Expression Levels of Plasma Angiogenic Factors during Early Pregnancy in Hanwoo

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ABSTRACT

This study was conducted to compare the expression pattern of the specific factors associated with pregnancy and angiogenesis during early pregnancy in Hanwoo. Synchronized female Hanwoo (4~6 year-old) were inseminated artificially. After 10 weeks after artificial insemination (AI), the pregnancy was tested by rectal palpation method. Three pregnant and non-pregnant Hanwoo were used in this experiment, respectively. The plasma progesterone level was measured by ELISA. Western blot analysis was performed to detect the expression of pregnancy associated glycoprotein (PAG) or angiogenic factors (VEGF, B-FGF, ANP-1, and TIE-2). The plasma P4 level was increase gradually in pregnant group and maintained high level. The concentration of PAG was significantly higher from 5th weeks in pregnant group compared to that of non-pregnant group (p<0.05). The concentrations of the VEGF (p<0.05), B-FGF (p<0.05), and ANP-1 (p<0.05) were significantly increased from 6th or 7th week after AI in pregnant group, respectively. And the intensity of TIE-2, ANP-1 receptor, was well matched with ANP-1 (p<0.05). Taken together, it can be postulated that the blood vessels connected with fetus and dam were formed dramatically around 40 days after AI, because the expression levels of the angiogenic factors were increased significantly from this time in pregnant Hanwoo.

(Key words : P4, Early pregnancy, Angiogenic factors, Pregnancy associated glycoprotein)

INTRODUCTION

The implantation is the process in which the blastocyst physically and physiologically comes into intimate contact with uterus, and than embryo is development with placenta. In this stage, fetal is depend on maternal circulation for gases, nutrients, and wastes that exchanged between the maternal and fetal systems are transported via the placenta (Ramsey, 1982; Faber and Thornburg, 1983). During this time, pregnancy associated glycoprotein (PAG) is specifically stimulated in placenta and detected in blood of pregnant cow in the fourth week of pregnancy (Butler *et al.*, 1982; Sasser *et al.*, 1986; 1989).

Also there is marked proliferation of blood vessels at the fetal-maternal interface. This rapid proliferation of blood vessels, referred to angiogenesis, is mediated by placental and endometrial factors and continues in association with placental development (Reynolds *et al.*, 1992).

A large number of growth factors, receptors and transcription factors have been implicated in angiogenic processes during implantation and early pregnancy. The important growth factors and angiogenic factors are known as vascular endothelial growth factor (VEGF) (Ferrara and Davis-Smyth, 1997; Neufeld *et al.*, 1999) basic fibroblast growth factor (B-FGF) (Itoh *et al.*, 2004), and angiopoietin-1 (ANP-1) (Miyamoto *et al.*, 1992; Mandriota & Pepper, 1998; Oh *et al.*, 1999).

Recently, many studies have been reported for early pregnant and implantation stage of fetal or placental development by histologically or anatomically (Lee and DeMayo, 2004; Reynolds *et al.*, 2005). However, little is known about the pregnancy and angiogenesis factors in blood during early pregnancy. Therefore, this study was conducted to figure out the expression pattern of the factors associated with pregnancy specific and angiogenic factors in Hanwoo.

MATERIALS AND METHODS

Animals

Female Hanwoo (4~6 year-old) were treated for estrous synchronization. Briefly, all cows received a CIDR device containing 1.9 g of progesterone (InterAg, Ha-

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milton, New Zealand) at random stages of the estrous cycle (day 0). On the morning and afternoon of day 6, donor cows were treated twice (22.5 mg/each) with prostaglandin $F_2\alpha$ ($PGF_2\alpha$, im; Lutalyse, Pharmacia, Kalamazoo, MI, USA). Progesterone implants were removed in the morning of day 7. After day 8, cows were observed for estrous behavior and inseminated at 12 h after the onset of estrous. After 10 weeks from artificial insemination (AI), the pregnancy was tested by rectal palpation method. Three pregnant and non-pregnant Hanwoo were used in this experiment, respectively.

Collection of Blood Samples

Blood samples were collected from the jugular vein using heparinized vacutatiner tubes from 0 (the day of AI) to 7 week after AI. Immediately after collection, the plasma was separated by centrifugation at 1,500 g for 20 minutes at 4℃ and stored at −20℃ until assayed.

Analysis of Plasma Progesterone Level

The plasma progesterone (P4) level was analyzed using ELISA kits (Endocrine Technologies, Inc., USA) according to manufacturer's protocol and calculated the concentration using Precision microplate reader (Molecular Device, USA).

Western Blot Analysis

For SDS-page, the 30 μg/ml of plasma samples were loaded 10 μl each well with 350 mM 2-mercaptoethanol (Bio-Rad, Hercules, CA, USA). After SDS-page, total proteins in the gel were transferred onto PVDF membrane (Bio-Rad) in transfer buffer containing 25 mM Tris (Sigma, St. Louis, USA), 193 mM glycine (Sigma) and 5% (v/v) methanol. After transfer, the PVDF membrane was washed three times with Tris buffered saline with Tween-20 [TBST, 20 mM Tris-HCl; pH 7.6; 154 mM NaCl; 1% (v/v) Tween-20 (Bio-Rad)] and incubated with TBST containing 3% (w/v) non-fat dry milk (Bio-Rad) for 1 hour at room temperature. The membrane was incubated with primary antibody (PAG, VEGF, B-FGF, ANP-1, and TIE-2) (1:1,000) in TBST containing 5% non-fat dry milk (w/v) for 1 hour, respectively. All the primary and secondary antibodies were purchased from Abcam (Cambridge, UK), except for PAG (Proteintech Group, Inc., Chicago, USA). After incubation, the membrane was washed five times with TBST and then incubated secondary antibody (mouse or rabbit) (1:5,000) in TBST containing 1% non-fat dry milk for 30 minutes, the membrane was washed five times with TBST. For visualization of antigen-antibody complexes, the membrane was treated with chemiluminescent detection reagent (ECL, Amersham Biosciences) and exposed onto X-ray film for 0.5 to 5 minutes. The X-ray film was developed and fixed using automatic film processor (JPI Co. Ltd, Seoul, Korea). The band intensity was calculated by Quantity One Software (Bio-Rad).

Statistical Analysis

Differences among treatment means were determined by student's *t*-test using statistical analysis system (SAS Institute, Cary, NC, USA). All data were expressed as mean ± stand error (SE). Differences were considered significant at $p<0.05$.

RESULTS

The plasma P4 level was increase gradually and maintained high level in pregnant group, the P4 level in non-pregnant group, however, was changed up and down according to estrous cycle periodically (Fig. 1).

The concentration of PAG was significantly higher from 3rd week in pregnant group compared to that of non-pregnant group ($p<0.05$) (Fig. 2).

The expression patterns of angiogenic factors were shown in Fig. 3 (VEGF), Fig. 4 (B-FGF), and Fig. 5 (ANP-1), respectively. The concentrations of the three angiogenic factors were significantly increased from 6th or 7th week after AI, ($p<0.05$), respectively.

And TIE-2, ANP-1 receptor, level was also significantly increased during 1~2 week and from 6th week after AI ($p<0.05$) (Fig. 6), respectively.

DISCUSSION

The continuity for pregnant or prepared next estous cycles have been connected with the corpus luteum

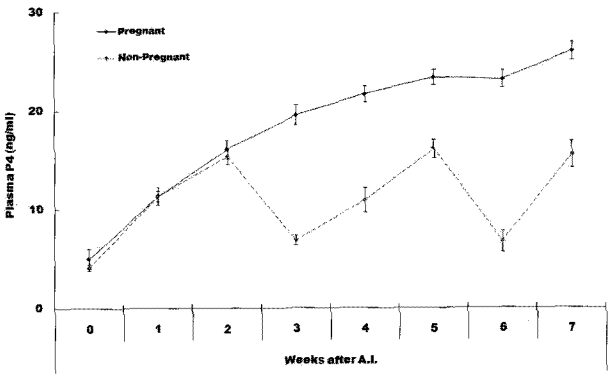

Fig. 1. The plasma progesterone level during early pregnancy. The blood was collected from the recipients pregnant by AI, respectively. The concentration of plasma P4 level was measured by ELISA. Data was expressed as mean ± standard error (S.E.).

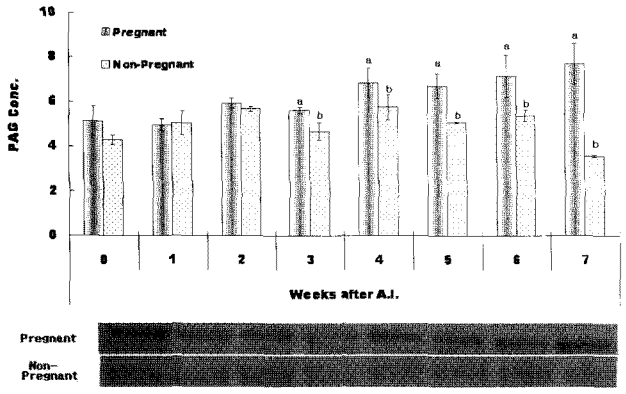

Fig. 2. The intensity of pregnancy specific protein during early pregnancy. 30 ug/ml of serum samples were loaded in each well. The band intensity was normalized by BSA. $^{a\ vs\ b}$; $p<0.05$.

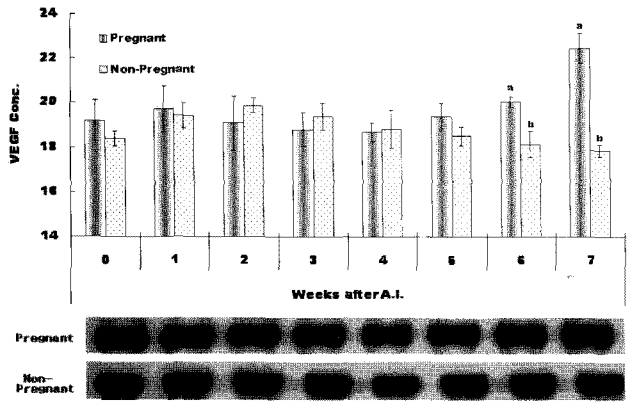

Fig. 3. The intensity of VEGF during early pregnancy. 30 ug/ml of serum samples were loaded in each well. The band intensity was normalized by BSA. $^{a\ vs\ b}$; $p<0.05$.

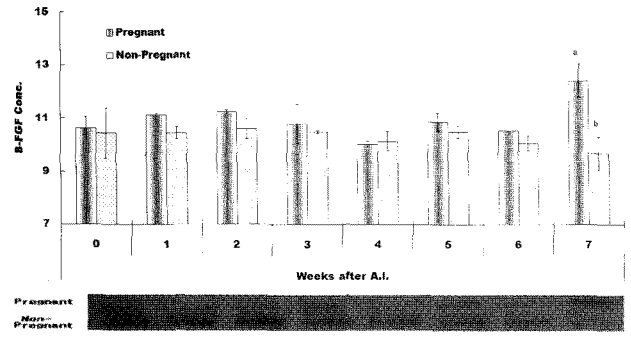

Fig. 4. The intensity of B-FGF during early pregnancy. 30 ug/ml of serum samples were loaded in each well. The band intensity was normalized by BSA. $^{a\ vs\ b}$; $p<0.05$.

(C.L) which is made from granulosa and theca cells within the ruptured follicle undergo luteinization after ovulation, and secretes progesterone (Downey 1980).

In this study, the P4 level in pregnant group was started to increase from 2nd week after AI and maintained high level during early pregnancy. Progesterone is very crucial for the maintenance of pregnancy as

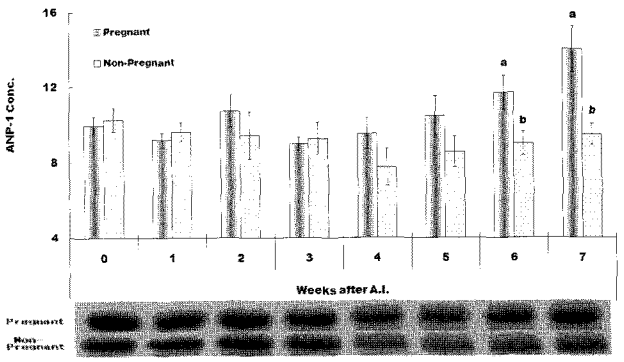

Fig. 5. The intensity of ANP-1 during early pregnancy. 30 ug/ml of serum samples were loaded in each well. The band intensity was normalized by BSA. $^{a\ vs\ b}$; $p<0.05$.

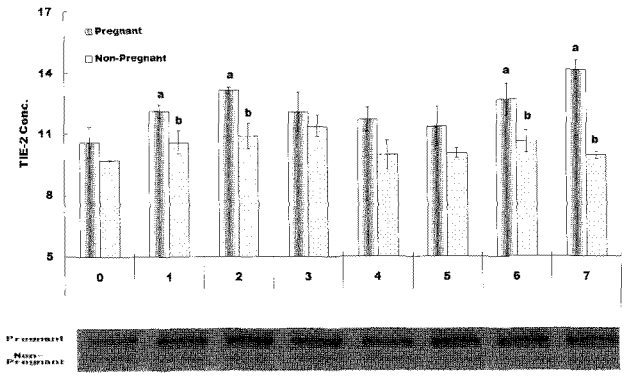

Fig. 6. The intensity of TIE-2 during early pregnancy. 30 ug/ml of serum samples were loaded in each well. The band intensity was normalized by BSA. $^{a\ vs\ b}$; $p<0.05$.

well as parturition in bovine (Hwang *et al.*, 2008 a,b). During early pregnancy, the maintenance of higher level of P4 was very important, or corpus luteum will be regressed and follicular development will be started by the secretion of follicular stimulating hormone from anterior pituitary.

In 1988, a gonadotropin-like molecule was identified from bovine placenta and purified until apparent homogeneity (Beckers *et al.*, 1988). This protein was more appropriately referred as pregnancy-specific protein-B (PSP-B) (Sasser *et al.*, 1989) or pregnancy-associated glycoprotein (PAG) (Zoli *et al.*, 1991; 1992). Little, however, is known about the function of this protein according to implantation. The intensity of PAG, in the present study, was increased from 5th week after AI in pregnant group, like previous reports (Butler *et al.*, 1982; Sasser *et al.*, 1986; 1989). It can be postulated that the increasing intensity of PAG implies the changes of environments in uterus such as the formation of placenta.

Angiogenesis is defined as the generation of new blood vessels through sprouting from already existing blood vessels in process involving the migration and proliferation of endothelial cells from pre-existing ve-

ssels. In adult angiogenesis occurs rarely with exception such as the female reproductive system, wound healing and cancer (Reynolds and Redmer, 1998).

During early pregnancy, the newly formation of blood vessels in placenta is very important for normal development of fetus. Several angiogenic factors, such as VEGF (Ferrara *et al.*, 1996; Carmeliet *et al.*, 1996; Matsumoto and Claesson-Welsh, 2001), B-FGF (Baird *et al.*, 1986; Itoh *et al.*, 2004), and ANP-1 (Maisonpierre *et al.*, 1997; Suri *et al.*, 1998) and TIE-2 (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997) were very necessary for the formation of the placenta. The implantation of the bovine conceptus is being around day 19 of gestation and a tenuous attachment has formed between the trophobast and maternal tissue by day 30 (King *et al.*, 1981). After this period, a blood vessel proliferation is very fast (Carmeliet *et al.*, 2000).

Here we performed western blotting analysis to detect the expression pattern of these angiogenic factors. In the present study, the expression pattern of these three angiogenic factors was very similar. Based on our results, it can be speculated that the formation of placenta in Hanwoo is dramatically started from 6th week after AI (about 40 days after AI), because the angiogenic factors closely related to blood vessel formation were stimulated simultaneously.

Taken together, it can be postulated that the blood vessels connected with fetus and dam were formed dramatically around 40 days after AI, because the expression levels of the angiogenic factors were increased significantly from this time in pregnant Hanwoo.

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(Received: 1 December 2008 /Accepted: 12 December 2008)