Progestins inhibit expression of MMPs and of angiogenic factors in human ectopic endometrial lesions in a mouse model

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Abstract: Progestins are successfully used in the treatment of endometriosis; however, the exact mechanisms of their action are still unsolved. We here focused on the effect of different progestins on parameters of extracellular matrix degradation and angiogenesis involved in the establishment and maintenance of ectopic endometrial lesions. Human endometrium was intraperitoneally transplanted into nude mice. After 7 and 28 days of treatment with progesterone, dydrogesterone, or its metabolite dihydrodydrogesterone, respectively, ectopic lesions were evaluated for proliferation and apoptosis. Expression of estrogen receptor α, progesterone receptor-AB, the angiogenic factors, cysteine-rich angiogenic inducer (CYR61), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGFA) and the matrix metalloproteinase (MMP)-2, -3, -7 and -9 was investigated. Functional impact on angiogenesis was evaluated by density of microvessels and of vessels stabilized by pericytes within the ectopic lesions. Although dydrogesterone significantly reduced proliferation of endometrial stromal cells after 28 days, suppression of apoptosis was independent from progestins. Expression of MMP-2 was significantly reduced by all progestins and MMP-3 by dydrogesterone. In the grafted endometrial tissue, transcription of bFGF was suppressed by progesterone and dihydrodydrogesterone, and VEGFA and CYR61 by dihydrodydrogesterone and dydrogesterone. In parallel, microvessel density was slightly suppressed by progestins, whereas number of stabilized vessels increased. Thus, progestins regulate factors important for the establishment and maintenance of ectopic endometrial lesions.

Key words: progestins / dydrogesterone / MMP / angiogenesis / endometriosis

Introduction

Endometriosis, defined by the presence of endometrium-like tissues outside the uterus, is one of the most frequent benign gynaecological diseases and affects an estimated 10–15% of all women of reproductive age and >30% of women suffering from infertility or pelvic pain (Cramer and Missmer, 2002; Giudice and Kao, 2004). The widely accepted mechanism for the pathogenesis of this disease involves that, following retrograde menstruation, shed endometrial tissues attach, invade the peritoneal surface, become vascularized and form established endometriotic lesions (Sampson, 1940). This multifactorial process involves angiogenic as well as extracellular matrix degrading systems (Gilbert-Estellés et al., 2007). Anomalous expression levels of matrix metalloproteinases (MMPs) have been identified in endometriotic lesions which could promote the establishment of ectopic lesions at peritoneal sites (Osteen et al., 2002). Angiogenesis represents a crucial step during maintenance of these lesions, since endometriotic implants require neovascularization to guarantee oxygen and essential nutrient supply (Groothuis et al., 2005). Elevated levels of angiogenic growth factors, particularly VEGF, have been demonstrated in the peritoneal fluid as well as in eutopic and ectopic endometrial tissues from endometriosis patients (Laschke and Menger, 2007).

Progestins are successfully used in the treatment of endometriosis since they lead to regression of this disease and to a relief of pain symptoms in women with endometriosis (Olive and Pritts, 2002; Moore et al., 2003; Vercellini et al., 2003). Progestogens are generally...
well-tolerated, have a limited metabolic impact compared with drugs interfering with the pituitary regulation, are inexpensive and may be used on a long-term basis. Besides progesterone, dydrogesterone, a potent, orally active progestogen indicated in a wide variety of gynaecological conditions, is used in the therapy of endometriosis (Overton et al., 1994). Because the exact mechanisms of progestin action on endometriotic lesions are poorly understood, in this study, we focused on the effect of the progestins, progesterone, dydrogesterone and its main metabolite dihydrodydrogesterone on proliferation, angiogenic factors and extracellular matrix degrading enzymes.

Since occurrence of spontaneous endometriosis is restricted to humans and non-human primates, we used an animal model by xenotransplantation of human endometrial fragments into the peritoneal cavity of immunodeficient nude mice (Grümm et al., 2001). These fragments implant and form endometriotic-like lesions which resemble lesions found in patients in terms of macroscopic and histological appearance (Nisolle et al., 2000; Grümm et al., 2001), steroid responsiveness (Bruner-Tran et al., 2002) and vascularization (Grümm et al., 2001; Hull et al., 2003). Using this nude mouse experimental model, we obtained evidence that MMPs as well as angiogenic factors are suppressed by progestins in ectopic endometrial lesions.

Materials and Methods

Human endometrial tissue

Endometrium was obtained from 20 premenopausal women undergoing endometrial biopsy or hysterectomy at the Department of Gynecology, University Hospital Essen, Germany. Institutional ethical approval was obtained and all women provided written informed consent. All endometrial tissues were obtained from the proliferative phase of the menstrual cycle from patients undergoing endometrial biopsy for diagnostic reasons because of infertility (9 patients) or hysterectomy because of uterus myomatosis (11 patients). The stage of the menstrual cycle was confirmed by histological staging according to Noyes et al. (1950). None of the patients had been diagnosed for endometriosis or had undergone any hormonal treatment for at least 3 months before surgery. Age of patients was 36.95 ± 7.9 years. Explanted endometrial tissue was cut into fragments of 1–2 mm in diameter under sterile conditions and left for 1 h in culture medium (DMEM-Ham’s F12 1:1; Biochrom KG, Berlin, Germany) supplemented with Pen/Strep (Gibco BRL, Karlsruhe, Germany) and transcribed into cDNA by reverse transcription with M-MLV Reverse Transcriptase (Invitrogen) using an oligo(dT)16 primer in a total volume of 25 μl. PCR amplifications were obtained using gene-specific primers (Table I). The reaction mixture consisted of 2 μl cDNA, 1 μl dNTP-mixture (10 mM each), 2.5 μl 10× PCR buffer (GeneCraft, Ludinghausen, Germany), 1.25 U Taq-Polymerase (GeneCraft) and sterile water in a total volume of 25 μl. The amplification programme consisted of the following steps: 4 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C and 90 s at 72°C. The amplification programme ended with a final step of 4 min at 72°C. The generated PCR products were size-fractionated on a 2% agarose gel and detected by ethidium bromide staining.

Animals

Athymic female nude mice (Han:nMRI nu/nu) were maintained in a barrier unit in a controlled pathogen-free environment and regulated light/dark cycles (12/12 h). All equipment and food entering the barrier was autoclaved. Mice had free access to food and water. All experiments were carried out in accordance with German laws for animal protection and with permission of the state.

Transplantation of fragments and tissue processing

For each experiment, tissue of the same patient was transplanted into four naturally cycling mice. Each mouse received four endometrial tissue fragments which fixed intraperitoneally to the lateral abdominal wall of each nude mouse by laparotomy with surgical sutures as described before (Fechner et al., 2007). Implanted endometrial lesions were dissected 7 (40 lesions/group) or 28 days (40 lesions/group) after transplantation, respectively. Size of lesions was determined. Two lesions of each mouse were frozen directly in liquid nitrogen for RNA extraction, and two lesions of each mouse were fixed with formalin and embedded in paraffin. For each experimental approach, endometrial tissue of at least 10 different patients has been transplanted.

Application of drugs

Progesterone (Sigma Aldrich, Munich, Germany), dydrogesterone and dihydrodydrogesterone (Solvay Pharmaceuticals, Hannover, Germany) were diluted in benzylbenzoate (Synopharm GmbH, Barsbüttel, Germany)/castor oil (Fisher Scientific GmbH, Niddereu, Germany) 1:4 and injected subcutaneously in a volume of 100 μl/animal. Mice were injected 50 μg/day from transplantation of human tissue onwards. Doses administered were derived from the respective subcutaneous human dose of medroxyprogesterone acetate (MPA) (Schlaff et al., 2006). To account for a faster metabolism in mice, a daily injection was chosen in this study. Animals in the control group were treated with vehicle only. Each drug was applied to at least 10 mice transplanted with endometrium of 10 different patients.

RNA extraction and RT–PCR

Isolation of total RNA from endometrial tissue was performed using the E.Z.N.A. Kit (Omega, Doraville, GA, USA) according to the manufacturer’s instructions. The concentration of RNA was determined spectrophotometrically and the RNA was stored at −80°C until use.

One microgram of total RNA was digested with DNase I (Invitrogen, Germany) and transcribed into cDNA by reverse transcription with M-MLV Reverse Transcriptase (Invitrogen) using an oligo(dT)_14 primer in a total volume of 25 μl. PCR amplifications were obtained using gene-specific primers (Table I). The reaction mixture consisted of 2 μl cDNA, 1 μl dNTP-mixture (10 mM each), 2.5 μl 10× PCR buffer (GeneCraft, Ludinghausen, Germany), 1.25 U Taq-Polymerase (GeneCraft) and sterile water in a total volume of 25 μl. The amplification programme consisted of the following steps: 4 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C and 90 s at 72°C. The amplification programme ended with a final step of 4 min at 72°C. The generated PCR products were size-fractionated on a 2% agarose gel and detected by ethidium bromide staining.

Real-time PCR

Samples were further analysed by quantitative real-time RT–PCR using the qPCR MasterMix for SYBR Green I (Applied Biosystems, Darmstadt, Germany) in a 20 μl volume according to the supplier’s instructions applying the ABI Primer 5700 Sequence Detection System (Applied Biosystems). The amplification of gene-targets was accomplished using 40 ng cDNA and 0.15 μl gene-specific oligonucleotides (25 pmol/μl) per reaction (Table I). To determine the concentration of the PCR fragments, serially diluted standard cDNAs generated for each gene were amplified in separate tubes in each run. The amplification programme consisted of the following steps: 10 min at 95°C, 40 cycles at 15 s at 95°C, 1 min at 60°C. Finally, the temperature was raised gradually (0.1°C/s) from 60 to 90°C for the melting curve analysis to verify the specificity of the amplification. Due to the diversity in the RNA quality, the amplification products were normalized to the expression of β-actin run in separate tubes.

Immunohistochemical staining for Ki67 and activated caspase-3

Paraffin sections were cut to a thickness of 7 μm and mounted on protein-coated glass slides. After dewaxing in xylene and rehydration in a series of
alcohols, endogenous peroxidase activity in the tissue was blocked with 5% hydrogen peroxide for 5 min. This was followed by incubation with the primary antibodies caspase-3 cleaved (polyclonal antibody, 1:200; Zytomed Systems, Berlin, Germany) or Ki67 (monoclonal antibody, 1:1200; Zytomed Systems, Berlin, Germany). After incubation, the sections were incubated for 1 h at room temperature with the primary antibodies: endothelia of mouse smooth muscle actin (αSMA, Neomarkers, Fremont, CA, USA, undiluted). As secondary, a biotinylated rabbit anti-rat antibody (Zytomed Systems) was used for α SMA, and a biotinylated goat anti-rabbit immunoglobulin (E0432, Dako) for anti-α SMA (RB-9010-R7, Neomarkers, Fremont, CA, USA, undiluted). As secondary, a biotinylated rabbit anti-rat antibody (E0468, Dako, Denmark) was used for panendothelial cell antigen, and a biotinylated goat anti-rabbit immunoglobulin (ED432, Dako) for anti-α SMA. The chromogenic reaction was carried out by incubating the sections with the peroxidase substrate 3,3′-diaminobenzidine for 5 min, sections were rinsed in PBS, dehydrated in ascending ethanol concentrations and coverslipped. To demonstrate specificity of the staining, consecutive sections were stained using the same protocol, except that the primary antibody was omitted. To show the tissue morphology and to confirm both orientation and localization parallel sections were stained with haematoxylin–eosin (HE).

**Table I Oligonucleotides used for real-time PCR**

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<thead>
<tr>
<th>Primer</th>
<th>GenBank accession no.</th>
<th>Sequence</th>
<th>Product size (bp)</th>
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</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>NM_001101.2</td>
<td>5′-ACCAACTGGGACGACATGGA; 3′-CCAGAGGCCTACAGGATG</td>
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<tr>
<td>ERα</td>
<td>NM_001025.2</td>
<td>5′-GTCGCTGCTAGAGATTGTCC; 3′-GATCTCCACATGTCCTCTA</td>
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<tr>
<td>PR(AB)</td>
<td>NM_009926.2</td>
<td>5′-AGCCCACATAACAGCCTCCAG; 3′-TTTGCAAAGGCAAGGAC</td>
<td>253</td>
</tr>
<tr>
<td>bFGF</td>
<td>NM_002006.3</td>
<td>5′-CGACCCTCATCAAGCTACAA; 3′-CCAGGTAACGGTTAGCACACACT</td>
<td>61</td>
</tr>
<tr>
<td>VEGFA</td>
<td>NM_003376.4</td>
<td>5′-TCTGCTGCTCTGGTGCATT; 3′-ACCAGGGGCTCCAGTGATG</td>
<td>168</td>
</tr>
<tr>
<td>CYR61</td>
<td>NM_001554.3</td>
<td>5′-TAAGGAGCTGGGGATTGCAGT; 3′-TCTGGGCTTGAAAGGGTTG</td>
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<tr>
<td>MMP-3</td>
<td>NM_002422.3</td>
<td>5′-GCAGGTTGCTGCTACCTATCC; 3′-GAGGTGCGGAGTCCAGCTCT</td>
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</tr>
<tr>
<td>MMP-7</td>
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<td>MMP-2</td>
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<tr>
<td>MMP-9</td>
<td>NM_004994.2</td>
<td>5′-TTGCACAGCGACAAGAAGTG; 3′-GCCATTCACGTCCTTAT</td>
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**Microvessel density** was determined by counting the number of CD31- or αSMA-positive microvessel structures, respectively, or of vessels containing Ki67-positive endothelial cells within the ectopic endometrial lesions. Area of lesions was measured using a Zeiss Axiohot microscope with NIS-Elements BR 3.0 software. Lesion area was quantified from one representative section of 10 different patients per experimental group. Microvessel density was calculated and expressed as vessel per squared millimetre.

**Staining for endothelial cells and pericytes and determination of microvessel density**

Immunostaining for panendothelial antigen was performed on ethanol-fixed cryostat sections from freshly frozen tissues, for alpha smooth muscle actin (αSMA) on paraffin sections after dewaxing as described above. All sections were rinsed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin to reduce non-specific antibody binding, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide. After washing in PBS, the sections were incubated for 1 h at room temperature with the primary antibodies; endothelia of mouse vessels were stained with a rat-anti-mouse panendothelial cell antigen (MECA-32, Pharmingen, Hamburg, Germany, 1:100), mature pericytes with a rabbit anti-mouse αSMA (RB-9010-R7, Neomarkers, Fremont, CA, USA, undiluted). As secondary, a biotinylated rabbit anti-rat antibody (E0468, Dako, Denmark) was used for panendothelial cell antigen, and a biotinylated goat anti-rabbit immunoglobulin (ED432, Dako) for anti-αSMA. The chromogenic reaction was carried out by incubating the sections with the peroxidase substrate 3,3′-diaminobenzidine for 5 min, sections were rinsed in PBS, dehydrated in ascending ethanol concentrations and coverslipped. To demonstrate specificity of the staining, consecutive sections were stained using the same protocol, except that the primary antibody was omitted. To show the tissue morphology and to confirm both orientation and localization parallel sections were stained with haematoxylin–eosin (HE).

**Statistical analysis**

Exploratory data analysis and the non-parametric analyses of variances (Mann–Whitney test) were performed applying the programs SPSS for windows (Vers. 14). Differences with $P \leq 0.05$ were regarded as statistically significant.

**Results**

**Influence of different progestins on growth, proliferation and apoptosis in ectopic endometrial lesions**

Human endometrial fragments cultured in the peritoneal cavity of nude mice revealed a well-preserved morphology even 28 days after implantation (Fig. 1). The lesions show typical endometrial histomorphology with endometrial glands surrounded by stroma. Glands dilate with time of culturing, possibly due to an accumulation of secretion products of the glandular cells. With time of culturing, size of ectopic lesions generally decreased and fragments revealed to be smaller at 28 days in comparison to 7 days after implantation (Fig. 2). Neither morphology nor size of lesions changed after treatment with the different progestins (Figs 1 and 2).

Rate of proliferation and apoptosis was determined by quantification of staining for Ki67 (Fig. 3A–C) and activated caspase-3 (Fig. 3D and E), respectively. Independent from progestin treatment, proliferation of human endometrial tissue was increased on Day 7 of transplantation compared with the freshly biopsied endometrium (Fig. 4A and B). On Day 28, proliferation rate was in general diminished (Fig. 4C and D). However, dydrogesterone significantly decreased proliferation in the stromal compartment when compared with the appropriate control tissue (Fig. 4D).
During the time course of intraperitoneal culturing, an increase in apoptosis as marked by activated caspase-3 staining was seen in the endometrial epithelial cells compared with the native tissue at 7 and at 28 days after inoculation (Fig. 4E and F). In contrast, activated caspase-3 was significantly reduced in the stromal cells after 7 as well as after 28 days compared with the not transplanted endometrium (Fig. 4G and H). In both compartments, the different progestin treatments had no significant effect on caspase-3 staining intensity.

**Steroid hormone receptors**

Transcription of estrogen receptor (ER) α and progesterone receptor (PR)-AB was investigated in ectopic human endometrial lesions after treatment with different progestins for 7 days. Quantitative PCR (qPCR) analysis revealed a slight reduction of ERα expression by dihydrodydrogesterone and dydrogesterone (Fig. 5A). Expression of PR-AB was significantly suppressed only by dihydrodydrogesterone (Fig. 5B).

**MMPs and angiogenic factors**

The effect of different progestin treatments on the expression levels of MMP-2, -3, -7 and -9 was analysed at Day 7 after xenograft transplantation. Treatment with progesterone, dihydrodydrogesterone as well as dydrogesterone significantly decreased the expression of MMP-2 compared with lesions grown in vehicle-treated control mice (Fig. 5C). Furthermore, also MMP-3 transcripts decreased upon all progestins tested, however, significantly only after dydrogesterone treatment (Fig. 5E). Neither progestin treatment had a significant
effect on the expression of MMP-7 and -9 in the human ectopic endometrial lesions (Fig. 5D and F).

The angiogenic factors VEGFA, bFGF and CYR61 were regulated by progestin treatment in the endometrial lesions. Transcription of the human angiogenic factor bFGF was significantly reduced by progesterone and by trend by dihydrodydrogesterone (Fig. 5G), whereas dihydrodydrogesterone and dydrogesterone in addition suppressed VEGFA (Fig. 5J) as well as CYR61 (Fig. 5H).

**Effect on angiogenesis**

To evaluate the functional effect of progestin treatment on angiogenesis, endothelia of mouse vessel grown into the human fragments were stained for CD31 (Fig. 6A) and mature pericytes were identified by staining for αSMA (Fig. 6B), and microvessel density has been calculated. Density of CD31-positive microvessels was slightly but not significantly decreased after 7 days by all progestins (Fig. 6C). However, on Day 28 after inoculation, the density of vessels was only reduced by treatment with dydrogesterone and dihydrodydrogesterone (Fig. 6D). In contrast, density of αSMA-positive mature pericytes (Fig. 6E and F) was significantly increased after 28 days of treatment by progesterone and dydrogesterone, respectively (Fig. 6F). No significant effect of progestins, however, could be seen in regard to density of microvessels containing endothelial cells stained positive for the proliferation marker Ki67 or for activated caspase-3. In general, density of vessels with proliferating as well as apoptotic endothelial cells was higher after 7 days of cultivation (Fig. 7A and C) than after 28 days where only few proliferating cells and no caspase-3-positive cells could be detected (Fig. 7B and D).

**Discussion**

Progestin-based therapies are well established in the treatment of endometriosis since they lead to a regression of the disease and reduction of pain (Olive and Pritts, 2002). However, their exact mode of action is still poorly understood. We here describe the effect of different progestins used in endometriosis therapy in regard to their effect on growth and on locally expressed endometriosis-related molecules in ectopic endometrial tissue using the nude mouse model. This heterologous animal model for endometriosis represents one of the most widely used and well-established models for the investigation of mechanisms involved in the development of endometriosis (Grümmer, 2006). Furthermore, the model is appropriate to investigate hormonal responses since the expression of both receptors, ERα and PR-AB, is well preserved in these ectopic endometrial lesions (Grümmer et al., 2001). In the present study, it could be shown that human endometrial tissue fragments decreased in size during the culture period of 4 weeks, but neither growth nor morphology was changed by progestin treatment. Proliferation of epithelial as well as stromal cells increased in the ectopic fragments within the first week but decreased to levels below those of the native tissue after 28 days. Over the culture period, epithelial cells showed a higher rate of apoptosis compared with the non-transplanted tissue, whereas there was a significant decrease of activated caspase-3 in stromal cells during intraperitoneal culturing in mice. However, besides a suppression of stromal proliferation by dydrogesterone after 28 days, no effect of progestin treatment could be shown in regard to proliferation or apoptosis in the ectopic endometrial lesions.

In regard to steroid hormone receptor expression, no significant effect of progestin treatment on of ERα transcription could be observed, whereas PR-AB mRNA was significantly suppressed by dihydrodydrogesterone. These results confirm our previous investigations that PR-AB was down-regulated by MPA, but not by dydrogesterone (Fechner et al., 2007). Thus, in this regard, dihydrodydrogesterone seems to be the more effective progestin.

An obvious effect of progestin on the expression of MMP-2 and -3 as well as on the angiogenic factors bFGF, VEGFA and CYR61 was...
demonstrated. Recent MMP-related research has suggested that the mechanisms of ectopic endometrial growth in endometriosis are combined with invasive events similar to metastatic neoplasms, and MMPs are required for extracellular matrix degradation during penetration of the peritoneal mesothelium and invasion of the host tissue (Osteen et al., 2002). They have been associated with the establishment of endometriosis since they have been found to be increased in ectopic endometrial lesions (Spuijbroek et al., 1992; Saito et al., 1995; Wenzl and Heinzl, 1998; Chung et al., 2001). Progesterone suppresses the expression of MMP-1 (Marbaix et al., 1995), -3 and -7 (Osteen et al., 1994) in human endometrium and decreases the amount of MMP-2 and -9 released in the medium by explants of human endometrium (Marbaix et al., 1992). In addition, it was demonstrated that progesterone treatment inhibits expression of MMP-3 and -7 in human endometrial fragments cultured in mice treated with progesterone (P), dihydroxyprogesterone (DHD), or dydrogesterone (Dydro), for 7 or 28 days from transplantation of human tissue onwards.

Figure 4 Rate of proliferation (A–D) and apoptosis (E–H) in human endometrial fragments cultured in mice treated with progesterone (P), dihydroxyprogesterone (DHD), or dydrogesterone (Dydro), for 7 or 28 days from transplantation of human tissue onwards. Animals in the control group were treated with vehicle only (C). Seven days after transplantation, proliferation of epithelial cells increased compared with the native, not transplanted endometrium (EM) independent from progestin treatment (A). This increase in proliferative activity was less in the stroma (B). After 28 days of treatment, proliferation rate of epithelial and stromal cells was lowered at least to the initial values of the native endometrium (C). In addition, in the stromal compartment, dydrogesterone significantly decreased proliferation rate compared with vehicle control. Expression of activated caspase-3 showed a significant increase in epithelial cells compared with the native endometrial tissue (EM) 7 and 28 days after transplantation (E and F). In contrast, caspase-3 was significantly reduced in the stromal cells after 7 as well as after 28 days (G and H). Expression of caspase-3 was independent from progestin treatment in both the epithelial and the stromal compartment. *Significant difference compared with non-transplanted endometrium (EM) and **significant difference compared with non-transplanted endometrium as well as to vehicle-treated control (C) (P < 0.05).
endometrium and prevents the establishment of ectopic lesions in a nude mouse model (Bruner-Tran et al., 2002). In contrast to MMP-7 and -9, we could demonstrate a significant suppression of MMP-2 transcription by all progestins tested, and a significant down-regulation of MMP-3 by dydrogesterone. Since species-specific primers for β-actin have not been addressed, the quantity for those marker genes could be shifted due to different ratios between mouse and human tissue compartments. In addition, it has to be evaluated to what extend the regulation of transcription correlates to the secretion of active enzymes.

Furthermore, it is discussed that MMPs and angiogenic factors act in concert during the establishment of endometriotic lesions, since controlled extracellular proteolysis is required for sprouting of capillaries.

Figure 5  Quantitative real-time RT–PCR analysis of ERα and PR-AB in human endometrial fragments cultured in nude mice.
Mice were subcutaneously injected progesterone (P), dihydrodydrogesterone (DHD) or dydrogesterone (Dydro) daily for 7 days from transplantation of human tissue onwards. Animals in the control group were treated with vehicle only and were set 1. Although no obvious effect could be seen on expression of ERα (A), qPCR analysis revealed a significant suppression of PR-AB by dihydrodydrogesterone compared with controls (B). Treatment with all three progestins significantly decreased the expression of MMP-2 compared with lesions grown in vehicle-treated control mice (C). All progestins tested also decreased the expression of MMP-3 and this suppression was significant for dydrogesterone (E). Either progestin treatment had no obvious effect on the expression of MMP-7 and -9 (D and F). Transcription of human bFGF was reduced by P and DHD (G), whereas treatment with DHD and Dydro, respectively, led to a suppression of VEGFA (J) as well as CYR61 (H). *Significant difference compared with control ($P < 0.05$).
from pre-existing vessels (Hyder and Stancel, 1999). The development of new blood vessels represents a crucial step during the establishment of endometriosis because endometriotic implants require neovascularization to guarantee oxygen and essential nutrient supply (Groothuis et al., 2005). Correspondingly, a typical clinical feature of endometriotic lesions as well as their surrounding areas is the dense vascularization (McLaren, 2000). The interaction between the ectopic endometrium and the peritoneal tissue is a prerequisite for the induction of angiogenesis and the maintenance of endometriosis. Although in the nude mouse model this is a cross-species interaction which may not fully represent the molecular interactions that occur in human disease, in this model, molecular similarities to the human disease could be demonstrated by Hull et al. (2008).

It could be shown before in this nude mouse model that ectopic human endometrium acts on the endothelial cells within the mouse peritoneum to attract murine vessels from the immediate environment (Grümmer et al., 2001; Hull et al., 2003), suggesting that the endometrial tissue transmits angiogenic signals that cause destabilization of the murine vessels and induce angiogenesis. Inhibitors of angiogenesis effectively interfere with the maintenance and growth of endometriosis by decreasing microvessel densities and number of established ectopic endometrial lesions in this model (Nap et al., 2004). These angiogenic factors may include VEGFA as well as bFGF and CYR61.

VEGF is the most potent angiogenic factor and has been shown to be produced by endometriotic lesions (Shifren et al., 1996), whereby VEGFA seems to be the factor predominantly involved (McLaren, 2000; Takehara et al., 2004). It is significantly elevated in eutopic endometrium of endometriosis patients (Donnez et al., 1998) and is contained in significantly greater amounts in the peritoneal fluid of patients with endometriosis compared with healthy controls.

Figure 6 Evaluation of amount of vessels stained for CD31 or αSMA human endometriotic lesions cultivated in nude mice.

Immunohistochemical staining for CD31 (A) and αSMA (B). Density of CD31-positive microvessels was slightly decreased by all progestins after 7 days (C), however, after 28 days only by DHD and Dydro (D). Although only Dydro by trend decreased amount of αSMA-positive vessels after 7 days (E), an increase in amount of mature pericytes was observed after 28 days of treatment which was significant for P and Dydro (F). P, progesterone; DHD, dihydrodydrogesterone; Dydro, dydrogesterone.

Scale bar = 60 μm. *Significant difference compared with vehicle-treated controls (C) (P < 0.05).
Suppression of MMPs and angiogenic factors by progestins

(Hull et al., 2003) showed a suppression of vessel formation by anti-VEGFA antibodies in human ectopic endometrial lesions in the nude mouse model which corresponds to our results of diminished vessel formation after treatment with dydrogesterone and dihydrodydrogesterone which both decrease VEGFA-mRNA. Transcription of the angiogenic factor bFGF on the other hand was suppressed by progesterone. bFGF is expressed in human endometrium (Ferriana et al., 1993), but although it is present in peritoneal fluid, its concentration does not change during the menstrual cycle nor in the presence of endometriosis (Seli et al., 1998). Thus, this factor could play a minor role compared with VEGF-A.

Recently, the pro-angiogenic protein CYR61 was shown to be one of the most up-regulated genes in endometria of women with endometriosis and in ectopic endometrium (Absenger et al., 2004). CYR61 (CCN1) belongs to the CCN proteins which are secreted and known to promote cell adhesion, migration and neovascularization (Brigstock, 2002). Proof for its pro-angiogenic role comes from Cyr61 knockout mice missing branching vessels in the placenta which leads to death in utero (Mo et al., 2004). Here, we demonstrate a down-regulation of CYR61 in ectopic endometrial tissue by dydrogesterone and dihydrodydrogesterone, but not by progesterone. The reduction of vessel formation after 28 days of treatment with dydrogesterone and dihydrodydrogesterone, respectively, may be due to the combined inhibition of VEGFA and CYR61 in the ectopic lesions and seems to be accompanied by an increase in vessel stabilization.

An interaction of VEGFA and MMP-3 (Gilabert-Estellés et al., 2007) and a role of MMP-2 and -9 (Ria et al., 2002) in the establishment of angiogenesis in endometriosis have been proposed. Progesterone, and possibly even to a greater extend the progestin dydrogesterone and its main metabolite dihydrodydrogesterone, may have an inhibitory effect on implantation and growth of regurgitated endometrial tissue by inhibiting expression of MMPs and angiogenesis. By targeting the expression of endometrial MMPs and angiogenic factors therapeutically, it may be possible to regulate critical molecules that are necessary for the establishment and progression of endometriosis, perhaps resulting in more successful treatments for this disease. Progestins including dydrogesterone (Vercellini et al., 2003; Trivedi et al., 2007; Somigliana et al., 2009) are used for effective treatment of endometriosis. However, discussing progestin action on ectopic endometrial lesions, it has to be taken into account that a reduced responsiveness, or an intrinsic resistance, to progesterone could be demonstrated in the eutopic endometrium of women with endometriosis (Bruner-Tran et al., 2002; Klemmt et al., 2006; Burney et al., 2007). Thus, it has to be evaluated to which extent these mechanisms also work in endometrium of women with endometriosis. Nevertheless, deciphering the locally produced paracrine factors mediating ectopic endometrial responses to progestins may strengthen the success of progestin-based treatments for endometriosis. Since different progestins obviously regulate such factors to a different degree, a panel of progestins should be evaluated in regard to their effect on the expression of MMPs and angiogenic factors, which are essentially involved in the persistence of endometriotic lesions, to identify those progestins or progestin compositions most effective in the therapy of endometriosis.
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