Gene expression analysis reveals an angiogenic profile in uterine leiomyoma pseudocapsule

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ABSTRACT: The pseudocapsule (PC) of the uterine leiomyoma (UL) is an anatomic entity that surrounds the myoma separating it from the myometrium (UM). Although a number of microarray experiments have identified differences in gene expression profile in the UL when compared with the UM, there is a lack of systematic studies on the PC. In this study, quantitative RT–PCR analysis was performed on 18 matched PC, UL and UM specimens and results showed that the PC displays a specific gene expression profile. The low expression level of insulin-like growth factor (IGF-2), a fibroid specific marker, that we found in the PC and the UM when compared with the UL, clearly indicates that the PC is in structural continuity with the UM. However, the significant increase in endoglin expression level in PC with respect to the UL and UM indicates that an active neoangiogenesis is present in PC. Conversely, other angiogenic factors such as von Willebrand factor (vWF) and vascular endothelial growth factor A (VEGF-A) seem to have little influence on the PC angiogenesis. Because the endoglin is preferentially expressed in proliferating endothelial cells, whereas the vWF and VEGF-A are preferentially expressed in preexisting endothelial cells, our idea is that the angiogenic activity in the PC is linked to wound healing. The angiogenic activity is also sustained by intermediate expression level of cystein-rich angiogenesis inducer 61, connective tissue growth factor and collagen 4α2 genes all involved in the neoangiogenesis, that we detected in the PC. Taken together our data demonstrate that the specific expression pattern observed in the PC could be the response of the uterine wall’s smooth cells to the tension imposed by the tumor. As a consequence, a neovascular structure is generated involving regenerative processes. For these reasons, we suggest that the laparoscopic intracapsular myomectomy (LIM), a new surgical technique that preserves the PC during the UL removal, should always be preferred, to favor a faster and proper uterine healing.

Key words: angiogenesis / fertility / fibroid pseudocapsule / intracapsular myomectomy / uterine leiomyoma / endoglin (CD105)

Introduction

Uterine leiomyoma (UL) is the most common benign tumor of the female reproductive tract, affecting 20–50% of women in the procreative age (Parker, 2007). Although most are asymptomatic, clinical symptoms are present in 25% of cases, manifesting as menstrual bleeding, pelvic pain and possible infertility. The high diffusion rate among female population makes UL the main indication for surgery in the gynecological field. Currently, UL surgery treatment by laparoscopic technique has replaced laparotomy myomectomy because it shows numerous advantages, including shorter hospitalization, faster recovery, fewer adhesions and less blood loss. Good pregnancy outcomes confirmed the validity of the laparoscopic approach (Seracchioli et al., 2000). Anyway, a number of cases of uterine rupture have been reported after laparoscopic myomectomy, suggesting that the quality of uterine myometrium (UM) healing may be still suboptimal. The utilization of high wattage electrocoagulation for realizing hemostasis and inadequate reapproximation of the uterine edges after myomectomy have been postulated to interfere with the wound healing and to increase the risk of rupture. However, it is also possible that individual wound healing characteristics may predispose to uterine ruptures (Parker et al., 2010). A specific feature of all ULs is the presence of a pseudocapsule (PC), a structure anatomically distinguishable from UL, that appears as an echogenic line of 5 mm or less surrounding the fibroid (Hsu et al., 2007). The recognition of this anatomic entity has permitted the development of the laparoscopic intracapsular myomectomy (LIM), a new surgical approach that allows the enucleation of UL from the PC. As a consequence, the PC is left in situ and...
the hemostasis is achieved by the selective and gentle coagulation of PC vessels by low wattage. The myometrium is closed in a single or double layer according to the surgeon’s choice (Tinelli et al., 2009, 2010). A large prospective observational study with a follow-up of 5 years has assessed the surgical complaints and the reproductive outcomes of LIM. Among the 235 women who underwent subserous and intramural LIM for indications of pelvic pain, menstrual disorders, a large growing myoma or infertility, no late complications, such as bleeding, urinary tract infection or bowel lesion, were observed. Of the women who underwent myomectomy for infertility, 74% have conceived with no case of uterine rupture, demonstrating a full recovery of the uterine functionality (Tinelli et al., 2012a). To assess the quality of the uterine healing process after LIM, two-dimensional power Doppler ultrasound was used to monitor the uterine scar and the resistance index (RI) of the uterine arteries, after myomectomy. The uterine examination showed a significant progressive reduction in uterine scar area from 78% of the previous UL location at the first day, to 4% at the 45th day, corresponding to an optimal reconstruction of the damaged tissue and a high quality muscle scar. Furthermore, increase in RI value at the scar area from 0.65 on the first post-operative day to 0.83 after 7 days followed by a decrease to 0.61 on the 45th post-operative day reflects a neangiogenic process that occurs in the uterine wall just after myomectomy, performed with pseudocapsule sparing (Tinelli et al., 2012b). In spite of the possible regenerative properties of the PC, the literature is still lacking in systematic studies concerning the molecular and biochemical characteristics of the PC and its functional correlation with UM and UL. Conversely, both differential display and microarrays have been used to examine gene expression differences between UL and adjacent UM. In particular, over-expression of IGF-2 in UL represents one of the most consistent findings of gene array to date (Catherino et al., 2003; Wang et al., 2003; Hoffman et al., 2004; Arslan et al., 2005). For this reason IGF-2, known as a potent mitogen factor, has been closely associated with the tumor growth (Peng et al., 2009). Published data also indicate the down-regulation of CYR61/CCN1 and CTGF/CCN2 genes (Ahn et al., 2003; Hoffman et al., 2004; Quade et al., 2004; Arslan et al., 2005) and the up-regulation of COL4A2 in UL when compared with adjacent UM (Winston et al., 2003). The genes belonging to the CCN gene family are involved in promoting proliferation, cellular differentiation and angiogenesis (Babic et al., 1999; Chen et al., 2001). COL4A2 gene, implicated in extracellular matrix formation, encodes for collagen α, one of the main components of the endothelial basement membrane (Kuhn, 1995). It is also described as an anti-angiogenic factor for the presence of the canstatin domain, designated as an angiogenesis inhibitor (Kamphaus et al., 2000). The expression of this triad of genes has been interpreted as an anti-angiogenic profile in fibroid tissue (Winston et al., 2003), in accordance with the reduced microvessel density, based on anti-von Willebrand factor (vWF) immunostaining, observed in ULs with respect to normal myometrium (Poncelet et al., 2004). However, data regarding VEGF-A expression, the most potent angiogenic factor, in ULs are conflicting, with some reports supporting its up-regulation and others that do not observe any significant difference when compared with matched UM (Harrison-Woolrych et al., 1995; Gentry et al., 2001).

In this study, we performed, by quantitative real-time RT–PCR method (qRT–PCR), a gene expression analysis of PC, matching it with the same analysis in UL and UM, evaluating the expression levels of IGF-2, used as tumoral marker and COL4A2, CYR61/CCN1, CTGF/CCN2, VEGF-A and vWF, known to be involved in angiogenic processes. We added endoglin/CD105 to our investigation because it has been recently identified as a specific marker of neo-formed vessels (Dallas et al., 2008). Our aim is to define the structural and functional features of PC that could explain its possible roles in the uterine regenerative process.

**Materials and Methods**

**Sample acquisition and preparation**

At the Department of Gynecology and Obstetric of Vito Fazzi Hospital of Lecce, Italy, a group of 18 patients underwent LIM between January 2010 and November 2011. Patients were selected based on their symptoms as pain or pressure, menstrual disorders, large and growing myomas, infertility or reproductive dysfunction. The median age of the women was 48.3 years (range, 32–70). The number, size and location of ULs were assessed for all patients: 11 women had subserous UL (61.1%), whereas 7 women had intramural UL (38.9%); ULs were single in 10 patients (55.5%) and multiple in 8 patients (44.4%) with the dominant fibroid measured between 3 and 12 cm in diameter. Patients with suspected adenomyosis or adenomyoma were excluded. Pedunculated, small and submucous ULs were also excluded. Preoperative treatment with gonadotrophin releasing hormone analogs (GnRHa), known to induce distortion of the UL pseudocapsule (De Falco et al., 2009; Mettler et al., 2012), has also been considered as an exclusion criterion for the purposes of this study. All women were to be subjected to intracapsular myomectomy, the protocol was primarily explained to the patients and full written consent to operation and protocol was obtained prior to surgery.

Pre-surgical transvaginal ultrasound was performed to determine the presence of the PC as a white ring surrounding the UL. Fig. 1 shows TV EcoColor Doppler of UL vascularization, where PC appears as a ‘ring of fire’ around the UL.

LIM was performed as depicted by Tinelli and colleagues (Tinelli et al., 2012a).

Tissues sampling from the fibroids were taken from approximately 20 mm in depth, including full thickness of the UL, UL-PC interface and the surrounding UM; samples of UL-PC interface were taken by scissors from its surface, as soon as good hemostasis was reached, whereas UM tissues were biopsied at 2 cm distance from UL. All samples were orientated as surgeons proceed in case of cervical conization. All samples were collected and stored in saline solution, then sent to the laboratory in a dry-ice container for successive analysis.

**RNA extraction and cDNA synthesis**

Samples were aliquoted in pieces of 100–200 mg in weight and stored in 0.5 ml of RNA later (Ambion, Inc.) prior to RNA extraction or freezing. Samples were homogenized in Trizol reagent (Invitrogen) for total RNA extraction, according to the manufacturer’s protocol. RNA was suspended in RNAase-free water. The quality of the extracted RNA was checked by electrophoresis based on the identification of 18S and 28S ribosomal RNA (rRNA) bands. A NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to determine RNA purity and concentration. Only high quality RNA was used (A260/A280 > 1.95).

First strand cDNA synthesis was performed by reverse transcription of 2 μg of total RNA per sample, primed with 1 μl of oligodT (0.5 μg/μl) using 1 μl of dNTP mix (10 mM), 2 μl DTT (100 mM) and 1 μl of murine leukemia virus (MLV)-reverse transcriptase (200 U/μl) (Invitrogen) in the recommended buffer in a final volume of 20 μl. Using a
DNA thermal cycler, reagents were incubated at 65°C for 5 min, at 37°C for 50 min and heated at 70°C to inactivate the reaction. cDNA was stored at −20°C for successive analysis.

**Quantitative RT–PCR**

Quantitative real-time reverse transcription PCR (qRT–PCR) was used to quantify IGF-2, COL4A2, CYR61/CCN1, CTGF/CCN2, VEGF-A, vWF and endoglin/CD105 transcript levels. GAPDH transcript level was used as an internal control. Sequence-specific primers were designed at the turn of two contiguous exons, to avoid genomic amplification, using the Web-based primer design program Primer3 and obtained commercially (Invitrogen). The sequences of the primers and their relative annealing temperature and amplicons length are given in Table 1.

Primer pairs related to each target gene were all first tested on a randomly selected cDNA, by standard PCR. PCR product of the correct length was purified using the PureLink PCR Purification Kit (Invitrogen-Life Technologies) and cloned in a TA-vector. Each clone was sequenced using a commercial service to confirm the specificity of the insert and then used to obtain a standard curve.

Transcriptional level determinations were carried out in triplicate, using Smart Cycler apparatus (Cepheid®). Each reaction was performed with cDNA equivalent to 100 ng of RNA, 10 μM each of forward and reverse primers and SYBR Green real-time PCR Mix (Euroclone) according to the manufacturer’s protocol. The cycling parameters were activation

<p>| <strong>Table 1</strong> Primers for mRNA detection used in qRT–PCR. |
|-------------------------------|------------------|----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th><strong>Gene (Acc.Nr)</strong></th>
<th><strong>Primers</strong></th>
<th><strong>Position</strong></th>
<th><strong>Sequences of primers</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>GADPH (NM_002046)</td>
<td>Forward</td>
<td>Ex 8</td>
<td>5'-GGCATTCACTGGGCTACACTGA - 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Ex 9</td>
<td>5'-TCCACACCTTGTTGCTAG-3'</td>
</tr>
<tr>
<td>IGF-2 (NM_000612)</td>
<td>Forward</td>
<td>Ex 6</td>
<td>5'-CTTACGCCCTAGTGAGACC - 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Ex 7</td>
<td>5'-ACTTGGCAGGGTAGCACAG - 3'</td>
</tr>
<tr>
<td>COL4A2 (NM_001846.2)</td>
<td>Forward</td>
<td>Ex 1</td>
<td>5'-GCCGCGGCGGATGAGACTTG - 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Ex 2</td>
<td>5'-CTGTTGCCGTAGATGGGCCAGG - 3'</td>
</tr>
<tr>
<td>CYR61/CCN1 (NM_001554)</td>
<td>Forward</td>
<td>Ex 4</td>
<td>5'-ACCCTGATGCGGCTTGATGA - 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Ex 5</td>
<td>5'-CCTGACTGTGTCGGGGGATT - 3'</td>
</tr>
<tr>
<td>CTGF/CCN2 (NM_001901)</td>
<td>Forward</td>
<td>Ex 3</td>
<td>5'-TGTCAGACCACTGCTGTTTC - 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Ex 4</td>
<td>5'-CAGAGGGGTTGGTATTGTTGTA - 3'</td>
</tr>
<tr>
<td>VEGF-A (NM_001171623)</td>
<td>Forward</td>
<td>Ex 2</td>
<td>5'-CATGAGGGAGGGAGGCAAT - 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Ex 3</td>
<td>5'-ATCGCATCAGGGGCCACACAG - 3'</td>
</tr>
<tr>
<td>ENG/CD105 (NM_000118.2)</td>
<td>Forward</td>
<td>Ex11</td>
<td>5'-GCCCTTACCTCACAGCCCAAAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Ex12</td>
<td>5'-GCTTCACACAGTTGCCCTTG-3'</td>
</tr>
<tr>
<td>vWF (NM_000552.3)</td>
<td>Forward</td>
<td>Ex48</td>
<td>5'-GTGGAGATGGTTGTCCTACG - 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Ex49</td>
<td>5'-GTTCATCAAGGGTGGGCAC-3'</td>
</tr>
</tbody>
</table>

**Figure 1** Fibroid pseudocapsule. (A) uterine transvaginal ultrasound section evidencing the pseudocapsule vascularization as a ‘ring of fire’ around the fibroid by transvaginal EcoColor Doppler; (B) enhancing of pseudocapsule fibrovascular network, during removal of leiomyoma from uterus. PC surrounding UL is marked by white arrows.
at 95 °C for 10 min (one cycle), denaturation at 95 °C for 30 s, annealing at temperature specific for each primers pair (Table 1) for 30 s and extension at 72 °C for 30 s (45 cycles). mRNA levels were quantified using the standard curve method (Larmonov et al., 2005), normalized to GAPDH gene expression and expressed as arbitrary ‘mRNA relative units’. The melting curve analysis, obtained at the end of each real-time reaction, allowed us to confirm the specificity of the amplification products.

**RT–PCR analysis and cloning**

For the VEGF-A isoforms determination, 2 μl of cDNA related to UL, UM and PC of each patient was added in a PCR reaction mixture containing 2 μl of forward (KU-VEGF: 5′-ATGGAATTTCTGCTGTCTT-3′) and reverse primers (XL-VEGF: 5′-CCTCTTCTCTTCATTTCAGG-3′) (10 μM each) designed respectively on the 5′UTR and 3′UTR of VEGF-A gene, 2 μl of MgCl2 50 mM, 1 μl of dNTP mix (10 mM), 1 μl of Taq Platinum (Invitrogen) in the recommended buffer, in a final volume of 50 μl. The first-round synthesis was carried out for 18 cycles, each consisting of 94 °C for 30 s, 55 °C for 50 s and 72 °C for 50 s, with an initial denaturation step of 95 °C for 5 min and a final elongation step of 72 °C for 10 min.

One microliter of the first-round PCR mixtures was used as a template for a second round of amplification, whose reaction mixture differs from the previous one only for the forward primers (U2-VEGF: 5′-CAGAGGAGGAGGCAGAAT-3′) that is designed on the second exon that is shared by all VEGF-A isoforms. The second-round synthesis was carried out for 25 cycles, each consisting of 94 °C for 30 s, 55 °C for 40 s and 72 °C for 40 s, with an initial denaturation step of 95 °C for 5 min and a final elongation step of 72 °C for 10 min. PCR products were purified, cloned into StrataClone PCR Cloning Vector and sequenced using a commercial service.

Splicing efficiency of each VEGF-A variant was calculated from densitometric scan of gels as the ratio between the intensity of each band divided by the sum of the intensity of all the bands obtained. Densitometric determinations were carried out using the KODAK Gel Logic 100 software.

**Western blot**

Sample tissues of PC, UL and UM were homogenized in SDS lysis buffer (50 mM Tris-HCl pH 6.8, 6% SDS, 1 mM EDTA) containing EDTA-free complete mini protease inhibitors (Roche Diagnostics, Italy). The homogenate was centrifuged and the Bio-Rad Protein Assay (Bio-Rad-Italy) was used for the protein quantification of the supernatant. Fifty micrograms of total protein, extracted from PC, UL and UM tissues, related to eight different patients, were resolved on a 12% SDS-PAGE under reducing conditions alongside the SDS-PAGE Molecular Weight Standards, Broad range (BIO-RAD). Gels were equilibrated for 15 min in transfer buffer (28 mM Tris, 192 mM Glycin and 10% Methanol) before blotting at 300 mA for 2 h onto a nitrocellulose membrane (Immobilon™-P Millipore) using a Transblot apparaturus (BIO-RAD). Blots were blocked with 1% BSA in TNT buffer (10 mM Tris-HCl pH 8, 150 mM NaCl and 0.05% Tween-20) over night, at room temperature and then incubated for 2 h with a rabbit anti-VEGFA or anti β-actin primary polyclonal antibodies (Santa Cruz Biotechnology Inc., dilution 1:200 in TNT buffer) at room temperature. After incubation, blots were washed four times in TNT buffer and incubated with goat anti-rabbit IgG-AP secondary antibody (BIO-RAD), diluted 1:1000 in TNT buffer, for 1 h at room temperature. Finally, the membranes were developed with AP Conjugate Substrate Kit (BIO-RAD).

Relative abundance of each VEGF-A protein variant was calculated from densitometric scan of the membrane as the ratio between the intensity of each band divided by the sum of the intensity of all the VEGF bands. Densitometric determinations were carried out using the KODAK Gel Logic 100 software.

**Statistical analysis**

Data related to expression levels of the target genes in each sample were obtained in triplicate. Statistical evaluations as mean values and standard deviation were carried out. All data were analyzed and graphed using MedCalc software. Most of the variables examined in this study appeared to be arranged in a non-normal, positively skewed distribution, making conventional parametric statistics misleading. Therefore, data are described by the medians and interquartile ranges (IQRs), and the degree of variability among groups was determined using the Kruskal–Wallis non-parametric algorithm. Mann–Whitney test was used for pairwise comparisons. The level of significance was set at $P < 0.05$.

**Results**

**Quantitative RT–PCR of selected genes**

To examine the molecular characteristics of PC, the expression levels of CYR61/CCN1, CTGF/CCN2, COL4A2, VEGF-A, vWF, endoglin/CD105 and IGF-2 genes were analyzed by quantitative RT–PCR in 18 matched PC, UM and UL specimens. The last gene was chosen because it is known to be a tumor proliferation marker in UL pathology; the others were selected for their involvement in angiogenesis.

As shown in Fig. 2, IGF-2 expression varied significantly among the three tissues ($P = 0.0011$ by Kruskal–Wallis test). In particular, it was significantly higher in UL (0.0165 relative units mean) when compared with UM (0.0028 relative units mean; $P = 0.033$ by Mann–Whitney test) and PC (0.0026 relative units mean; $P = 0.032$ by Mann–Whitney test). The difference between UM and PC was not significant ($P = 0.916$ by Mann–Whitney test).

Expression level analysis of endoglin/CD105 revealed a significant difference among the three tissues ($P = 0.016$ by Kruskal–Wallis test). In particular, there was a significant over-expression in PC (0.031 relative units mean) with respect to both UL (0.013 relative units mean, $P = 0.020$ by Mann–Whitney test) and UM (0.008 relative units mean; $P = 0.021$ by Mann–Whitney test). The difference between UL and UM was not significant ($P = 0.23$ by Mann–Whitney test).

Both CYR61 and CTGF showed a significant degree of variability among the three tissues in our samples ($P = 0.044$ and $P = 0.021$ respectively by Kruskal–Wallis test). The down-regulation of CYR61 gene in UL (0.0278 relative units mean) with respect to UM (0.0588 relative units mean) was statistically significant ($P = 0.032$ by Mann–Whitney test), whereas difference with respect to PC (0.0479 relative units mean) did not reach significance ($P = 0.067$ by Mann–Whitney test). The difference between UM and PC was not significant ($P = 0.92$ by Mann–Whitney test).

The down-regulation of CTGF in UL (0.0049 relative units mean) with respect to UM (0.012 relative units mean) was significant ($P = 0.006$ by Mann–Whitney test), whereas no significant differences were found in the CTGF mRNA level between UL and PC (0.0111 relative units mean; $P = 0.090$ by Mann–Whitney test) and between PC and UM ($P = 0.15$ by Mann–Whitney test).

Similarly, COL4A2 expression is significantly different across the three tissues ($P = 0.018$ by Kruskal–Wallis test). Moreover in this case, COL4A2 was expressed significantly higher in UL (2.313 relative units mean) than in UM (1.157 relative units mean, with $P = 0.007$ by Mann–Whitney test), whereas the difference when compared with
PC (1.658 relative units mean) was not significant ($P = 0.137$ by Mann–Whitney test). The difference between UM and PC was not significant too ($P = 0.154$ by Mann–Whitney test).

VEGF-A and vWF mRNA levels showed no significant differences across the three tissues ($P = 0.73$ and $P = 0.24$, respectively, by Kruskal–Wallis test).

**Detection of alternative splicing**

Alternative splicing is a key element in the regulation of the pro-angiogenic VEGF-A factor, leading to the production of at least seven different isoforms that are characterized by various combinations of exons 6, 7 and 8 or part of these (Ferrara et al., 2003; Ladomery et al., 2007). To assess the isoforms that occur in mRNA samples from UL, PC and UM, we performed a two-step RT–PCR assay.

As shown in Fig. 3, three different products were detected in all the three tissues. The sequence analysis of the cloned products shows that they correspond to VEGF$_{189}$, VEGF$_{165}$ and VEGF$_{121}$ isoforms. The average rate of VEGF-A isoforms expression in UL derived from three independent experiments in the same patient was 40.5% for VEGF$_{121}$, 27.5% for VEGF$_{165}$ and 32% for VEGF$_{189}$. In UM, VEGF$_{121}$ represents 38.2% of all VEGF-A isoforms, VEGF$_{165}$ is 32.2%, whereas VEGF$_{189}$ is 28.6%. The average rate of VEGF-A isoforms expression in PC was 40.4% for VEGF$_{121}$, 32.1% for VEGF$_{165}$ and 27.5% for VEGF$_{189}$.

These differences in VEGF-A isoforms expression did not reach statistical significance.

The same profile of alternative exons and average rate was confirmed in all the analyzed patients (data not shown).
Western blot analysis

To examine the expression profile of VEGF isoforms, western blot analysis with the anti-VEGF antibody on protein extracts of UL, PC and UM specimens was performed. A representative image of the VEGF labeling on protein extracts of PC, UM and UL specimens of two patients together with β-actin labeling (43 Kd), used as the loading control, is provided in Fig. 4.

In accordance with the RT–PCR analysis, three VEGF bands of the approximate molecular mass of 27 kDa, 23 kDa and 18 kDa were detected in all the tissues. By comparison with the work of Cressey and colleagues (Cressey et al., 2005), we were able to refer them as VEGF189, VEGF165 and VEGF121 isoforms, respectively.

The average rate of VEGF-A isoforms expression in UL that resulted from three independent experiments in the first patient was 40.7% for VEGF121, 38.3% for VEGF165 and 20.9% for VEGF189. In UM, VEGF121 represents 40.7% of all VEGF-A isoforms, VEGF165 is 35.8%, whereas VEGF189 is 23.8%. The average rate of VEGF-A isoforms expression in PC was 39.6% for VEGF121, 36.5% for VEGF165 and 23.6% for VEGF189. The same profile was observed in the second patient and in the other six patients analyzed.

The differences in VEGF-A protein isoforms expression did not reach a statistical significance. There are no significant differences even between average of each protein isoform and its corresponding transcriptional variant.

Discussion

The data generated in this study clearly indicate that the PC, a structure anatomically distinguishable from the UM and surrounding the UL, displays a significant and specific gene expression profile. We can affirm this, through a number of observations listed below. The PC, like the UM, exhibits a significantly reduced expression of the IGF-2 gene, known to be a tumor growth marker (Peng et al., 2009), when compared with the UL, suggesting that it has a non-fibroid origin and that it has a structural continuity with UM. The PC also showed a statistical relevant over-expression of the endoglin/CD105 gene, when compared with the UM and with the UL. The endoglin is an integral membrane glycoprotein, expressed predominantly on the surface of proliferating endothelial cells, activated monocytes, macrophages and vascular smooth muscle cells of the neoformed blood vessels, where it works as a part of the transforming growth factor-beta (TGF-β) receptor complex (Cheifetz et al., 1992). Because the vascular expression of the endoglin is limited to proliferating cells, this factor is considered a more selective marker, than traditional vascular markers, such as CD31 and von Willebrand factor, to identify native or newborn vessels involved, predominantly, in pathological conditions such as tumor growth and wound healing (Dallas et al., 2008; Valluru et al., 2011). Based on these findings, the over-expression of the endoglin gene, rather than of other angiogenic genes, seems to indicate the presence of an active angiogenesis correlated with reparative process in the PC.

We found in the UM, significantly increased expression level of the CYR61 and CTGF genes, when compared with the UL, but not with the PC, in agreement with the literature data (Ahn et al., 2003; Hoffman et al., 2004; Quade et al., 2004; Arslan et al., 2005). The CYR61 and CTGF genes have been reported as pro-angiogenic genes that play an important role in angiogenic remodeling. In particular, they have been indicated as the main responsive angiogenic genes...
in the mechanically challenged cells (Tamura et al., 2001). In fact, recent in vitro studies indicate that the smooth muscle cells are induced to express a number of early responsive genes such as those encoding for the CYR61 and CTGF molecules, in response to mechanical stimuli such as shear stress, tension and hydrostatic pressure (Chaqour and Goppelt-Struebe, 2006). Even if the two genes are homologous and can be co-induced by mechanical stretch, a higher strength level is required for CTGF induction, so that the CYR61 gene is expressed earlier and at higher level than CTGF (Tamura et al., 2001; Chaqour et al., 2006). Therefore, we suppose that the presence of a consistent CYR61 and less consistent CTGF expression levels in UM is the response of the uterine wall to the tensions imposed by the UL growth, by activating genes involved in signaling of angiogenesis. Because we did not detect statistically significant differences in the expression levels of CYR61 and CTGF genes, between UM and PC and between UL and PC, we can infer that intermediate transcript levels of the two genes are present in the PC.

In accordance with the literature (Weston et al., 2003), we also found a significantly increased expression level of the COL4A2 gene in the UL with respect to the UM, but not with the PC. Again, we did not detect statistically significant differences in the expression level of the COL4A2 gene between UL and PC and between UM and PC, so it can be said that intermediate transcript level of this gene is present in the PC. The expression of the COL4A2 gene in the PC could be correlated with the extracellular matrix reconstruction during angiogenesis. In fact, it has been demonstrated that collagen type IV is necessary for stability of basement membranes under conditions of mechanical stress (Poschl et al., 2004).

We found no difference in the VEGF-A and in the vWF expression level among the three tissues. In addition, using the RT–PCR sequencing, the VEGF189, VEGF165 and VEGF121 splicing variants of the VEGF-A gene were detected in PC as well as in UM and PC, with no statistically significant difference among the three tissues. The presence of these three isoforms is in agreement with literature data that describe these variants as the most commonly detected in all the cellular types expressing the vascular growth factor (Ferrara and Davis-Smyth, 1997). Expression of VEGF189, VEGF165 and VEGF121 isoforms in UL, UM and PC was confirmed in our samples also at the protein level with no difference in the three tissues. The uniformity of the VEGF expression in UL and UM correlates well with the results from Harrison-Woolrych (Harrison-Woolrych et al., 1995) who reported no difference in VEGF mRNA content between UL and UM.

Conversely, our data conflict with previous reports on microvessel density based on vWF factor that was found lower in UL than in UM (Poncelet et al., 2004). Our hypothesis to explain this discrepancy is that the expression of vWF, unlike VEGF, may depend on the physiopathological state of the myometrium. The wide range of the vWF mRNA levels present in the UM when compared with that in PC and UL (Fig. 2) could support our hypothesis. In addition, the analysis was conducted on matched PC, UM and UL specimens in the current study, whereas Poncelet et al. (2004) used UM and UL samples that were derived from different groups of women. All together, these data clearly depict the PC as a site of intense angiogenesis strictly linked to the endoglin activation, rather than other angiogenic factors such as VEGF-A or vWF. The presence of an active angiogenesis is concordant with the histological studies that describe a parallel array of extremely dense capillaries in the PC and in the adjacent UM that are absent in UL (Malvasi et al., 2011). The appearance of the PC can thus be seen as the adaptive response of the uterus to mechanical overload, aimed to maintain uterus functionality, promoting muscular repair and regeneration.

Based on these observations, we strongly recommend the LIM as a surgical approach because the preservation of the PC, with regenerative properties, in the scar site may explain the optimal reconstruction of the damaged tissue and the high quality of the muscle scar exhibited by patients undergoing this surgical technique.

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**Authors’ roles**

S.D.T. carried out the molecular studies, data acquisition, statistical analysis and wrote the manuscript; S.M. conceived and coordinated the study and contributed in drafting the article; M.P.B. and A.M. revisited the manuscript critically; A.T. conceived the study, operated patients and gave his final approval to the version to be published.

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**Conflict of interest**

None declared.

**References**


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References


