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Anti-angiogenic effects of green tea catechin on an experimental endometriosis mouse model

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BACKGROUND: The development of new blood vessels plays an essential role in growth and survival of endometriosis. Epigallocatechin gallate (EGCG) from green tea has powerful anti-angiogenic properties and our aim was to evaluate these properties in experimental endometriosis.

METHODS AND RESULTS: Eutopic endometrium from endometriosis patients was transplanted s.c. to severely compromised immunodeficient mice, randomly treated i.p. with EGCG (anti-angiogenic and -oxidant), Vitamin E (a non-angiogenic antioxidant) or saline for 2 weeks. The endometrial implant, including adjacent host outer skin and subcutaneous layers plus inner abdominal muscle and peritoneum, was collected. New microvessels were determined by species-specific immunohistochemistry. Angiogenic factors in lesions and abdominal muscle were detected by quantitative real-time PCR. Apoptosis was studied by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling and quantitative real-time PCR. In saline control, endometrial implants developed new blood vessels with proliferating glandular epithelium and were tightly adhered to host subcutaneous and abdominal muscle layers. After EGCG, endometriotic lesions were smaller than control ($P < 0.05$), and glandular epithelium was smaller and eccentrically distributed. Angiogenesis in lesions from the implant and adjacent tissues was under-developed, and microvessel size and density were lower (both $P < 0.01$) than control. mRNA for angiogenic vascular endothelial growth factor A, but not hypoxia inducible factor 1, alpha subunit, was significantly down-regulated in lesions after EGCG ($P < 0.05$). In addition, apoptosis in the lesions was more obvious, and nuclear factor kappa B and mitogen activated protein kinase 1 mRNA levels were up-regulated ($P < 0.05$) after EGCG treatment. No differences were observed with Vitamin E treatment.

CONCLUSIONS: EGCG significantly inhibits the development of experimental endometriosis through anti-angiogenic effects.

Key words: angiogenesis / vascular endothelial growth factor / green tea / catechin / endometriosis

Introduction

Angiogenesis has an essential role in development, reproduction and repair. However, pathological angiogenesis occurs not only in tumour formation, but also in a range of non-neoplastic diseases that could be classed together as 'angiogenesis-dependent diseases' (Carmeliet and Jain, 2000; Simons, 2005). Endometriosis is a chronic disorder characterized by the implantation of endometrial glands and stroma outside the uterine cavity. Despite different hypotheses regarding the pathogenesis of endometriosis, it is widely accepted that angiogenesis plays an essential role in the growth and survival of endometriotic lesions (Shifren *et al*., 1996; Donnez *et al*., 1998; Becker *et al*., 2004). Numerous peritoneal blood vessels are observed around the active endometriotic lesions at laparoscopy and the implant itself is strongly vascularized under histological examination (Nisolle *et al*., 1993; Nap *et al*., 2004). Moreover, increased angiogenic activity has been demonstrated in peritoneal fluid from women with endometriosis and strong expression of angiogenic factors has been shown in active lesions (Nisolle *et al*., 1993; Donnez *et al*., 1998). Further evidence of the pathogenesis of endometriosis is provided by studies on animal models. Experiments involving implantation of human endometrium in immunodeficient mice have shown that endometriotic lesions derive their blood supply from the surrounding vascular network. I.p. injection of angiostatic compounds or overexpression of angiostatin inhibit the growth of endometrial implants by disrupting their vascular supply (Dabrosin *et al*., 2002; Nap *et al*., 2004).

Green tea is one of the most widely consumed beverages in the world. The water-extractable fraction of green tea catechins, especially epigallocatechin gallate (EGCG), exhibits potent antioxidant capacity;

tumour inhibition and also suppressive effects on microvascular endothelium both *in vitro* and *in vivo* (Mukhtar *et al*., 1994; Stoner and Mukhtar, 1995; Cao and Cao, 1999; Tang and Meydani, 2001). The aim of our study was to evaluate the anti-angiogenic effects of EGCG on the growth and survival of experimental endometrosis in severely compromised immunodeficient (SCID) mice. EGCG was administrated to the mice with human endometrial tissues from endometriosis patients, whereas Vitamin E, as potent antioxidant but non-angiogenic agent, was also included for comparison.

Materials and Methods

Human endometrium

The use of human tissue from patients for this study was approved by Human Research Ethics Committee of The Chinese University of Hong Kong. Eutopic endometrium, ~15 mm^2 in size or 100 mg wet weight, was obtained by dilatation and curettage from women of reproductive age (n=10) undergoing laparoscopic surgery for ovarian endometriosis. All endometriosis patients were classified as in ASRM Stage III according to the American Society for Reproduction Medicine revised classification of endometriosis 1996 (American Society for Reproduction Medicine, 1997). Patients had regular menstrual cycles and were in the proliferative phase at the time of surgery. None of them had received any hormonal treatment for at least 3 months before surgery and sample collection. Endometrium collected was washed twice in cold sterile Dulbecco's phosphate-buffered saline (PBS) (Sigma, CA, USA) to remove cellular debris and excessive blood, then the tissue was cut into 1 mm^2 pieces under sterile conditions using a stereomicroscope and suspended in PBS with 200 IU/ml of penicillin and 200 μg/ml of streptomycin (both Sigma). The presence of endometrial glands in the tissues was confirmed by histological examination retrospectively.

Experimental endometriosis

Hetero-transplants of human tumours into SCID mice, which are deficient in T and B lymphocyte function, maintain their tumorigenicity, morphology and hormone responsiveness. Hetero-transplantation of human eutopic endometrium into subcutaneous pockets in SCID mice has been successful, as the mice were shown to develop lesions resembling those in humans and the endometriotic implants survived for 10 weeks (Aoki *et al*., 1994; Awwad *et al*., 1999). In this study, SCID mice were used to examine the pathogenesis of endometriosis as well as to test the effects of potential therapeutic compounds on human tissues.

Six-week-old, non-obese diabetic/SCID female mice, weighing about 20 g, were housed in disease-free conditions in a separate barrier facility with individually ventilated filter cages. Ambient temperature was monitored, and cycles of light and darkness were regulated. Mice were fed with laboratory chow and sterile water *ad libitum* for at least a week as an acclimatization period before experimentation. Under general anaesthesia and sterile conditions, a small skin incision, <3 mm in length, was made on the ventral midline of lower abdomen and a small subcutaneous pocket created. Five pieces of human eutopic endometrium were transplanted into each subcutaneous pocket within 60 min of endometrial tissue procurement. The pockets were closed without thread and the cutis was sutured with a 4/0 nylon thread. The animals were allowed to rest on warm bedding for total recovery.

Study interventions

Following the day of operation, immediately after the induction of endometriosis, the animals were randomly divided into three groups to receive (i) green tea catechin EGCG (Sigma) at 50 mg/kg i.p.; (ii) vitamin E (Sigma) at 20 mg/kg i.p.; or (iii) sterile normal saline i.p. daily for 2 weeks. The respective doses of EGCG and Vitamin E were selected as they had been proven to have significant anti-amgiogenic and anti-oxidative effects in mice *in vivo* (Villayandre *et al*., 2004; Sang *et al*., 2005). To demonstrate the dose-dependent anti-angiogenic effects, EGCG at the lower dose of 5 mg/kg i.p. (Nakae *et al*., 2008) was also included for histological and immunohistochemical examination.

Oral administration is the usual route in human use but less than half of green tea catechins can only be absorbed from the intestine and they are quickly metabolized to glucuronide derivatives (Harada *et al*., 1999). Subcutaneous administration can exert a local effect on target endometriotic lesions but does not provide valuable pharmacokinetic data. Both i.v. and i.p. administration provide a systemic effect and effective dose for study intervention but the i.v. route is difficult in small animals and may increase risk of infection in SCID mice. To achieve higher concentrations of EGCG and Vitamin E in subcutaneous layer of the animals (Chu *et al*., 2006; 2007), i.p. injection was employed as the administration route in current study.

Endometrial samples from the same patient were used for the animal randomization and study intervention. Ten animals for each intervention were required in order to detect, at 8 mm^2, a mean endometriotic lesion size difference with a 5 mm^2 SD, where the Type I error is 0.05 and Power is 90%. In total, 10 patients were recruited and endometrial samples were collected; 30 animals were randomized for each study intervention. At the end of the experimentation period, the size of the endometrial implants was assessed. First, laparotomy was performed to explore the experimental endometriotic lesion between subcutaneous and abdominal muscle layers. The size of the endometriotic lesion was determined by measuring the longest length and perpendicular width in millimetre. Then the endometriotic lesion, including the adjacent host outer skin and subcutaneous layers and the inner abdominal muscles and peritoneum, was excised for further laboratory studies. Tissues were either fixed in 10% formalin (Sigma) and embedded in paraffin wax for histological and immunohistochemical analyses, or further divided by separating skin/subcutaneous layer, endometriotic lesion and abdominal muscle/peritoneal layer under dissecting stereomicroscope (Leica, Microsystems, Germany) and placed individually in RNA later solution (Qiagen, CA, USA) for RNA study.

Histology and immunohistochemistry

Paraffin sections were prepared for staining with haematoxylin and eosin for histological examination. Immunohistochemical staining was performed by the immunoperoxidase method using MACH 3 HRP Polymer Kit (Biocare Medical, Concord, CA, USA). The sections were incubated with anti-human pan-cytokeratin antibody (Santa Cruz Biotechnology Inc., CA, USA) for endometrial gland epithelium from engrafted human endometrium (Awwad *et al*., 1999), and with anti-human CD34 antibody (AbCam, Cambridge, UK) and anti-mouse CD34 antibody (AbCam) for both engrafted and host vascular endothelium as primary antibodies (Mai *et al*., 2008) in a humid chamber overnight at 4°C, followed by the second antibody and peroxidase-labelled avidin for 30 min. Sections were then incubated with 3,3-diaminobenzidine (Biocare Medical) and counterstained with methyl green. Negative controls without primary antibodies were included.

Endometrial gland and microvessel density

Endometrial gland and microvessel density of the lesions was determined by observing the number and the size of the cytokeratin-stained endometrial and CD34-stained microvessel structures within the endometriotic lesion. Size was computed by the area measurement using Leica DM5500B Microscope with Application Suit Software (Leica). Mean number and size were calculated from five separated sections from each of the 10 animals

within a group. The five best sections showing complete histological features of (i) outer skin and subcutaneous layers; (ii) middle endometriotic lesion; and (iii) inner abdominal muscles and peritoneum were firstly evaluated and then selected for final detailed examination by two observers. Each section was evaluated by both observers blinded to the experimental groups and results. Mean number of endometrial glands and microvessels in the section were calculated and presented as numbers per lesion, whilst size of the endometrial glands and microvessels in the section was expressed as micrometer square per gland or lesion.

Quantitative real-time PCR

Total RNA was extracted separately from frozen endometriotic lesions and host abdominal muscle layers by RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The quality and quantity of total RNA were determined by Nanodrop spectrophotometry (Wilmington, USA) at A260 and A280 nm. The integrity was assessed by comparing the 18S and 28S ribosomal peaks with Agilent 2100 Bioanalyzer (Agilent, Germany). One hundred nanogram total RNA samples were hybridized with oligo(dT) primer and then reverse-transcribed SuperScript III (Invitrogen, CA, USA) at 70°C for 10 min, 50°C for 60 min and then 70°C for 15 min. One microliter of complementary DNA was subjected to quantitative real-time PCR using *Power* SYBR® Green PCR Master Mix in 7900HT Fast Real-Time PCR system (Applied Biosystems, CA, USA) as previously described (Ufer *et al*., 2008). β-actin (human ACTB & mouse Actb) was used as housekeeping gene to calculate relative gene expression. Primer pairs for factors involved in angiogenesis (vascular endothelial growth factor A—human VEGFA & mouse Vegfa; Hypoxia Inducible Factor 1, alpha subunit—human HIF1A & mouse Hif1a); invasion (Plasminogen Activator, Urokinase—human PLAU & mouse Plau, or uPA); adhesion (Membrane-type Matrix Metalloproteinase 2—human MMP2 & mouse Mmp2; Integrin, alpha V beta subunit 3—human ITGAVβ3 & mouse Itgavβ3) and apoptosis (Nuclear Factor Kappa B—human NFKB & mouse Nfkb; Mitogen Activated Protein Kinase 1—human MAPK1 & mouse Mapk1) were designed using Primer Express Version 3.0 for Windows (Applied Biosystems). To avoid non-specific amplification of mRNA from different origins, primer sequences specific for human or mouse targeted genes were used (Table I) and specificity checked by gel electrophoresis (Fig. 1).

The cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 60 s and 72°C for 45 s. Dissociation curves were maintained at 95°C for 15 s, 60°C for 15 s and 95°C for 15 s. The threshold cycle (C_T) was determined as the fractional PCR cycle number at which the fluorescence emission passed the threshold intersect with the exponential region of the amplification curve by the Fast System Software (Applied Biosystems). The results were analysed using the comparative C_T method, and the values were normalized to the β-actin expression by subtracting mean C_T of β-actin from mean target C_T for each sample to obtain the mean delta C_T. The mean delta C_T values were then converted into fold change values, based on a doubling of PCR product in each PCR cycle, according to the manufacturer's guidelines.

Tunel assay

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) was carried out using a ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Temecula, CA, USA) and performed according to the manufacturer's instructions as previously described (Ufer *et al*., 2008). Sections were washed in PBS, and then endogenous peroxidase activity was exhausted by incubation with 0.3% H_2O_2 in methanol for 15 min at room temperature. DIG-labelling reaction was performed using 70 μl TUNEL TdT enzyme for each sample and incubated for 1 h in a humidifying chamber at 37°C. No TdT enzyme was added to the negative control. The sections were then incubated with anti-dioxigenin peroxidase for 30 min in a humidifying chamber at room temperature. After washing, development for localization of cells containing labelled DNA strand

Table I Primer sequences used for quantitative real-time PCR analysis

Markers	Genes	Human	Product size	Genes	Mouse	Product size
Angiogenesis	VEGFA	FW:5-CACTGCCTGGAAGATTCA-3 RV: 5-TGGTTTCAATGGTGTGAGGA-3	226 bp	Vegfa	FW: 5-ACACGGTGGTGGAAGAAGAG-3 RV: 5-CAAGTCTCCTGGGGACAGAA-3	146 bp
	HIF1A	FW: 5-AGGAGGATCACCCTCTTCGT-3 RV: 5-AAAGGCAAGTCCAGAGGTG-3	168 bp	Hif1a	FW: 5-TCAGTGCACAGAGCCTCCT-3 RV: 5-GCGGAGAAAGAGACAAGTCC-3	210 bp
Invasion	PLAU	FW: 5-TCACCACCAAAATGCTGTGT-3 RV: 5-AGGCCATTCTCTTCCTTGGT-3	223 bp	Plau	FW: 5-CTAGAGCCCTGGAGCCTCTT-3 RV: 5-TGGGATGGTCTATGCTGTCA-3	192 bp
Adhesion	ITGAVβ3	FW: 5-TTTGCCTTATTGGCAGCTCT-3 RV: 5-TCTCCCTTTGAGGAAAAGCA-3	165 bp	Itgavβ3	FW: 5-ATCTTCAGATGACGCCAGGA-3 RV: aCACATTTCCCCACACTCGT-3	191 bp
	MMP2	FW:5-CCTCTCCACTGCCTTCGATA-3 RV: 5-GCCTGGGAGGAGTACAGTCA-3	182 bp	Mmp2	FW:5-ACGGGCCCTATCATCTTCAT-3 RV: 5-CACAAAAAGAAGCCACCCTCT-3	137 bp
Apoptosis	MAPK1	FW:5-ATGCTCACAGGCCTCACTTT-3 RV: 5- TGTCGGGGCTTCTTTGTTAG-3	190 bp	Mapk1	FW: 5-TTTGCATAGGGAGGTCCAAG-3 RV: 5-GGTGCCATCATCAACATCTG-3	150 bp
	NFKB	FW: 5-CTGGGGATGAGGTTGCTTAC-3 RV: 5-TGATGCTGTGGTCAGAAGGA-3	149 bp	Nfkb	FW: 5-GCACAGACGGTGTCTAGCAA-3 RV: 5-CGGAGGGACAGCAGTAACA-3	130 bp
Housekeeping gene	ACTB	FW: 5-GGGACCTGACAGACTACCTCAT-3 RV: 5-GTCAGGCAGCTCATAGCTCTTC-3	185 bp	Actb	FW: 5-GGGACCTGACAGACTACCTCAT-3 RV: 5-GTCAGGCAGCTCATAGCTCTTC-3	185 bp

VEGFA, vascular endothelial growth factor A; HIF1A, hypoxia inducible factor 1, alpha subunit; PLAU, plasminogen activator, urokinase; MMP2, membrane-type matrix metalloproteinase 2; ITGAVβ3, integrin, alpha V beta subunit 3; NFKB, nuclear factor kappa B; MAPK1, mitogen activated protein kinase 1; ACTB, β-actin.

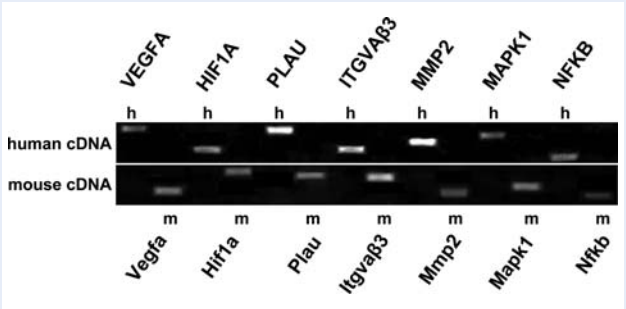

Figure 1 Specificity of primers designed for PCR.
Human (h) and mouse (m)-specific primers were tested with human complementary DNA (cDNA) and mouse cDNA prepared from muscle tissues and checked by gel electrophoresis. VEGFA, vascular endothelial growth factor A; HIF1A, hypoxia inducible factor 1, alpha subunit; PLAU, plasminogen activator, urokinase; ITGAVβ3, integrin, alpha V beta subunit 3; MMP2, membrane-type matrix metalloproteinase 2; MAPK1, mitogen activated protein kinase 1; NFKB, nuclear factor kappa B.

breaks was performed by incubating with 3,3-diaminobenzidine. Slides were counterstained with methyl green prior to permanent mounting.

Statistical analysis

Quantitative data are expressed as mean ± SD. The significant differences between the control and treatment groups were evaluated by parametric Student's *t*-test, and $P < 0.05$ was considered significant.

Results

Ectopic endometriotic lesions

Animals were all healthy and active after endometrium implantation. No signs of stress, intolerance to anaesthesia and implantation, or toxic response from the saline, Vitamin E or EGCG administration were observed. No significant difference in weight change over the experimentation period was observed between groups of mice. There was no observable swelling or hair loss at the abdominal implantation sites. At laparotomy, the implanted endometriotic lesions were all clearly located between abdominal muscle and subcutaneous layer (Fig. 2A, upper panels). The lesions were smooth and well defined, with no signs of inflammation and spread (Fig. 2A, lower panels). The length and width of the lesions were measured using venire caliper (Fig. 2A, arrows). The size of the lesions was calculated by multiplying the length and width. Compared with the control group, lesion size in the Vitamin E and EGCG groups was smaller, but the difference was significant only in the EGCG group (Fig. 2B, $P < 0.05$).

The histological changes in the endometriotic lesions with adjacent host structures (inner abdominal muscle and peritoneal layers and outer subcutaneous and skin layers) were examined and compared (Fig. 3, upper panels). In control saline group, but not in Vitamin E and EGCG groups, the lesions were in close contact with outer skin and subcutaneous layers and inner abdominal muscle and peritoneum, i.e. tightly adhered to the host structures. Endometrial glandular tissue was confirmed in all the implanted endometriotic lesions in three groups (Fig. 3, lower panels). Compared with the control group, endometrial glands in Vitamin E group were fewer (16.3 ± 3.5/lesion versus 8.9 ± 0.4/lesion, $P = 0.053$) whilst in the EGCG group there were significantly fewer of them (16.3 ± 3.5/lesion versus 2.5 ± 0.8/lesion, $P = 0.001$), they were smaller ($61.2 \pm 19.5 \times 10^3 \mu m^2$/gland versus $5.81 \pm 0.71 \times 10^3 \mu m^2$/gland, $P = 0.015$) and eccentrically distributed.

Anti-angiogenesis

In the implants themselves, new microvessels, as indicated by anti-human CD34 positive stains, had developed within the endometrial stroma next to the glands (Fig. 4A, upper panels). The mean total number and size of these human endometrial microvessels were significantly reduced with EGCG treatment (Fig. 4B, left panel,

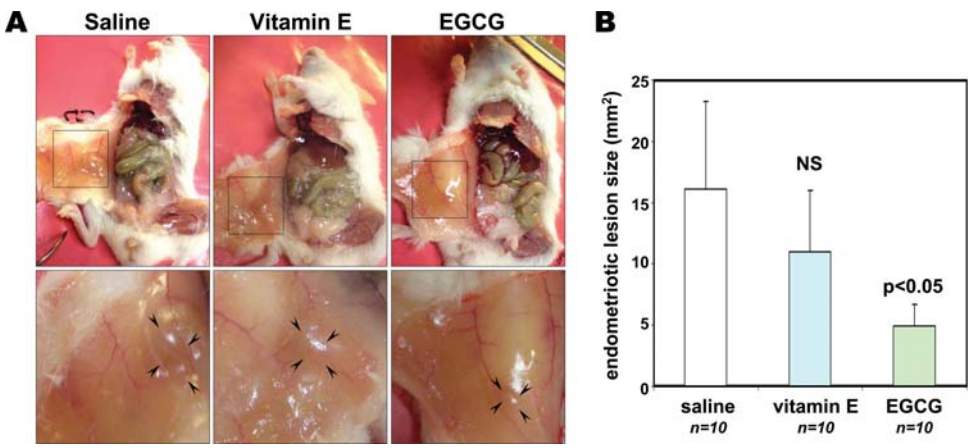

Figure 2 Endometriotic lesions.
(A) Upper panels show a severely compromised immunodeficient mouse with the lesions in right ventral abdominal wall under laparotomy. Lower panels show the close-up of the lesions. Arrows indicate the greatest length and perpendicular width of the lesions for lesion size calculation. **(B)** Bar charts of the lesion size in different groups. EGCG, epigallocatechin gallate. NS refers to no significant difference and *P*-values are indicated above the bar when compared with saline control group.

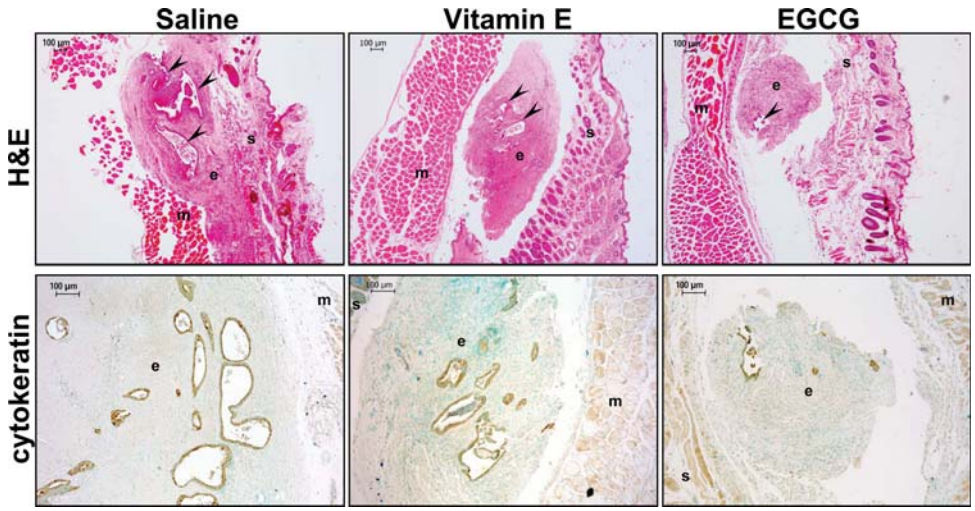

Figure 3 Histological characteristics of the endometriotic lesions.
Upper panels show the sandwich structures of outer skin and subcutaneous layers (s), middle endometriotic lesion (e) and inner abdominal muscle and peritoneum (m). Arrows, endometrial glands. Lower panels show the immunoreactive human pan-cytokeratin endometrial glandular epithelium in the lesions. H&E, haematoxylin and eosin.

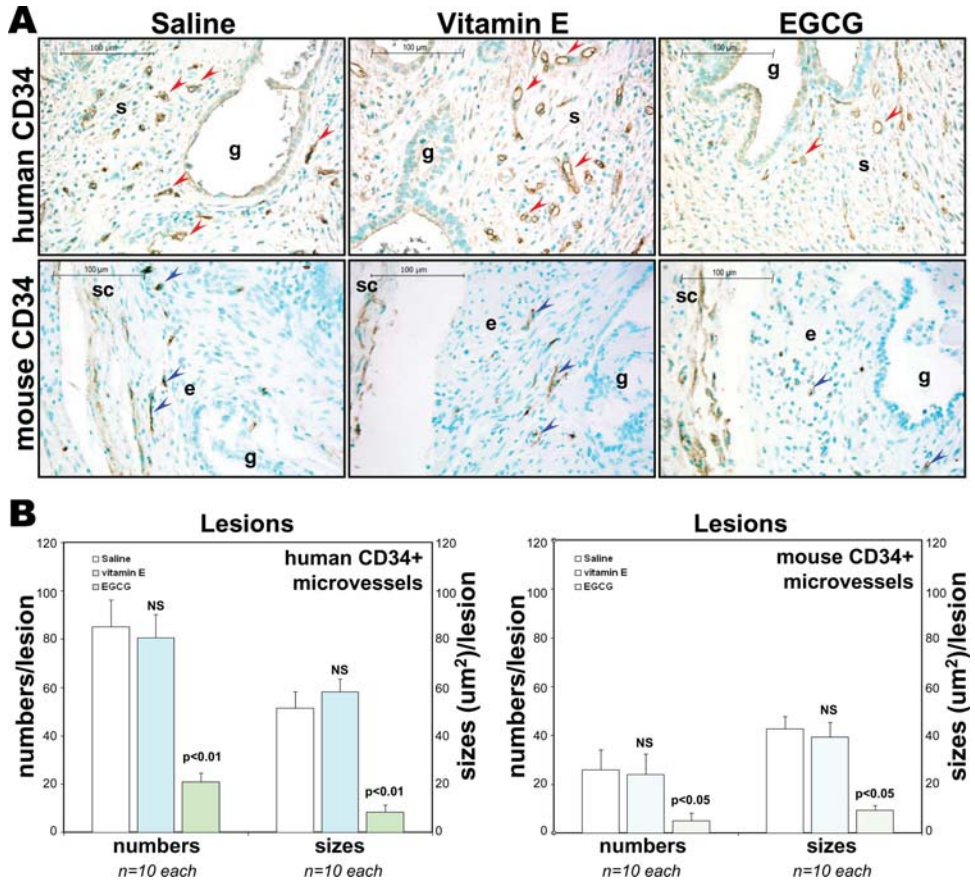

Figure 4 Anti-angiogenesis effect of EGCG in endometriotic lesions.
(A) Immunostaining of anti-human CD34 (upper panels) and anti-mouse CD34 (lower panels) in the lesions. Red arrows, microvessels from the endometrial implants; blue arrows, microvessels from the host adjacent tissues; s, endometrial stromal; g, endometrial glands; e, endometriotic lesions; sc, subcutaneous layer. **(B)** Bar charts of mean number and size of graft origin microvessels (human, left panel) and host origin microvessels (mouse, right panel) in the active lesions.

$P < 0.01$). New microvessels also developed from the surrounding host tissues, as indicated by anti-mouse CD34 positive stains, mainly from host subcutaneous layer and growing into the endometrial stroma (Fig. 4A, lower panels). Similarly, the mean total number and size of these microvessels of mouse origin were significantly reduced with EGCG treatment (Fig. 4B, right panel, $P < 0.05$). The decreases in endometrial microvessel number and size were 75.4% and 83.6%, whereas the decreases of host microvessels into the endometriotic lesion in number and size were 83.6% and 78.4%, respectively. No significant change was found in Vitamin E group. With lower dose EGCG treatment at 5 mg/kg i.p. ($n = 2$), the decreases of human endometrial and host microvessel formation in the endometriotic lesions became less obvious (Fig. 5).

Human VEGFA mRNA levels in the lesions with EGCG treatment were significantly down-regulated and the decrease was 65.8% versus control; whilst mouse Vegfa mRNA expression was also significantly down-regulated and the decrease was 55.7% (Fig. 6A, $P < 0.05$). The decreased human VEGFA and mouse Vegfa mRNA in the lesions with Vitamin E treatment were 33.1% and 30.5%, respectively, however, the decreases were not significant. Both human HIF1A and mouse Hif1a mRNA levels in EGCG and Vitamin E group were not significantly different from control saline (Fig. 6B).

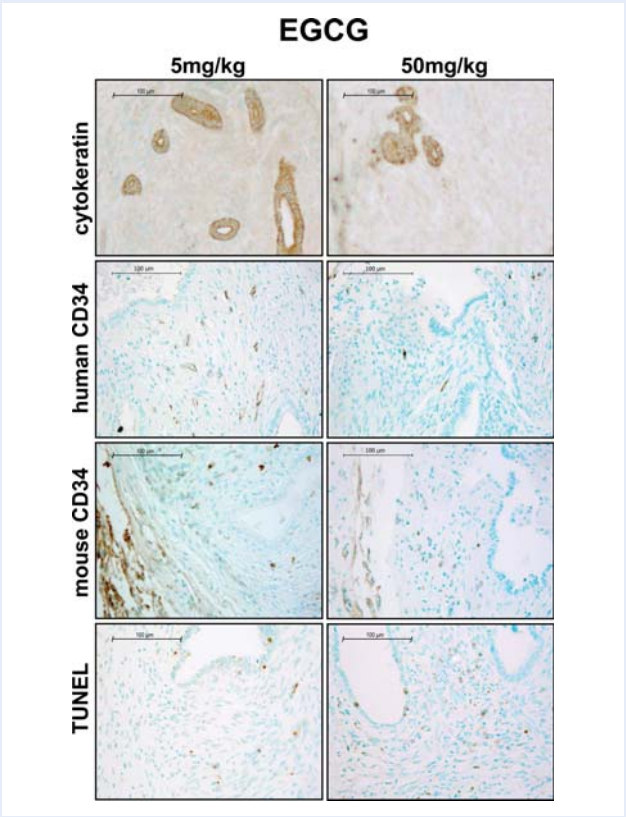

Figure 5 Dose-dependent anti-angiogenesis effect of EGCG on endometriosis.
Glandular epithelial growth (cytokeratin staining), endometrial microvessels (human CD34 staining), host microvessels (mouse CD34 staining) and apoptotic bodies terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL assay) with EGCG treatment at low dose (5 mg/kg i.p. left panels) and high dose (50 mg/kg i.p. right panels) are shown.

Lesion survival

Survival of the endometriotic lesions was assessed by TUNEL assay. Apoptotic bodies were found in both endometrial stroma and glandular epithelium in all the active lesions (Fig. 7A). With EGCG treatment, apoptosis bodies were more obvious in the endometrial stroma and were eccentrically distributed. Human MAPK1 and NFKB mRNA levels were significantly up-regulated in the lesions with EGCG exposure (Fig. 7B, left panels, $P < 0.05$). In contrast, mouse Mapk1 and Nfkb mRNA in host abdominal muscle and peritoneum were significantly down-regulated in animals with EGCG treatment when compared with controls (Fig. 7B, right panels, $P < 0.05$). No significant change was found in Vitamin E group.

Invasion and adhesion

As shown in the histological examination above, the anatomical relationship between the endometriotic lesion and surrounding host tissues was loose, with no tight attachment. Human PLAU and MMP2 mRNA levels in the active lesions were significantly down-regulated with EGCG treatment compared with the control group but not with Vitamin E (Fig. 8, left panels, $P < 0.05$ for PLAU, $P < 0.001$ for MMP2). In contrast, mouse Plau, Mmp2 and Itgavβ3 mRNAs in the host muscles and peritoneum were up-regulated with EGCG treatment, but the changes were not statistically significant (Fig. 8, right panels).

Discussion

VEGF is the most prominent and most studied pro-angiogenic factor in endometriosis and is the main stimulus for angiogenesis. It is strongly expressed by endometriotic lesions in both glandular epithelial and stromal cells, and in activated macrophages and neutrophils in the proliferative phase (Shifren *et al.*, 1996; Mueller *et al.*, 2000). Significantly increased VEGF levels have been found in the peritoneal fluid and in pelvic lesions of patients with endometriosis compared with that in eutopic endometrium or in normal controls (Donnez *et al.*, 1998). Various polymorphisms in the VEGF family have been associated with an increased risk of endometriosis (Hsieh *et al.*, 2004; Bhanoori *et al.*, 2005; Kim *et al.*, 2005).

The growth of newly formed blood vessels is of pivotal importance in the development of endometriosis, so inhibition of angiogenesis may offer a new opportunity for treatment. Significant decrease in microvessel density and number of established endometriotic lesions has been reported with the use of anti-angiogenic agents, including TNP470, endostatin, anginex and anti-human VEGF antibody (Nisolle *et al.*, 2000; Nap *et al.*, 2004). Experimental studies using a variety of tumour angiogenic models, have shown that green tea extract, and EGCG in particular, have strong anti-angiogenic effects (Adhami *et al.*, 2004; Fassina *et al.*, 2004; Liao *et al.*, 2004). The underlying mechanisms seem to be associated with inhibitory effects on VEGF production and VEGF receptor activity (Jung *et al.*, 2001; Lamy *et al.*, 2002; Kondo *et al.*, 2002; Sartippour *et al.*, 2002; Kojima-Yuasa *et al.*, 2003; Basini *et al.*, 2005). In the present study, EGCG treatment caused a dose-dependent reduction in the number

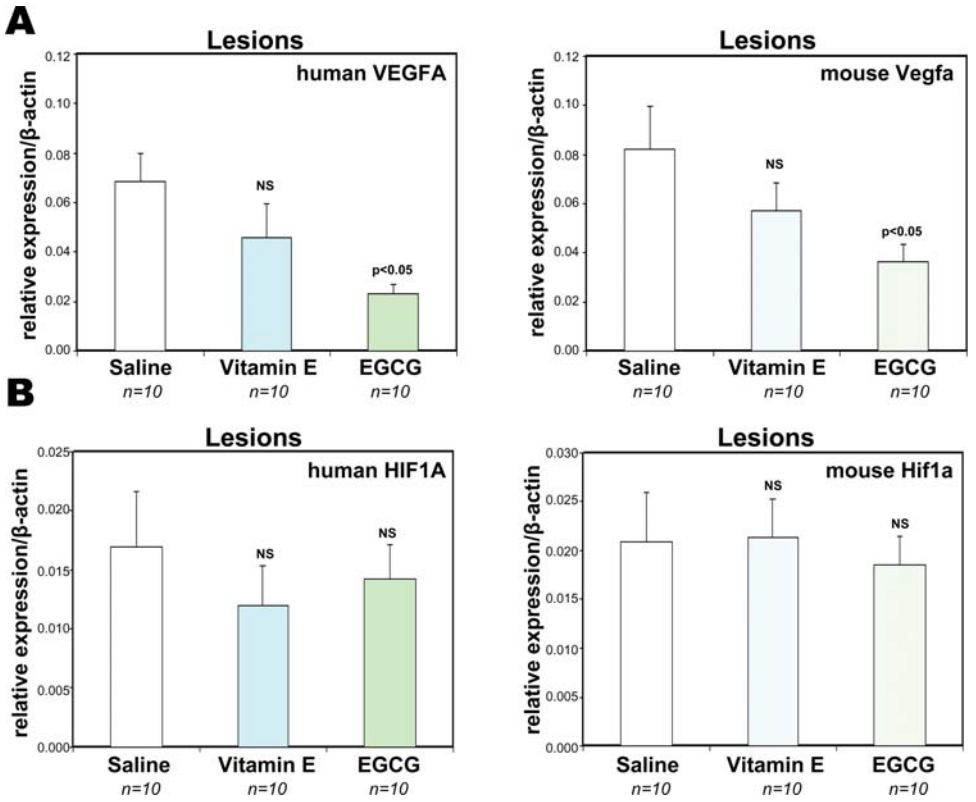

Figure 6 Angiogenesis factors in endometriotic lesions.
Relative human (left) and mouse (right) VEGFA/Vegfa **(A)** and HIF1A/Hif1a **(B)** mRNA levels in the lesions. *P*-values are indicated above the bar when compared with saline control group.

and size of microvessels in the experimental endometriosis model. Both blood vessels within the engrafted endometriotic lesion and those from the host subcutaneous layer were reduced in the EGCG group when compare with controls. Angiogenic VEGFA mRNA originating from both human and mouse tissues was significantly down-regulated, but HIF1A was not affected. The binding of HIF1A to hypoxia-responsive element in the VEGF promoter is a predominant enhancer of VEGF production. VEGF protein binds to VEGF receptors on endothelial cells, and these mediate its angiogenic functions (Semenza, 1999). The selective inhibition of angiogenic factors suggests that green tea EGCG has an anti-angiogenic effect in endometriosis specifically through VEGF suppression.

On the other hand, EGCG treatment has also been shown to inhibit both adhesion and invasion in tumour metastasis and thereby exerts anti-angiogenic activity. Adhesions of endometrial cells onto the peritoneal surface followed by invasion are important steps in the development of endometrial lesions. MMPs and PLAU are the key invasion enzymes that break down the endometrial stroma and extracellular basement proteins, allowing further growth and spread of endothelial cells, playing a major regulatory role in extracellular matrix reorganization and the initiation of angiogenesis (Rabbani, 1998; Kähäri and Saarialho-Kere, 1999). ITGAVβ3 is a cell adhesion molecule that functions in both cell–cell and cell–substratum adhesion. Vascular expression of ITGAVβ3 is associated with angiogenesis in other tissues (Brooks *et al.*, 1994). In our study, the expression of MMP2 and PLAU in the endometriotic lesions was significantly lower in mice treated with EGCG than in the controls but expression of ITGAVβ3 was unaffected. It indicates that the anti-angiogenic effects of EGCG interfere with cell adhesion and invasion mainly through MMP2 and PLAU suppression, respectively.

EGCG significantly inhibited angiogenesis, adhesion and invasion of the endometriotic lesions, and also reduced the lesion size, severely decreasing the survival of endometriosis as a consequence. In our TUNEL study, apoptotic bodies occurred more frequently in the stroma of ectopic endometrium in the EGCG group than amongst controls, with significant activation of NFKB and MAPK1. NFKB and MAPK1 play a critical role in the regulation of cell survival, proliferation and apoptosis (Schwartz *et al.*, 1999; Joyce *et al.*, 2001). NFKB pathway activates many of the target genes that are critical to the initiation and establishment of the early and late stages of endometriosis, including the induction of expression of several key pro-inflammatory, chemokine, angiogenic, prostanoid and cell cycle genes (Karin and Lin, 2002). NFKB is constitutively activated in eutopic endometrium and ectopic endometrial lesions, and peritoneal macrophages of women with endometrosis. Hormonal endometriosis therapies, including progesterone, danazol and GnRH agonist, appear to suppress the NFKB pathway (Surh *et al.*, 2001). In addition, EGCG is a known NFKB inhibitor (Ahmad *et al.*, 2000; Kuo and Lin, 2003). Interestingly, in our study, mouse Nfkb and Mapk1 were significantly inhibited in surrounding host tissues only, but human NFKB and

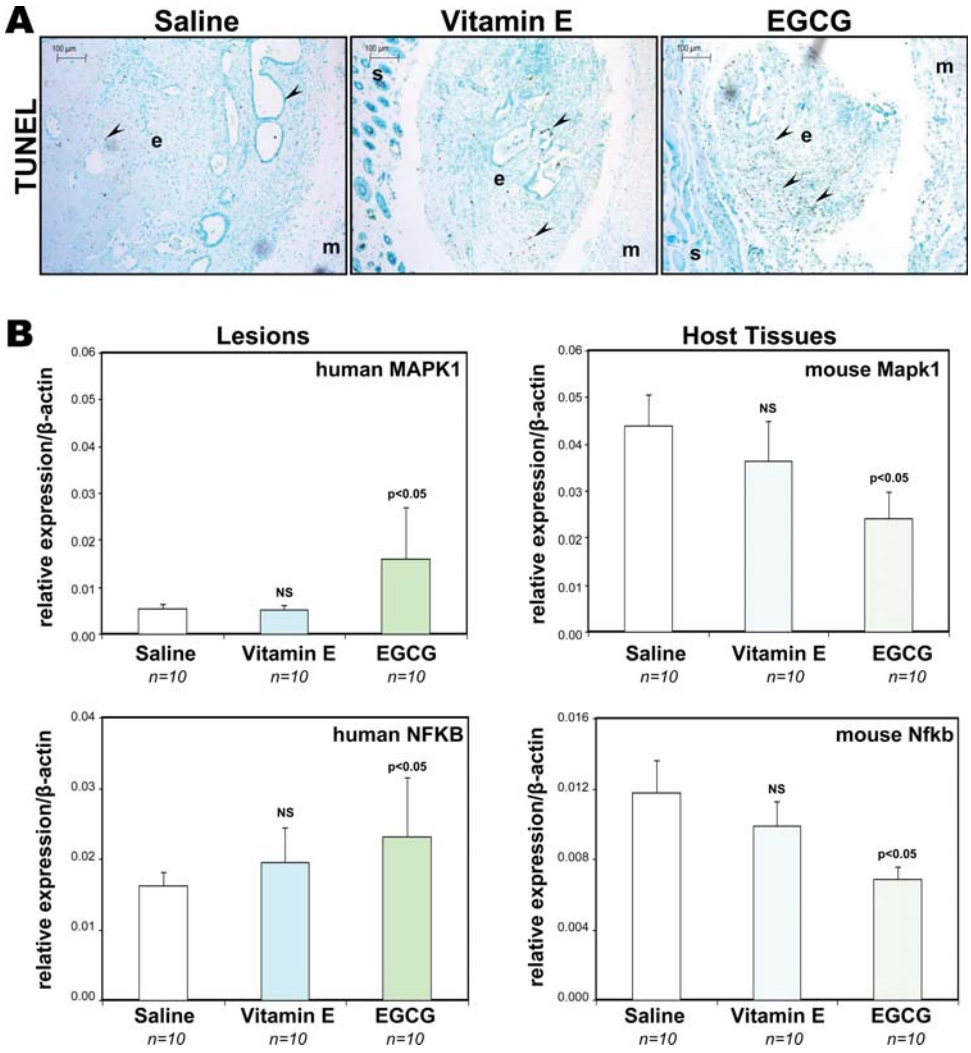

Figure 7 Survival of endometriotic lesions.
(A) TUNEL analysis of the lesions. Arrows, apoptotic bodies in the endometrial stroma and glandular epithelium; s, skin and subcutaneous layers; m, abdominal muscles and peritoneum; e, endometriotic lesions. **(B)** Relative human and mouse MAPK1/Mapk1 (upper panels) and NFKB/Nfkb (lower panel) mRNA levels in the lesions (left) and host tissues (right). *P*-values are indicated above the bar when compared with saline control group.

MAPK1 were significantly up-regulated in the endometriotic lesions. This may be because the animal model used in this study is immunocompromised, and other non-immunocompromised models may be required to investigate the detailed effects of EGCG on the NFKB pathway. A non-human primate (D'Hooghe, 1997) might be a very good alternative model.

EGCG is also a potent antioxidant. To ensure that this effect on the angiogenesis was not solely related to its antioxidant properties, Vitamin E (another potent antioxidant) was included in the comparison as a second control group. With Vitamin E treatment, even though endometriotic lesions were also smaller in size, and angiogenic, adhesive and invasive molecules were down-regulated, these differences were not significant. This suggests that the anti-angiogenic effect of EGCG on the endometriotic growth is independent from its anti-oxidative property, and an antioxidant alone may not be helpful in endometriosis.

Currently, concentrated efforts in this area of research are leading to the discovery of a growing number of pro- and anti-angiogenic molecules, some of which are already in clinical trials. The complex interactions among these molecules and how they affect vascular structure and function in different environments are now beginning to be elucidated. In the present study in mice, EGCG in the higher dose (50 mg/kg), but not in the lower dose (5 mg/kg), showed a beneficial effect on the experimental endometriosis and the effect observed resembles the ability of EGCG to prevent establishment of endometriosis. To achieve similar concentrations of EGCG as found in human plasma, 600 mg/kg of EGCG is required to be administered to animals orally, which is equivalent to 30 cups of tea in the human (Chu *et al*., 2006, 2007). Green tea catechins possess many beneficial properties, including a large distribution volume, reasonable half-life and wide safety margin. Thirty cups of tea a day will not lead to any major adverse effect apart from some caffeine impedance in individuals

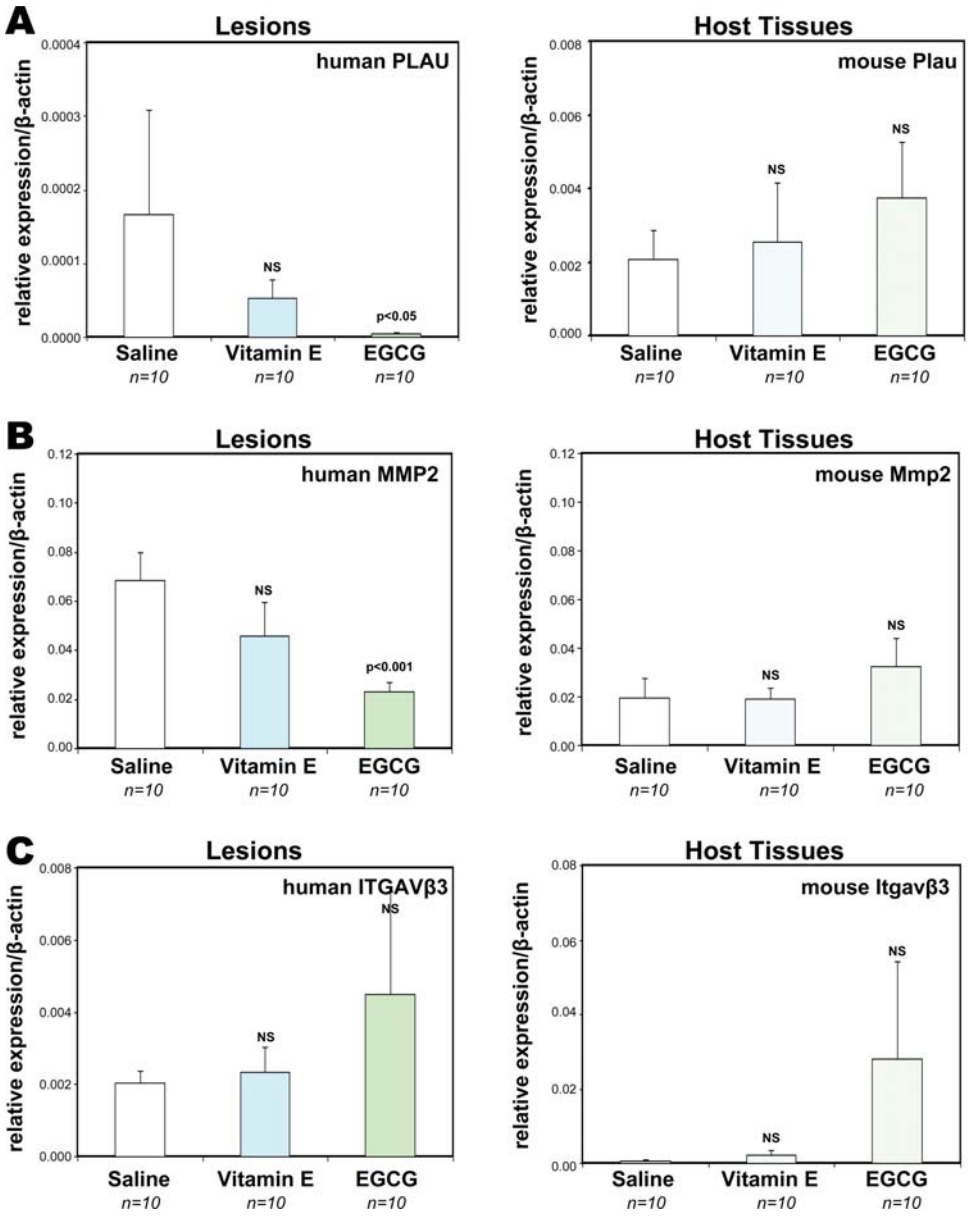

Figure 8 Invasion and adhesion in endometriotic lesions.
Relative human and mouse PLAU/Plau **(A),** MMP2/Mmp2 **(B)** and ITGAVβ3/Itgavβ3 **(C)** mRNA levels in the lesions (left) and host tissues (right). *P*-values are indicated above the bar when compared with saline control group.

(Mitscher *et al*., 1997) and the required EGCG concentrations could be easily achieved by taking some commercial green tea extracts which are very popular as nutrient supplements nowadays; i.e. potentially practical and feasible for clinical use.

The limitation of the endometriosis model in mice is that they do not undergo menstruation and never develop spontaneous endometriosis. Moreover, the subcutaneous pocket explants of human endometrium are rather remote from endometriosis in women. The surgically induced mouse model of endometriosis allows allogeneic or autogeneic endometrium to be transplanted. The hormonal regulation and estrous cycle in mice provide support for the growth of the engrafted endometrium, and the endometriotic lesions generate a blood supply by recruiting new blood vessels from the surrounding vasculature, resembling the situation in human. This provides a useful model for the study of the endometriosis growth and associated angiogenesis *in vivo*. However, the potential therapeutic benefit of green tea catechin for prevention and treatment of endometriosis could only be defined by a more relevant preclinical animal model, such as baboon (D'Hooghe, 1997), and a large scale epidemiological study or randomized clinical trial.

In conclusion, our study suggested that green tea EGCG has a significant inhibitory effect on the development of endometriosis. This inhibitory effect is mediated mainly through its selective suppression of VEGF-A in both ectopic endometrium and in the host abdominal

wall, and results in fewer new blood vessels, thus limiting the ability of the engrafted endometriotic lesion to invade host tissues.

References

Adhami VM, Siddiqui IA, Ahmad N, Gupta S, Mukhtar H. Oral consumption of green tea polyphenols inhibits insulin-like growth factor-I-induced signaling in an autochthonous mouse model of prostate cancer. *Cancer Res* 2004;**64**:8715–8722.

Ahmad N, Gupta S, Mukhtar H. Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor kappaB in cancer cells versus normal cells. *Arch Biochem Biophys* 2000;**376**: 338–346.

American Society for Reproduction Medicine. Revised American Society for Reproduction Medicine classification of endometriosis: 1996. *Fertil Steril* 1997;**67**:817–821.

Aoki D, Katsuki Y, Shimizu A, Kakinuma C, Nozawa S. Successful heterotransplantation of human endometrium in SCID mice. *Obstet Gynecol* 1994;**83**:220–228.

Awwad JT, Sayegh RA, Tao XJ, Hassan T, Awwad ST, Isaacson K. The SCID mouse: an experimental model for endometriosis. *Hum Reprod* 1999;**14**:3107–3111.

Basini G, Bianco F, Grasselli F. EGCG, a major component of green tea, inhibits VEGF production by swine granulosa cells. *Biofactors* 2005; **23**:25–33.

Becker CM, Bartley J, Mechsner S, Ebert AD. Angiogenesis and endometriosis. *Zentralbl Gynakol* 2004;**126**:252–258.

Bhanoori M, Arvind Babu K, Pavankumar Reddy NG, Lakshmi Rao K, Zondervan K, Deenadayal M, Kennedy S, Shivaji S. The vascular endothelial growth factor (VEGF) +405GNC 5′-untranslated region polymorphism and increased risk of endometriosis in South Indian women: a case control study. *Hum Reprod* 2005;**20**:1844–1849.

Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin avb3 for angiogenesis. *Science* 1994;**264**:569–571.

Cao Y, Cao R. Angiogenesis inhibited by drinking tea. *Nature* 1999; **398**:381.

Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;**407**:249–257.

Chu KO, Wang CC, Chu CY, Chan KP, Rogers MS, Choy KW, Pang CP. Pharmacokinetic studies of green tea catechins in maternal plasma and fetuses in rats. *J Pharm Sci* 2006;**95**:1372–1381.

Chu KO, Wang CC, Chu CY, Choy KW, Pang CP, Rogers MS. Uptake and distribution of catechins in fetal organs following in utero exposure in rats. *Hum Reprod* 2007;**22**:280–287.

Dabrosin C, Gyorffy S, Margetts P, Ross C, Gauldie J. Therapeutic effect of angiostatin gene transfer in a murine model of endometriosis. *Am J Pathol* 2002;**161**:909–918.

D'Hooghe TM. Clinical relevance of the baboon as a model for the study of endometriosis. *Fertil Steril* 1997;**68**:613–625.

Donnez J, Smoes P, Gillerot S, Casanas-Roux F, Nisolle M. Vascular endothelial growth factor (VEGF) in endometriosis. *Hum Reprod* 1998; **13**:1686–1690.

Fassina G, Vene R, Morini M, Minghelli S, Benelli R, Noonan DM, Albini A. Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by epigallocatechin-3-gallate. *Clin Cancer Res* 2004;**10**:4865–4873.

Harada M, Kan Y, Naoki H, Fukui Y, Kageyama N, Nakai M, Miki W, Kiso Y. Identification of the major antioxidative metabolites in biological fluids of the rat with ingested (+)-catechin and (-)-epicatechin. *Biosci Biotechnol Biochem* 1999;**63**:973–977.

Hsieh YY, Chang CC, Tsai FJ, Yeh LS, Lin CC, Peng CT. T allele for VEGF gene-460 polymorphism at the 5′-untranslated region: association with a higher susceptibility to endometriosis. *J Reprod Med* 2004;**49**:468–472.

Joyce D, Albanese C, Steer J, Fu M, Bouzahzah B, Pestell RG. NF-kappaB and cell-cycle regulation: the cyclin connection. *Cytokine Growth Factor Rev* 2001;**12**:73–90.

Jung YD, Kim MS, Shin BA, Chay KO, Ahn BW, Liu W, Bucana CD, Gallick GE, Ellis LM. EGCG, a major component of green tea, inhibits tumor growth by inhibiting VEGF induction in human colon carcinoma cells. *Br J Cancer* 2001;**84**:844–850.

Kähäri VM, Saarialho-Kere U. Matrix metalloproteinases and their inhibitors in tumour growth and invasion. *Ann Med* 1999;**31**:34–45.

Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol* 2002;**3**:221–227.

Kim SH, Choi YM, Choung SH, Jun JK, Kim JG, Moon SY. Vascular endothelial growth factor gene +405 C/G polymorphism is associated with susceptibility to advanced stage endometriosis. *Hum Reprod* 2005; **20**:2904–2908.

Kojima-Yuasa A, Hua JJ, Kennedy DO, Matsui-Yuasa I. Green tea extract inhibits angiogenesis of human umbilical vein endothelial cells through reduction of expression of VEGF receptors. *Life Sci* 2003;**73**:1299–1313.

Kondo T, Ohta T, Igura K, Hara Y, Kaji K. Tea catechins inhibit angiogenesis in vitro, measured by human endothelial cell growth, migration and tube formation, through inhibition of VEGF receptor binding. *Cancer Lett* 2002;**180**:139–144.

Kuo PL, Lin CC. Green tea constituent (-)-epigallocatechin-3-gallate inhibits Hep G2 cell proliferation and induces apoptosis through p53-dependent and Fas-mediated pathways. *J Biomed Sci* 2003;**10**:219–227.

Lamy S, Gingras D, Beliveau R. Green teas catechins inhibit vascular endothelial growth factor receptor phosphorylation. *Cancer Res* 2002; **62**:381–385.

Liao J, Yang GY, Park ES, Meng X, Sun Y, Jia D, Seril DN, Yang CS. Inhibition of lung carcinogenesis and effects on angiogenesis and apoptosis in A/J mice by oral administration of green tea. *Nutr Cancer* 2004;**48**:44–53.

Mai KT, Teo I, Al Moghrabi H, Marginean EC, Veinot JP. Calretinin and CD34 immunoreactivity of the endometrial stroma in normal endometrium and change of the immunoreactivity in dysfunctional uterine bleeding with evidence of 'disordered endometrial stroma. *Pathology* 2008;**40**:493–499.

Mitscher LA, Jung M, Shankel D, Dou JH, Steele L, Pilllai SP. Chemoprotection: a review of the potential therapeutic antioxidant properties of green tea (Camellia Sinensis) and certain of its constituents. *Med Res Rev* 1997;**17**:327–365.

Mueller MD, Lebovic DI, Garrett E, Taylor RN. Neutrophils infiltrating the endometrium express vascular endothelial growth factor: potential role in endometrial angiogenesis. *Fertil Steril* 2000;**74**:107–112.

Mukhtar H, Katiyar SK, Agarwal R. Green tea and skin-anticarcinogenic effects. *J Invest Dermatol* 1994;**102**:3–7.

Nakae Y, Hirasaka K, Goto J, Nikawa T, Shono M, Yoshida M, Stoward PJ. Subcutaneous injection, from birth, of epigallocatechin-3-gallate, a component of green tea, limits the onset of muscular dystrophy in mdx mice: a quantitative histological, immunohistochemical and electrophysiological study. *Histochem Cell Biol* 2008;**129**:489–501.

Nap AW, Griffioen AW, Dunselman GAJ, Bouma-Ter Steege JCA, Tijssen VJL, Evers JLH, Groothuis PG. Antiangiogenesis therapy for endometriosis. *J Clin Endocrinol Metab* 2004;**89**:1089–1095.

Nisolle M, Casanas-Roux F, Anaf V, Mine JM, Donnez J. Morphometric study of the stromal vascularization in peritoneal endometriosis. *Fertil Steril* 1993;**59**:681–684.

Nisolle M, Casanas-Roux F, Donnez J. Early-stage endometriosis: adhesion and growth of human menstrual endometrium in nude mice. *Fertil Steril* 2000;**74**:306–312.

Rabbani SA. Metalloproteases and urokinase in angiogenesis and tumor progression. *In Vivo* 1998;**12**:135–142.

Sang S, Lambert JD, Hong J, Tian S, Lee MJ, Stark RE, Ho CT, Yang CS. Synthesis and structure identification of thiol conjugates of (-)-epigallocatechin gallate and their urinary levels in mice. *Chem Res Toxicol* 2005;**18**:1762–1769.

Sartippour MR, Shao ZM, Heber D, Beatty P, Zhang L, Liu C, Ellis L, Liu W, Go VL, Brooks MN. Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. *J Nutr* 2002;**132**:2307–2311.

Schwartz SA, Hernandez A, Mark Evers B. The role of NF-kappaB/IkappaB proteins in cancer: implications for novel treatment strategies. *Surg Oncol* 1999;**8**:143–153.

Semenza GL. Regulation of mammalian oxygen homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol* 1999;**15**:551–578.

Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, Jaffe RB, Taylor RN. Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *J Clin Endocrinol Metab* 1996;**81**:3112–3118.

Simons M. Angiogenesis: where do we stand now? *Circulation* 2005; **111**:1556–1566.

Stoner GD, Mukhtar H. Polyphenols as cancer chemopreventive agents. *J Cell Biochem* 1995;**22**:169–180.

Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, Lee SS. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. *Mutat Res* 2001;**480**: 243–268.

Tang FY, Meydani M. Green tea catechins and vitamin E inhibit angiogenesis of human microvascular endothelial cells through suppression of IL-8 production. *Nutr Cancer* 2001;**41**:119–125.

Ufer C, Wang CC, Fähling M, Schiebel H, Thiele BJ, Billett EE, Kuhn H, Borchert A. Translational regulation of glutathione peroxidase 4 expression through guanine-rich sequence-binding factor 1 is essential for embryonic brain development. *Genes Dev* 2008;**22**:1838–1850.

Villayandre BM, Paniagua MA, Fernandez-Lopez A, Chinchetrua MA, Calvo P. Effect of vitamin E treatment on N-methyl-d-aspartate receptor at different ages in the rat brain. *Brain Res* 2004; **1028**:148–155.

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