Physiological role of Kallikrein-Kinin system in Implantation

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Physiological role of Kallikrein-Kinin system in Implantation

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논 문 개 요

생쥐에서 수정란이 포배기로 발생하는 동안 자궁은 난소내 스테로이드 호르몬의 합성량 조절과 자궁내막의 생리적 변화를 통하여 임신 4일 자정 무렵에 착상이 가능하게 한다. 자궁내막층의 기질세포는 착상을 신호로 염색성이 약한 탈락막세포로 분화하며 세포질에 글리코겐등의 물질 축적을 진행한다. 영양막세포는 분열능력이 왕성한 세포성영양막(cytotrophoblast)으로 발달하며, 바깥쪽의 융합체세포는 증식능력이 없는 두꺼운 합포성영양막(syncytiotrophoblast)으로 발달한다. 합포성영양막에 세포가 많아지면 작은 공포가 형성되며, 이들은 서로 합쳐져 혈액이 차는 공간인 열공을 만든다. 또한 합포성영양막이 태반이 형성되는 동안 자궁내막을 침식하게 된다. 합포성영양막은 혈관이 잘 발달된 자궁내막강을 계속 침식하기 때문에 모체의 혈관을 침식하게 된다. 결과적으로 혈액이 열공으로 들어가 모체 혈액이 발생중인 배아에게 영양을 효율적으로 공급할 수 있게 된다. 포배가 자궁내막 상피에 부착하고 이후 침입하는 과정에서 배아는 적절한 산소의 공급이 요구되며 현재 이와 관련하여 착상 부위에 혈관의 분포와 혈액 공급이 증가하는 현상이 관찰 보고 되었다. 그러나 이러한 현상을 유발하는 기작과 혈관의 재분포 및 혈액공급 증가에 관한 기작은 잘 알려져 있지 않다. VEGF를 매개한 혈관 재분포를 설명 하려는 노력이 있으나 불충분하다. 흥미롭게도 칼리크레인-키닌 시스템은 생물학적인 기능으로는 국소지역 혈액의 흐름과 신생 혈관 형성, 조직 침입, 유사분열의 조절 등에 관여한다고 잘 알려져 있다. 따라서 본 연구에서는 칼리크레인-키닌 시스템이 착상 시 관찰되는 혈관의 재분포에 관여함을 알아보고자 하였다. 먼저 칼리크레인 집단의 유전자 발현, 그 기질인 키니노겐들의 발현, 대사산물인 키닌의 세포막 수용체들의 발현을 착상시기 전후의 자궁, 배아, 태반에서 알아보았다. 그리고 자궁에서 착상시기 전후에 발현되는 유전자를 선별하여 스테로이드 호르몬에 의해 발현이 조절되는가

를 알아보았다. 한편, 브레디키닌 수용체 길항제를 이용하여 혈관 분포조절에서의 역할을 확인하였다. 그 결과 칼리크레인 1, 2, 4, 5, 6, 12와 키니노겐 1, 2 그리고 브래디키닌 2 수용체가 임신 5일을 기준으로 착상 전 후에 자궁에서 발현되었다. 배아에서는 칼리크레인 4, 5, 6, 7이 초기 배아 발달시기와 착상 시기에 발현되었다. 그리고 키니노겐 1, 2가 5.5 p.c.d에 발현되었다. 하지만 브래디키닌 1, 2 수용체는 모두 발현되지 않았다. 태반에서는 칼리크레인 4만 8.5 p.c.d와 11.5 p.c.d에서 발현되었으며, 키니노겐 2 와 브래디키닌 2 수용체가 발현되었다. 난소를 절제한 생쥐의 자궁에서 칼리크레인 1, 2는 프로게스테론에 의해 발현이 유도되었다. 칼리크레인 4, 5, 6, 12와 브래디키닌 2 수용체는 에스트로겐에 의해 발현이 유도되었다. 한편, 키니노겐 1의 mRNA 발현은 에스트로겐에 의해 발현이 급증하였으며 프로게스테론에 의해서 어느정도 유도 되었고, 에스트로겐 노출 전 자궁에서 프로게스테론에 의해 발현이 감소되는 특징을 보였다. 키니노겐 2 mRNA 발현의 경우는 키니노겐 1과는 다르게 에스트로겐에 노출된 자궁에서 프로게스테론에 의해 발현이 억제되지는 않았다. 흥미롭게도 이들 유전자 중 일부는 다른 호르몬에 의하여 발현이 억제 되는 복잡한 조절 기작이 있음을 알 수 있다. 착상지연유도 모델을 통해 착상 유도와 관련해서 칼리크레인-키닌 시스템의 유전자 발현을 본 결과 에스트로겐 주사 후 6시간에 칼리크레인 4, 5, 6의 발현이 급증하였다. 키니노겐 2와 브래디키닌 2 수용체는 1시간 내에 발현양이 증가하기 시작했다. 한편, 브래디키닌 2 수용체의 길항제인 WIN 64338의 농도 의존적으로 배아 주변의 혈관 분포 정도가 감소하였다. 그러나 브래디키닌 1 수용체의 길항제인 R715의 처리에서는 감소하지 않았다. 이러한 결과를 바탕으로 칼리크레인 키닌 시스템이 브래디키닌 2 수용체를 통하여 착상 시 혈관의 재형성에 관여한다는 것을 알 수 있다.

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INTRODUCTION

Implantation has been known to be a steroid hormone dependent process coordinated action of both estrogen and progesterone are necessary for the preparation and process of implantation (Carson et al., 2000). The process of implantation consists of apposition between the trophoectoderm layer of the blastocyst with the luminal epithelium, attachment of these layers and finally invasion of the uterine luminal epithelium by the embryo. Upon embryo invasion, the uterine stromal cell is rapidly remodeled in the process of decidualization (Cheon et al., 2002 ; Lee et al., 2007).

Adequate fetal development requires the maturation of placenta and the replacement of the contractile spiral arteries by saccular dilated vessels and an extensive and intact surface of exchange of oxygen, nutrients and waste products between the fetal and the maternal blood (Corthorn et al., 2006). The mechanism by which localized vascular permeability and angiogenesis occur at the sites of implantation is not clearly understood. This increased vascular permeability and attachment reaction occur in the evening (2200 - 2300 hr) of day 4 of pregnancy (Das et al., 1994). This is followed by localized decidualization of the stroma at the site of implantation (Parr et al., 1987). Facilitating invasion of trophoblast cells through the underlying basement membrane occurs after adhesion (Schlafke et al., 1975). These processes are accompanied by remodeling of the extracellular matrix and angiogenesis in the stromal bed. So far, the molecular mechanisms by which increased localized va scular permeability and angiogenesis occur in the uterus at the site of implantation are poorly defined.

Vasculogenesis is the formation of new blood vessels and angiogenesis is

formation of blood vessels from the preexisting ones. Angiogenesis is required for proliferative processes in the reproductive tract, placental growth, tissue regeneration in wound healing, as well as in pathological conditions such as solid tumor growth, rheumatoid arthritis, and retinopathies (Folkman et al., 1987).

NO is a well known requirement for vascular permeability and angiogenesis. At the time of embryo implantation, the levels of NO is increased. It is originated from embryo, and suggested as a key factor for embryo development in mammals. It means that NO originated from embryo can induce angiogenesis or vasodilation of the site of implantation (Gouge et al,. 1973).

Early placentation (<10 - 12 weeks of gestation) occurs in an environment characterized by relatively low concentrations of oxygen (17.9mm Hg) as compared with the endometrium (39.6mm Hg) (Kingdom et al., 1999). Under hypoxic conditions, the trophoblast layer assumes a proliferative trophoblast phenotype and an invasive trophoblast phenotype (James et al., 2006). However, there is no corresponding abrupt change in the level of any angiogenic growth factor or inhibitor at this point (Zhang et al., 1999). This suggests that the regulation of these factors is determined by additional factors although oxygen and oxidative stress may play a role for some (Charnock et al., 2004).

The kallikreins (Klks) encode proteins that activate a wide range of substrates including ECM proteinases and growth factors (Clements et at., 1997). Kallikrein-kinin system involved in many biological functions such as local blood flow, angiogenesis, tissue invasion and mitogenesis (Bhoola et al., 1992). This increase is specifically located in the implantation sites, in contrast to interimplantation sites (Valdes et al., 1996). It is suggested that

kallikrein-kinin systems are linked with NO and VEGF. From the DNA-chip based analysis, Klks are identified in the pregnant uterus. Based on them, in this study we expression profiles of Klks, kininogens (Kig 1, 2), bradykinin receptors (B1, 2 receptor) were analysed and examined their possible roles on the rearrange of blood vessel.

MATERIALS AND METHODS

Experimental animals

All experiment animals were studied according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and under the Experimental Animals Committee of Sungshin Women's University. Animals were maintained under the standard conditions at animals house in Sungshin Women's University with diurnal rhythm kept under the 14 L : 10 D schedule with light-on at 0600hr and clean room system. Animals were fed a standard rodent diet and water *ad libitum* from weaning at 21 days after birth.

Ovariectomy and steroid administration

6 weeks old CD-1 female mice were ovariectomized and administrated with sex steroid hormones after 2 wks later. 17β-estradiol (Sigma, E_2, 2μg/kg, BW) or P_4 (Sigma, 40 μg/kg BW) were administrated into subcutaneous for 3 days at 0830. E_2+P_4 were prepared one day E_2 administration and two days more P_4 administration. The mice were sacrificed at next day. And ICI 182,780 (Tocris, 0.0027mg/0.1ml), RU 486 (Tocris, 0.405mg/0.1ml) were used for E_2, P_4 antagonist.

Delayed implantation model

Female CD-1 mice were superovulated by injection of 2.5 IU of PMSG and followed by injection of 2.5IU of hCG after 48hr and then followed by mating with stud male mice. The next day morning, these mice were examined for the presence of vaginal plug, and this was defined day 1 of pregnancy. At day 4 of

pregnancy, ovariectomized at the morning (0830) and day5, 6, 7 of pregnancy were P_4 (2mg/ 0.1ml sesame oil) sc injected and followed 17β-estradiol (25ng/ 0.1ml sesame oil) injected at the morning (0830). The mice were sacrificed to collect uteri on time dependent (P_4 only, 0.5 hr, 1 hr, 1.5 hr, 3 hr, 6 hr, 9 hr, 12 hr post E_2).

Embryo collection and uterus, placenta sampling

6 weeks old CD-1 female mice were mated with fertile males of the same strain to induce pregnancy and were checked for vaginal plugs on the following morning. Various stage peri and post implantation uterus, embryo (embryonic day 2.5, 3.0, 3.5, 4.0, 4.125 4.25, 4.5, 5.5, 8.5 and 11.5) and placenta (embryonic day 8.5, 11.5) were collected.

Total RNA isolation and first strand cDNA synthesis

Total RNA of embryos were extracted using RNeasy® Micro Kit (QIAGEN, CA USA) according to the manual of manufacturer. Total RNA 5ug were used to perform reverse transcription. First Strand cDNA Synthesis Kit (Agilent, CA, USA) according to the manual of manufacturer. Briefly, reaction reagents are total RNA 5ug, 5.0μl Accuscript buffer (10X), 6.0μl oligo dT primer (0.5μg/μl), 1.0μl random primer (0.1μg/μl), 2μl dNTP mix (100mM), 1μl RNase-free water. Reaction mixture was incubated at 65℃ for 5min, placed the tube at RT to allow the primers to anneal to RNA for 10 min and then added 4.0μl DTT (100mM), 1μl Accuscript multiple temperature RT, 2μl RNase block ribonuclease inhibitor (40U/ml). The mixture was incubated at 42℃ for 1 hr and 72℃ for 15 min to terminate cDNA synthesis.

Real-Time RT-PCR

Transcripts of target gene were amplified using real time-PCR (TaKaRa, TP800) and the specific primers (Table 1). The primer parameters were 50% GC contents, avoiding repeat base pair and lengthening 20-24mer. For Quantitative RT-PCR (qPCR) was performed using SYBR Premix Ex Taq™ (TaKaRa) (Table 2). Each reaction was run in triplicate and consisted of 1.0 μl cDNA. Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature. The fold change in gene expression was calculated using the ΔΔCt method with the housekeeping genes, 36B4.

Immunofluorescence

Dissected mouse uterine horns were cut into 0.5 cm segments, fixed in 10% formaldehyde for 24 hr and embedded in paraffin. 4μm sections were mounted on glass slides. Endogenous peroxidase activity was blocked in 0.3% hydrogen peroxide in H_2O for 15 min. Briefly, tissue were incubated with 1% normal blocking serum in 0.1% PBST with 0.1% BSA for 1 hr and then incubated with the Rabbit polyclonal B2R antibody (dilution 1:200) 2hr at room temperature. After washing in 0.1% PBST, tissues were incubated with biotinylated anti-rabit IgG diluted 1:200 in 0.1% PBST with 0.1% BSA for 2hr at room temperature. Tissues were washed with 0.1% PBST and incubated with Hoe 33238 (dilution 1:200) 5min for counterstaining. Slides were washed with 0.1% PBST for 5 min and mounted.

FITC-dextran injection

6 weeks old CD-1 female mice were superovulated by injection of 5IU of PMSG and followed by injection of 5IU of hCG after 48hr and then followed by mating with stud male mice. The next day morning, these mice were examined for the presence of vaginal plug, and this was defined day1 of pregnancy. At day4 (0900, 1700), 5 (0900) of pregnancy, sc injected with B1 receptor Antagonist R715 (Tocris, 500μg, 100μg / 0.1ml), B2 receptor Antagonist WIN 64338 (Tocris, 500μg, 100μg / 0.1ml), Bradykinin(BK) (Sigma, 500μg/ 0.1ml) and combination B1+B2 receptor (500μg, 100 μg, 50μg / 0.2ml), B1+B2 receptor+BK (300μg+300μg+500μg / 0.2ml).

At day5 (1200) of pregnancy, injected with 0.1ml of FITC-dextran (sigma, 500-kDa ; 1.25mg / 0.1ml) into the tail vein. After 10min, these animal were sacrificed by cervical dislocation and the uterus freezing for cryosection. And we performed immunostaing for colocalization of CD31 (cy3-anti-goat IgG).

Statistics

The t-test was used to evaluate the difference between control and experiment group. Results were presented as mean SEM. Values of $P < 0.05$ were considered significant.

Table 1. Primers list for real time PCR ananlysis

Gene		Primer sequence (5′-3′)	Amplified length (bp)
Klk1	s	CCT CCT GTC CAG TCT CGA ATT GTT	237
	as	AGG GAT GGC TTT GCT GAC AA	
Klk2	s	CAT GTG GTT CCT GAT CCT GTT CCT A	206
	as	CAT AAT AGC AGT GGG CAG CTG TGA	
Klk3	s	CAT TGA TCG TCG ACC TCC TGT	221
	as	ATT CCA AGA TGA ACC CTC TGC TC	
Klk4	s	AGA AGC AGC AGG AGC CAA CAT	204
	as	ACC AAG ACT CCC GAG CAG AAA	
Klk5	s	TAC CCT GAT CAC AAC CCT GGT TCT	313
	as	GAT TCT GAA CAC TGG CTT TCT GC	
Klk6	s	ACA ATG AAG ATG CTG GCC CT	273
	as	GTC CAC TGA GAT TTG CCT TTG G	

Gene		Primer sequence (5′-3′)	Amplified length (bp)
Klk7	s	CAA GTC ACT CCC AGC TCT CTC ATC T	233
	as	ACC CAG TAT AAG TCC ACC AGC AC	
Klk8	s	TCC ATC CTC CAG CAA GAC TCA A	185
	as	CCC ATG AAC AGA AGC AGA AGG AT	
Klk9	s	CTG CCA ATG ACC ACA ATG ATG AT	216
	as	GGC AGA GCT TGT TGT CCA GAA TG	
Klk10	s	TTC TTC ACC TCT CCA CTG CCT CT	206
	as	CGC ACA TTG GAA CTG GAG GTT A	
Klk11	s	ACT TCA ACA ACA GCC TCC CCA A	272
	as	CAG CAT GGT GTC TGT GAT GTT G	
Klk12	s	CAT TCT CTT GCT CCT GTG TGC TGT	244
	as	CAA CTG TTC TGT CCA GTC CAG CTT	

Gene		Primer sequence (5′-3′)	Amplified length (bp)
Klk13	s	CTT GGG AAA GAG GTG ACG AAC A	207
	as	CAC TAG TCC CAT TGG TAC CAT TGA G	
Klk14	s	CCC AGA GCC AAG GAG ATC ATA A	216
	as	GAG TCG CAA CCC ACC AAC TTA TGT T	
Klk15	s	ATG TGG CTT CTC CTT GCT TTC G	203
	as	ACT CTC ATG AAG CGG GTT TGG	
Kig1	s	CGA CTG CAA TGC TAA CGT GTA CA	221
	as	GGG TCC TTG TTC AGT TTC TGC A	
Kig2	s	CGA CTG CAA TGC TAA CGT GTA CA	270
	as	GGA CAT TGC TGG GAC TAC TGC TT	
B1 receptor	s	GCT TGG CCT CCT ATA CTC TCT GTC A	328
	as	CCA GCA CAA ACA CCA GAT CAG A	

Gene		Primer sequence (5′-3′)	Amplified length (bp)
B2 receptor	s	TTT CGA AGG ACA ACT GCC CA	264
	as	ACA ACA CCT CTC CAA ACA CCC A	
36B4	s	CGA CCT GGA AGT CCA ACT ACT TCC T	254
	as	GCA CCT TAT TGG CCA ACA GCA T	

Table 2. Thermal cycler schedule

Step		Temperature (℃)	Time
Hold	Hold	94	30 sec
3 step PCR (45 cycle)	Denaturation	95	1 min
	Annealing	59	30 sec
	Extension	72	1 min
Dissociation	Denaturation	95	15 sec
	Annealing	60	30 sec
	Extension	95	15 sec
Hold		4	5 min

RESULTS

Expression profiling of Klk, Kig and B2 receptor during periimplantation stages

Among the Klk 2, 3, 4, 5, 6, and 12 were detected during periods in uterus. Kig 1 and 2 expressed on day 4 and 5 of gastation. B2 receptor was detected on day 4 and 5 of gastation, during periimplantation periods (Table 3). In the embryos, Klk 5 and Kig 2 were expressed at 5.5 p.c.d stage. These was no expression of B1, 2 receptor (Table 4). The Klk which were expressed in the differentiating placenta was only Klk 4. Kig 2 and B2 receptor were detected (Table 5).

Table 3. Profiling the expression of Klk, Kig, B1, 2 receptor in uteri

Uterus	8-c	mor	bla	4.0d	4.125d	4.25d	4.5d	5.5d	8.5d	11.5d
KLK1							+++	+++		
KLK2					+++			+++	+++	+++
KLK3										
KLK4										+++
KLK5			++				+	++	+++	+++
KLK6								++		++
KLK7										
KLK8										
KLK9										
KLK10										
KLK11										
KLK12				+						
KLK13										
KLK14										
KLK15										
Kig1								+		
Kig2					+++		+++	++		
B1 receptor										
B2 receptor				+++	+++			++	+++	+++

Table 4. Profiling the expression of Klk, Kig, B1, 2 receptor in embryo

Embryo	8-c	mor	bla	4.0d	4.125d	4.25d	4.5d	5.5d	8.5d	11.5d
KLK1										
KLK2										
KLK3										
KLK4										
KLK5		++	++				++	++		
KLK6										
KLK7	+									
KLK8										
KLK9										
KLK10										
KLK11										
KLK12										
KLK13										
KLK14										
KLK15										
Kig1										
Kig2								+++		
B1 receptor										
B2 receptor										

Table 5. Profiling the expression of Klk, Kig, B1, 2 receptor in placenta.

Placenta	8.5d	11.5d
KLK1		
KLK2		
KLK3		
KLK4	+++	+++
KLK5		
KLK6		
KLK7		
KLK8		
KLK9		
KLK10		
KLK11		
KLK12		
KLK13		
KLK14		
KLK15		
Kig1		
Kig2	+++	+++
B1 receptor		
B2 receptor	+++	

Expression levels of Klk, Kig and B2 receptor were regulated by steroid hormones

Based on the screen results, the expression regulation of Kig, Klk and B2 receptor in uterus by steroid hormones were examined. As mentioned in materials and methods, ovx mice were administrated with estrogen and progesterone. Expression of Klk 1 and 2 was induced by progesterone. Their expression was suppressed by RU 486. Interestingly the expression of them was induced by suppression of E_2 receptor (Fig. 1A, B). In the case of Klk 4, 5, and 6 their expression was induced by estrogen. Their expression was completely decreased by ICI 182,780 (Fig. 1C, D, E). On the other hand, Klk 12 was reduced by treatment E_2 cotreatment with ICI 182,780 and E_2, and RU 486 and P_4. It means that Klk 12 expression regulation may be more complex than other Klk (Fig. 1F). Kig 1 and 2 were expressed by the administration of E_2 and P_4 (Fig. 2A). Interestingly Kig 1 expression was also induced by ICI 182,780 and RU 486 (Fig. 2A). On the other hand, Kig 2 expression was totally suppressed by ICI 182,780 and RU 486. It means that Kig 2 expression induced through E_2 - ER and P_4 - PR. But Kig 1 expression is under the control of a more complex network. B2 receptor expression was similar with Kig 1 (Fig. 3).

Expression levels of Klk, Kig and B2 receptor were controlled by steroid hormones during the implantation period

The expression patterns of Kig, Klk and B2 receptor were various by physiological status. So far, the delayed-implantation model was employed to evaluate the meanings of the expression in ovx mice. Klk 1 and 2 were expressed after 0.5 hr of estrogen administration and dramatically increased from 1.5 hr of estrogen administration. The expression of Klk 4, 5 and 6 were peaked of 6 hr post E_2 administration. The expression of Klk 12 was induced from 0.5 hr and the levels were continuously decreased. Kig 1 expression peaked at 0.5 hr, and Kig 2 expression peaked at 6 hr (Fig. 5). Expression of B2 receptor was peacked at 6 hr (Fig. 6). Such expression patterns of Klk, Kig and B2 receptor, means 6 hr at kallikrein-kinin system may involved in embryo implantation.

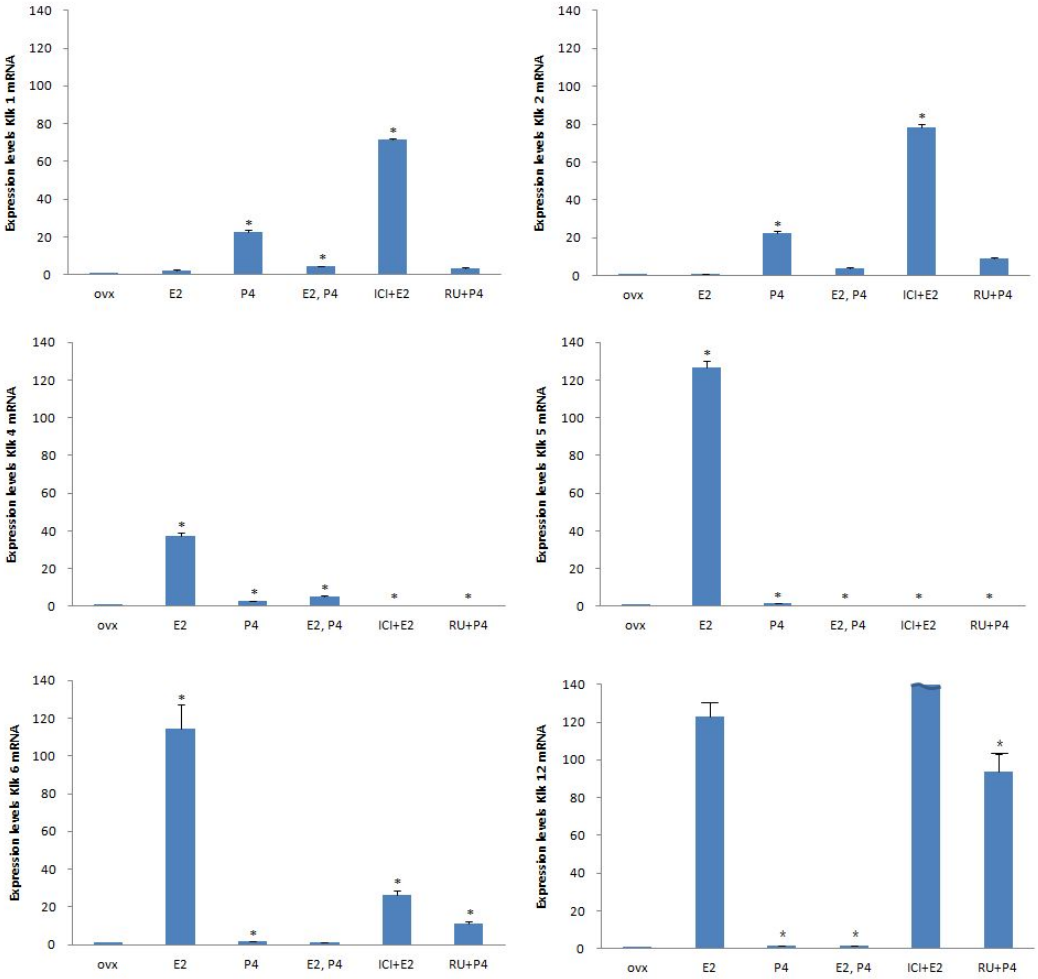

Figure 1. Steroid hormones regulate the expression of Klks in the uterus.

The differential gene expression screen was employed to identify the genes that regulated by steroid hormones in the uterus used ovx model. $P<0.5$

A, B : Klk 1, 2 were regulated by P_4 then E_2.

C, D, E, F : Klk 4, 5, 6, 12 were regulated by E_2 then P_4.

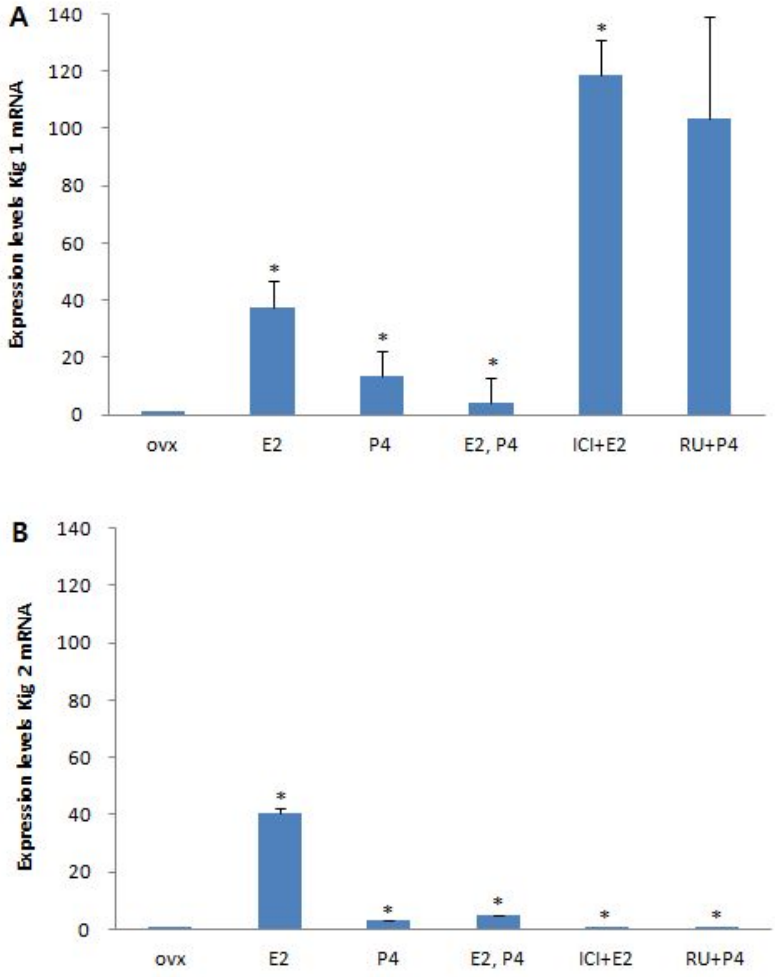

Figure 2. Steroid hormones regulate the expression of kig 1, 2 in the uterus.

The differential gene expression screen was employed to identify the genes that regulated by steroid hormones in the uterus used ovx model. $P<0.5$

A, B : Kig 1, 2 were regulated by E_2 then P_4.

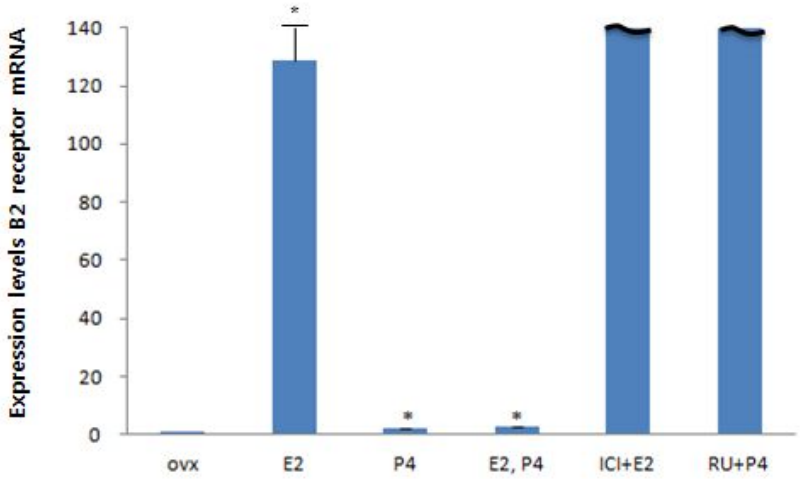

Figure 3. Steroid hormones regulate the expression of B2 receptor in the uterus.

The differential gene expression screen was employed to identify the genes that regulated by steroid hormones in the uterus used ovx model. B2 receptor was regulated E_2 then P_4. $P<0.5$

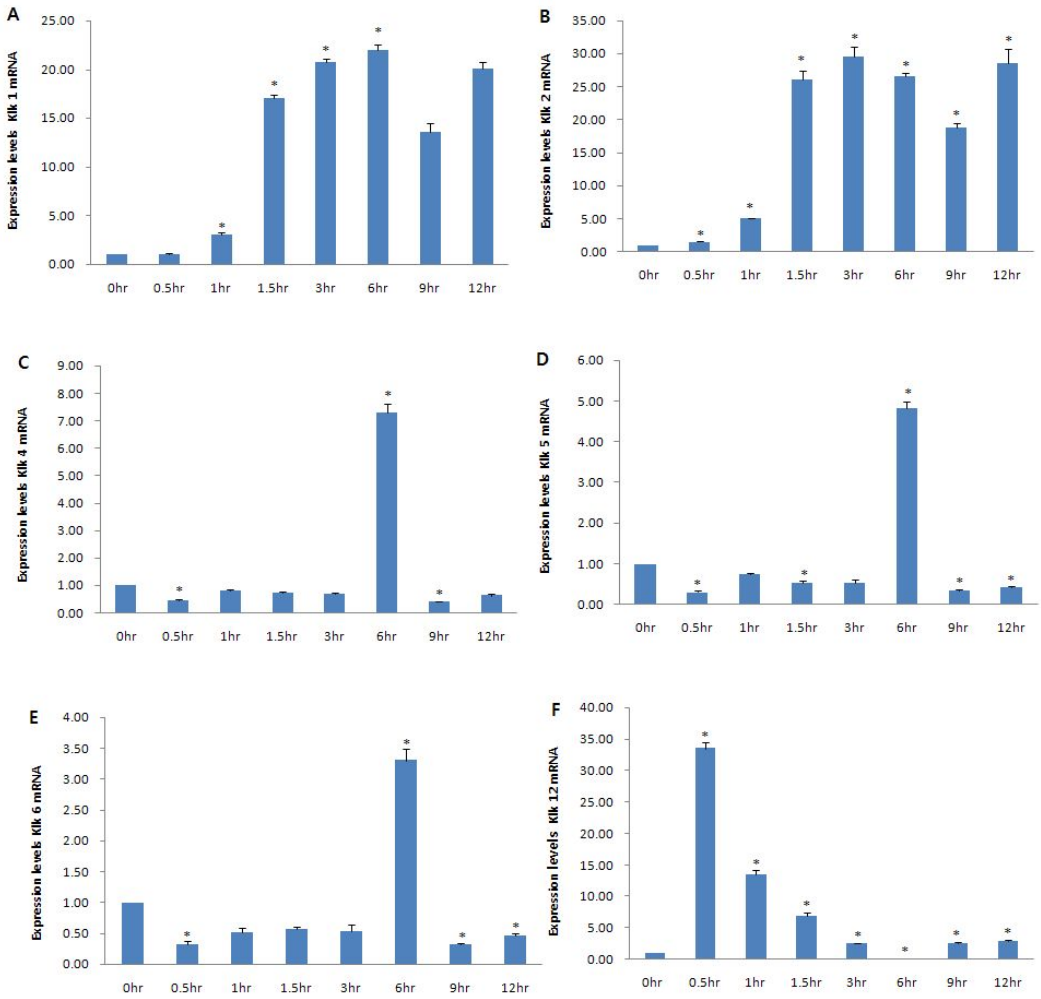

Figure 4. Steroid hormonal regulation of Klks expression in the uterus of the delayed inplantation mice.

A, B : Klk 1 and 2 were expressed in all stage. But C, D, E : Klk 4, 5, 6 was peaked at 6 hr after E_2 injected. On the other, F : Klk 12 was highly expressed in 0.5 hr but low levels in 6 hr after E_2 injected. $P<0.5$

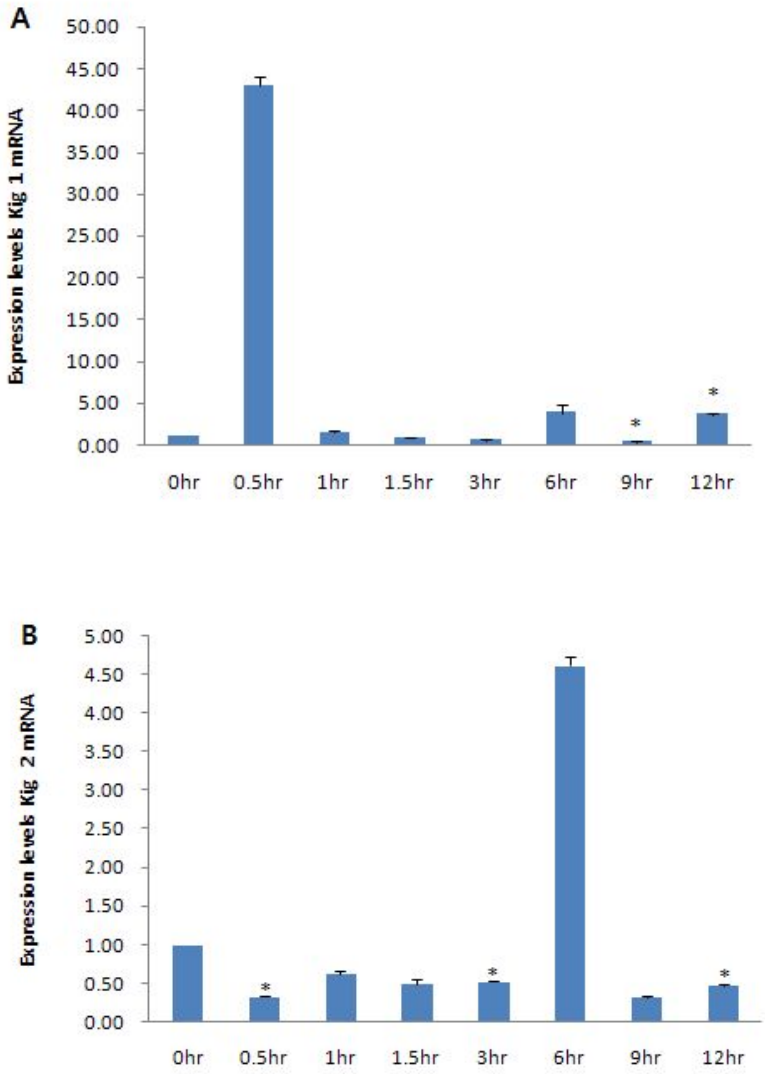

Figure 5. Steroid hormonal regulation of Kig 1, 2 expression in the uterus of the delayed implantation mice.

A: Kig 1 was peaked at 0.5 hr after E_2 injected but B: Kig 2 was peaked at 6 hr after E_2 injected. $P<0.5$

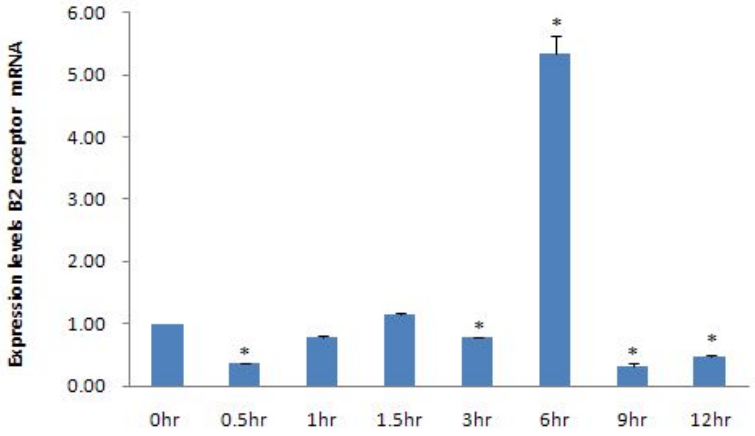

Figure 6. Steroid hormonal regulation of B2 receptor expression in the uterus of the delayed implantation mice.

B2 receptor was peaked at 6 hr after E_2 injected. $P<0.5$

Localization of B2 receptor in natural pregnancy day 3.5, 4.5 and 5.5.

At embryonic stage 3.5, B2 receptor specific signal was not detected in the uterus near the embryo (Fig. 7A). B2 receptor specific signal was detected in the stromal layer near the embryo at 4.5 p.c.d embryo stage (Fig. 7B). During invasion B2 receptor strongly localized at ectoplacental corn area (Fig. 7C).

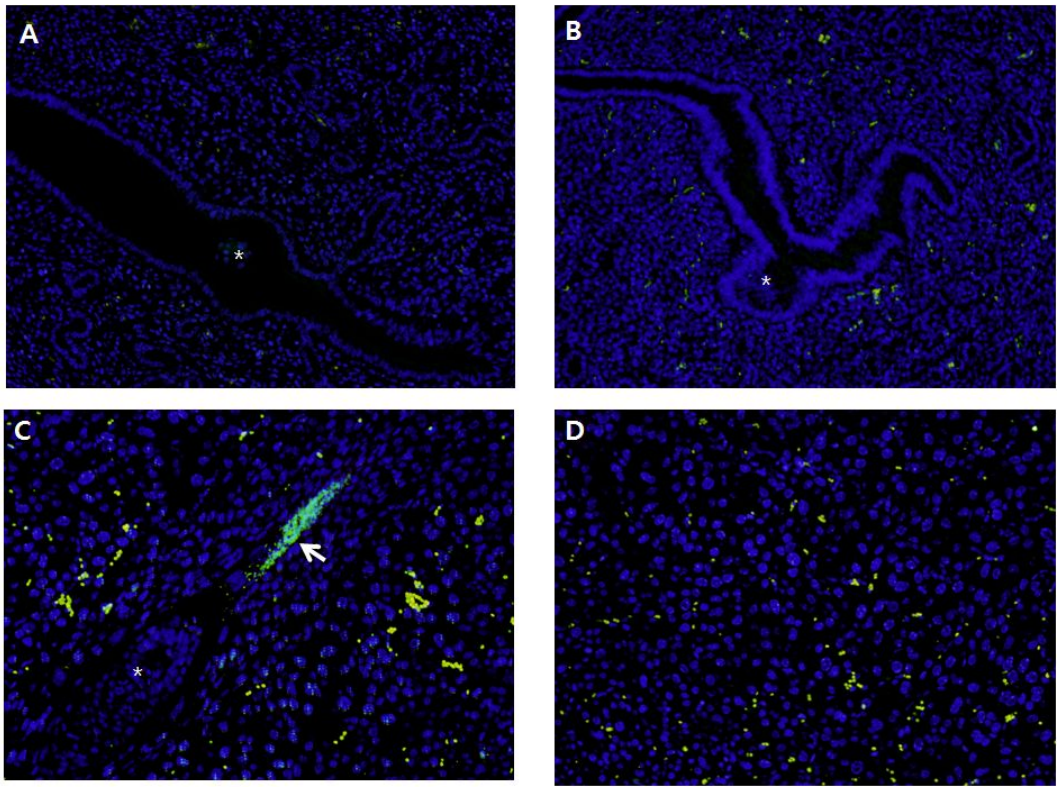

Figure 7. Localization of B2 receptor in the implanting site on day 3.5, 4.5, and 5.5 (x200).

Uterus were collected of natural pregnant uterus 3.5 p.c.d, 4.5 p.c.d, 5.5 p.c.d . A : 3.5 p.c.d uterus, B : 4.5 p.c.d, C : 5.5 p.c.d. D : Rabbit-IgG. Nucleus was stained with DAP1 (blue). Star indicates embryo.

Change the distribution of blood vessels by B2 receptor at the implanting sites

Endothelial cell specific CD31 anti body and FITC-dextran were employed the identify the blood vessels. Because CD31 expressed in decidualized cells edema occures in the implant area. CD31 was localized on the near the implantation sites 4.5 p.c.d and 5.5 p.c.d. The number of colocalized sites were not reduced by B1 receptor antagonist (Fig. 8D, E) of agonist (Fig. 8K). However, its nuclear was decreased by B2 receptor antagonist in concentration dependent manner. In the case of cotreatment of BK with B1 receptor antagonist and B2 receptor antagonist, the number of colocalized sites was decreased (Fig. 8F, I).

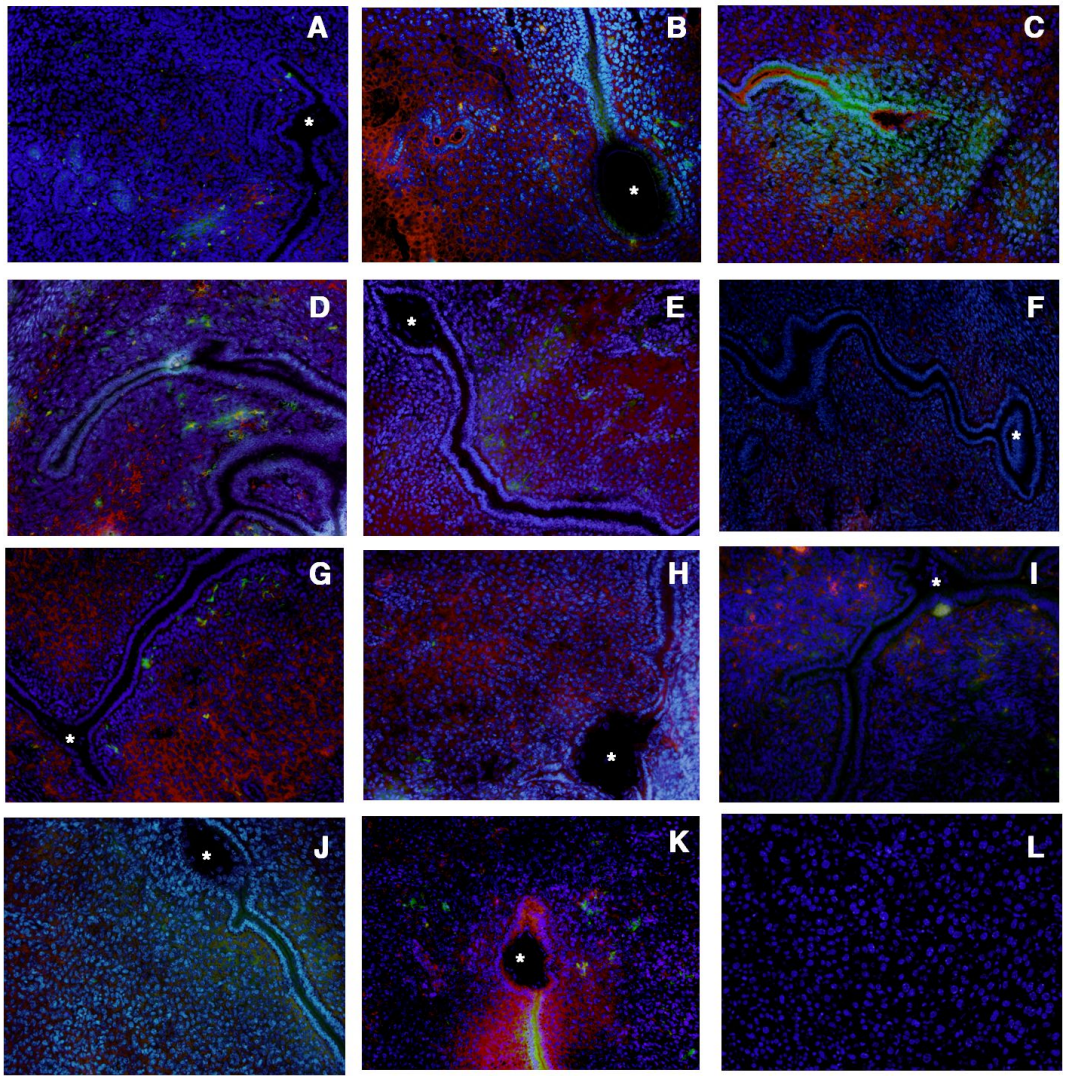

Figure 8. Change the distribution of blood vessels by B1 receptor and B2 receptor at the implanting sites (x200). A: 3.5 p.c.d, B: 4.5 p.c.d, C: 5.5 p.c.d, D: B1 receptor antagonist 100ug, E: B1 receptor antagonist 500ug, F: B1+B2 receptor antagonist 100ug, G: B2 receptor antagonist 100ug H: B2 receptor antagonist 500ug, I: B1+B2 receptor antagonist 500ug, J: B1+B2 receptor+BK (300μg+300μg+500μg), K: BK (500μg), L: Goat-IgG. Star indicates embryo.

Discussion

Implanting embryo encounter hypoxic environment during implantation. To survive during implantation, rearrangement of blood vessels is needed. Histrionically, the implanting area can be easily detected after adhesion of blastocyst to uterine endometrium by increased blood supply and intense arrange of blood vessels. Although such a rearrangement of blood vessel is important for successful embryo development, the mechanisms for control of it is not much uncovered.

In hypoxia condition, trophoblast express endothelial nitric oxide synthase (ecNOS) mRNA (Seligman, 1997). It induce trophoblast differentiation and expression of hypoxia-induced transcriptional and post-transcriptioanl regulation of angionetic factors, such as vascular endothelial growth factor and placental gorwth factor (Charnock-Jones and Burton, 2000). Using DNA microarray analysis, the clue for the angiogenesis and vasodilation can explored, the expression of Kig is expected at the time of implantation in the uterus (Cheon et al., 2002). In this study, the expression profiles of the factors of kallikrein-kinin system was screened with the nest RT-PCR during periimplantation stages. Klk 1, 2, 4, 5, 6 and 12 expressed at the time of implantation. Kig 1 and 2, substrates of Klk, expressed at the time of implantation. Besides, B2 receptor also expressed at the time of implantation. On the other hand, in the embryos of periimplantaton stages, Klk are detected but not B1 receptor and B2 receptor. As expected all the factors of kallikrein-kinin system were detected at placenta. It means that the elements of kallikrein-kinin system only expressed at uterus during periimplantation stages.

Pregnancy is controled and maintained mainly by sex steroid hormones. Therefore it is important to know whether these factors are under the control of sex steroid hormones. Expression of Klk, Kig and B2 receptors were analyzed in the uterus of ovariectomized mice. Expressions of Klk 4 and 5 were under the control of estrogen and its receptor. The expression of Klk 1, 2 and Kig 2 was induced by progesterone and ICI 182,780. In the case of Klk 12, its expression was induced by estrogen and by ICI 182,780 and RU 486. Such a curious regulation pattern was also observed in Kig 1 and B2 receptor. In delayed-implantation model mice, the expression of Klk 1 and 2 expressed from 0.5 hr after estrogen administration and dramatically increased from 1.5 hr and stayed the levels. Klk 4, 5 and 6 peaked at 6 hr post estrogen administration. In the case of Klk 12, its expression was dramatically increased at 0.5 hr and decreased continuously after then. Kig 1 and 2 peaked at 0.5 hr and 6 hr, respectively. B2 receptor peaked at 6 hr after implantation induction. These showed the complexity of the expression control of kallikrein-kinin system in the pregnant uterus by their physiological status. Based on these results, it is suspected that kallikrein-kinin system may one of the key factor in implantation.

The evidences regarding the angiogenetic potency of kallikrein-kinin system is accumulated and the roles of B1,2 receptors in angiogenesis is suggested (Moreau et al., 2005). In reproductive tissues, the expression of Klk and its receptors were explored especially in the area of the blood flow regulation and the development of new vessels (Valdés and Corthorn, 2011). At the time of implantation, maternal vessel increase in number and diameter in order to supply the fetal growing (Matsumoto

et al., 2002). During decidualization, angiogenesis such as the development of new capillaries from preexisting vessels is induced and controlled tightly with the embryo development and decidualization (Sharkery et al., 2003). One of the characters of angiogenesis at the implantation site is the localized uterine vascular permeability along with the development of maternal vessels (Matsumoto et al., 2002). As seen in the results, the blood vessels were identified with CD31 and FITC-dextran because CD31 expressed in the decidualized cell and FITC-dextran translocalied into the extracellular fluid. The number of blood vessel was increased near the implanting embryos. It showed that the possibility of the role of B1, 2 receptor in angiogenesis at the time of implantation.

Previously it was suggested that nitric oxide may play an improtant role in embryo implantation. Because, inducible nitric oxide synthase (iNOS) and enodthelial nitirc oxide synthase (eNOS) is strongly localized in implantation sites compared to other sites from day 6 of gestation. iNOS labelled cells are localized within the decidua, around myometrial vessels, and within the ectoplacental cone. eNOS was localized in vessels of the primary decidual zone adjacent to the embryo. Neuronal nitric oxide synthase (nNOS) is localized mainly in the mesomtrium and myometrium and do not appear to change through the peri-oimplantation period (Purcell, 1999).

Interstingly, the numbers of blood vessel specific sites were decreased by the B2 receptor antagonist. On the other hand, the agonist BK increased the intensity of FITC-dextran in the implantation site. From this study, it is revealed that the factors for kallikrein-kinin

system expressed at the stages of periimplantation and stayed under the control of sex steroid hormones. It is suggested that the role of kallikrein-kinin system at the time of implantation may the inducer of angiogenesis and vasodilation.

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ABSTRACT

Physiological role of kallikrein-kinin system in Implantation

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Increased capillary permeability at the site of blastocyst attachment has been shown to be a requisite for implantation in all species. We suggest that B2 receptor which is known to increase blood flow and affect capillary permeability at higher concentrations, is an important embryonic factor involved in the initiation of the implantation process.

A differential gene expression screen was employed to identify the genes that regulated by steroid hormones in the uterus during pregnancy. We isolated mRNAs from uteri of ovx-model (E2, P4, and E2 after P4 injection) and delayed implantation model (pregnant day 4 ; ovx and E2 injection) mice. Expressions of Klk 4 and 5 were under the control of estrogen and its receptor. The expression of Klk 1, 2, and Kig 2 was induced by progesterone and ICI 182,780. In the case of Klk 12, its expression was induced by estrogen and by ICI 182,780 and RU 486. Such a curious regulation pattern was also observed in Kig 1 and B2

receptor. In delayed-implantation model mice, the expression of Klk 1 and 2 expressed from 0.5 hr after estrogen administration and dramatically increased from 1.5 hr and stayed the levels. Klk 4, 5 and 6 peaked at 6 hr post estrogen administration. In the case of Klk 12, its expression was dramatically increased at 0.5 hr and decreased continuously after then. Kig 1 and 2 peaked at 0.5 hr and 6 hr, respectively. B2 receptor peaked at 6 hr after implantation induction.

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