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Original article

Analysis of integrins and vascular endothelial growth factor isoforms mRNA expression in the canine uterus during perimplantation period

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Abstract

Integrins are the major receptors within the extracellular matrix (ECM) that mediate several functions connected with cell life and metabolism, such as cell adhesion, migration, cytoskeletal organization, proliferation, survival, and differentiation. A vascular endothelial growth factor (VEGF) is one of the most important angiogenic factors. It has been suggested that the expression of this gene may play crucial physiological roles in reproductive organs.

All investigated endometrial tissues were isolated on day 10-12 after mating. Control bitches, used in this study, were in metestrus, which was determined according to the vaginal cytology and progesterone level in blood. Early pregnancy was verified by flushing the uterine horns with PBS. Total RNA was isolated from the bitches endometrium by means of the Chomczyński and Sacchi method, treated by DNase I, and reverse-transcribed into cDNA. A quantitative analysis of integrins $\alpha 2b$, $\beta 2$ and $\beta 3$, VEGF 164, 182 and 188 cDNA was performed by RT-PCR.

In results we have shown an increased expression of all investigated genes (integrins $\alpha 2b$, $\beta 2$ and $\beta 3$, VEGF 164, 182, and 188) in pregnant bitches uterus as compared to non-pregnant females ($P < 0.001$).

Our results indicated that the expression of genes encoding integrins and vascular endothelial growth factors is different in relation to the time of the embryo implantation and it is increased in the first period of this process. This may be associated with the induction of specific mechanisms responsible for receptivity of uterus following the embryo attachment. In addition, all of investigated genes are up-regulated in a pregnancy-specific manner and the increased expression of these genes may regulate the uterus function during the implantation of canine embryos.

Key words: integrins, vascular endothelial growth factor, uterus, pregnancy, bitch

Introduction

The implantation is the process characterized by the synchronous interaction between the implantation-stage blastocyst and maternal endometrium. This process depends on the complex series of molecular and cellular mechanisms that are induced in the uterus by several species-specific paracrine and autocrine regulations (Joyce et al. 2007, Germeyer et al. 2009, Seshagiri et al. 2009, Aguilar and Mitchell 2010, Paidas et al. 2010). The process of successful implantation is divided into three steps, including the apposition, adhesion and invasion (Chavatte-Palmer and Guillomot 2007, Demir et al. 2010, Robertson 2010). The apposition, described also as the initial step of implantation, is characterized by a specific interaction between micro-protrusions, also called pinopodes, from the apical uterine epithelium surface and microvilli on the apical syncytiotrophoblast surface of the preimplantation blastocyst. During the second step of implantation adhesion, the physical contact between the blastocyst and uterine epithelium is much closer. Prior to this stage the blastocyst area is oriented to the epithelium. At the last stage of invasion begins the penetration of the syncytiotrophoblast through the epithelium that is also followed by the infiltration of cytotrophoblast. It was proved experimentally that the implantation is a process that needs adequate hormonal stimulation and activation of several protein factors. The factors encompass the growth factors and several cell-adhesion molecules, including integrins.

The integrins are transmembrane proteins, which may bind to extracellular matrix (ECM) proteins and mediate several processes important for the cells life, including cell adhesion, migration, differentiation and possess the ability to transduce the signals into the cell, including mammalian oocytes (Hynes 1992, Burghardt et al. 1997, Kempisty et al. 2009, Antosik et al. 2010). Since the integrins exist as the receptors for matrix proteins, they may also play an important role in the interaction between trophoblast and uterine epithelium (Bowen and Hunt 2000). The expression of integrins in the uterus is regulated spatially and temporally by hormones secreted during different phases of the reproductive cycles.

Vascular endothelial growth factor (VEGF) is mitogenic primarily for vascular endothelial cells and it is structurally related to the platelet-derived growth factor. The expression of VEGF in reproductive organs is highly related to proper ovarian angiogenesis as well as the normal growth of endometrium during the ovulatory cycle in several species of mammals, including rodents and human. Moreover, it was suggested that this protein is an important factor regulating the changes in endometrium during pregnancy in rodents and may play a pivotal role during the implantation (Cullinan-Bove and Koos 1993).

There are limited experimental data indicating the molecular basis of a dog embryo implantation. The results in most cases embrace the experiments using humans, rodents, pigs, sheep, goats and cattle models (Bowen et al. 1996, Aplin et al. 1997, Burghardt et al. 1997, Johnson et al. 2001, Garcia et al. 2004, Kaczmarek et al. 2009, Antosik et al. 2009, 2010). Therefore, the aim of the study was to evaluate the integrins as well as VEGF isoforms mRNA expression in uterus of non-pregnant and pregnant bitches.

Materials and Methods

Animals

Female pregnant (n=15) and non-pregnant (n=23) dogs were used in this study. In the experiment bitches of different breeds and of a similar age of 5.5 years, brought by their owners for routine castration, were used. The bitches were characterized as early pregnant (day 10-12 after mating). The non-pregnant, used as a control were in metestrus cycle, according to the vaginal cytology and progesterone level in the blood serum. The females identified as early pregnant were examined by embryo flushing after ovariohysterectomy (days 10-12 after mating; Schafer-Somi et al. 2008). All females were spayed under general anaesthesia. In all of the cases the spaying was performed as a contraceptive measure. The pregnancy was confirmed by flushing the uterus after ovariohysterectomy with PBS. Uterine tissues were collected from the middle part of the left horn from early pregnant and non-pregnant animals, including interplacental and placentation sites, as described previously by Schafer-Somi et al. (2008). Immediately after the tissue collection, the extraction of total RNA was applied, as described below.

The experiment was approved by the Local Ethical Committee.

RNA extraction from tissues

Total RNA was isolated from the bitches' endometrium by means of Chomczyński and Sacchi method (Schlafke and Enders 1967). Two ml of TRIzol (Invitrogen, USA) were added to approximately 30 mg of homogenized pieces of the uterine tissue. The samples were homogenized using a Virtishear homogenizer (Virtis Company, Inc., Gardiner, NY, USA). Thereafter, they were incubated at room temperature for 5 min and 1 ml chloroform was added to the sample. Samples were incubated at room temperature for 3 min and then centrifuged at 4°C for 30 min at 5000 g. The aqueous phase was transferred into a fresh tube, 2.5 ml isopropyl alcohol was added, and then the

Table 1. Oligonucleotide sequences used for RT-PCR analysis.

Transcript	Sequence (5'-3' direction)	Gene accession no.	Product size (bp)
VEGF 164	AGCAAGGCAAGAAAATCCC CGTTTAACTCAAGCTGCCT	AF133248	103 bp
VEGF 182	GCAAGAAATCCCCTCCCTG CCTGGGCTTGTCACATCTG	AF133249	161 bp
VEGF 188	CGGTATAAGTCCTGGAGCGTT TCGTTTAACTCAAGCTGCCTC	AF133250	140 bp
Integrin α 2b	GGGTTTTCACTGGACTTCT TGTGTCGGGTCTCGTCATT	NM001003163	178 bp
Integrin β 2	TGGTCAAAGGAGAGAGCGG GGAAGAGCAAAGAGCAGG	XM849290	122 bp
Integrin β 3	TGAGTCCTGTGTGTGCCTG ATGCGGACCTCACTGATGG	NM001003162	133 bp
GAPDH	CGCCATCAATGACCCCTTC ACTCAGCACCAGCATCACC	NM001003142	105 bp
β -actin	AGCCGTGTTCCCCTCCATC AGGATGCCCCGCTTGCTCT	NM001003349	111 bp
28S ribosomal protein S15	ATCCAATCGCGGCCCTTC AAGTTTCCCCTTCTCAGCC	XM 532559	105 bp

samples were placed in a -80°C freezer overnight. The samples were subsequently centrifuged at 4°C for 30 min at 22 500 g. The supernatant was discarded and the pellet was washed with 3 ml of 75% ethanol and then air-dried for 5 min. Total RNA was resuspended in 500 μl of diethyl pyrocarbonate (DEPC)-treated water and further purified by phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation. Samples were treated with DNase I (Invitrogen, USA) according to the manufacturer's protocol to eliminate a possible DNA contamination. Total RNA was quantified with a spectrophotometer at wavelength of 260 nm and purity was verified based on the ratio of 260:280. For reverse transcription PCR reaction (RT-PCR) 1.5 μg of total RNA was used.

RT-PCR analysis of integrins and vascular endothelial growth factor isoforms cDNA expression

RT-PCR was conducted in a LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR[®] Green I as a detection dye, and target cDNA was quantified using a relative quantification method. For the amplification, 2 μl of total (20 μl) cDNA solution was added to 18 μl of QuantiTect[®] SYBER[®] Green PCR Master Mix Qiagen GmbH (Hilden, Germany) and primers (Table 1). One RNA sample of each preparation was processed without RT-reaction to provide a negative

control in subsequent PCR. The housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin, were amplified as references for mRNA quantification. To quantify a specific gene expression in the endometrium, the levels of expression of specific endometrial mRNAs in each sample were calculated relative to GAPDH and β -actin. To ensure the integrity of these results, an additional housekeeping gene, 28S ribosomal protein S15 mitochondrial precursor (S15mt), (MRP-S15) was used as an internal standard to ensure that GAPDH and β -actin mRNA expression was not regulated in the groups of animals. This gene has been identified as an appropriate housekeeping gene for the use in quantitative PCR studies. The expression of GAPDH, β -actin and 28S ribosomal protein S15 mRNA was measured in cDNA samples from endometrium. The expression of GAPDH and β -actin did not vary when normalized against 28S ribosomal protein S15 (results not shown).

Statistical analyses

All results are given as means \pm SEM. Since data were not normally distributed, the non-parametric Mann-Whitney *U*-test was chosen for the comparison of the relative abundance of mRNA expression between the group of pregnant and non-pregnant bitches. All calculations were carried out with the SPSS software (Version 14 for Windows; SPSS Inc., Chicago, IL, USA). A p-value of $P < 0.05$ was considered statistically significant.

Results

After RNA isolation from the tissues, it was reversely transcribed into cDNA and analyzed by RT-PCR assay. RT-PCR analysis detected an increased expression of all VEGF 164, VEGF 182 and VEGF 188 transcripts isoforms in the endometrial tissue in pregnant bitches as compared to the non-pregnant ones. These results were statistically significant ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively). The level of integrin $\alpha 2b$, integrin $\beta 2$ and integrin $\beta 3$ transcript in collected tissues was higher in pregnant females as compared to non-pregnant ones. These results were also statistically significant ($P < 0.01$, $P < 0.01$, $P < 0.001$, respectively), (Fig. 1B).

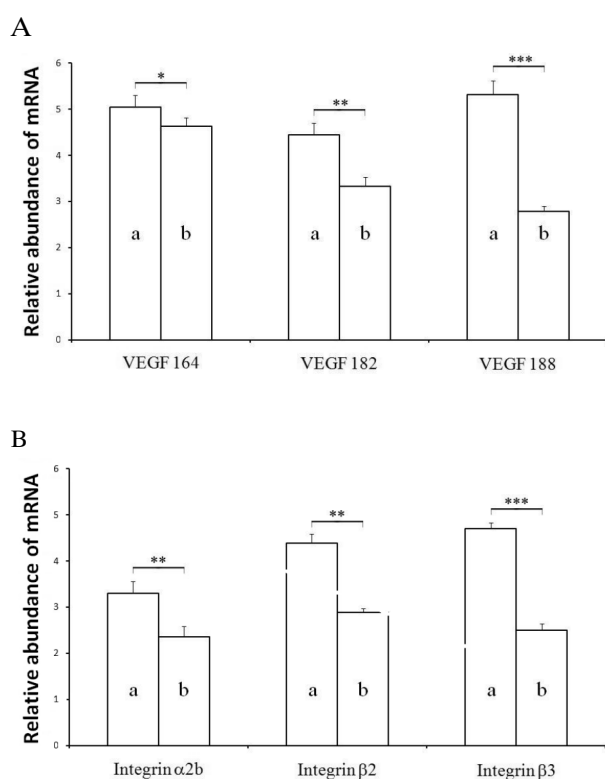


Fig. 1. Relative abundance of VEGF-164, VEGF-182, VEGF-188 as well as integrin $\alpha 2\beta$, integrin $\beta 2$ and integrin $\beta 3$ transcripts in endometrial tissues of pregnant and non-pregnant bitches.

Each sample was determined in triplicate from three independent tissue collections from 38 separate animals. Results are presented as mean \pm SEM with the level of significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discussion

Since Enders and Schlafke (1967) described the definition of implantation in rodents as “implantation can be considered to start when a fixed position of the blastocyst in relation to the uterus is established” many authors observed several modifications according to

species-specific mechanisms. This definition is also acceptable in dogs. The first easily observable change during a dog implantation is when the blastocysts reach the definitive position in the uterus and the endometrial edema undergoes morphological changes that indicate the position. When the blastocyst localized the endometrial edema, the embryos are hatched from the zona pellucida and the embryonic disc is differentiated into the primitive streak (Holst and Phemister 1971). Contrary to these observations, Gier (1950) suggested that the embryos are still in a free-floating blastocyst stage prior to the formation of somites. In relation to all these observations it is strongly suggested that the implantation of an embryo in the uterus is a complex process involving the biochemical (changes in immune-specific proteins such as cytokines or growth factors expression) and morphological changes within the endometrial tissues. Therefore, the aim of this study was to detect and determine the differences in integrins (molecules possibly involved in several adhesion mechanisms) and vascular endothelial growth factors genes expression in pregnant bitches.

There are no available published data that reported the expression of VEGF in the bitch uterus during pregnancy, although there are some information about the expression of this gene in other species of mammals, including pigs, rodents and human (Douglas et al. 2009, Kaczmarek et al. 2009, Kalkunte et al. 2009, Bolat et al. 2010, Chen et al. 2010, Souza et al. 2010). It was previously clearly demonstrated that the activation of VEGF-related biochemical pathway induces the processes of proper angiogenesis and vascularization. The expression of these genes and the activation of their metabolic pathway is also specific for reproductive organs, such as ovary and uterus, as it is presented in this study. It was also proved that the inactivation of this VEGF-specific pathway may be a main reason for the impaired implantation of the embryo or prevent proper pregnancy development (Douglas et al. 2009). In this study we (i) identified the expression pattern of VEGF transcript isoforms (164, 182, 188) in the canine uterus during pregnancy and (ii) we proved that this expression is increased during this stage as compared to the controls. Since the implantation of the embryo forms a specific structure named decidua by the activation of proliferation and differentiation of uterine stromal cells, we suggested that the increased expression of VEGF mRNA isoforms may play a central role in the regulation of decidual angiogenesis. It was previously partially described by Douglas et al. (2009), who demonstrated that VEGF and its specific receptor VEGFR-2 pathway and its regulation are crucial for the maintenance of early pregnancy through regulation of angiogenesis in the uterine decidua. Our observations were also proved by Kalkunte et al. (2009), who demonstrated that one of the isoform of VEGF (VEGF-C) produced by uNK cells support endovascu-

lar processes and play a central role in the pregnancy-compatible immunovascular processes during placentation and early fetal development. The VEGF and its specific receptor (VEGFR-1) is expressed not only in the ovary, endometrium or placenta, as it was previously demonstrated. These genes are also expressed in the porcine conceptus. Kaczmarek et al. (2009) investigated the expression of those genes in the porcine endometrium during the estrous cycle and conceptus. They found an increased expression of VEGF-164 mRNA isoform during the porcine conceptus development. They concluded that the higher expression of VEGF and its receptor in endometrial tissues during pregnancy suggests their role in the vascular remodeling and regulation of this process, which is crucial for the successful embryo implantation as well as regulation of vascularization. The observation from this study supports the hypothesis that VEGF and its receptor may play a critical role in the normal development of the porcine conceptus during pregnancy.

Integrins are described as transmembrane heterodimeric receptors for extracellular matrix and cell surface proteins. The binding of integrins to ligands in the extracellular matrix regulate several specific cellular processes as cell attachment, which in turn activates various cytosolic signal cascades to promote the cell migration, survival, proliferation, and differentiation. It was clearly proved that the cytoplasmic domains of integrins are essential for cell adhesion (Chang et al. 1997). Zhang et al. (2001) showed that Krev Interaction Trapped 1 (krit1), which was originally identified through its interaction with the Ras-family GTPase krev1/rap1 also strongly interacts with the integrin cytoplasmic domain-associated protein-1 (icap1). They suggested that beta1 integrin and krit1 may be involved in the same biochemical pathway, which regulates the epithelial cells functions through the mechanism of integrin beta1-dependent angiogenesis. This observation suggests that integrins are also involved in angiogenesis, what may explain their expression in the uterus during the stage of early pregnancy. There are several notes indicating the role of the integrins expression during pregnancy and embryo implantation in several mammalian species (Johnson et al. 2001, Roberts et al. 2009, Massuto et al. 2010). However, there are no available published data concerning these mechanisms during dog pregnancy. Zhang et al. (2001) using the rat endometrial cells during non-receptive, pre-receptive and receptive phases of the uterus as the model, demonstrated that the expression of the alpha(V)beta(3) integrin may be a useful marker of endometrial epithelial cells (EEC) differentiation phases. Moreover, they found that the integrins expression may be used as a marker of endometrial receptivity and it is necessary for the proper embryo attachment during the implantation. Our observations were partially proved by Lin et al. (2007). Using the pig as the model for the embryo

attachment during the implantation, they showed the expression of alphaV and beta3 integrin in endometrium in all investigated stages of pig pregnancy (on Days 0, 12, 18, and 25 of pregnancy). However, an increased expression of this gene on day 18 of pregnancy was identified. They suggested that the specific increased expression of this integrin mRNA in the porcine endometrium may play a central role during the process of the embryo attachment during implantation stages.

Conclusions

Here we identified the mRNA expression pattern of VEGF-164, VEGF-182, VEGF-188 as well as integrin $\alpha 2\beta$, integrin $\beta 2$ and integrin $\beta 3$ in the dog uterus during pregnancy. We suggested that the specific increased expression of these genes during pregnancy may indicate the crucial role of these genes in uterine-conceptus interactions during the perimplantation period.

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