Mast cell degranulation in rat uterine cervix during pregnancy correlates with expression of vascular endothelial growth factor mRNA and angiogenesis

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Abstract

Vascular growth of the uterine cervix during pregnancy is associated with mast cell (MC) degranulation. To better understand the mechanism underlying this process, uterine cervixes of intact pregnant rats were dissected and endothelial cell proliferation was measured by a bromodeoxyuridine incorporation technique. Total vascular endothelial growth factor (VEGF) mRNA expression and the relative abundance of VEGF splice variants (120, 164, and 188) were determined by RT-PCR. VEGF protein expression was evaluated by immunohistochemistry. To investigate the role of MCs on cervical angiogenesis, a second set of pregnant animals were treated with an MC stabilizer (disodium cromoglycate) to inhibit MC degranulation. Furthermore, 17 β -estradiol (E₂) serum levels were established by RIA. In intact pregnant rats, VEGF mRNA expression was positively correlated with endothelial cell proliferation and circulating E₂ levels. All selected splice variants of VEGF gene were detected and their relative abundance did not show any change throughout pregnancy. Animals treated with disodium cromoglycate showed a decrease in endothelial cell proliferation and in VEGF mRNA expression compared with controls. Relative abundance of VEGF mRNA splice variants and E₂ serum levels showed no differences between these experimental groups. These results show a time-dependent correlation between VEGF mRNA expression and E₂ serum levels in the uterine cervix of intact pregnant rats, while MC stabilizer-treated animals reduced the VEGF expression without modifying E₂ serum levels. We suggest that cervical angiogenesis during pregnancy could be regulated by a mechanism which involves endogenous E₂ and chemical mediators stored in MC granules via a VEGF-dependent pathway.

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Introduction

Vascular supply is a fundamental requirement for reproductive functions (Ferrara & Davis-Smyth 1997, Goth *et al.* 2003). Angiogenesis occurs regularly in association with cyclical changes in adult female endometrium and also during implantation and subsequent placentation (Heryanto & Rogers 2002). During pregnancy and parturition, the uterine cervix undergoes adaptation processes, such as polymorphonuclear leukocyte infiltration and collagen remodeling (Luque *et al.* 1996, 1998). All these processes demand energy and a vascular system providing sufficient nutrient supply is essential. Using intact pregnant rats, we have demonstrated that the vascular tree of the uterine cervix is an active cellular compartment showing striking changes in the relative area occupied by vessels and in endothelial proliferation (Varayoud *et al.* 2004). These

cellular events reflect an active angiogenic process throughout gestation.

Considerable attention has been focused on the regulation of angiogenesis and vascular permeability in the female reproductive tract (Goede *et al.* 1998, Rabbani & Rogers 2001). One of the best-studied proangiogenic mediators is the vascular endothelial growth factor (VEGF) that often represents a critical rate-limiting step in physiological processes (Hyder *et al.* 1996). VEGF-A (often referred to as simply VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor constitute the platelet-derived growth factor subfamily of the cysteine knot proteins. They show high structural homology but they represent distinct gene products (Halder *et al.* 2000, Byrne *et al.* 2005, Roy *et al.* 2006). VEGF-A mRNA and protein exist as multiple isoforms resulting from alternative splicing of the eight coding exons named according to the amino

acid number of the monomer. VEGF188 includes all eight exons, whereas deletion of exon 6 or exons 6 and 7 generates VEGF164 and VEGF120 isoforms respectively (Cullinan-Bove & Koos 1993, Hyder *et al.* 1996, Mowa *et al.* 2004). Other alternative spliced forms have also been reported in rodents, such as VEGF144 and VEGF115 (Sugihara *et al.* 1998, Ding *et al.* 2006). VEGF isoforms differ primarily in their bioavailability, which is conferred by heparin and heparan sulfate-binding domains encoded by exons 6 and 7. VEGF120 might diffuse relatively free in tissue, whereas approximately half of the VEGF164 binds to cell surface heparan sulfate proteoglycans (HSPGs). VEGF188 remains almost completely sequestered by HSPGs in the cell surface and in the extracellular matrix, making HSPGs a reservoir of VEGF that can be mobilized through proteolysis (Ferrara *et al.* 2003, Puxeddu *et al.* 2005). These differences in the biochemical properties of the splice variants could promote different physiological roles and bring a complex network that regulates the angiogenesis in the female reproductive tract.

A close relationship between endocrine function and angiogenesis has been observed during estrous cycle (Karuri *et al.* 1998) and early pregnancy (Walter *et al.* 2005). The steady-state level of VEGF mRNA increases rapidly in the uterus following estrogen (Eg) treatment (Cullinan-Bove & Koos 1993, Hyder *et al.* 2000). This effect suggests that Eg regulates vascular permeability of uterine blood vessels through modulation of VEGF expression. In agreement, Karuri *et al.* (1998) showed that a twofold increase in VEGF mRNA content in the rat uterine tissue at proestrous is exhibited, coincident with the highest 17β -estradiol (E_2) levels. Moreover, a localized increase in uterine vascular permeability and angiogenesis are the hallmarks of initiation and progression of blastocyst implantation and it is likely that VEGF signaling influences these events (Halder *et al.* 2000). The angiogenesis of the rodent uterus is also regulated by progesterone (Pg) by stimulating endothelial cell proliferation and VEGF expression (Cullinan-Bove & Koos 1993, Ma *et al.* 2001, Walter *et al.* 2005). Based on these results, VEGF expression is likely to be under the control of both steroid hormones; however, there is a need for a better understanding of the endocrine regulation of angiogenesis in the reproductive tract under physiological conditions.

Besides endocrine control, the immune system also plays an important role in the regulation of angiogenesis. Several studies indicate that leukocytes can induce vascular growth and specific leukocyte-derived cytokines have been identified as proangiogenic factors (Camussi *et al.* 1997, Griffioen & Molema 2000, Ribatti *et al.* 2001). We previously demonstrated that mast cell (MC) degranulation correlates with new blood vessel formation in the rat uterine cervix during the second half of gestation (Varayoud *et al.* 2004). This physiological control could be orchestrated with other immune cells, like macrophages,

since MC degranulation influences macrophage content of the uterine cervix (Bosquiazzo *et al.* 2005). Therefore, based on our previous work and taking into account that MCs actively participate in the angiogenesis of tumors through the production of VEGF (Aoki *et al.* 2003), we suggest that VEGF could be a mediator of MC-induced angiogenesis in the rat uterine cervix.

To better understand the regulation of angiogenesis in the uterine cervix during pregnancy, we evaluated the correlation between endothelial cell proliferation and VEGF gene expression. Since MCs promote cervical angiogenesis by a degranulation-mediated process (Varayoud *et al.* 2004), we tested here whether VEGF mRNA content is also influenced by MC degranulation.

Materials and Methods

Animals

Female adult rats (200–250 g body weight (BW)) of a Wistar-derived strain bred at the Department of Human Physiology (Santa Fe, Argentina) were used. Animals were maintained under controlled environment ($22 \pm 2^\circ\text{C}$; lights on from 0600 to 2000 h) and had free access to pelleted rodent chow (Cooperación, Buenos Aires, Argentina) and tap water. To obtain pregnant specimens, proestrous females were caged overnight with males with proven fertility. Pregnancy was confirmed by the presence of spermatozoa in the vaginal smear the morning after coitus, defined as day 1 of pregnancy (D1; Freeman 1988, Montes & Luque 1988). In this colony, delivery occurred on D23. To determine serum E_2 levels, peripheral blood was collected from jugular vein on D3–D5, D12, D14, D15, D18, D22, and D23 (three to four animals per day) of gestation. Serum samples were stored at -20°C until used for hormone assays. All rats were handled in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the US National Academy of Sciences.

Treatment and experimental design

Pregnant rats were randomly assigned to each experimental group (at least six animals per group). To evaluate endothelial cell proliferation and VEGF mRNA expression during pregnancy, whole cervixes were dissected on D12, D14, D18, and D22 of gestation. Another set of pregnant rats was used to evaluate the effects of MC stabilizer treatment on VEGF mRNA expression. These rats were treated either with the MC stabilizer, disodium cromoglycate (i.p. 87 mg/kg BW, Sigma Chemical Co) or with saline solution (control) daily from D12 to D18 and killed 3 h after the last injection. Day 12 of gestation was selected as onset of administration of cromoglycate because it is coincident with a high level of MC infiltration in the rat uterine

cervix (Varayoud *et al.* 2004) and to avoid putative interferences with implantation and/or early developmental stages. The absence of acute or chronic toxicity signs in treated mothers and the effectiveness of this experimental protocol on the inhibition of MC degranulation were previously evaluated in our laboratory (Varayoud *et al.* 2004).

All animals were injected intraperitoneally with the thymidine analog bromodeoxyuridine (BrdU, 60 mg/kg BW, Sigma) 2 h before killing. Animals were killed by decapitation, blood was collected, and serum stored at -20°C until used for hormone assays. Whole uterine cervix was either fixed by immersion in 10% (v/v) buffered formalin for 6 h at 4°C or flash frozen in liquid nitrogen. Fixed tissue was embedded in paraffin for immunohistochemistry. Frozen tissue was stored at -80°C until RNA extraction.

RT and competitive PCR

Synthesis of competitor DNA fragments

PCR was performed using a template purified λ phage DNA (λ DNA) and specific oligonucleotide primers for the rat VEGF mRNA (Table 1 and Fig. 1). Forty cycles of amplification were carried out at low annealing temperatures by allowing the oligonucleotide primers to bind randomly to λ DNA generating DNA fragments with different molecular weights. The reaction tube contained *Pyrococcus furiosus* DNA polymerase (Promega Corporation), 2 mM MgCl_2 , 0.2 mM of each of the four deoxy-NTPs (Invitrogen), and 20 pmol of each primer (Invitrogen) in a final volume of 25 μl of $1\times$ PCR buffer (Triton X-100, Tris-ClH, KCl, $(\text{NH}_4)_2\text{SO}_4$, BSA, and Cl_2Mg). After initial denaturation at 97°C for 5 min, the reaction mixture was subjected to successive cycles of denaturation at 96°C for 45 s, annealing at 42°C for 1 min, and extension at 72°C for 1 min. A final extension cycle at 72°C for 5 min was included. The generated fragments were resolved on 3% (w/v) agarose gels containing ethidium bromide (Sigma) and molecular weights were determined by comparing with DNA standards (Cien Marker, Biodynamics, Buenos Aires, Argentina). A DNA fragment with a very similar molecular weight to the cDNA target was selected and purified using the Wizard-SV Gel and PCR Clean-Up System (Promega). This fragment was inserted and cloned

into a plasmid vector (pBluescript SK, Stratagene, La Jolla, CA, USA) and used as a competitor in subsequent PCRs for VEGF mRNA quantitation. The competitor DNA concentration was assessed with a spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA) at 260 nm.

Reverse transcription (RT)

Following manufacturer's instructions, RNA extractions were performed using TRIzol reagent (Invitrogen). The quantity of total RNA was assessed with a spectrophotometer at a wavelength of 260 nm. Equal quantities (4 μg) of total RNA were RT into cDNA with 300 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Promega) using 0.5 μg random primers (Invitrogen). Twenty units of RNase inhibitor (RNAout, Invitrogen) and 10 nmol deoxy-NTP mixture were added to each reaction tube in a final volume of 30 μl of $1\times$ MMLV-RT buffer (Promega). RT was performed at 37°C for 60 min. Reactions were terminated by heating at 80°C for 5 min and cooling at 4°C , followed by dilution of the reverse-transcribed cDNA with RNase-free water to a final volume of 60 μl . RNA incubated under identical conditions, but without reverse transcriptase, served as a negative control.

Competitive PCR to evaluate VEGF mRNA expression

An optimized competitive PCR protocol was employed to analyze the expression levels of VEGF mRNA transcripts. Total uterine cervix cDNA was pooled from pregnant females (two animals per pool and three pools per gestational day) and competitive PCR was performed with each pool of cDNA. Constant amounts of target cDNA were added to all reaction tubes together with crescent quantities of competitor DNA (0.88–10.7 attomoles). Each reaction mixture contained 2.5 U Taq DNA polymerase, 1 mM MgCl_2 , 0.2 mM of each of the four deoxy-NTPs, and 20 pmol of each VEGF specific primer (all from Invitrogen; Table 1) in a final volume of 25 μl of $1\times$ PCR Taq buffer (Invitrogen). After initial denaturation at 97°C for 5 min, the reaction mixture was subjected to successive cycles of denaturation at 96°C for 45 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. A final extension cycle at 72°C for 5 min was included. The optimal number of cycles, determined in the

Table 1 Sequence of PCR primers.

Primer	Sequence	Product size
VEGF sense	5'-CTGCTCTCTTGGGTGCACTGG-3'	320 bp
VEGF antisense	5'-GGTTTGATCCGCATGATCTGCAT-3'	
VEGF isoform sense	5'-CATGCGGATCAAACCTCACC-3'	VEGF120: 126 bp VEGF164: 258 bp VEGF188: 330 bp
VEGF isoform antisense	5'-CACCGCCTTGGCTTGTCACA-3'	
L19 sense	5'-GAAATCGCCAATGCCAACTC-3'	290 bp
L19 antisense	5'-ACCTTCAGGTACAGGCGTTG-3'

Figure 1 Illustration of the vascular endothelial growth factor (VEGF) gene amplification by RT-PCR. Primers 1 and 2 were used to quantify the VEGF mRNA by competitive PCR, whereas primers 3 and 4 were applied to detect the major splice variants. The expected sizes of cDNA fragments after PCR amplification are shown in Table 1.

exponential phase of the reaction was 30. The sequence of primers for VEGF mRNA was selected based on the published cDNA sequences of the rat VEGF mRNA (Kashida *et al.* 2001). In all assays, negative controls without cDNA target were performed to minimize the introduction of potential artifacts. Using the TA cloning kit (Invitrogen), PCR products corresponding to VEGF were cloned, and specificity was confirmed by DNA sequencing (data not shown). The generated fragments (cDNA target and competitor DNA) were resolved on 3% (w/v) agarose gels containing ethidium bromide (Sigma) and their molecular weight determined by comparing with DNA standards (Biodynamics). Agarose gel images were digitized using a Sony ExwaveHAM color video camera (Sony Electronics Inc., Park Ridge, NJ, USA) and the Image Pro-Plus 4.1.0.1 image system analyzer (Media Cybernetics, Silver Spring, MD, USA). The integrated optical density (IOD) for each PCR product was obtained by densitometry. The logarithm of the ratio of competitor and VEGF-IOD was plotted as a function of the logarithm of the concentration of competitor added to each PCR tube. The concentration of VEGF mRNA for each day of gestation in the uterine cervix was determined when the ratio of competitor/VEGF-IOD was equal to 1.

To confirm that changes in VEGF mRNA were specific, steady-state levels of a constitutively expressed cellular mRNA (L19 ribosomal protein) were measured using specific primers (Table 1; Chan *et al.* 1987).

Analysis of VEGF mRNA splice variants by RT-PCR

To detect the main VEGF mRNA splice variants (120, 164, and 188), RT reactions were carried out as mentioned previously. Total uterine cervix cDNA was pooled from six rats (two animals per pool) on each gestational day and PCRs were performed as described previously, using an annealing temperature of 61 °C. To co-amplify the VEGF mRNA variants, the sense and antisense primers were located flanking the alternative splicing sites corresponding to exons 6 and 7 of the VEGF cDNA sequence (GenBank accession no.: NM_031836, Table 1 and Fig. 1). Melting curves showed

the absence of heteroduplex formation and unspecific amplification products (Eckhart *et al.* 1999). The PCR products were resolved on 2% (w/v) agarose gels, cloned, and sequenced (data not shown). The relative expression of each splice variant was evaluated determining the IOD by densitometry.

Hormone assays

Serum levels of E₂ were determined by RIA using [2,4,6,7,16,17-³H] E₂ (Perkin-Elmer Life and Analytical Sciences Inc., Boston, MA, USA) and a specific antibody provided by Dr GD Niswender. E₂ concentration was measured after ethyl ether extraction (Merck; Kass *et al.* 2004). The sensitivity of the assay was 1.6 pg/ml, and the intra- and interassay coefficients of variation were 10.16 and 7.90% respectively.

Immunohistochemistry and morphometry

BrdU incorporation was used to evaluate proliferating endothelial cells (Kass *et al.* 2000) rat mast cell proteinase-I (RMCP-I) to identify MCs (Gibson & Miller 1986) and anti-VEGF to determine the cellular sites and expression patterns of VEGF. Immunostaining for MCs was performed following the immunoperoxidase technique after periodic acid and sodium borohydrate incubation to block endogenous peroxidase activity. Detection of BrdU incorporation was performed as previously described, including the acidic hydrolysis for DNA denaturation and microwave (MW) pretreatment for antigen retrieval (Kass *et al.* 2000). VEGF immunodetection was done following the previously described protocol (Bosquiazzo *et al.* 2005). Primary antibodies were incubated overnight at 4 °C, and dilutions used were anti-BrdU 1:100 (clone 85-2C8, Novocastra Laboratories Ltd, Newcastle-upon-Tyne, UK), anti-RMCP-I 1:200 (Moredun Scientific Ltd, Edinburgh, Scotland), and anti-VEGF 1:40 (clone C-1, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Reaction was developed using the avidin-biotin peroxidase method and diaminobenzidine (Sigma) was used as a chromogen substrate. Samples were counterstained with Harris hematoxylin (Biopur, Rosario, Argentina) for BrdU and Mayer's hematoxylin for RMCP-I and VEGF. The slides were dehydrated and mounted with permanent mounting medium (PMYR Buenos Aires, Argentina). Each immunohistochemical run included positive and negative controls. For negative controls, the primary antibody was replaced with nonimmune rabbit serum (Sigma) or the immunohistochemical run was performed in samples from animals that did not receive BrdU. Suppliers had tested the specificity of the primary antibodies used.

The evaluation of tissue sections was done using an Olympus BH2 microscope (Olympus Optical Co. Ltd,

Tokyo, Japan) with a Dplan 100 $\times$ objective (Olympus). The percentage of proliferating endothelial cells was measured on sections immunostained with an anti-BrdU antibody and was calculated by dividing the number of BrdU-positive endothelial cells by the total number of endothelial cells. Two sections of each uterine cervix were analyzed and approximately 200 vessels/section were evaluated.

To evaluate the effect of MC-stabilizing treatment, the percentage of degranulated MCs in immunohistochemical sections was compared between MC stabilizer-treated and control rats on D18 of pregnancy. MC was considered as degranulated, with more than three granules outside of the cell shape or with empty cavities in the cytoplasm (Gunin & Sharov 1998, Varayoud *et al.* 2004). VEGF immunostaining expression was established using a semiquantitative method.

Statistical analysis

The statistical analysis was performed by Kruskal–Wallis one-way ANOVA and significance between groups was determined by Dunn's *post hoc* test. Correlations were performed using Pearson analysis. To assign probabilities of the difference between two groups, the Mann–Whitney *U* test was used (Siegel 1956).

Results

VEGF mRNA expression, endothelial cell proliferation, and serum levels of E_2 are positively correlated during pregnancy

The design of the primers and the competitive RT-PCR were suitable to quantify VEGF mRNA expression in the uterine cervix throughout gestation. The slope and r^2 of the competitor/VEGF rate plotted as a function of increasing amounts of competitor did not differ between the different time points analyzed, indicating high reproducibility and accuracy of the amplification process (Fig. 2). The VEGF mRNA expression levels changed significantly during pregnancy showing higher expression on D14 and D18 of gestation when they were compared with D12 and D22 ($P < 0.05$; Fig. 3A). A similar temporal pattern was exhibited by endothelial cell proliferation during pregnancy, with higher BrdU incorporation rates on D14 and D18 of gestation compared with D12 (Fig. 3B). VEGF mRNA expression was positively correlated with endothelial cell proliferation (Fig. 3C). Figure 3D shows a BrdU-positive endothelial cell in the uterine cervix blood vessels.

E_2 serum levels of pregnant animals belonging to our colony are shown in Fig. 4A. Positive correlations were found between E_2 serum levels and VEGF mRNA (Fig. 4B) or BrdU incorporation (Fig. 4C) on D12, D14, D18, and D22.

Figure 2 Competitive RT-PCR of VEGF mRNA in the uterine cervix during pregnancy. Representative agarose gels stained with ethidium bromide from each studied day of gestation showed VEGF target and competitor products. VEGF transcripts were obtained from total RNA using primers upstream from the alternative splicing site. Arrows indicate the log of the equivalent points (defined as the IOD ratio of competitor and VEGF equal to 1) and values on the abscissa mark the corresponding VEGF mRNA copy numbers.

Figure 3 VEGF mRNA expression and endothelial proliferation during pregnancy. (A) Levels of VEGF mRNA determined by competitive RT-PCR. (B) Endothelial cell proliferation measured by BrdU incorporation on D12, D14, D18, and D22 of pregnancy. (C) VEGF mRNA expression was positively correlated with endothelial cell proliferation from D12 to D22 of pregnancy. (D) Photomicrograph of rat uterine cervix showing blood vessels with BrdU-positive (arrow) and BrdU-negative (arrowhead) endothelial cells. Values are mean $\pm$ S.E.M. of at least six animals per group. Bars with different letters differ significantly ($P < 0.05$). bv, blood vessels. Scale bar represents 30 μ m.

Alternative splice variants of VEGF mRNA do not change during pregnancy

We examined the expression of alternative splice variants of VEGF mRNA by RT-PCR using oligonucleotide primers that encompass exons 6 and 7 (Fig. 1 and Table 1). Three different splice variants of VEGF mRNA (VEGF120, VEGF164, and VEGF188) were detected by RT-PCR in the rat uterine cervix during the second half of gestation (Fig. 5A). The relative expression levels of these transcripts were as follows: VEGF164 > VEGF188 > VEGF120, and the relative contribution of each other to the total expression did not show any change clearly suggesting that the splicing mechanism of the VEGF mRNA was not modified throughout gestation (Fig. 5B).

MC degranulation correlates with VEGF expression in the uterine cervix during pregnancy

To investigate whether MCs could regulate angiogenesis via a VEGF-dependent pathway; pregnant rats were treated with an MC stabilizer from D12 to D18 of gestation. A polyclonal antibody against RMCP-I was successfully used to identify both non-degranulated and degranulated MCs (Fig. 6A). Densely packed granules were found in the cytoplasm of immunostained non-degranulated MCs (Fig. 6B). Evidence of degranulation were defined by the presence of granules released to the pericellular space and of cytoplasmic halos in MCs (Fig. 6C). Pregnant rats treated with MC stabilizer had more than 40% of non-degranulated MCs, whereas controls showed signs of degranulation in the majority of the MCs (Fig. 6D). These results provide evidence that the MC stabilizer treatment is effective.

To discard possible effects of disodium cromoglycate treatment on the Eg synthesis and metabolism, E_2 serum levels were measured on D18 of pregnancy showing no

changes in E_2 concentration in MC stabilizer-treated rats compared with controls (Fig. 7A). In contrast, endothelial cell proliferation and VEGF mRNA expression significantly decreased when MC degranulation was inhibited (Fig. 7B and C). Even though total VEGF mRNA decreased, no differences in the splicing process of VEGF gene following MC stabilizer treatment were detected and VEGF164 remained as the main isoform (Fig. 7D). Using immunohistochemistry, an intense cytoplasmic VEGF staining was detected in epithelial, endothelial, and smooth muscle cells, while no expression was detected in MCs. VEGF was localized largely in the uterus pole of the uterine cervix rather than in the vaginal pole. It is interesting to emphasize that animals treated with MC stabilizer showed a decreased expression of VEGF protein in the epithelial cells (Fig. 7E and F).

Discussion

In adulthood, angiogenesis occurs infrequently in physiological situations and the endothelium of most tissues is an extremely stable population of cells with a low mitotic rate. Exceptions are found in the female reproductive system where vascular growth and development should occur, for example, during pregnancy (Reynolds *et al.* 2002). Recently, we reported that the active angiogenic process that takes place in the rat uterine cervix during the second half of gestation is regulated, at least in part, by secretion of contents stored in MCs granules (Varayoud *et al.* 2004). Here, mechanistic information was added regarding this event. We suggest that MC degranulation may regulate the angiogenic process in the uterine cervix by modulating VEGF expression. The control of this process occurs at the primary transcript level because no changes in the processing of the alternative splice variants were found.

Figure 4 E_2 serum levels are associated with angiogenic parameters during pregnancy. (A) Serum E_2 concentrations from pregnant rats determined by RIA. E_2 levels were positively correlated with VEGF mRNA expression (B) and endothelial cell proliferation (C). Values are mean $\pm$ S.E.M. of at least six animals per group.

It has been demonstrated that E_2 regulates VEGF mRNA expression acting at the transcriptional level and involving E_2 receptors (Kazi *et al.* 2005). Moreover, Karuri *et al.* (1998) showed physiological variations of the VEGF mRNA content in the rat uterus during the estrous cycle with a positive correlation with endogenous E_2 serum levels. In the present study, total

Figure 5 RT-PCR analyses of VEGF mRNA splice variants in the uterine cervix during pregnancy. (A) Representative 2% (w/v) agarose gel stained with ethidium bromide showing RT-PCR products from uterine cervix obtained during gestation (D12, D14, D18, and D22). Bands correspond to three major variants of VEGF mRNA: 120 (126 bp), 164 (258 bp), and 188 (330 bp). MW, molecular weight marker. (B) Relative amounts of VEGF mRNA variants quantified by densitometry. Bars represent mean values $\pm$ S.E.M. of six animals per group.

VEGF mRNA expression and E_2 serum levels were positively correlated suggesting that the estrogenic pathway could play a pivotal role in VEGF expression control in the uterine cervix during pregnancy.

Although VEGF has an important role in angiogenesis, very little is known about the regulation of VEGF mRNA processing in the rat uterine cervix during pregnancy. Recently, Mowa *et al.* (2004) found that the uterine cervix of pregnant rats clearly expresses two VEGF splice variants (VEGF164 and VEGF120) and they identified a faint band corresponding with the molecular weight of another variant (VEGF188). Previous work using rat uterine horns has described three splice variants of VEGF mRNA (188, 164, and 120; Cullinan-Bove & Koos 1993, Long *et al.* 2001). In accordance with these results, we have identified the same three splice variants in the rat uterine cervix during pregnancy. The predominant splice variant was VEGF164 as it was reported by Cullinan-Bove & Koos (1993) and Mowa *et al.* (2004).

Regarding endothelial cell proliferation in the uterine cervix, BrdU incorporation was positively correlated with the VEGF mRNA pattern expression. Other authors (Phillips *et al.* 1990, Shweiki *et al.* 1993) observed a similar temporal relationship between VEGF mRNA expression and the formation of blood vessels in the reproductive system. Supporting these findings, the main

Figure 6 Immunohistochemical detection of MCs using anti-RMCP-I antibody. Low-magnification photomicrograph showing immunostained MCs (arrows) distribution in the uterine cervix on D12 of pregnancy; cells are usually localized near blood vessels (arrowhead) (A). MCs with densely packed granules showing no signs of degranulation (B) and MCs with features of degranulation showing empty cavities in the cytoplasm (asterisk) (C). Percentage of non-degranulated MCs was quantified as defined in Materials and Methods (D). Values are mean $\pm$ S.E.M. of six animals per group. Scale bar represents 50 μ m in (A) and 10 μ m in (B) and (C).

activity of VEGF is to induce endothelial cell mitogenesis and to increase vascular permeability, which is believed to provide essential serum factors for new blood vessel growth (Norrby 1997, Robinson & Stringer 2001, Ferrara *et al.* 2003). VEGF mediates its signals via high-affinity receptors with tyrosine kinases activity (VEGF-R) which results in the synthesis and release of proteases and specific integrins required for the initiation of endothelial cell proliferation and migration (Neufeld *et al.* 1999). Temporal agreement between the patterns of VEGF mRNA described here and the levels of VEGF receptors (VEGF-R1 and VEGF-R2) described by Mowa *et al.* (2004) are observed in the uterine cervix of pregnant rats. Therefore, taking into account that VEGF mRNA expression is regulated by E_2 and that VEGF is a potential mediator of endothelial proliferation, we suggest that

E_2 -VEGF-VEGF-R pathway could regulate blood vessel formation in the rat uterine cervix during pregnancy.

The other aim of this study was to investigate whether chemical mediators contained in MC secretory granules could regulate VEGF expression in the rat uterine cervix. Using a previously described experimental model of pregnant rats treated with an MC stabilizer (Varayoud *et al.* 2004), here a significant fall was observed in the steady-state levels of VEGF mRNA expression in association with a decrease in endothelial cell proliferation. Moreover, when MC stabilizer-treated rats were compared with controls, the observations suggest that MCs regulated VEGF mRNA expression acting at the primary transcript level as there were no changes in the processing of the splice variants. In addition, no expression of VEGF protein was observed in MCs. VEGF was localized in epithelial, endothelial, and smooth muscle cells, showing in MC stabilizer-treated animals a decreased expression in luminal epithelium when compared with controls. Protein expression decrease correlated with the decrease in VEGF mRNA. Furthermore, VEGF mRNA expression was positively correlated with E_2 levels during D12 to D22 of pregnancy (control animals), while MC stabilizer-treated animals reduced the VEGF expression without exerting any variation in E_2 serum levels. Therefore, it is suggested that during gestation, VEGF expression and endothelial cell proliferation may be regulated by a mechanism controlled by both endogenous E_2 levels and chemical mediators released from MCs. MCs release many mediators in response to non-immunogenic and immunogenic stimuli (Garfield *et al.* 2000, Rivera & Gilfillan 2006). Estrogen promotes MC degranulation and histamine release and enhances the IgE-dependent MC activation. Since it has been shown that MCs express estrogen receptors and E_2 enhances MC degranulation by genomic and non-genomic pathways (Cocchiara *et al.* 1992, Zaitzu *et al.* 2006), we could not discard the hypothesis that the MCs degranulation process and the consequent VEGF increase described here could be regulated by E_2 . Further investigations are necessary to elucidate this event.

Recently, we showed that inhibition of MC degranulation affected the angiogenic process in the rat uterine cervix during pregnancy, suggesting that a hypoxic microenvironment may have been generated (Varayoud *et al.* 2004). *In vitro* studies showed that hypoxia induces macrophage's activation stimulating the release of angiogenic cytokines and factors, along with proteolytic enzymes that might play a role in the promotion of neovessel formation (Crowther *et al.* 2001). In accordance with these results, we observed that inhibition of MC degranulation increased macrophage numbers in rat uterine cervix and this effect was sustained beyond the end of treatment perhaps as a compensatory mechanism to warrant new vessel formation (Bosquiazzo *et al.* 2005).

Figure 7 Effect of treatment with disodium cromoglycate on endothelial cell proliferation and VEGF expression in uterine cervix at D18 of gestation. (A) Serum levels of 17 β -estradiol (E_2). (B) Endothelial proliferation determined by BrdU incorporation. (C) Amounts of VEGF mRNA in control and treated animals, quantified by competitive RT-PCR. (D) Relative expression of the splice variants of VEGF mRNA determined by RT-PCR. (E and F) Photomicrographs of rat uterine cervix showing the expression pattern of VEGF in controls and disodium cromoglycate-treated animals respectively. Animals treated with MC stabilizer showed a decreased expression of VEGF protein in the epithelial cells (arrow). Arrowhead points out a VEGF-immunostained blood vessel. Bars represent means values $\pm$ S.E.M. of at least six animals per group. Means with different letters represent statistically significant differences ($P < 0.05$). Scale bar represents 50 μ m.

Mast cells can produce, store, and release many kinds of chemical mediators, including histamine, typtase, chymase, heparin proteoglycan, growth factors (bFGF, VEGF, and TGF- β), and cytokines (TNF- α , IL-6, and IL-8). These cytokines have been involved in normal as well as in tumor-associated angiogenesis (Ribatti *et al.* 2001). *In vitro* studies showed that TNF α enhanced the production of IL-8, VEGF, and bFGF (Yoshida *et al.* 1997). Furthermore, previous reports indicate that VEGF mRNA expression can also be regulated by a number of cytokines and growth factors, such as TNF α , TGF- β , bFGF, IL-1, and IL-6 (Neufeld *et al.* 1999, Taylor *et al.* 2001, Xie *et al.* 2004, Puxeddu *et al.* 2005). In this work, we used a physiological model to evaluate whether MC degranulation affects VEGF expression in the uterine cervix of pregnant rats. Based on present knowledge, this regulation could be exerted on one hand through two different signaling pathways: 1) direct pathway (autocrine): MCs could modulate their own VEGF synthesis and release or 2) indirect pathway (paracrine): MCs could produce a mediator (such as cytokines) that could regulate VEGF expression by other cells. On the other hand, Mowa *et al.* (2004) proposed that angiogenic and sensory neuronal factors may be important in regulating the dynamic microvasculature in the ripening cervix and they observed that VEGF is downregulated in the uterine

cervix of pregnant rats after pelvic neurectomy. Therefore, a possible effect from vasoactive neuropeptides on MC regulation of angiogenic process in the uterine cervix could not be ruled out. Finally, the MC-stabilizing agent used in our work blocks the release of many substances from MC (Shin *et al.* 2004) and all of them could modulate angiogenesis. It may therefore be difficult to dissect the effect of a particular MC chemical mediator in our *in vivo* model.

Furthermore, relaxin is present in high levels in the peripheral circulation throughout the second half of pregnancy and plays a key role in promoting growth and softening the rat uterine cervix (Sherwood 1994). Relaxin is involved in blood vessel promotion in the uterine cervix during pregnancy (Burger & Sherwood 1998) and stimulates the expression of VEGF in the genital tract (Unemori *et al.* 1999). Based on these data, neither a role of relaxin on angiogenesis during second half of pregnancy nor a possible effect of disodium cromoglycate treatment on the relaxin synthesis could be ruled out.

In conclusion, these results suggest that angiogenesis of the uterine cervix during gestation may be regulated by a mechanism which involves a control of endothelial proliferation by endogenous E_2 levels and an MC degranulation-dependent pathway that implies the action of chemical mediators released from MCs. Both pathways

mediate an upregulation of VEGF gene transcription, constituting an important regulatory step in cervical angiogenesis during pregnancy. Since cervical ripening entails a controlled inflammation-like process (Luque & Montes 1989, Luque *et al.* 1996, Muñoz-de-Toro *et al.* 2003) and angiogenesis is intimately associated with inflammation, it is suggested that angiogenesis could be a significant component in the physiological mechanisms of cervical ripening. Consequently, and considering the well-established role of VEGF in angiogenesis (Norrby 1997, Hyder *et al.* 2000, Ferrara *et al.* 2003), this growth factor could play a significant role in remodeling cervical microvasculature for a normal parturition. These data provide the groundwork for future studies, which will examine the impact of the inhibition of MC degranulation and the decrease of VEGF expression on cervical ripening and parturition.

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