IGF-Ⅱ and IGFBP-1 reversely regulate blastocyst implantation in mouse

TIAN Yongqiang[1,2], TIE Guodong[1], CAO Yujing[1], ZHAO Xingxu[2] & DUAN Enkui[1]

1. State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China;
2. College of Vet Medicine, Gansu Agricultural University, Lanzhou 730070, China

Correspondence should be addressed to Duan Enkui (e-mail: duane@panda.ioz.zc.cn)

Abstract **Insulin-like growth factor (IGF)-Ⅱ and IGF binding protein (IGFBP)-1, members of IGF family, are important in the cyclic development of endometrium and the blastocyst implantation. In the present study, the indirect immunofluorescence showed that IGF-Ⅱ and IGFBP-1 were specifically expressed at the maternal-fetal interface. In a co-culture system, IGF-Ⅱ significantly enhanced the attachment and outgrowth of the blastocyst on monolayer of uterine epithelial cells, while IGFBP-1 did not affect the blastocyst attachment, but markedly inhibited the blastocyst outgrowth. The results of zymography showed that IGF-Ⅱ enhanced the activities of MMP-2 and MMP-9, while IGFBP-1 did not affect the activities of MMP-2 and MMP-9. In conclusion, the equilibrium between the invasion of trophoblast and the inhibition of deciduas may be regulated by the interaction between the IGF-Ⅱ-expressing invading cytotrophoblast and maternal deciduas-derived IGFBP-1.**

Keywords: **IGF-Ⅱ, IGFBP-1, implantation, MMP-2, MMP-9.**

The insulin-like growth factor family is composed of IGF-Ⅰ, IGF-Ⅱ, the cellular IGF receptors (type Ⅰ, type Ⅱ), and the soluble high affinity IGF binding proteins (IGFBP-1 to IGFBP-6). IGFs participate in the regulation of cellular growth, differentiation and function, by virtue of their varied biological actions that include mitogenic, differentiating, metabolic, and anti-apoptotic effects. IGFBPs bind IGFs with equal or higher affinity than the cellular IGF receptors and, mostly, limit the bioavailability of IGF peptides to the cellular receptors in target tissues.

While IGF-Ⅰ and IGF-Ⅱ are both expressed in early pregnancy, the latter is the main peptide expressed at the maternal-fetal interface in human. However, the decidual stromal cells do not express IGF-Ⅱ mRNA in the first trimester of intrauterine pregnancies, but the trophoblast synthesizes at a high level. Interestingly, the high expression of IGF-Ⅱ mRNA corresponds to the columns of the invading intermediate trophoblast, where there is a gradient of IGF-Ⅱ mRNA abundance, with great levels expressed at the invading front[1]. Meanwhile, IGFBP-1 mRNAs are expressed at the maternal-fetal interface primarily by the decidual tissue, with the trophoblast showing no expression. IGFBP-1 is also by far the most abundantly expressed IGFBPs in late secretory endometrium and continues to be expressed in pregnancy deciduas at much higher concentrations than any of the other IGFBPs[2]. Thus, the distinct spatial pattern of IGF-Ⅱ and IGFBP-1 at the maternal-fetal interface is suggestive of paracrine interactions between the IGF-Ⅱ-expressing invading cytotrophoblast and maternal decidua-derived IGFBP-1. In addition, IGFBP-1 has an RGD sequence similar to that of integrin. IGFBP-1 has been shown to inhibit the invasion of trophoblast cells via its RGD sequence interaction with $\alpha5\beta1$ integrin[3]. Clinical observations showed the higher concentrations of immunoreactive IGFBP-1 at the decidua-placental interface in the case of severe pre-eclampsia, compared with normal pregnancies, which suggests a potential contribution of local IGFBP-1 to shallow placental invasion in pre-eclampsia[4].

The present study was aimed at investigating the impact of IGF-Ⅱ and IGFBP-1 on the adhesion and outgrowth of blastocyst, and to explore their mechanism during the implantation in mouse.

1 Materials and methods

(ⅰ) Reagents. IGF-Ⅱ and IGFBP-1, FITC-conjugated anti-rabbit secondary antibody from the goat, paraformaldehyde (PFA), bovine serum albumin (BSA) and propidium iodide (PI) were purchased from Sigma. Polyclonal goat antibodies against IGF-Ⅱ and IGFBP-1 were from Santa Cruz. Hams-F12 medium, fetal calf serum (FCS) and trypsin were from Gibco BRL.

(ⅱ) Animals. Adult mice of Kunming white strain (5—6 weeks old, 25—30 g) were purchased from the Experimental Animal Center of the Institute of Heredity Science, Chinese Academy of Sciences. The animals were raised at room temperature (25℃) and with a constant photoperiod (light∶dark cycle, 12L∶12D). Food and water were provided *ad libitum*.

(ⅲ) Indirect immunofluorescence and confocal microscopy. Mice uterus were collected on 6, 7 and 8 d post coitum and made into frozen sections. The frozen sections were fixed for 30—60 min at 4℃ with 4% paraformaldehyde containing 0.2% Triton X-100 and, then, washed three times with PBS. Nonspecific protein binding was blocked with 10% BSA in PBS for 1 h at room temperature in a humidified chamber. The sections were then incubated with properly diluted antibodies against IGF or IGFBP-1 at 4℃ overnight. Subsequently, the sections were incubated with secondary antibody conjugated with FITC at 1∶100 dilutions for 1 h at 37℃. Nuclei were stained with PI at 1∶100 dilution in PBS for 10 s. Finally, the sections were rinsed in PBS and viewed under a confocal laser scanning microscope.

(ⅳ) Co-culture. The co-culture protocol was referred to Zeng et al.[5]. Briefly, mouse uterine epithelial cells were obtained from pregnancy D4 mouse, and cultured in the F12 media with 10% FCS. After uterine epithelial cells attached and formed monolayer onto the plate (24 h), the blastocysts were transferred in wells (about 40 blastocysts each well). The same experiment was repeated at least three times. The medium in control group (C) was F12-medium complemented with 0.4% BSA, 2.2 mmol/L calcium lactate, 2.05 mmol/L glutamine, 12.5 mmol/L NaHCO3 and 400 IU/mL gentamycin sulfate. The media in the treatment group were F12-medium containing IGF-Ⅱ or IGFBP-1 at concentrations of 2, 20 and 200 ng/mL. After 12, 24, 36 and 48 h co-culture, the attachment and outgrowth of blastocysts was observed and defined under a phase-contrast microscope (Olympus) according to the following criteria. The plate was shaked gently and briefly, and observed. If the blastocyst was at the same place, it was designated as "attachment". If primary giant trophoblast cells were visible around the attachment site, the blastocyst was designated "outgrowth".

(ⅴ) Zymography of gelatin-degrading MMPs. MMP activity in conditioned media was determined by gelatin degrading zymography. Briefly, 50 μL samples were incubated in SDS sample buffer without β-mercaptoethanol and electrophoresed on 10% polyacrylamide gels co-polymerised with 1 mg/mL gelatin. Gels were then washed in 2.5% Triton X-100 and incubated for 24 h at 37℃ in 50 mmol/L Tris-HCl containing 10 mmol/L CaCl2 (pH 7.8). Gelatinolytic activities were visualized as clear bands after staining with 0.5% Coomassie Blue R-250 and destaining.

(ⅵ) Statistical analysis. All the results are shown as mean ± SE. Data were analyzed by *t*-test, and a value of $P < 0.05$ was considered to be significant.

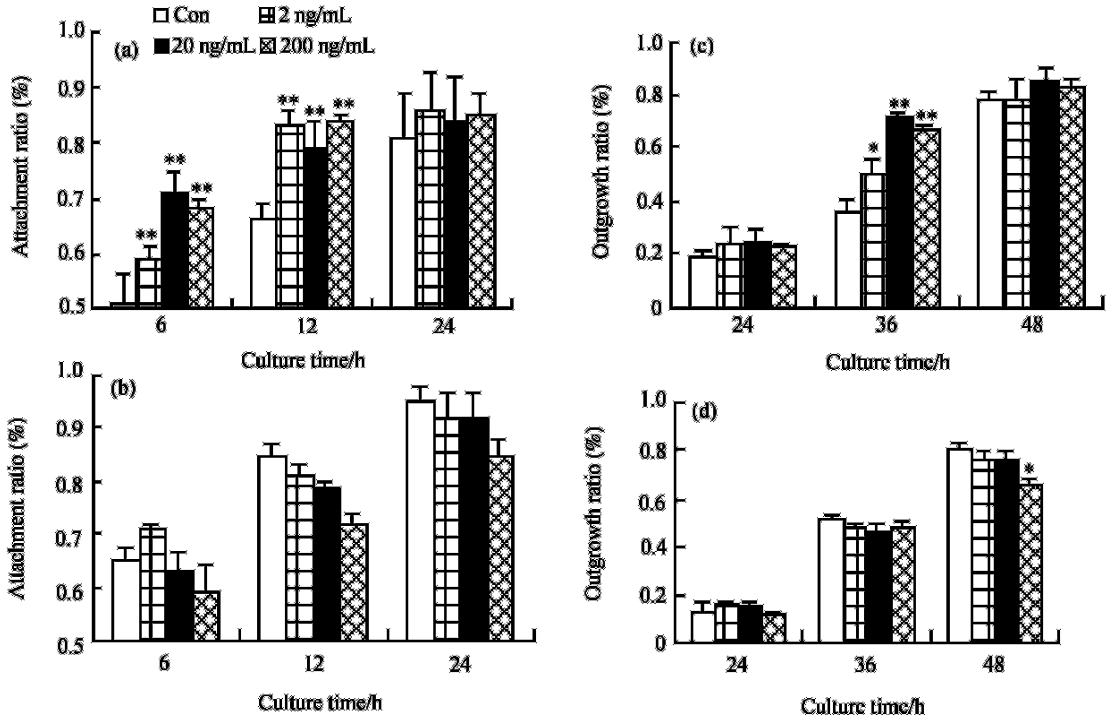

Fig. 1. Effect of IGF-Ⅱ and IGFBP-1 on blastocyst adhesion and outgrowth. (a) and (b) represent the effect of IGF-Ⅱ and IGFBP-1 on blastocyst adhesion respectively. (c) and (d) represent the effect of IGF-Ⅱ and IGFBP-1 on blastocyst adhesion respectively. * and ** show significant differentation (* $P < 0.05$, ** $P < 0.01$).

2 Results

(ⅰ) Expression of IGF-Ⅱ and IGFBP-1 proteins. In the present study, we used a double-staining technique. The nucleus was stained by PI, which showed red under the confocal microscope. The FITC-conjugated secondary antibody bound specifically with the primary antibody (IGF-Ⅱ and IGFBP-1) showed green, which manifested the location of the detected antigen. As shown in Plate Ⅰ, IGF-Ⅱ and IGFBP-1 are specifically concentrated in the maternal-fetal interface on D6 and D8, and the expressions keep consistent.

(ⅱ) Effect of IGF-Ⅱ and IGFBP-1 on blastocyst

adhesion and outgrowth. As shown in fig. 1, at 6 h co-culture, the attachment ratios in treatment groups were significantly increased by addition of IGF-Ⅱ with different concentrations ($P < 0.05$); at 12 h, these were increased most significantly ($P < 0.01$). However, IGFBP-1 had no obvious effect on blastocyst attachment. After 36 h of co-culture, IGF-Ⅱ with different concentrations promoted the blastocyst outgrowth ($P < 0.05$), among which, 20 and 200 ng/mL IGF-Ⅱ had great effect on blastocyst outgrowth ($P < 0.01$); whereas IGFBP-1 had an inhibitory effect on blastocyst outgrowth, and 200 ng/mL IGFBP-1 had an obvious effect ($P < 0.05$).

(iii) IGF-Ⅱ/IGFBP-1 effect on MMP activity. Zymographic analysis of gelatin-degrading activities in media from blastocysts was shown in fig. 2. After 6 h blastocyst culture on fibronectin, MMP-2 and MMP-9 activities were significantly improved by IGF-Ⅱ of different concentrations ($P < 0.05$). After 12 h culture, all different concentrations of IGF-Ⅱ most significantly improved MMP-2 and MMP-9 activities ($P < 0.01$). After 24 h culture, IGF-Ⅱ had no influence on MMPs activities. Moreover, MMPs activities showed no significant changes by treatment with IGFBP-1 (fig. 3).

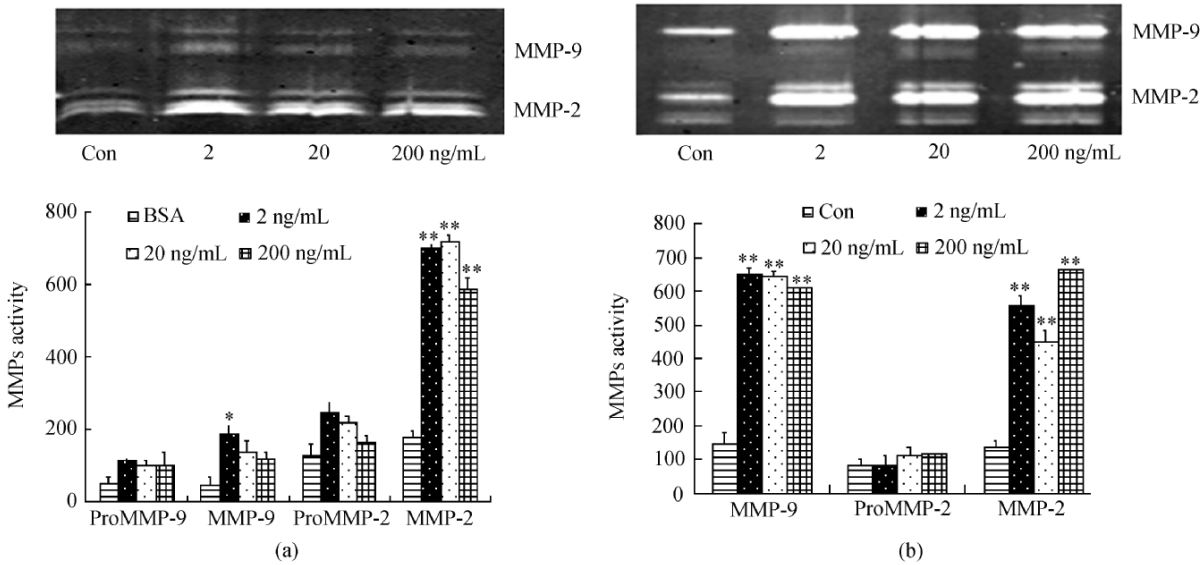

Fig. 2. (a) Effect of IGF-Ⅱ on MMPs activity after 6 h culture; (b) effect of IGF-Ⅱ on MMPs activity after 12 h culture. The upper are the figures of Zymographic analysis; the lower are the figures of quantitative analysis. * and ** show significant differentation, * $P < 0.05$, ** $P < 0.01$.

3 Discussion

In a number of mammalian species including human, the blastocyst must cross the uterine epithelium and invade the underlying endometrial stroma, glands, and blood vessels in order to establish proper fetomaternal exchange of molecules. This process requires that specific trophoblast cells exhibit an invasive phenotype. During further development of the placenta in human, cytotrophoblast stem cells in the chorionic villi give rise to a subset of highly proliferative, migratory, and invasive population that break out of the villi and invade the deciduas and the uteroplacental arteries. The optimal extent of trophoblast invasion of uterus was believed to result from multilevel factors, including steroid hormones, cytokines, growth factors, adhesive molecules, and proteinases. Especially, some paracrine and autocrine factors at the fetomaternal interface play a key role in influencing trophoblast proliferation, migration and invasion. For example, transforming growth factor (TGF) β reduces proliferation, migration and invasion of extravillious trophoblast cells propagated *in vitro*[6], whereas leukaemia inhibitor factor (LIF), interleukin-1, and transforming growth factor (TGF) α enhance adhesion and outgrowth of mouse blastocysts, and in addition, promote proliferation and migration of trophoblast cells[7].

Both IGF-Ⅰ and IGF-Ⅱ may promote cell proliferation and migration. During blastocyst implantation, IGF-Ⅰ is primarily expressed during the proliferative phase, whereas IGF-Ⅱ predominates in secretory endometrium, thus, IGF-Ⅱ may have a more important role in blastocyst implantation. In the present study, results from historical sections of uterus showed that IGF-Ⅱ and IGFBP-1 proteins are expressed in early pregnancy in mice, particularly at the fetomaternal inteface. Han et al.[1] have shown that IGF-Ⅱ mRNA expression is most abundant in trophoblastic columns of the anchoring villi, par-

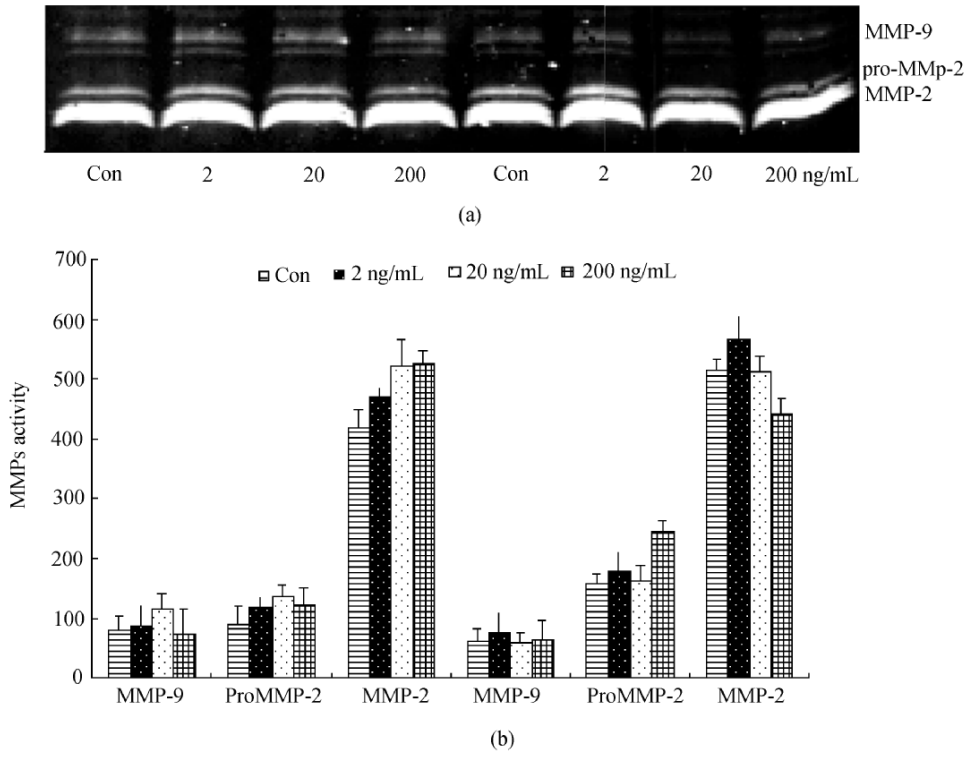

Fig. 3. Effect of IGFBP-1 on MMPs activity after 12 and 24 h culture. (a) The figure of zymographic analysis; (b) the figure of quantitative analysis. There is no significant difference between treatment and contral groups.

ticularly in cells at the leading edge of the column, suggesting that IGF-Ⅱ may play a role in trophoblast invasion of the endometrium. Furthermore, IGFBP-1, an important IGF binding protein in pregnancy, is expressed at the fetomaternal interface primarily by the decidual tissue. Thus, the distinct spatial pattern of expression of IGF-Ⅱ and IGFBP-1 at the fetomaternal interface is suggestive of paracrine interactions between the IGF-Ⅱ-expressing invading cytotrophoblast and maternal deciduas-deriving IGFBP-1. This idea is proved by our results that IGF-Ⅱ enhances the adhesion and outgrowth of mouse blastocysts, whereas IGFBP-1 inhibits outgrowth of mouse blastocysts without significantly affecting the adhesion. It is well known that adhesive molecules play an important role in blastocyst adhesion, and that integrin is one of the important molecules in adhesion onto endometrium[8]. Samani et al.[9] have shown that IGFs induce the expression of integrins αvβ3, αvβ5, α3β1, α5β1 and α6β1, suggesting that IGF-Ⅱ may promote blastocyst adhesion by inducing the expression of integrins. IGF-Ⅱ promoting blastocyst outgrowth may be contributed to two factors. One is to enhance the proliferation and migration of trophoblast cells; the other is to improve the expression and activity of matrix metalloproteinase. The later speculation is accord with our following results. In addition, the results from IGFBP-1 supports the study of Irvin et al.[4] that IGFBP-1 has an inhibitory effect on the invasion of trophoblast cells by interaction with integrin α5β1 located on the surface of trophoblast cells.

Trophoblast invasion plays an important role in blastocyst implantation, and MMPs that degrade the constituents of the extracellular matrix is the main dynamic of trophoblast invasion. MMP-2 and MMP-9 are thought to be the speed-limited enzymes of the proteolysis of extracellular matrix. We examined the effect of IGF-Ⅱ on MMP-2 and MMP-9 by the blastocyst culture *in vitro* and zymography, and the results showed that IGF-Ⅱ significantly enhanced the activities of MMP-2 and MMP-9, which coincides with the results from human umbilical vein endothelial cells that the activity of MMPs was increased by IGF-Ⅱ[10]. Similarly, we have indicated that LIF and EGF increased the expressions and activities of MMP-2 and MMP-9. Therefore, at the course of blastocyst invasion IGF-Ⅱ promote trophoblast invasion not only by enhancing the proliferation and migration of trophoblast cells but also by increasing the secretion of MMPs to degrade extracelluar matrix[11,12]. It is well known that IGFBP-1 is one of the important regulators of

IGF-Ⅱ in many tissues, particularly at the fetomaternal interface. In the present study IGFBP-1 did not have significant influence on the activity of MMPs. Bischof et al.[13] have obtained the same conclusion in human trophoblast cells *in vitro*. So IGFBP-1 regulates the activity of MMPs by regulation of IGF-Ⅱ. Furthermore, Manes et al.[14] have shown that MMP-11 (stromelysin) can cleave IGFBP-1 *in vitro*. This enzyme is present at the maternal-fetal interface[15]. However, this enzyme functions only to the form of non-phosporylation IGFBP-1[14], whereas IGF-Ⅱ is the unique factor to induce the phosphorylation IGFBP-1[16].

In summary we have demonstrated that the specific expressions of IGF-Ⅱ and IGFBP-1 at the maternal-fetal interface play an important role in blastocyst implantation. IGF-Ⅱ is mainly expressed in the trophoblast cells and functions at the maternal-fetal interface with paracrine fashion. IGF-Ⅱ stimulates the expression of adhesive molecules for increasing the adhesion of blastocyst in endometrium, and enhances the expression and activity of metalloproteinase to degrade extracellular matrix for promoting trophoblast invasion and outgrowth, which ensure successful implantation of blastocyst. Whereas IGFBP-1, as a regulative protein of IGF-Ⅱ, is highly expressed in the decidual tissue and may function by the interaction with IGF-Ⅱ at the maternal-fetal interface to inhibit trophoblast invasion. Hence, the equilibrium between the invasion of trophoblast and the inhibition of deciduas may be regulated by the interaction between the IGF-Ⅱ-expressing invading cytotrophoblast and maternal deciduas-derived IGFBP-1.

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