Resveratrol is a potent inhibitor of vascularization and cell proliferation in experimental endometriosis

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STUDY QUESTION: Does the phytochemical compound resveratrol inhibit vascularization of endometriotic lesions?

SUMMARY ANSWER: Resveratrol suppresses the development of new microvessels in endometriotic lesions by inhibiting endothelial cell proliferation.

WHAT IS KNOWN ALREADY: Establishment and progression of endometriosis is crucially dependent on angiogenesis. Resveratrol is a pleiotropic agent, which dose-dependently suppresses the development of new blood vessels.

STUDY DESIGN, SIZE, DURATION: This was a randomized study in a mouse model of endometriosis. Twenty female BALB/c mice with surgically induced endometriosis were treated with resveratrol (40 mg/kg/day, n = 10) or vehicle (n = 10) for 4 weeks.

MATERIAL, SETTING, METHODS: Peritoneal and mesenteric endometriotic lesions were surgically induced by uterine tissue transplantation into the abdominal cavity of BALB/c mice. The animals were daily treated with resveratrol (40 mg/kg) or vehicle by oral gavage. Lesion growth, vascularization, apoptosis and cell proliferation were subsequently analyzed by means of high-resolution ultrasound imaging, caliper measurements, histology and immunohistochemistry throughout an observation period of 4 weeks.

MAIN RESULTS AND THE ROLE OF CHANCE: Resveratrol inhibited angiogenesis in peritoneal and mesenteric endometriotic lesions, as indicated by a significantly reduced microvessel density when compared with controls. Additional immunohistochemical analyses revealed that this was caused by a decreased proliferating activity of CD31-positive endothelial cells in the newly developing microvasculature of the lesions. In line with these findings, lesions in resveratrol-treated mice exhibited a reduced growth rate and a smaller final size than controls. This was associated with lower numbers of proliferating cell nuclear antigen- and Ki67-positive stromal and glandular cells. Apoptotic cells were not detectable in either group. To limit the role of chance, the experiments were conducted under standardized laboratory conditions with appropriate controls. Statistical significance was accepted for a value of P < 0.05.

LIMITATIONS, REASONS FOR CAUTION: Endometriotic lesions were surgically induced by uterine tissue transplantation without the use of pathological endometriotic tissue of human origin. Therefore, the results obtained in this mouse model may not fully correlate to human patients with endometriosis.

WIDER IMPLICATIONS OF THE FINDINGS: Resveratrol is a potent inhibitor of vascularization in endometriotic lesions. This, most probably, causes the suppression of lesion growth. Accordingly, resveratrol represents a promising candidate therapy for future phytochemical treatment of endometriosis.

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Key words: endometriosis / resveratrol / angiogenesis / proliferation / ultrasound

Introduction

Endometriosis represents a common gynecological disorder, which is characterized by the presence and growth of endometriotic lesions consisting of endometrial glands and stroma outside the uterine cavity (Galle, 1989). Affected patients often suffer from chronic pelvic pain and infertility, resulting in a markedly reduced quality of
life and high costs for the health-care system (Kennedy et al., 2005; Simoens et al., 2012).

It is widely accepted that peritoneal endometriotic lesions develop from endometrial tissue fragments, which are retrogradly shed through the Fallopian tubes during menstruation (Sampson, 1927). During the last years, numerous studies could demonstrate that the establishment and survival of these lesions is crucially dependent on the formation of blood vessels, which guarantee their oxygen supply (Groothuis et al., 2005; Laschke and Menger, 2007; May and Becker, 2008). Accordingly, anti-angiogenic agents of different substance groups are currently discussed as promising candidates for future endometriosis therapy. Besides specific growth factor antagonists, endogenous angiogenesis inhibitors, statins, cyclooxygenase-2 inhibitors and immunomodulators, these anti-angiogenic agents also include several phytochemical compounds (reviewed by Laschke and Menger, 2012). The latter ones have been successfully used in traditional medicine without inducing severe side effects. Thus, in view of their favorable risk profile, they may be also highly preferable for the safe treatment of endometriosis patients.

Resveratrol (trans-3,5,4′-trihydroxystilbene) is a phytochemical compound of grapes, red wine, nuts and different berries, which has previously been shown to exert beneficial effects in a variety of pathological conditions, such as cardiovascular diseases and cancer (Baur and Sinclair, 2006). Resveratrol acts as a typical pleiotropic agent, affecting multiple cellular processes, including proliferation, apoptosis and oxygen radical formation (Athar et al., 2009). Moreover, resveratrol dose-dependently suppresses the development of new blood vessels (Chen and Tseng, 2007).

Recently, Bruner-Tran et al. (2011) reported for the first time that resveratrol inhibits the establishment of endometriotic lesions. They found that daily oral gavage of resveratrol significantly reduced the number and size of endometriotic lesions, which were induced by human endometrial tissue transplantation into the peritoneal cavity of nude mice. This was associated with decreased proliferative activity and up-regulation of apoptotic cell death inside the lesions. However, whether resveratrol contributes to the regression of ectopic endometrial tissue via its anti-angiogenic activity has not been analyzed so far.

Therefore, the aim of the present study was to evaluate the effect of resveratrol on vascularization and growth of endometriotic lesions. For this purpose, we used a well-established mouse model of surgically induced intraperitoneal endometriosis, which allows for the analysis of developing endometriotic lesions by means of high-resolution ultrasound imaging, histology and immunohistochemistry (Körbel et al., 2010).

Materials and Methods

Animals

Ten- to 14-week-old female BALB/c mice with a body weight of 18–20 g were used for the experiments. They were housed 10 per cage in a temperature-controlled environment under a 12 h/12 h light–dark cycle and received standard pellet food (Altromin, Lage, Germany) and water ad libitum. All experiments were approved by the local governmental animal care committee and were conducted in accordance with the German legislation on protection of animals and the NIH Guidelines for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, USA).

For the induction of endometriosis, we used cycling donor and recipient animals with intact ovaries, which were not treated with exogenous steroids. To exclude discrepancies between individual animals owing to different sex hormone levels, we only selected animals in the stage of estrus. For this purpose, the cycle stage was evaluated by pipetting 15 μl of 0.9% saline into the vagina. The suspension was then transferred on a glass slide and cytologically examined under a phase contrast microscope (CH-2; Olympus, Hamburg, Germany).

Model of intraperitoneal endometriosis

Intrapерitoneal endometriotic lesions were surgically induced by suturing uterine tissue samples from donor mouse to the abdominal wall and the mesentery of recipient mice, as described previously (Rudzitis-Auth et al., 2012). Briefly, both uterus horns were removed from anesthetized donor mice (75 mg/kg body weight (bw) ketamine i.p. (Pharmacia GmbH, Erlangen, Germany) and 15 mg/kg bw xylazin i.p. (Bayer, Leverkusen, Germany)) and transferred into a Petri dish containing Dulbecco’s modified Eagle medium (DMEM; 10% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin; PAA, Colbe, Germany). The horns were opened longitudinally and 2 mm tissue samples were removed using a dermal biopsy punch (Stiefel Laboratorium GmbH, Offenbach am Main, Germany). After a midline incision, tissue samples were fixed with a 6–0 Prolene suture (Ethicon Products, Norderstedt, Germany) to the right and the left side of the abdominal wall (peritoneal lesions; n = 2) and to the intestinal mesentery (mesenteric lesions; n = 2) of anesthetized recipient animals. Subsequently, the laparotomy was closed with running 6–0 Prolene muscle and skin sutures. In contrast to the mesenteric lesions, peritoneal endometriotic lesions were not affected by respiratory movements or peristalsis of the intestine. Therefore, peritoneal lesions were used for detailed repetitive analyses of growth and cyst formation by means of high-resolution ultrasound imaging throughout an observation period of 28 days.

High-resolution ultrasound image acquisition and analysis

Mice were anesthetized with 2% isoflurane in oxygen and fixed in the supine position on a heated stage with ECG electrodes and heart rate display (THM100; Indus Instruments, Houston, TX, USA). After chemical depilation (Nair hair removal lotion; Church & Dwight Canada Corp., Mississauga, ON, Canada) of the abdomen to prevent air trapped in the fur from interfering with ultrasound coupling into the animal, ultrasound coupling gel (Aquasonic 100; Parker, NJ, USA) was applied to the skin.

Ultrasound imaging of peritoneal lesions was performed with the Vevo 770™ high-resolution in vivo micro-imaging system (VisualSonics, Toronto, ON, Canada) and a real-time microvisualization (RMVTM) 704 Scanhead (VisualSonics) with a center frequency of 40 MHz and a focal depth of 6 mm (Laschke et al., 2010). The ultrasound images were analyzed by means of a three-dimensional reconstruction and analysis software licensed to VisualSonics for distribution with the Vevo 770™ high-resolution imaging system. The analyses included the determination of the overall volume of endometriotic lesions, their stromal tissue and cysts (in mm³) by manual image segmentation. For this purpose, boundaries of endometriotic lesions and their cysts were manually outlined in parallel slices, which were separated by a step size of 200 μm in the three-dimensional ultrasound images. Based on the outlined areas, volumes were subsequently computed by the VisualSonics software. Moreover, we calculated the growth of lesions and stromal tissue by dividing the measured lesion and stromal tissue volumes at individual time points by the initial lesion and stromal tissue volumes at Day 0 (in %) and we assessed the fraction of cyst-containing lesions (in % of all analyzed lesions).
After the last ultrasound analysis at Day 28, the animals were anesthe-
tized by i.p. injection of ketamine and xylazine and carefully laparotomized under a stereo-microscope to measure the largest and perpendicularly aligned smallest diameters of peritoneal and mesenteric endometriotic lesions by means of a digital caliper. The lesion sizes were then calculated with the formula \( D1 \times D2 + \pi/4 \) (Becker et al., 2008).

**Experimental protocol**

The mice were randomly divided into two groups (n = 10) receiving either 40 mg/kg resveratrol (dissolved in 100 \( \mu \)l 25% ethanol; Biomol, Hamburg, Germany) or vehicle (control) by oral gavage once a day. The treatments were initiated at the time of induction of experimental endometriosis (d0) and were continued over a period of 28 days until the end of the experiments. Ultrasound image analyses of developing peritoneal endometriotic lesions were performed directly after tissue transplantation (d0) as well as at Day 7, 14, 21 and 28 to measure the volume of cysts and stromal tissue. At the end of the experiments, the size of peritoneal and mesenteric endometriotic lesions was additionally assessed by means of a digital caliper. Subsequently, the lesions were excised for further histological and immunohistochemical analyses.

**Histology and immunohistochemistry**

Formalin-fixed specimens of endometriotic lesions from Day 28 were em-
bedded in paraffin. Two-micrometers-thick sections were cut and stained with hematoxylin and eosin according to standard procedures. Cross-
sectional areas of endometriotic lesions were measured by computer-
assisted planimetry using a BZ-8000 microscope with a Biozero analysis software (version 3.60; Keyence, Osaka, Japan). To assess the overall volume of each lesion (in mm\(^3\)) as well as its stromal tissue volume (in mm\(^3\)) and cyst volume (in mm\(^3\)), trapezoidal bodies were calculated according to: \( V_t = \frac{1}{2} \times (A + B) \times d \), where \( A \) and \( B \) represent the neighboring section areas and \( d \) the interslice distance. The sum of all trapezoidal bodies yielded the total volumes.

Apoptotic cells within the tissue samples were detected immuno-
chemically using a polyclonal rabbit anti-cleaved caspase-3 antibody (1:100; Cell Signaling Technology, Boston, MA, USA) as primary antibody. Proliferating cells were stained with a monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody (1:200; Dako Cytomation, Hamburg, Germany) and a rabbit polyclonal anti-Ki67 antibody (1:2000; Dako Cytomation, Hamburg, Germany) or vehicle (control) by oral gavage once a day. The treatments were initiated at the time of induction of experimental endometriosis (d0) and were continued over a period of 28 days until the end of the experiments. Ultrasound image analyses of developing peritoneal endometriotic lesions were performed directly after tissue transplantation (d0) as well as at Day 7, 14, 21 and 28 to measure the volume of cysts and stromal tissue. At the end of the experiments, the size of peritoneal and mesenteric endometriotic lesions was additionally assessed by means of a digital caliper. Subsequently, the lesions were excised for further histological and immunohistochemical analyses.

**Statistics**

Data were first analyzed for normal distribution and equal variance. In case of parametric data, differences between the two experimental groups were assessed by the unpaired Student’s t-test. In case of non-parametric data, differences between the two experimental groups were assessed by the Mann–Whitney rank sum test. To test for time effects within each experimental group, analysis of variance (ANOVA) for repeated measurements was applied. This was followed by the Dunnett post hoc test (SigmaStat; Jandel Corporation, San Rafael, CA, USA). All data are given as means ± SEM. Statistical significance was accepted for a value of \( P < 0.05 \).

**Results**

**Growth and cyst formation of endometriotic lesions**

High-resolution ultrasound imaging showed that uterine tissue samples, which were sutured to the abdominal wall of vehicle-treated and resveratrol-treated animals, exhibited a comparable initial size of \( \sim 1 \text{ mm}^3 \), providing standardized conditions for further analyses of growth and cyst formation in developing endometriotic lesions (Fig. 1C). Throughout the further time course of the experiment, the size of lesions from the control group progressively increased, finally presenting with a volume of \( \sim 3 \text{ mm}^3 \) at Day 28 (Fig. 1A, C and D). This was associated with the growth of the stromal tissue fraction (Fig. 1E and F) and endometrial cysts (Fig. 1G and H).

In contrast, resveratrol treatment markedly suppressed the develop-
ment of endometriotic lesions. In fact, at Day 28 they still exhibited a lesion volume, which did not differ from that of Day 0 (Fig. 1B–D). This was due to the regression of the stromal tissue. Accordingly, the stromal tissue volume and the growth of these lesions were signifi-
cantly reduced between Days 14–28 when compared with controls (Fig. 1E and F). In addition, although the fraction of cyst-containing lesions was comparable in both experimental groups (Fig. 1H), endo-
metrial cysts in resveratrol-treated lesions presented with lower volumes until the end of the experiment (Fig. 1G). In general, there was no resveratrol-treated or control animal that exhibited complete regression of a lesion.

Histological analyses at Day 28 proved that the transplanted uterine tissue samples of both groups had developed to typical endometriotic lesions consisting of cyst-like dilated endometrial glands and vascular-
ized stroma, independently of their localization at the peritoneal wall or in the mesentery (Fig. 2A–D). However, peritoneal and mesenteric lesions of resveratrol-treated animals exhibited a markedly decreased size when compared with vehicle-treated controls (Fig. 2A–D). This was confirmed by quantitative measurements of lesion sizes by means of a caliper (Fig. 2E). More detailed histomorphometric ana-
lyses revealed that the reduced lesion sizes in resveratrol-treated animals were caused by a lower stromal tissue volume and cyst volume (Table I). Of interest, volumes measured by histology were markedly underestimated when compared with the ultrasound and caliper measurements, because the volume of endometriotic lesions drastically decreases during the processing of tissue samples for hist-
ology (Laschke et al., 2010).
Apoptosis and cell proliferation in endometriotic lesions

Because we did not detect any cleaved caspase-3-positive apoptotic cells in endometriotic lesions of both groups at Day 28 (data not shown), we speculated that the observed differences in lesion size were primarily caused by a reduced cell proliferation in resveratrol-treated animals. To test this hypothesis, tissue sections were additionally stained for the proliferation markers PCNA and Ki67. By this, we
found that treatment with resveratrol resulted in a significantly reduced number of PCNA-positive stromal cells in peritoneal and mesenteric lesions when compared with controls (Fig. 3A–E), whereas PCNA expression in glandular cells did not show marked differences between the two groups (Fig. 3A–D and F).

As expected, numbers of Ki67-positive cells in endometriotic lesions were substantially lower (Fig. 4A–F), because Ki67 is known to be a more specific marker of proliferation than PCNA (Kordek et al., 1996). Ki67 staining of peritoneal and mesenteric lesions revealed a comparable number of positive stromal cells in both groups (Fig. 4A–E). In contrast, numbers of Ki67-positive glandular cells were significantly decreased in resveratrol-treated animals when compared with controls (Fig. 4A–D and F).

Vascularization of endometriotic lesions

Treatment with resveratrol effectively inhibited angiogenesis in peritoneal and mesenteric endometriotic lesions. This was indicated by a significantly reduced density of CD31-positive microvessels at Day 28 (Fig. 5A–E). More detailed immunohistochemical analyses showed that this was caused by a reduced proliferating activity of the microvascular endothelium in resveratrol-treated lesions, exhibiting less PCNA-positive endothelial cells when compared with controls (Fig. 6A–E).

Discussion

Resveratrol represents one of the most frequently analyzed phytochemical compounds in life sciences during the last decades. Because resveratrol exerts a broad spectrum of beneficial effects under various pathological conditions, it has been suggested as a promising therapeutic agent for the treatment of cancer as well as several inflammatory, metabolic and cardiovascular diseases (Beaudeux et al., 2010; Petrovski et al., 2011; Aluyen et al., 2012). Recently, Bruner-Tran et al. (2011) reported for the first time that resveratrol may also be suitable for the prevention and treatment of endometriosis by inhibiting the establishment and growth of endometriotic lesions. Herein, we now provide the novel finding that the anti-angiogenic activity of resveratrol crucially contributes to this observation.

Animals were orally treated with 40 mg/kg resveratrol over 4 weeks, because this dose and route of application has previously been shown to effectively suppress the formation of new blood vessels in tumors (Tseng et al., 2004; Harikumar et al., 2010). In this context, it should be noted that resveratrol acts as a typical pleiotropic agent, which targets several key steps of the angiogenic process (Chen...
Figure 3 (A–D) Immunohistochemical staining of endometriotic lesions at Day 28 after surgical induction by fixation of uterine tissue samples to the peritoneal wall (A and B) or the intestinal mesentery (C and D) of BALB/c mice, which received vehicle (control; A and C) or 40 mg/kg resveratrol (B and D). Sections were stained with an antibody against PCNA for the detection of proliferating cells in the endometrial stroma (arrows) and glands (arrowheads). Note that resveratrol-treated lesions exhibit a reduced number of PCNA-positive stromal cells (arrows) when compared with controls. Scale bars: 50 μm. (E and F) PCNA-positive (%) stromal (E) and glandular (F) cells of peritoneal and mesenteric endometriotic lesions of control (white bars; n = 10) and resveratrol-treated BALB/c mice (black bars; n = 10). Mean ± SEM; *P < 0.05 versus control (unpaired Student’s t-test; Mann–Whitney rank sum test).

Figure 4 (A–D) Immunohistochemical cross sections of endometriotic lesions at Day 28 after surgical induction by fixation of uterine tissue samples to the peritoneal wall (A and B) or the intestinal mesentery (C and D) of BALB/c mice that received vehicle (control; A and C) or 40 mg/kg resveratrol (B and D). Sections were stained with an antibody against Ki67 for the detection of proliferating cells in the endometrial stroma (arrows) and glands (arrowheads). Note that resveratrol-treated lesions exhibit a reduced number of Ki67-positive glandular cells (arrowheads) when compared with controls. Scale bars: 50 μm. (E and F) Ki67-positive (%) stromal (E) and glandular (F) cells of peritoneal and mesenteric endometriotic lesions of control (white bars; n = 10) and resveratrol-treated BALB/c mice (black bars; n = 10). Mean ± SEM; *P < 0.05 versus control (unpaired Student’s t-test).
and Tseng, 2007). In fact, resveratrol has been shown to inhibit hypoxia-mediated activation of Erk1/2 and Akt, resulting in decreased expression of hypoxia-inducible factor-1α and vascular endothelial growth factor (Cao et al., 2004; Tseng et al., 2004; Zhang et al., 2005). Moreover, resveratrol reduces the activity of matrix metalloproteinase-2 and -9 (Ganapathy et al., 2010; Kaneko et al., 2011), which are both involved in extracellular matrix disruption in the early angiogenic phase of vascular bud and sprout formation (Carmeliet, 2000). In addition, resveratrol directly inhibits the proliferation and migration of endothelial cells and vascular smooth muscle cells (Hu et al., 2007; Lee et al., 2009). Accordingly, we found in the present study that resveratrol-treated endometriotic lesions exhibited a reduced microvessel density when compared with vehicle-treated controls, independently of their localization at the peritoneal wall or in the intestinal mesentery. More detailed immunohistochemical analyses further revealed that this was caused by a decreased proliferating activity of endothelial cells in the newly developing microvasculature of the lesions.

For the non-invasive evaluation of peritoneal endometriotic lesions, we used the technique of high-resolution ultrasound imaging. Using...
this approach, it is possible to distinguish between the proliferation of stromal tissue and the formation of endometriotic cysts as possible causes for increasing lesion volumes over time (Laschke et al., 2010). We could demonstrate that resveratrol treatment reduced the secretory activity of endometriotic cysts, resulting in decreased cyst volumes when compared with controls. Furthermore, resveratrol treatment induced a regression of the stromal tissue, which was confirmed by additional histomorphometric analyses. The latter observation may be attributed to an up-regulation of apoptotic cell death, as previously reported by Bruner-Tran et al. (2011), studying the growth of ectopic human endometrial tissue in nude mice. However, unexpectedly we could not detect any caspase-3-positive apoptotic cells inside our endometriotic lesions. These discrepant results may be due to the different tissue types that were transplanted in these studies for the induction of endometriotic lesions. In fact, in the present study endometriotic lesions were surgically induced by transplantation of uterine tissue from donor mice into the peritoneal cavity of recipient animals without the use of endometrium or pathological endometriotic tissue of human nature. Because there may be marked differences between human and mouse tissue, the results obtained in our mouse model may thus not fully correlate to human patients with endometriosis. On the other hand, it is well known that there is a biphasic dose-dependent effect of resveratrol on apoptosis (Mukherjee et al., 2010). Because we treated mice with a resveratrol dose, which was ≈8-fold lower than that used by Bruner-Tran et al. (2011), it may thus be speculated that the resveratrol concentrations achieved in our study were not sufficient to induce programmed cell death.

In addition, we analyzed the proliferative activity of stromal and glandular cells. By this, we found that resveratrol treatment suppressed the proliferation of both cell types in peritoneal and mesenteric lesions. However, our data from the analyses of PCNA- and Ki67-stained tissue sections did not correlate well. Numbers of Ki67-positive cells were in general much lower in endometriotic lesions than those of PCNA-positive cells. Moreover, marked differences in proliferating activity of PCNA-stained lesions were found in stromal cells, whereas Ki67 expression mainly differed in the glandular cells. This may be explained by a prolonged half-life of PCNA or by the finding that growth factors are capable of stabilizing PCNA mRNA, resulting in the accumulation of the protein also in non-proliferating cells (Hall et al., 1990; Kordek et al., 1996). Besides, PCNA has also been shown to be expressed in cells with increased DNA repair activity (Gramantieri et al., 2003). Nonetheless, despite these discrepancies both markers indicated an inhibitory effect of resveratrol on the ectopic endometrial tissue.

In summary, we could demonstrate that resveratrol suppresses the development of new microvessels in endometriotic lesions by inhibiting endothelial cell proliferation. This is associated with a decreased lesion size, further supporting the concept that the establishment and progression of endometriosis is crucially dependent on angiogenesis (Feng et al., 2012). Importantly, we used in our study a resveratrol dose that is in the dose range of clinical trials (Scott et al., 2012; Smoliga et al., 2011). Accordingly, we are optimistic that the observed beneficial effects of resveratrol may be also reproducible under clinical conditions. Further studies have to clarify now whether this succeeds without exerting severe side effects in endometriosis patients.

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**Authors’ roles**

J.R.-A. contributed toward designing of the study, acquisition of data, and analysis and interpretation of data and participated in the drafting and final approval of the article. M.D.M. contributed toward designing of the study and interpretation of data and participated in the revision and final approval of the article. M.W.L. contributed toward designing of the study, supervision of experiments and interpretation of data and participated in the revision and final approval of the article.

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**Conflict of interest**

None declared.

**References**


Resveratrol and endometriosis

1347


