, which may inhibit tumor growth via multiple intracellular signaling pathways. Herein, we studied whether EGCG may also be effective in the treatment of endometriosis. METHODS: We investigated the effect of EGCG on activation by estradiol (E₂), proliferation and vascular endothelial growth factor (VEGF) expression of isolated hamster endometrial stromal cells and glandular cells in vitro using the water-soluble tetrazolium (WST)-1 colorimetric assay and western blot analysis. In the dorsal skinfold chamber model of Syrian golden hamsters, which were treated for 14 days with EGCG or vehicle, we further analyzed angiogenesis, blood perfusion and tissue integrity of both endometriotic lesions and ovarian follicles by intravital fluorescence microscopy and histology. RESULTS: We found that EGCG suppresses E₂-stimulated activation, proliferation and VEGF expression of endometrial cells in vitro (all P<0.05). Furthermore, EGCG selectively inhibited angiogenesis and blood perfusion (P<0.05) of endometriotic lesions in vivo without affecting blood vessel development in ovarian follicles. Histology confirmed that EGCG-treatment induces regression of the endometriotic lesions. CONCLUSIONS: Our data indicate that EGCG might be a promising therapeutic agent in the treatment of endometriosis, preventing the establishment of new endometriotic lesions.

Keywords: epigallocatechin-3-gallate; green tea; endometriosis; angiogenesis; intravital fluorescence microscopy

Introduction

Green tea represents one of the most widely consumed beverages in the world and is believed to exert many beneficial effects on health. Its major chemical component is the polyphenol epigallocatechin-3-gallate (EGCG) (Yang et al., 2006). In numerous studies, this catechin with high antioxidant capacity has been shown to be effective in the treatment of different tumor types (Lambert et al., 2005; Ju et al., 2007; Yang et al., 2007). Thereby, EGCG acts as a pleiotropic substance, which influences multiple mechanisms that are involved in carcinogenesis such as mutation, cell proliferation, cell invasion and apoptotic cell death (Beltz et al., 2006; Khan et al., 2006). Importantly, EGCG has also been demonstrated to inhibit angiogenesis in vitro and in vivo (Kondo et al., 2002; Tang et al., 2003; Zhu et al., 2007; Shankar et al., 2008). On the basis of these findings, we hypothesized that EGCG may not only be effective in the prevention and therapy of cancer, but may also represent a promising therapeutic agent in the treatment of endometriosis.

Endometriosis is one of the most frequent gynecological diseases with a prevalence of >10% in the group of women of reproductive age (Viganò et al., 2004). Histopathologically, endometriosis is defined as the presence of endometrium-like tissue outside the uterine cavity, comprising proliferating functional endometrial glands and stroma (Galle, 1989). According to the implantation theory (Sampson, 1927), these lesions originate from endometrial fragments which are retrogradely shed through the Fallopian tubes into the peritoneal cavity during menstruation, where they attach and proliferate at ectopic sites. Even though endometriosis is a benign disease, it shares important similarities with cancer. For instance, similar to tumors or metastases, endometriotic lesions release a variety of angiogenic growth factors, which stimulate the ingrowth of new blood vessels from the surrounding tissue (Taylor et al., 2002). This angiogenic process is a major prerequisite for the long-term survival of the ectopic tissue and for the estrogen-induced growth of the lesions (Groothuis et al., 2005; Becker and D’Amato, 2007).

To analyze whether the anticancer properties of EGCG may also be useful for the treatment of endometriosis, we tested in the present study the effect of EGCG on activation by
17β-estradiol (E2), proliferation and vascular endothelial growth factor (VEGF) expression in endometrial glandular cells (EGCs) and endometrial stromal cells (ESCs) in vitro. In addition, we analyzed the effect of EGCG on angiogenesis and development of endometriotic lesions in vivo in a well-established endometriosis model (Laschke et al., 2005; Laschke and Menger, 2007).

Materials and Methods

Animals
All experiments were conducted in accordance with the German legislation on protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, USA) and were approved by the local governmental animal care committee.

Eight- to 10-week-old female Syrian golden hamsters with a body weight of 60–90 g were used for the study. The animals were kept one per cage within a temperature-controlled environment on a 12 h/12 h light–dark cycle and had free access to water and standard pellet food (Altromin, Lage, Germany). To exclude discrepancies between individual animals due to different sex hormone levels, all animals were hormonally synchronized according to the method of Gross by two s.c. injections of 55 μg/kg body weight E2 (17β-estradiol; Sigma, Taufkirchen, Germany), given 24 h apart, followed by one injection of 7.5 mg/kg body weight of progesterone (Sigma), given 20 h after the last E2 injection (Gross, 1977). For all experiments, endometrial and ovarian tissue was harvested at estrus.

Preparation and primary cultures of ESCs and EGCs
For in vitro analyses, ESCs and EGCs were isolated from the uterus of 10 hamsters. For this purpose, the animals were laparatomized under sodium pentobarbital anesthesia (50 mg/kg body weight i.p.) and both uterine horns were aseptically removed. The explanted horns were placed in a 30-mm-diameter plastic Petri dish, containing 37°C warm Dulbecco’s modified Eagle’s medium [DMEM; 10% fetal calf serum (FCS), 1 U/ml penicillin, 0.1 mg/ml streptomycin; PAA, Colbe, Germany]. Subsequently, the uterine horns were opened longitudinally and small endometrial fragments (~1–2 mm³) were carefully dissected from the uterine muscle under a stereo microscope. The tissue samples were then incubated for 2 h at 37°C in DMEM containing 0.07% collagenase V (Sigma) and 0.017% DNase I (Sigma). After enzymatic digestion ESCs, which were present as single cells or small aggregates, could be isolated by passing through a 40 μm cell strainer (BD Biosciences, Heidelberg, Germany), whereas endometrial glands, which were still intact, were retained. Glands were recovered from the cell strainer by backwashing with DMEM. To disperse glands into a single-cell suspension, the gland-containing fraction was first resuspended in 3 ml of 0.25% trypsin/EDTA solution (PAA). Glands were disrupted by thoroughly pipetting. The trypsin was inactivated with DMEM/10% FCS. The isolated ESCs and EGCs were pelleted, resuspended in DMEM/10% FCS and cultured for 2 and 5 days in humidified atmosphere of 5% CO₂ at 37°C to be grown to subconfluency for western blot experiments. Moreover, 2 × 10⁴ ESCs or 4 × 10⁴ EGCs per well were transferred to 96 well plates (BD Biosciences) to perform water-soluble tetrazolium (WST)-1 assays.

WST-1 assay
To assess the cellular activity of cultured ESCs and EGCs, an in vitro colorimetric assay (WST-1 assay; Roche, Mannheim, Germany) was used. This assay is based on the finding that living cells are capable of cleaving slightly red colored WST salt to dark red colored formazan by mitochondrial dehydrogenases (Hamasaki et al., 1996; Ishiyama et al., 1996). Formazan is then excreted into the culture medium and the absorbance can be measured with a microplate reader. The WST-1 assay was carried out according to the manufacturer’s instructions. The cells were cultured in presence of 40 μM EGCG (Cayman Chemical Company, Ann Arbor, MI, USA), 1 μM E₂ (Sigma) or a combination of both. The dose of 40 μM EGCG has previously been shown to inhibit cell growth and VEGF expression of tumor cells (Chen et al., 1998; Jung et al., 2001). Cells exposed to vehicles [dimethylsulfoxide (DMSO) and corn oil] served as controls. All experiments were performed in quadruplicate. After 48 h, 10 μl of WST-1 reagent/100 μl medium was added to each well. After 1 h at 37°C, absorption was measured at 450 nm with 620 nm as reference using a microplate reader and corrected to blank values (wells without cells).

Western blot analysis
To investigate the effect of EGCG on protein expression of proliferating cell nuclear antigen (PCNA) and VEGF in cultured ESCs and EGCs, the cells were exposed to 40 μM EGCG, 1 μM E₂ or a combination of both. Cells exposed to vehicles (DMSO and corn oil) served as controls. All experiments were performed in triplicate. After 48 h, cells were harvested with accutase (PAA) and stored in liquid nitrogen for western blot analysis. For extraction of the whole protein fraction, frozen cells were incubated for 30 min in lysis buffer [10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton-X 100, 0.02% NaN₃, 0.2 mM phenylmethylsulfonyl fluoride and Protease-Inhibitor-Cocktail (1:100 v/v; Sigma)] on ice and centrifuged to 16 000 g (4°C). The supernatant was saved as whole protein fraction. Protein concentrations were determined using the Lowry assay with bovine serum albumin as standard. Fifteen micrograms protein/lane were separated discontinuously on 10% sodium dodecylsulfate polyacrylamide gels and transferred to a polyvinylidifluoride membrane (BioRad, München, Germany). After blockade of non-specific binding sites, membranes were incubated for 2 h with a mouse- monoclonal anti-PCNA antibody (1:2000; DAKO Cytomation, Hamburg, Germany) and a rabbit-polyclonal anti-VEGF antibody (1:100; Santa Cruz, Heidelberg, Germany) followed by the corresponding horse-radish peroxidase-conjugated secondary antibodies (1:5000; GE Healthcare Amersham, Freiburg, Germany). Protein expression was visualized using luminol-enhanced chemiluminescence and exposure of membranes to blue light-sensitive autoradiography film (Hyperfilm ECL, GE Healthcare Amersham). Signals were densitometrically assessed (Geldoc, Quantity one software, BioRad) and normalized to β-actin signals (mouse anti-β-actin antibody, 1:15 000; Sigma) to correct for unequal loading. For VEGF, the two bands detected (isoforms 189 and 165) were included in the densitometric analysis.

In vivo endometriosis model
To investigate the effects of EGCG on angiogenesis and development of endometriotic lesions in vivo, we used the dorsal skinfold chamber model (Fig. 1A), which allows for repetitive analyses of morphological and microhemodynamic parameters of the microcirculation in endometriotic lesions by means of intravital fluorescence microscopy (Laschke et al., 2005; Laschke and Menger, 2007). Using this model, we were able to analyze simultaneously the influence of EGCG on endometriotic lesions and potential adverse effects on ovarian tissue by combined transplantation of both endometrium and ovarian follicles into the same chamber (Laschke et al., 2008).

The implantation procedure of the chamber has been described previously in detail (Menger et al., 2002). After chamber preparation, the
animals were laparotomized under sodium pentobarbital anesthesia, one uterine horn was aseptically removed and the laparotomy was closed with a two-layer 5–0 running suture. The explanted horn was placed in a 30 mm diameter plastic Petri dish, containing 37°C warm DMEM (10% FCS, 1 U/ml penicillin, 0.1 mg/ml streptomycin; PAA) and the fluorescent dye bisbenzimide H33342 (200 µg/ml; Sigma), which allows for an easy differentiation of the stained endometrium from the non-stained surrounding host tissue after transplantation into the dorsal skinfold chamber (Fig. 1). The uterus horn was opened longitudinally, and small tissue fragments of comparable size (≈0.25 mm²) were carefully dissected from the uterine muscle under a stereo microscope. Subsequently, the fragments were transferred into 37°C H33342-free DMEM.

For isolation of ovarian follicles, four hormonally synchronized donor animals were laparotomized under sodium pentobarbital anesthesia. Ovaries were aseptically removed and were also placed in Petri dishes filled with 37°C DMEM and H33342. After removing the surrounding tissue, ovaries were microdissected using 27 gage needles under a stereomicroscope. This handpicking procedure guaranteed single connective tissue-free follicles of comparable size (≈0.25 mm²) for transplantation. The isolated follicles were transferred into Petri dishes, which contained 37°C H33342-free DMEM.

For transplantation of endometrium and ovarian follicles, the cover glass of the dorsal skinfold chamber was temporarily removed, and two endometrial fragments and two follicles were placed onto the striated muscle within each chamber (Fig. 1B).

**Intravital fluorescence microscopy**

For intravital fluorescence microscopy, the animals were immobilized and the dorsal skinfold preparation was attached to the microscopic stage. After i.v. injection of 0.2 ml 5% fluorescein isothiocyanate-labeled dextran 150 000 (contrast enhancement by intravascular staining of plasma), intravital fluorescence microscopy was performed using a modified Leitz Orthoplan microscope with a 100 W mercury lamp attached to a Ploemo-Pak illuminator with blue, green and ultraviolet filter blocks (Leitz, Wetzlar, Germany) for epi-illumination. The microscope images were recorded by a charge-coupled device video camera (CF8/1 FMC; Kappa GmbH, Gleichen, Germany) and transferred to a DVD system for off-line evaluation. With the use of 4 ×, 6.3 ×, 10 × and 20 × long-distance objectives (Leitz) magnifications of ×86, ×136, ×216 and ×432, respectively, were achieved on a 14-inch video screen (PVM 1444; Sony, Tokyo, Japan).

Quantitative off-line analysis of the DVDs was performed by means of the computer-assisted image analysis system CapImage (Zeintl, Heidelberg, Germany) and included the determination of the size of the grafts (mm²), the size of the vascularized area (given as a percentage of the size of the grafts), the microvessel density, i.e. the length of red blood cell (RBC)-perfused microvessels per observation area (cm²), the diameters of the microvessels (µm) and the centerline RBC velocity $V_{RBC}$ (µm/s). Volumetric blood flow (VQ) of individual microvessels was calculated from $V_{RBC}$ and diameter (d) for each microvessel as $VQ = \pi \times (d/2)^2 \times V_{RBC}/K$ (pl/s), where $K$ (=1.3) represents the Baker/Wayland factor (Baker and Wayland, 1974), considering the parabolic velocity profile of blood in microvessels.

**Experimental protocol**

A total of 14 endometrial fragments and 14 ovarian follicles were transplanted into the dorsal skinfold chambers of seven female hamsters treated daily with EGCG (65 mg/kg body weight i.p. in 200 µl DMSO; Cayman Chemical Company). Jung et al. (2001) could previously demonstrate that the dose of EGCG used in our study effectively inhibits the growth of tumors in vivo. A total of 20 endometrial fragments and 20 ovarian follicles were transplanted.

![Figure 1: (A) Syrian golden hamster with a dorsal skinfold chamber (chamber weight ~4 g). (B) Observation window of the dorsal skinfold chamber at the day of tissue transplantation. Within each chamber, two endometrial fragments (e) and two ovarian follicles (f) were placed onto the host striated muscle tissue at a maximal distance from each other to ensure that angiogenesis of the grafts was unaffected by the neighboring tissue transplants. (C–F) Intravital fluorescence microscopy of an endometrial fragment (C and D) and an ovarian follicle (E and F) directly after transplantation into the dorsal skinfold chamber. Because the grafts were stained with the fluorescent dye bisbenzimide H33342 before transplantation, they could easily be distinguished from the non-stained surrounding host tissue of the chamber using ultraviolet light epi-illumination (C and E). Blue light epi-illumination with contrast enhancement by intravascular staining of plasma with 5% fluorescein isothiocyanate (FITC)-labeled dextran 150 000 i.v. allowed the visualization of the microvasculature within the chamber (D and F). Scale bars: A = 1.5 cm; B = 1600 µm; C–F = 125 µm.](https://academic.oup.com/humrep/article-abstract/23/10/2308/711779/figureintro?redirectedfrom=https://academic.oup.com/humrep/article-abstract/23/10/2308/711779/figureintro)
into the dorsal skinfold chambers of 10 DMSO-treated (200 μl/day i.p.) control hamsters. Intravital fluorescence microscope analysis was performed on Day 0 (day of transplantation) and Days 2, 4, 7, 10 and 14 after transplantation. At the end of the in vivo experiments, i.e. Day 14 after transplantation, the animals were sacrificed with an overdose of pentobarbital, and the dorsal skinfold chamber preparations were processed for histology.

To analyze the effect of EGCG on the eutopic endometrium and the ovary, additional animals were treated daily with EGCG (65 mg/kg body weight i.p. in 200 μl DMSO; n = 3) or DMSO (200 μl/day i.p.; control; n = 3). After 3 days, the uterine horns and the ovaries were isolated and expression of PCNA and VEGF was analyzed by the western blots, as described above.

**Histology**

For light microscopy, formalin-fixed specimens of the dorsal skinfold chamber preparations were embedded in paraffin. Four-micrometer-thick sections were cut and stained with hematoxylin and eosin according to standard procedures. Sections through the central plane of endometriotic lesions or ovarian follicles (i.e. those containing the largest cross-sectional area) were recorded on DVD, and cross-sectional areas of individual endometriotic lesions, their cysts and stroma as well as of follicles were measured by computer-assisted planimetry.

**Statistics**

Data were first analyzed for normal distribution and equal variance. Differences between the groups of the in vitro experiments were calculated by analysis of variance (ANOVA) followed by the appropriate post hoc comparison. Differences between the in vivo groups were calculated by the unpaired Student’s t-test. To test for time effects within each experimental group, ANOVA for repeated measures was applied. This was followed by a post hoc paired comparison, including correction of the α-error according to Bonferroni probabilities for repeated measurements (SigamStat; Jandel Corporation, San Rafael, CA, USA). All data are given as mean ± SEM. Statistical significance was accepted for a value of P < 0.05.

**Results**

**EGCG inhibits E2-induced activation, proliferation and VEGF expression of endometrial cells**

In the WST-1 assay, activation of cultured ESCs, which had been exposed to EGCG, did not show any differences when compared with controls (Fig. 2A). Stimulation of ESCs with E2 resulted in a significantly increased metabolic activity of the cells, which again could not be inhibited by an additional treatment with EGCG (Fig. 2A). Comparable results were found for unstimulated EGCs. They did not differ to controls when exposed to EGCG (Fig. 2B). Stimulation with E2 led to a significantly increased activation of EGCs. Interestingly, in contrast to ESCs, this effect could be abolished by the treatment with EGCG (Fig. 2B). These results indicate that EGCG effectively inhibits E2-induced activation of EGCs.

Western blot analysis of cultured ESCs and EGCs revealed that EGCG treatment did not affect protein expression of PCNA and VEGF in unstimulated cells when compared with controls (Fig. 3). For VEGF, the two bands detected represent isoforms 189 and 165. Stimulation with E2 resulted in a significant increase of PCNA and VEGF expression, which was most pronounced in EGCs. According to the results of the WST-1 assay, this E2-induced activation of cells could be suppressed by EGCG exposure (Fig. 3).

**EGCG inhibits angiogenesis of endometriotic lesions without affecting blood vessel development in ovarian follicles**

After isolation and transplantation into the dorsal skinfold chamber, endometrial fragments and ovarian follicles presented with a comparable size of ~0.25 mm² in both observation groups, excluding size-dependent differences in the angiogenic process.

In developing endometriotic lesions of EGCG-treated and control animals, blood perfused microvessels could already be observed at Day 2 after transplantation. During the following days, an increasing number of microvessels developed within the lesions of both groups, finally resulting in a vascularized area of ~90–100% at Day 14 (Fig. 4A, B and E). However, at this time point, endometriotic lesions of EGCG-treated hamsters exhibited a significantly reduced microvessel density when compared with controls, indicating that EGCG inhibits the angiogenic process within these lesions (Fig. 4A, B and G).
In contrast, vascularization of transplanted ovarian follicles was not affected by treatment with EGCG. First signs of angiogenesis, i.e. development of capillary sprouts, could be observed in transplanted follicles at Day 2 after transplantation (Fig. 4F). Subsequently, new microvascular networks developed in follicles of both EGCG-treated animals and controls, which presented with a vascularized area of 100% and a microvessel density of \( \frac{240}{\text{cm}^2} \) at Days 10 and 14 after transplantation (Fig. 4C, D, F and H).

EGCG decreases blood perfusion of endometriotic lesions, but not of ovarian follicles

Besides the analysis of angiogenesis, we also determined blood perfusion of microvessels within endometriotic lesions and ovarian follicles. Throughout the observation period of 14 days, the diameter of these microvessels ranged between 10 and 14 \( \mu \text{m} \) in endometriotic lesions and between 9 and 12 \( \mu \text{m} \) in ovarian follicles without significant differences between the EGCG-treated group and the control group (Table I).

In control animals, centerline RBC velocity and volumetric blood flow of endometriotic lesions progressively increased over time up to a maximum of 234 \( \mu \text{m/s} \) and 21 pl/s at Day 10 (Table I). Interestingly, treatment with EGCG markedly reduced blood perfusion in endometriotic lesions, as indicated by a decreased centerline RBC velocity and volumetric blood flow of only 135 \( \mu \text{m/s} \) and 14 pl/s, respectively (Table I). In contrast, EGCG treatment did not affect blood perfusion in ovarian follicles, presenting with a centerline velocity and a volumetric blood flow that were comparable with control animals throughout the observation period (Table I).
EGCG induces regression of endometriotic lesions

Histological examination of the dorsal skinfold chamber preparations at Day 14 demonstrated that the transplanted endometrial fragments had developed to endometriotic lesions of typical histomorphological appearance, i.e. cyst-like dilated glands surrounded by a vascularized stroma (Fig. 5A and B). However, treatment with EGCG resulted in the regression of the endometriotic lesions, as indicated by a flattened glandular epithelium, a decreased microvessel density and a significantly reduced lesion size when compared with controls (Fig. 5B and E). More detailed analysis of the size of cysts and stroma within these lesions revealed a significant tissue reduction in the stromal compartment, whereas the size of cysts remained unaffected during treatment with EGCG (Fig. 5E). Corresponding to our intravital microscopic findings, histological examination of transplanted ovarian follicles did not show any differences between EGCG-treated and control animals. In both observation groups, follicular grafts were densely vascularized and exhibited a comparable size (Fig. 5C, D and F).

EGCG inhibits VEGF expression in the eutopic endometrium, but not in the ovary

Western blot analysis of the eutopic endometrium and the ovaries showed that EGCG treatment did not affect protein
expression of PCNA when compared with controls (Fig. 6). However, VEGF expression was significantly reduced in the eutopic endometrium of EGC2-treated animals (Fig. 6). In contrast, ovaries of EGC2-treated animals and control animals did not differ in VEGF expression (Fig. 6).

**Discussion**

The main findings of the present study are that the green tea polyphenol EGC2 (i) inhibits E2-stimulated activation, proliferation and VEGF expression of endometrial cells, (ii) reduces angiogenesis and blood perfusion of ectopic endometrial tissue without affecting blood vessel development in ovarian follicles and (iii) induces regression of endometriotic lesions. Although EGC2 has previously been reported to exert distinct anticancer effects (Lambert et al., 2005; Ju et al., 2007; Yang et al., 2007), this is to our knowledge the first study demonstrating that EGC2 might also be a promising therapeutic agent in the treatment of endometriosis.

For our in vitro and in vivo experiments, we used dosages of EGC2 that have been shown to be growth inhibitory in various tumor studies (Chen et al., 1998; Jung et al., 2001; Fassina et al., 2004). However, these high micromolar concentrations are unlikely to be established in the bloodstream of individuals that simply drink green tea. In fact, pharmacokinetic studies conducted in humans reported that the physiologically relevant serum concentrations of EGC2 are exclusively in the high nanomolar range (Wiseman et al., 2001; Chow et al., 2003; Ullmann et al., 2003; Henning et al., 2004). Thus, further studies have to clarify whether regular green tea consumption might also have beneficial effects in endometriosis patients. Alternatively, these patients could be treated by high-dose supplementation therapy due to recent progress in the selective total synthesis of EGC2 (Nagle et al., 2006).

In our study, we found that EGC2 inhibits angiogenesis in endometriotic lesions in vivo, as indicated by a decreased microvessel density when compared with controls. The inhibition of angiogenesis might be the cause for the observed regression of the lesions. In fact, the development of new blood vessels is a major prerequisite for the establishment and long-term survival of endometriotic lesions (Hull et al., 2003; Nap et al., 2004). Therefore, endometriosis can be assigned to the group of angiogenic diseases. Accordingly, we and others have already demonstrated during the last years that application of anti-angiogenic agents might represent a promising treatment strategy in the therapy of endometriosis (Laschke et al., 2006a,b; Becker and D’Amato, 2007; Laschke et al., 2007). However, this novel approach may also have some limitations. For instance, older endometriotic nodules could be resistant to anti-angiogenic therapy, because they are mainly composed of sparsely vascularized fibromuscular tissue (Itoga et al., 2003). Thus, anti-angiogenic therapy may have a perspective in preventing new lesions rather than in eradicating established ones. In fact, application of anti-angiogenic agents in the post-operative treatment of endometriosis may help to increase the pain-free interval and to decrease the recurrence of the disease (Ferrero et al., 2006). In line with this view, the animals of our study were treated with EGC2 at the moment of transplantation of endometrial fragments into the dorsal skinfold chambers. Further studies have now to clarify whether EGC2 can also be used to treat already established endometriotic lesions.

The anti-angiogenic effect of EGC2 can be attributed to multiple mechanisms. EGC2 has been shown to reduce VEGF expression, VEGF receptor binding, VEGF receptor
phosphorylation, interleukin-8 production and matrix metalloproteinase activity (Cao and Cao, 1999; Tang and Meydani, 2001; Kondo et al., 2002; Lamy et al., 2002; Oak et al., 2005). Moreover, EGCG inhibits mitogenesis and ephrin-A1-mediated migration of endothelial cells and induces endothelial cell apoptosis (Yoo et al., 2002; Neuhaus et al., 2004; Tang et al., 2007). In line with these findings, we could show in the present study that EGCG attenuated VEGF expression in cultured ESCs and EGCs, however, only when these cells were stimulated with E2. This indicates that EGCG specifically blocks the E2-induced activation of endometrial cells, which could be due to the ability of EGCG to compete with E2 for binding to estrogen receptor-α and -β, as previously shown in human breast carcinoma cells (Goodin et al., 2002; Farabegoli et al., 2007). Moreover, Kao et al. (2000) reported that high-dose treatment of rats with EGCG reduced circulating E2. Considering the fact that endometriosis is an E2-dependent disease, we therefore suggest that EGCG causes the regression of endometriotic lesions by inhibiting E2-mediated signaling pathways in ectopic endometrial tissue. On the basis of these considerations, it is not surprising that endometriotic lesions of EGCG-treated animals also exhibited a significantly reduced blood perfusion when compared with controls. In fact, in a previous study, we could

Figure 5: Hematoxylin-eosin stained cross-sections of endometriotic lesions (A and B) and ovarian follicles (C and D) at Day 14 after tissue transplantation onto the striated muscle tissue (arrows) within the dorsal skinfold chamber of EGCG-treated (B and D) and control Syrian golden hamsters (A and C). In both groups, endometriotic lesions are characterized by cyst-like dilated endometrial glands (asterisks) surrounded by a vascularized endometrial stroma (A and B). However, treatment with EGCG results in a regression of endometriotic lesions (B), as indicated by a flattened glandular epithelium, a decreased microvessel density and a reduced lesion size when compared with controls (A). In contrast, transplanted ovarian follicles of both groups are densely vascularized and exhibit a comparable size (C and D). Scale bars: 50 μm. (E and F) Quantification of cross-sectional areas of individual endometriotic lesions, their cysts and stroma (E) as well as of ovarian follicles (F) within dorsal skinfold chambers of EGCG-treated animals (black bars; n = 7) and control animals (white bars; n = 10), as assessed by computer-assisted planimetry. Mean ± SEM. *P < 0.05 versus control animals.
already demonstrate that withdrawal of E2 by bilateral ovariectomy significantly decreases blood flow in endometriotic lesions (Laschke et al., 2005).

Interestingly, we could also show in our study that EGCG inhibits VEGF expression in the eutopic endometrium. Considering the hypothesis that endometrium of women with endometriosis exhibits an increased angiogenic activity (Healy et al., 1998), our findings indicate that EGCG treatment may not only induce regression of endometriotic lesions, but may also have a positive anti-angiogenic effect on the eutopic endometrium of endometriosis patients.

Besides the analysis of endometriotic lesions and the eutopic endometrium, we also investigated the effect of EGCG on transplanted ovarian follicles and the ovary in order to evaluate whether EGCG might have comparable effects in other organs of the reproductive tract. This is not unlikely, because the ovary and the uterus are the only organs in the adult, where angiogenesis takes place under physiological conditions and is crucial for a normal reproductive function (Reynolds et al., 1992). Accordingly, treatment of endometriosis with anti-angiogenic agents may bear the risk of inducing infertility in reproductive age women. Therefore, anti-angiogenic agents should be able to selectively inhibit angiogenesis in endometriotic lesions. Interestingly, we found that EGCG treatment affected neither angiogenesis and blood perfusion nor tissue integrity of ovarian follicles. Moreover, expression of PCNA and VEGF in the ovary was not reduced by EGCG treatment when compared with controls, indicating that EGCG might selectively affect endometrial tissue. However, the effect of EGCG on ovarian tissue might need some further analysis, because others recently reported that EGCG may negatively influence function of granulosa cells (Basini et al., 2005a,b). Thus, we suggest that more studies are necessary in order to gain more information about the effects of EGCG on reproductive function.

Because EGCG is a typical pleiotropic substance, we believe that beside the observed anti-angiogenic effect of EGCG on ectopic endometrial tissue, several other mechanisms might contribute to the regression of endometriotic lesions. For instance, we could demonstrate that EGCG also inhibits E2-induced endometrial cell proliferation in vitro, as indicated by a decreased expression of PCNA. Other studies have shown that EGCG targets multiple intracellular pathways that are involved in cell proliferation, differentiation and death by inhibition of signaling mediators such as nuclear factor-κB, mitogen-activated protein kinases or insulin-like growth factor-I (Khan et al., 2006). Interestingly, there is also increasing evidence that EGCG reduces the expression of cyclooxygenase-2, which is considered to be a potential therapeutic target for the treatment of endometriotic lesions (Ozawa et al., 2006; Laschke et al., 2007).

Taken together, the present study demonstrates for the first time that EGCG induces regression of endometriotic lesions. Indeed, application of EGCG inhibited E2-induced activation, proliferation and VEGF expression of endometrial cells in vitro and suppressed angiogenesis and blood perfusion of endometriotic lesions in vivo. Thus, this major component of green tea might be a promising therapeutic agent in the treatment of endometriosis, preventing the establishment of new endometriotic lesions.

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