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Expression of Epidermal Growth Factor-Receptor (EGF-R) on the Inner Cell Mass (ICM) of Bovine IVM/IVF/IVC Blastocyst

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체외생산된 소 배반포기배 ICM세포에서의 EGF-R 발현

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요 약

본 연구는 체외생산된 소 배반포기배의 inner cell mass (ICM) 세포에서 epidermal growth factor-receptor (EGF-R) 의 발현 유무를 immunosurgery와 indirect immunofluorescence (간접 면역 형광방법) 을 이용하여 조사하고자 실시하였다. 본 실험에 사용된 ICM 세포는 체외수정 후 7~8 일째에 회수된 소 배반포기배로 부터 immunosurgery 방법을 실시하여 얻어졌으며, 회수된 ICM세포는 live /dead 염색방법을 통한 생사 유무와 EGF-R 발현 유무 조사에 공시되었다. 특히, 배반포기배에 대한 immunosurgery를 위해 trophectoderm 세포에 대한 rabbit anti-bovine trophectoderm cell antibody (RABTE) 를 제조하여 사용하였다. 결과를 요약하면 다음과 같다. ICM세포의 회수율은 RABTE와 guinea pig serum (complement) 에 각각 15~30 분과 15~60 분동안 처리했을 경우 16.7~74.2% 였으며, 또한 처리시간이 각각 30분과 30분일 때 가장 높은 회수율 (74.2%)을 얻었다. Immunosurgery 후 얻어진 ICM세포의 생존 유무를 조사하기 위해 live /dead 염색 방법을 이용하였던 바, ICM세포의 생존율은 complement 가 60분 처리된 군 (69.3%)을 제외한 모든 처리군에서 84.0~91.6% 의 높은 생존율을 나타냈다. 또한, 회수된 ICM세포에 대한 EGF-R 발현 유무를 간접 면역 형광방법을 이용하여 조사하였던 바, ICM세포의 표면에서 EGF-R의 존재를 확인하였다. 따라서, ICM 세포에서의 EGF-R의 발현은 인위적으로 첨가된 EGF의 이용 가능성을 높임으로서 체외에서의 착상전 배 발달을 증진시킬 수 있을 것으로 사료된다.

(Key words : EGF-R, ICM, *In vitro*, Bovine)

I. INTRODUCTION

Control of growth and differentiation during mammalian embryogenesis may be regulated by growth factors from embryonic or maternal sources (Rappolee et al., 1988; Paria and Dey, 1990; Gandolfi, 1994; Harvey et al., 1995). Through the immunological techniques, ligand binding techniques and reverse transcription-polymerase chain reaction method, the expression of a number of growth factor ligand

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and receptor genes has been demonstrated within preimplantaion mouse and bovine embryos (Heyner et al., 1989; Werb,1990; Paria and Dey, 1990; Watson et al., 1992). In addition, it has been known that the bovine embryonic stages at which transcripts of growth factors and their receptors can be detected are distinctly different from mouse (Watson et al., 1992). Among the growth factors, epidermal growth factor (EGF) is one of the most biologically potent mitogen (Carpenter and Cohen, 1979). However, the transcripts for EGF is not detected even at the blastocyst stage in preimplantation embryos (Nexo et al.,1980; Rappolee et al., 1988; Watson et al., 1992). Nontheless, many researchers reported that EGF stimulates growth and protein synthesis in preimplantation embryo through the EGF-R (Wood and Kaye, 1989; Dardik and Schultz, 1991). In previous study (Kim et al., 1996), we showed that the expression of EGF-R on the bovine embryonic stage by indirect immunofluorescence presents after 4-cell stage and that the intensity of the EGF-R staining was variable with the development progression. Also, it showed that ICM and TE cell number was increased by the addition of EGF with developmental level.

Therefore, this study was to determine whether EGF-R was expressed to ICM cell obtained from bovine IVM/IVF/IVC blastocyst by immunosurgery and indirect immunofluorescence.

Ⅱ. MATERIALS AND METHODS

1. Production of bovine IVM/IVF/IVC blastocysts

The culture procedures employed in the production of preimplantation bovine blastocysts from follicular aspirates derived from slaughtered ovaries were as outlined by Park et al. (1996). Cumulus oocyte complexes (COCs) were collected from visible follicles (2~6mm) of ovaries, washed with TALP-HEPES (Parrish et al., 1988) and cultured into maturation medium composed of TCM-199 (Gibco) + 10% (v/v) fetal bovine serum (FBS) supplemented with sodium pyruvate (0.2 mM), follicle-stimulating hormone (1 μg/ml), estradiol-17β (1 μg/ml), and gentamycin (25 μg/ml) for 22~24 h at 39℃, 5% CO_2 incubator. After *in vitro* maturation, the oocytes were fertilized using highly motile sperm recovered from frozen-thawed semen separated on a discontinuous percoll column and heparin (2 μg/ml) and PHE (18.2 μM Penicillamine, 9.1 μM Hypotaurine and 1.8 μM Epinephrine) were also added in fertilization drop for sperm capacitation. Cleaved embryos were cultured in CR1 medium supplemented with fatty acid-free BSA (3 mg/ml) until day 4 after IVF and then transferred into CR1 medium supplemented with FBS. Blastocysts used for this experiment were obtained from day 7 to 9 after IVF according to the experimental purpose.

2. Detection and production of polyclonal antibodies to bovine trophectoderm cells (RABTE)

Antigens for production of RABTE were obtained from trophectoderm cells (TEs) of hatched blastocysts produced at day 8 to 9 after IVF. ICM and TEs were mechanically separated under a microscope using a needle and a fine bore pipette (60 μm). Total 1,120 hatched blastocysts (Fig. 1A) were obtained from 10,325 matured oocytes at day 8 to 9 after IVF and harvested about 700 TEs (Fig. 1B). Polyclonal antibodies were produced at the University of Wisconsin-Madison Medical School Animal Care Unit using 2 New Zealand white rabbits. Production of antiserum was confirmed by indirect immunofluorescence test. Also, fluorescent level of produced antiserum (RABTE) was compared

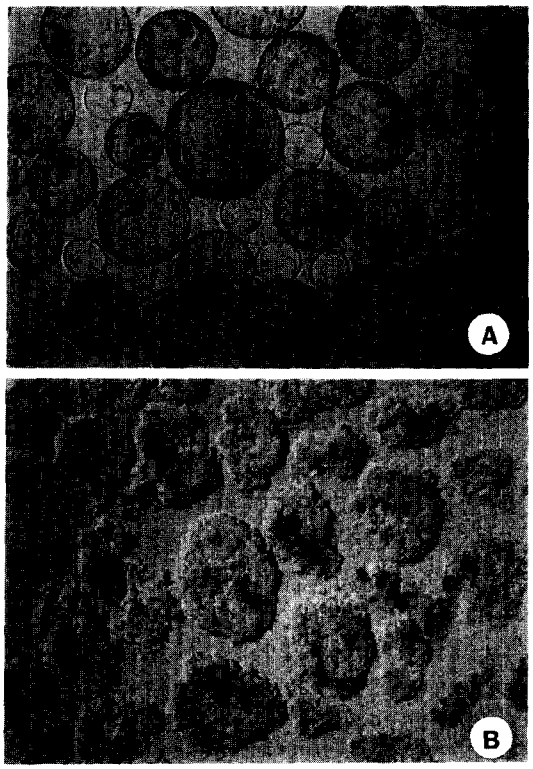

Fig. 1. Hatched blastocysts produced at day 8 to 9 after IVF. (A)×150. Trophectoderm cells mechanically separated from hatched blastocysts for production of antibody. (B)×200.

with positive control serum (B8270, Sigma). Assays were performed at 37℃. Briefly, zona-removed blastocysts were treated through the following steps; (1) 1;100 RABTE or positive control serum in TALP-HEPES, 30min.; (2) washing sufficiently; (3) 1;100 FITC-conjugated goat anti-rabbit IgG in TALP-HEPES, 30min. And then they were washed completely, transferred on a slide glass and observed immediately with an inverted phase-contrast microscope fitted with epifluorescence illumination.

3. Isolation of ICMs by immunosurgery

ICMs were isolated from blastocysts produced at day 7 to 8 after IVF. Immunosurgery was undertaken as the method described by Solter and Knowles (1975) following removal of the zona pellucidae with 0.5 % pronase solution. Assays were performed at 37℃. To optimize the condition of immunosurgery, firstly, blastocysts were treated in 10% rabbit anti-bovine trophectoderm cell antibody (RABTE) for 15~30 min., secondly, embryos were treated in 20% of guinea pig serum (Complement, Sigma) for 15~60 min. Isolated ICMs from immunosurgery were assayed immediately for cell viability and cell surface expression of EGF-R.

4. Assessment of viability of isolated ICMs by live/dead staining

To confirm the viability following immunosurgery, ICMs were treated in live/dead assay (Molecular Probes, Inc.). Briefy, when stained according to the instructions and viewed with UV microscope, the nuclei of live cells fluoresced intense uniform green, while those of cells with damaged plasma membranes fluoresced orange-red. Assays were performed at room temperature. Preincubated ICMs were treated with live/dead staining solution (final concentration; 2 μM of calcein-AM and 4 μM of ethidium homodimer) for 20 min. Then, ICMs were thoroughly washed with TALP-HEPES, transferred on a slide glass and observed immediately with fluorescence microscope.

5. Detection of EGF-R on ICMs by indirect immunofluorescence

To detect EGF-R, isolated ICMs from immunosurgery were treated with human EGF (Sigma), control medium (TALP-HEPES), goat anti-human EGF (Upstate Biotechnology Incorporated; UBI), and FITC-conjugated donkey anti-goat IgG (UBI). Assays were performed at

4℃. ICMs were processed through the following steps; (1) 100ng/ml EGF in PBS, 1h; (2) washing sufficiently; (3) antibody to EGF diluted 1:50 with TALP-HEPES, 2h; (4) washing completely; (5) FITC-conjugated donkey anti-goat IgG diluted 1:50 with TALP-HEPES, 4h. And then they were washed completely, transferred on a slide glass and observed immediately with fluorescence microscope.

6. Statistical analysis

Difference in recovery rate of ICMs according to the time variation of cytotoxicity treatment was compared using the Chi-square test ($p<0.01$).

Ⅲ. RESULTS AND DISCUSSION

1. Development of bovine IVM/IVF/IVC blastocysts

The rate of *in vitro* development of bovine embryos cultured in CR1 medium after *in vitro* fertilization through the 25 replicated experimentation was as follows; as shown in Table 1, the average cleavage rate was 76.8% and blastocysts rate at day 8 after *in vitro* fertilization was 30.8%.

2. Detection of rabbit anti-bovine trophectoderm cell antibody (RABTE) by indirect immunofluorescence (IIF) assay

Detection of antibody production against bovine TEs and comparison of fluorescent level between produced RABTE and postive control serum on TEs of zona removed blastocyst by IIF were indicated in Table 2. As presented in Table 2 and Fig. 2A, type B antibody showed highly fluorescent level which is similar to positive control serum and thus it was known that antibody production to bovine TEs was well carried out.

Table 1. Development of bovine IVM/IVF/IVC oocytes (r= 25)

No. of oocytes	≥2-cell (%)	Day 8 after IVF (%)		
		BL*	≥HgBL**	Total
3,172	2,437 (76.8)	565	186	751 (30.8)

*BL; Blastocyst, **HgBL; Hatching blastocyst

Table 2. Detection of RABTE by indirect immunofluorescence (IIF) assay

Treatment	Antibody			FITC-2nd Ab* (dilution)	IIF assay results**
	Blood sampling	Host type	Dilution		
RABTE	1st	A	1:100	1:100	−
		B	1:100	1:100	+
	2nd	A	1:100	1:100	−
		B	1:100	1:100	+++
	3rd	A	1:100	1:100	+
		B	1:100	1:100	+++
Control (B8270)	−	−	1:100	1:100	+++

* FITC-conjugated goat anti-rabbit IgG

** Intensity of immunofluorescence formed on surface of Z.P.: +++, very strong; +, weak; −, negative

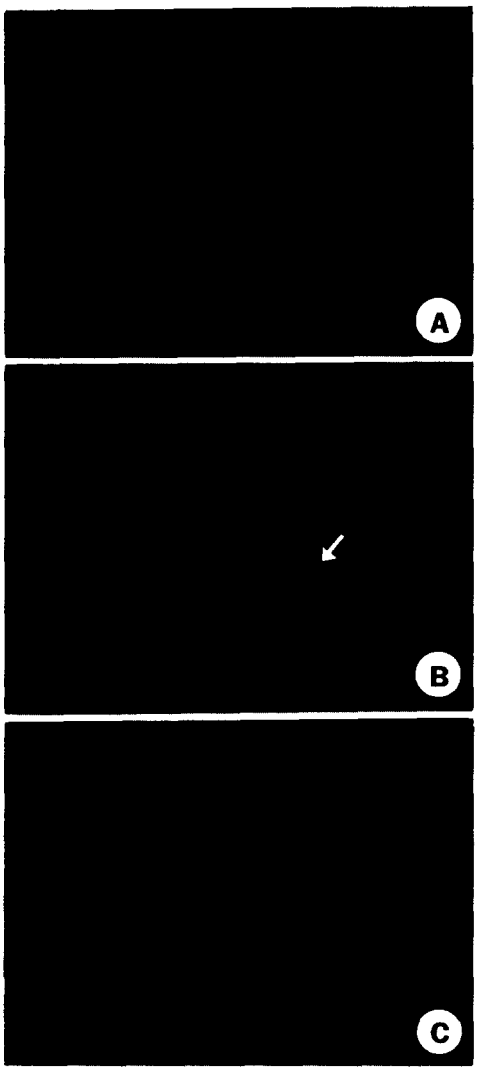

Fig. 2. Detection of polyclonal antibody against bovine trophectoderm cells by indirect immunofluorescence (IIF). (A)×150. Live/dead staining of ICMs with calcein (live-green) and ethidium homodimer (dead-red) (B)×300. It shows that trophectoderm cell is remainded in state of dead on ICMs post immunosurgery (arrow). IIF on ICMs with the binding between EGF and EGF-R (C)×300. All ICMs show that EGF-Rs are expressed on cell surface.

3. Optimization of recovery of ICMs following immunosurgery

Determination of exposure time to antiserum (RABTE) and complement for immunosurgically isolating ICMs was presented in Table 3. As indicated in Table 3, when blastocysts were exposed to RABTE for 15~30 min. and then transferred them to complement for 15~60 min., recovery rates of isolated ICMs were 16.7~74.2%. Especially, the best recovery (74.2%) of ICMs was obtained when exposure time to RABTE and complement was 30 min. and 30 min., respectively. In addition, when viability of isolated ICMs after immunosurgery was assessed by live (calcein; green) and dead (ethidium homodimer; red) staining method (Fig. 2B), in all groups viability (84.0~91.6%) of isolated ICMs was not heavily damaged except excessive complement treatment group (60 min.; 69.3%).

4. Detection of EGF-R on cell surface of ICM by indirect immunofluorescence

Expression of EGF-R on ICMs recovered from blastocysts post immunosurgery was examined (Fig. 2C). In Table 4, fluorescence emission of EGF treatment group was 71.9% when compared with control group (0.0%).

At present, in cattle, only a few study on the expression of EGF and EGF-R is reported yet (Watson et al., 1992). In the results, they suggested that gene products of most growth factors in bovine embryo may be important at even the very early stages of cleavage so that they are present before the maternal-zygote transition. Also, they demonstrated that EGF expressison in bovine embryos was not detected throughout preimplantation development. However, in previous study (Kim et al., 1996), we

Table 3. Cytotoxicity of rabbit anti-bovine trophectoderm cells antibody (RABTE) and guinea pig serum (complement) against bovine IVM/IVF/IVC blastocyst

Exposure time (min.)		No. of ICMs separated / Total no. of embryos examined (%)	Cell viability*(%)
RABTE	Complement		
15	15	12 / 72 (16.7)[a]	11 (91.6)
15	30	25 / 70 (35.7)[b]	21 (84.0)
30	30	52 / 70 (74.2)[c]	45 (86.5)
30	60	55 / 76 (72.4)[c]	38 (69.3)

* Cell viability was defined as the intense uniform green fluorescence formed on surface of ICM by live / dead staining method

[a-c] Means in the column without common superscripts are significantly different ($P<0.01$)

Table 4. Expression of EGF-R on ICMs of bovine IVM/IVF/IVC blastocyst

Treatment*	No. of blastocysts	No. (%) of isolated ICM	Rate (%) of fluorescent emission
Control	41	26 (63.4)	0 (0.0)
EGF (+)	45	32 (71.1)	23 (71.9)

* Control; ICMs were treated with TALP-HEPES, goat anti-human EGF and FITC-conjugated donkey anti-goat IgG.
EGF(+); ICMs were treated with human EGF, goat anti-human EGF and FITC-conjugated donkey anti-goat IgG.

demonstrated that the stimulating effect of exogeneous EGF on the development of IVM /IVF bovine embryos was significantly increased after 8-cell stage. In addition, we detected that the expression of EGF-R on the bovine embryonic stage by indirect immunofluorescence presents after 4-cell stage and that the intensity of the EGF-R staining was variable with the development progression. These results demonstrated that exogenous EGF and EGF-R are likely to play a role during embryo development *in vitro*. In this study, we confirmed that the expression of EGF-R were also presented on ICM cell. This result is similar to mouse data described by many researchers (Wiley et al., 1992; Brison and Schultz, 1996; Dardik et al., 1992). Therefore, these results indicate that EGF-R expression on the ICMs can stimulate the higher usability of exogeneous EGF in the ICMs to improve the preimplantation embryo development *in vitro*.

Ⅳ. SUMMARY

This study was undertaken to determine whether EGF-R was expressed to ICM cell obtained from bovine IVM /IVF /IVC blastocyst by immunosurgery and indirect immunofluorescence (IIF). ICM cells used for this experiment were obtained from immunosurgery of bovine blastocysts produced at day 7 to day 8 after IVF, and recovered ICMs were assayed for cell viability and expression of EGF-R. Especially, for immunosurgery, we produced rabbit anti-bovine trophectoderm cell antibody (RABTE). The results obtained in this experiment were summarized as follows: when blastocysts were exposed to RABTE for 15~30 min. and then transferred them to complement for 15~60 min., recovery rates of isolated ICMs were 16.7~74.2%. Especially, the

best recovery (74.2%) of ICMs was obtained when exposure time to RABTE and complement was 30 min. and 30 min., respectively. In addition, when viability of isolated ICMs after immunosurgery was assessed by live (calcein; green) and dead (ethidium homodimer; red) staining method, in all groups viability (84.0~91.6%) of isolated ICMs was not heavily damaged except excessive complement treatment group (60 min.; 69.3%). Also, we detected the expression of EGF-R on ICM cell by IIF. Therefore, these results indicate that EGF-R expression on the ICMs can stimulate the higher usability of exogeneous EGF in the ICMs to improve the preimplantation bovine embryo development *in vitro*.

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