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Expression of IGF-1 and Its Receptor Genes in the Oocytes and Preimplantation Embryos in Mouse

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생쥐 난자와 착상전 초기배아에서 IGF-1과 IGF-1 수용체 유전자 발현

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ABSTRACT : Insulin-like growth factors (IGF-1 and IGF-2) play an important regulatory role in preimplantation embryonic development. To study the role of IGF-1 during preimplantation embryonic development in mouse, the presence of mRNA transcripts for IGF-1 and IGF-1R in the oocytes and preimplantation embryos was examined. In this study, the transcripts of IGF-1 was detected in oocytes using primers for IGF-1. The PCR products were identified by Msp I restriction enzyme digest. We revealed that the transcripts of IGF-1 and IGF-1R were presented in the oocytes and preimplantation embryos. The highest mRNA levels in GV stage oocytes were decreased at 4- or 8-cell stage and then reincreased upto blastocyst. The presence of IGF-1 and IGF-1R in GV-oocytes suggests that the transcripts in the early stage embryos were derived from maternal genome. Additionally, the presence of IGF-1 and IGF-1R in the oocytes and preimplantation embryos suggests that IGF-1 plays an autocrine role during preimplantation embryonic development through IGF-1R as a signalling pathway.

Key words : Preimplantation mouse embryo, Insulin-like growth factor-1 (IGF-1), Insulin-like growth factor type-1 receptor (IGF-1R), RT-PCR.

요 약 : 인슐린 유사 성장 호르몬 1과 2 (IGF-1 & IGF-2)는 착상 전 초기배아 발생을 조절하는 중요한 요소이다. 생쥐 착상 전 초기배아에서 IGF-1의 역할에 관한 연구를 위해, IGF-1과 IGF-1 수용체의 전사물의 존재 여부를 난자와 착상 전 초기배아에서 조사하였다. 새로이 고안된 IGF-1 primer를 이용하여 난자에서 전사물을 검출하였다. 그리고, PCR 산물을 제한효소인 Msp I으로 절단하여 확인하였다. 이 실험에서 IGF-1과 IGF-1 수용체의 전사물이 난자와 착상 전 초기배아에서 모두 검출됨을 보였다. GV-난자에 다량 존재하는 mRNA는 4- 혹은 8-세포기까지 지속적으로 감소하다가 이후에 다시 증가하는 양상을 보였다. GV-난자에서 IGF-1과 IGF-1R 전사물이 존재한다는 것은 초기배아에 존재하는 전사물이 모계유래 산물임을 암시한다. 또한, 난자와 착상 전 초기배아에 IGF-1과 IGF-1 수용체 전사물이 존재하는 것으로 보아 착상 전 초기배아에서 IGF-1은 자가 분비되어 IGF-1 수용체의 신호전달 경로를 통하여 배아 발생에 작용하는 것으로 사료된다.

INTRODUCTION

Genes encoding polypeptide growth fators, and specifically insulin-like growth factors (IGFs), are known to be important in mammalian embryogenesis (Heyner et al., 1989a, 1993; Rappolee et al., 1992; Adamson, 1993; Schultz et al., 1993). IGF family are small mitogenic polypeptides with structural and functional homologies. IGF family consist of several ligands (insulin, IGF-1, and IGF-2), their receptors (insulin receptor, IGF-1R, and IGF-2R) and six IGF binding proteins (IGF-BP1 to IGF-BP6). The developmental importance of IGF family have demonstrated by gene mutation experiments in mice. Knockouts of IGF-1, IGF-2, insulin receptor, and IGF-1R all result in signifi-

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cantly reduced birthweight (40-60%) and are associated to neonatal lethality (Baker et al., 1993; Liu et al., 1993; Accili et al., 1996; Ludwig et al., 1996).

The transcripts for IGF-1 are not detected in the preimplantation embryos (Rappolee et al., 1992), but the transcripts of IGF-1R are detected first at eight-cell stage (Heyner et al., 1989; Rappolee et al., 1992; Schultz et al., 1992; Latham et al., 1994). In contrast, the transcripts for IGF-2 R and the ligand of IGF-2 are detected by RT-PCR at two-cell stage when the mouse zygotic genome is firstly transcribed (Rappolee et al., 1990; Schultz et al., 1990; Harvey & Kaye, 1991). Although it is known that the IGFs are supposed to be involved in several functions of preimplantation mouse embryogenesis, the specific functions and mechanisms of these growth factors in the oocytes and preimplantation embryos are still unclear. To examine the role of IGF-1 and IGF-1R on embryoinic development, in this study, the transcripts of IGF-1 and its cognate receptor in the oocytes and preimplantation embryos were investigated by RT-PCR. And also the origin of those transcripts in the early mouse embryos was investigated by the treatment of α-amanitin as a RNA polymerase Ⅱ alpha inhibitor.

MATERIALS AND METHODS

1. Collection of mouse oocytes and preimplantation embryos

The GV-oocytes were obtained from the ovarian follicles of the mouse (ICR strain) 48 hrs after injection of 5I.U. PMSG (Sigma). The ovulated-oocytes were collected from the oviducts of the mouse superovulated by injection of 5 I.U. PMSG and 5 I.U. hCG, 20 hrs post-hCG injection. The fertilized 1-cell, 2-cell, 4-cell, 8-cell, morula, and blastocyst embryos were collected from the females superovulated and mated with males 20, 44, 56, 68, 78, and 96 hr post -hCG injection by flushing either the oviduct or the uterus.

2. Total RNAs extraction

Total RNAs were extracted from 30 oocytes/embryos. The collected oocytes and embryos were washed through 3 drops of Ca^{2+}, Mg^{2+}-free PBS, counted and transferred in minimal volume to a chilled 1.5 ml microfuge tube on ice. 300 μl TRIzol (GibcoBRL) was added to each tube and the samples were vortexed vigorously. Prior to isolation of the RNA, 0.1 pg of rabbit α-globin mRNA (Gibco) was added per oocyte/embryo. The RNA was precipitated under the conditions described by the manufacturer and used in experiments or stored at −20℃.

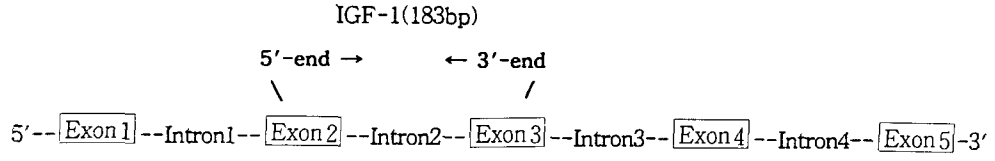
3. Reverse transcription (RT)-PCR

Total RNA was reversely transcribed to cDNA by MP480 thermocycler (Takara, Tokyo, Japan). Reverse transcription was performed with total RNA isolated from 30 oocytes/embryos. The reactions were carried out in 40 μl of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 5 mM $MgCl_2$ containing 1 mM each of 4 dNTPs, 25 unit of RNasin, and 2.5 pmol of oligo dT-M4 adaptor primer. The tubes were incubated at 37℃ for 2 min, 200 units of reverse transcriptase (AMV reverse transcriptase XL, Takara) were added, and the tubes were transferred to a MP480 thermocycler. Reverse transcription was performed for 1 hr at 42℃. The samples were then heated for 5 min at 99℃ and then placed on ice. At this point, the samples were either used directly for PCR or stored at −20℃. The AMV reverse transcript XL, reaction buffer, RNasin, and oligo (dT) primer were obtained from the Takara Corporation.

Based on the mouse IGF-1 cDNA sequence (Bell et al., 1986), primers for IGF-1 were designed (Fig. 1). The 5' and 3' primers for IGF-1 were 5'-CTGGTGGATGCTCTTCAGTTCG-3' and 5'-GGCTGCTTTTGTAGGCTTCAGTGG-3', respectively. The 5' and 3' primers for IGF-1R were 5'-ACTGACCTATGCGCATGTGCTGG-3' and 5'-CTCGTTCTTGCGGCCCCCGTTCAT-3' (Wada et al., 1993). The 5' and 3' primers for α-globin were 5'-GCAGCCACGGTGGCGAGTAT-3' and 5'-GTGGGACAGGAGCTTGAAAT-3' (Cheng et al., 1986). The IGF-1, IGF-1R, and α-globin primers gave rise to diagnostic fragments of 183 bp, 345 bp, and 257 bp, respectively.

The PCR reactions were performed in 40 μl of 10 mM Tris-HCl, pH 8.3, containing 50 mM KCl, 2.0 mM $MgCl_2$, 0.25 mM each of the 4 dNTPs, 2.5 units Taq polymerase (Takara), 25 pmol each of the appropriate 3' and 5' primers, and 5 μl of the reverse transcription reac-

A. Gene structure and primer binding sites of IGF-1



B. Patial cDNA sequence of IGF-1

5'-ctgag*ctggt ggatgctctt cagttcg*tgt gtggaccgag gggcttttac ttcaacaagc ccacaggcta
5'-end(sense)

tggctccagc attcggaggg cacctcagac aggcattgtg gatgagtgtt gctt[ccgg]ag ctgtgatctg

aggagactgg agatgtactg tgcc*ccactg aagcctacaa aagcagccc*g ctctatccgt gcccagcgcc
3'-end(anti-sense)

acactgacat gcccaagact cagaagtccc cgtccctatc gacaaacaag aaaacgaagc tgcaaaggag

aaggaaaggaag tacatttgaa gaacacaagt agaggaagtg caggaaacaa gacctacaga atgtaggagg

agcctcccac ggagcagaaa atgccacatc accgcaggat çctttgctgc ttgagcaacc tgcaaaacat

cgaaacacct-3'

Fig. 1. Representative diagram to illustrate the position of designed IGF-1 primer binding sites, the patial sequence of IGF-1 based on the mouse cDNA sequence(Bell et al., 1986). Both sense and anti-sense primers for IGF-1 were designed at 183 bp distance(underlined Italic). The primers were designed to cross the exon and intron regions. The size of PCR product using IGF-1 primers is shown in base pairs(bp)(Fig. 1A). One restriction enzyme site is shadow boxed(*Msp* I)(Fig. 1B).

tion. When IGF-1, IGF-1R, and α-globin were amplified, this volume was equivalent to 3 oocytes/embryos, respectively. The basic PCR program used was incubation at 95℃ for 3 min, followed by a cycle program of 95℃ for 30 sec, 60℃ 30 sec, and 72℃ 30 sec. The last cycle was concluded with a 72℃ incubation for 10 min extension. The number of cycles was 35 for α-globin, 40 for IGF-1 and IGF-1R. After PCR reaction, the tubes were chilled briefly on ice. The PCR products were electrophoresed in a 2.5% agarose gel at 100 volts for 40 min, followed by a 30-min staining in ethidium bromide (0.5 μg/ml) and destaining in deionized water for 30 min. Agarose gels were exposed to ultraviolet light.

4. Restriction enzyme digestion

The 183 bp IGF-1 product was identified by restriction enzyme digest. The 18 μl amplified PCR product eliquoted into a chilled 0.5 ml microfuge tube and added the 2 μl of reaction buffer (10X L-buffer, Promega). And then the *Msp* I (5 units) restriction enzyme was added into the tube and incubated for 3 hrs at 37℃. Finally, the tube was heated at 75℃ for 10 min and electrophoresed in a 2.5% agarose gel at 100 volts for 40 min. Agarose gels were exposed to ultraviolet light and photographed. The *Msp* I digest of IGF-1 yielded the two expected fragments of approximately 175 and 58 bp.

RESULTS

1. Detection and identification of IGF-1 and IGF-1R transcripts in the oocytes

Using the reverse transcription-PCR, the mRNA of IGF-1 was detected in GV stage oocytes, and the identity of the amplified PCR products was verified by the *Msp* I restriction enzyme digestion (Fig. 2). Because of the diagnostic fragment for IGF-1 should carry an *Msp* I site, so that restriction enzyme digestion ought to give rise to two fragments, one 125 bp and the other 58 bp for *Msp* I in length (Fig. 1). After the identification of IGF-1 transcripts, the number of cycle for IGF-1 and IGF-1R was adopted 35

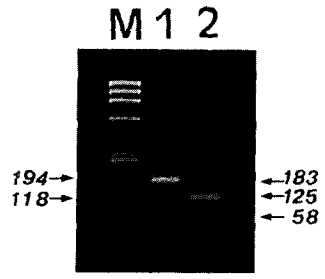


Fig. 2. Identification of amplified IGF-1 transcript by restriction enzyme. M, ϕX-174 Hae Ⅲ marker. Lane 1, amplified and undigested sample; the major band is 183 bp in length. Lane 2, amplified and digested sample by *Msp* I; a major band of 125 bp and a minor band of 58 bp.

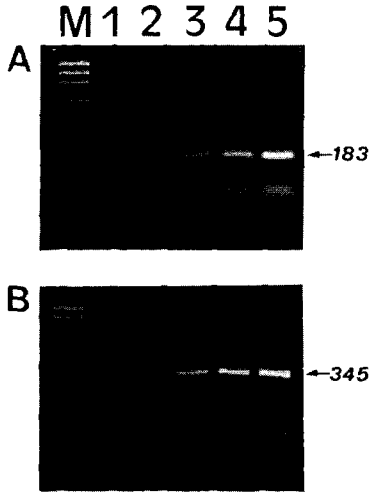


Fig. 3. Optimization of RT-PCR with regard to numbers of PCR cycle for IGF-1 (A) and IGF-1R (B). The amplified IGF-1 transcripts were electrophoresed in 2.5% agarose gel, stained with ethidium bromide, and photographed under UV-light. M, ϕX-174 Hae Ⅲ marker. Lane 1, 25 cycle; Lane 2, 30 cycle; Lane 3, 35 cycle; Lane 4, 40 cycle; Lane 5, 45 cycle.

for α-globin, 40 for IGF-1, and IGF-1R.

2. mRNA levels for IGF-1 and IGF-1R in the oocytes and preimplantation embryos

The transcripts of IGF-1 and IGF-1R were easily detected in the oocytes and preimplantation embryos in mouse. The detectable changes in the mRNA level of IGF-1 and IGF-1R were observed in the oocytes and preimplantation embryos in mouse.

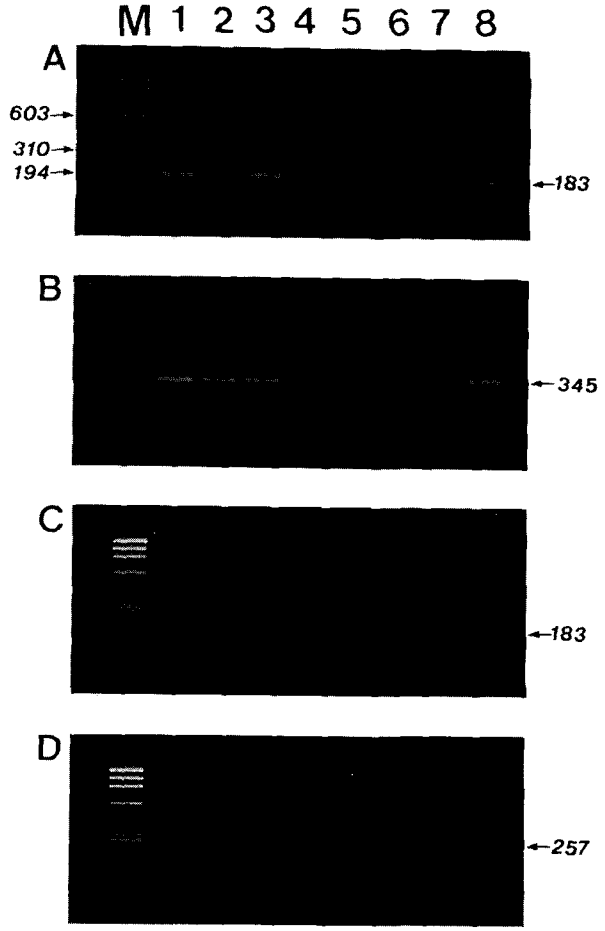


Fig. 4. Temporal pattern of IGF-1 and IGF-1R in mouse oocytes and preimplantation embryos. A: RNA isolated from oocyte and preimplantation embryo subjected to RT-PCR was analyzed for IGF-1 expression. B: As in A, but analyzed for IGF-1R expression. C: As in A, but not RT and analyzed for IGF-1. D: As in A, but analyzed for the amount of α-globin, which serves as an internal marker to monitor RNA recovery. M, ϕX174/Hae Ⅲ marker. : Lane 1, GV stage oocyte; Lane 2, metaphase stage oocyte; Lane 3, pronuclear stage 1-cell embryo; Lane 4, 2-cell stage embryo; Lane 5, 4-cell stage embryo; Lane 6, 8-cell stage embryo; Lane 7, morula; Lane 8, blastocyst.

Compared with amplified PCR product of α-globin, these changes were thought to reflect real differences in the amount of transcript present at different stages. The mRNA levels of IGF-1 and IGF-1R were highest in GV stage oocytes but decreased gradually to 4 or 8-cell stage, and thereafter increased upto blastocyst (Fig. 4).

DISCUSSION

It is commonly accepted that insulin and the IGF family play many roles in mammalian preimplantation embryo development (Heyner et al., 1989a, 1993; Rappolee et al., 1992; Adamson, 1993; Schultz et al., 1993). The ligands and receptors of this growth factor family have been shown to be expressed by pre- and post-implantation embryos (Heyner et al., 1989; Heyner & Kaye, 1991; Rappolee et al., 1992; Schultz et al., 1992; Latham et al., 1994). This is coincident with fundamental morphological changes leading to the formation of blastocyst. The IGF-1, which can activate both the IGF-1 receptor and insulin receptor, would act on these receptors; IGF-1 preferentially binds to the IGF-1 receptor and these receptors are present on the embryo starting at the 8-cell stage (Smith et al., 1993). The IGF-1R was shown to undergo ligand-induced autophosphorylation on tyrosine residues of the β-subunit. IGF-1 stimulated autophosphorylation of the β-subunit of the expressed receptor as well as phosphorylation of cellular substrates. Therefore, IGF-1 stimulates glucose uptake, glycogen synthesis, and DNA synthesis via the transfected IGF-1R.

In this study, using RT-PCR, we revealed that the transcripts of IGF-1 and IGF-1R genes are presented in the oocytes and preimplantation embryos in mouse. The temporal pattern of these transcripts was similar to β-actin (Bachvarova et al., 1989), in which IGF-1 and IGF-1R first decreases and then increases. The decrease is likely to be a consequence of the general degradation of maternal RNA that is initiated during oocyte maturation and is essentially terminated by the late 2-cell stage (Bachvarova et al., 1989). Following zygotic gene activation, which occurs during the 2-cell stage in the mouse (Bensaude et al., 1983; Flach et al., 1982), these maternal transcripts are replaced by zygotic ones. The observation, however, that the amount of IGF-1 and IGF-1R transcripts continue to decline until the 4- or 8-cell stage and increase afterward suggests that the onset of zygotic IGF-1 and IGF-1R transcription is delayed relative to the general activation of the embryonic genome as previous report (Lighten et al., 1997; Liu et al., 1997). The presence of IGF-1 and IGF-1R transcripts in the oocytes and preimplantation embryos suggest that those genes expression may regulate at various levels, e.g. posttranscriptional and posttranslational in the early mouse embryogenesis. In addition, the presence of IGF-1R transcripts in the preimplantation embryo suggests that IGF-1 may play an autocrine role in embryonic development through IGF-1R as a signaling pathway. In conclusion, we showed that the presence of IGF-1 and IGF-1R transcripts in the oocytes and preimplantation embryos in mouse was confirmed by RT-PCR.

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