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Study on Epidermal Growth Factor (EGF) and Expression of EGF-Receptor (EGF-R) in Mouse IVF/IVC Embryo

II. Expression of EGF-R on the Inner Cell Mass (ICM) of Mouse IVF/IVC Blastocyst

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체외생산된 생쥐배에 대한 EGF와 EGF-R 발현에 관한 연구
II. 체외생산된 생쥐 배반포기배 ICM세포에서의 EGF-R 발현

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= 국문초록 =

본 연구는 체외생산된 생쥐 배반포기배의 ICM 세포에서 EGF-R 발현유무를 immunosurgery와 indirect immunofluorescence (간접 면역 형광방법) 을 이용하여 조사하고자 실시하였다. 본 실험에 사용된 ICM 세포는 체외수정 후 96시간째에 회수된 생쥐 배반포기배를 immunosurgery 방법을 이용하여 얻어졌으며, 회수된 ICM세포는 생사유무와 EGF-R 발현유무 조사에 공시 되어졌다. 그 결과를 요약하면 다음과 같다. ICM세포의 회수율은 rabbit anti-mouse serum (antiserum) 과 guinea pig serum (complement) 에 각각 15 - 30 분과 15 - 60 분동안 처리 했을 경우 8.0 - 84.2% 였으며, 또한 처리시간이 각각 30분과 60분일 때 가장 높은 회수율 (84.2%) 을 얻었다. Immunosurgery 후 얻어진 ICM세포의 생존 유무를 조사하기 위해 live/dead 염색 방법을 이용하였던 바, 처리된 ICM세포중 93.8 - 100% 의 생존율을 나타내어 회수된 ICM세포는 유해한 영향을 받지 않았다는 것을 알 수 있었다. 또한, 간접 면역 형광방법을 이용하여 ICM세포에서 EGF-R가 발현되는 것을 확인 하였다. 따라서, ICM세포에서의 EGF-R의 발현은 인위적으로 첨가된 EGF의 이용가능성을 높임으로서 체외에서의 착상전 배 발달을 증진시킬 수 있을 것으로 사료된다.

INTRODUCTION

Growth factors produced by the preimplantation embryo and/or the reproductive tract are available to influence embryonic development and function in an autocrine/pararine manner (Rappolee *et al.*, 1988; Paria and Dey, 1990; Gandolfi, 1994; Harvey *et al.*, 1995). Addition of physiological levels of growth factors from the various gene family such as insulin, EGF, TGF to the culture medium of early mouse embryos results in a broad range of effects that include stimulation of RNA and protein synthesis, increased rate of cell division, increase in the cell number in blastocysts and increase in the percentage of cultured embryos that hatch from the zona pellucida (Heyner *et al.*, 1989; Wood and Kaye, 1989; Werb, 1990). Among the growth factors,

EGF is one of the most biologically potent mitogen (Carpenter and Cohen, 1979). Also, Wiley *et al.* (1992) demonstrated that EGF-R is expressed throughout preimplantation development. It is already known that EGF directly affect the rate of blastocoel expansion through the EGF-R (Dardik and Schultz, 1991). Paria and Dey (1990) demonstrated that the effect of EGF at blastocyst stage was confined to trophectoderm (TE) cell by autoradiographic immunolocalization of using ^{125}I-EGF binding. Recently, it reported that the EGF-Rs were also expressed in inner cell mass (ICM) cell as well as TE cell by using immunosurgery and reverse transcription-polymerase chain reaction (Dardik *et al.*, 1992; Brison and Schultz, 1996), although the direct effect of EGF on ICM at preimplantation embryo development was not proved yet. However, our previous results showed that exogenous EGF can stimulate the cell number of ICM (1997).

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

MATERIALS AND METHODS

1. Production of mouse blastocysts *in vitro*

In vitro fertilization (IVF) and blastocysts production were performed as the method described by Kim *et al.* (1997). Briefly, animals for IVF, from four to six week old F1 hybrid female mice (C57BL/6 x CBA) and from eight to ten week old F1 hybrid male mice of the same strain, were used. For induction of superovulation of female mice, 7.5 IU pregnant mare's serum gonadotrophin (Sigma) and 7.5 IU human chorionic gonadotrophin (Sigma) were injected with 50 h interval. Also, IVF and culture were undertaken in 50 *ul* drop of M16 medium at 5% CO^2 and 37℃ incubator. Assessment of fertilization after IVF was confirmed by 2-cell cleavage on day 1. Blastocysts used for this experiment were classified to early (ErB), middle (MB) and hatching blastocyst at 96 h after IVF according to the developmental morphology (Kim *et al.*, 1996).

2. Isolation of inner cell mass cells (ICMs) by immunosurgery

ICMs were isolated from blastocysts by immunosurgery (Solter and Knowles, 1975) following removal of the zona pellucidae with 0.5 % pronase solution. The embryos were handled during immunosurgery in HEPES- buffered Tyrode's medium (TALP-HEPES; Parrish *et al*, 1988) at 37℃. To optimize the condition of immunosurgery, firstly, blastocysts were treated in 10% of rabbit anti-mouse serum (Antiserum, Sigma) for 15 - 30 min., secondly, embryos were treated in 20% of guinea pig serum (Complement, Sigma) for 15 - 60 min. Also, several steps were taken to remove completely contaminating TE cells from the ICMs; blastocysts were incubated for 30 min. in working medium following immunosurgery to allow the lysed TE cells to slough off; a series of pipettes of progressively smaller internal diameter were used to strip away all of the remaining TE cells. Then, isolated ICMs were assayed immediately for cell viability and cell surface expression of EGF-R.

3. 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). Briefly, the assay permits the simultaneous determination of live and dead cells with two proves that measure two recognized parameters of cell viability, intracellular esterase activity and plasma membrane integrity. Live cells are distinguished by the presence of intracelluar esterase activity.

Esterase activity is measured by the fluorescence generated by the enzymatic hydrolysis of calcein-AM. The substrate hydrolysis yields the intensely fluorescent product, calcein. Fluorescence microscopy has shown that live cells generate an intense uniform green fluorescence. Also, dead cells are distinguished by the staining of nucleic acids. The nucleic acid stain, ethidium homodimer, is excluded from live cells. The nuclei of cells with damaged membranes label rapidly and fluoresce red. Assays were performed at room temperature. Preincubated ICMs were treated with live/dead staining solution (final concentration; 2uM of calcein-AM and 4uM of ethidium homodimer) for 20 min. Then, ICMs were thoroughly washed with TALP-HEPES, transferred on a slide glass and observed immediately with an inverted phase-contrast microscope fitted with epifluorescence illumination.

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

To detect EGF-R, ICMs were treated with mouse EGF (Sigma), control medium (TALP-HEPES), rabbit anti-mouse EGF (Sigma) and FITC- conjugated goat anti-rabbit IgG (Sigma). Assays were performed at 4℃. All antibody solutions were centrifuged immediately prior to use. Living embryos were processed through the following steps; (1) 100 ng/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 goat anti-rabbit 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.

Table 1. Rates of fertilization and development of mouse IVF embryo (r=4)

No. of oocytes	No. of 2-cell (%)	Development to blastocysts at 96 h after IVF (%)			
		>Bla.	ErB	MB	HgB
324	255 (78.7)	224 (87.8)	27 (12.1)	51 (22.8)	146 (65.2)

Bla.; Blastocyst, ErB; Early blastocyst, MB; Middle blastocyst, HgB; Hatching blastocyst

5. Statistical analysis

Difference in recovery rate of ICMs according to the time variation of cytotoxicity treatment was compared using the Chi-square test ($p<0.05$).

RESULTS AND DISCUSSION

1. Development of mouse preimplantation embryos

In vitro fertilization rate of mouse eggs through the 4 replicated experimentation was 78.7% and blastocysts rate at 96 h after IVF was 87.8% from the 2-cell embryos. Blastocysts used in this experiment were selected from middle to hatching blastocysts and development rates of each blastocysts were 22.8% and 65.2%, respectively.

2. Optimization of recovery of ICMs following immunosurgery

Determination of exposure time to antiserum

Table 2. Cytotoxicity of rabbit anti-mouse serum (antiserum) and guinea pig serum (complement) against mouse IVF/IVC blastocyst

Exposure time (min.)		No. of ICMs separated/Total no. of embryos examined (%)	Cell viability* (%)
Anti-serum	Complement		
15	15	2/25 (8.0)[a]	2 (100.0)
15	30	11/35 (31.4)[b]	11 (100.0)
30	30	20/35 (57.1)[c]	19 (95.0)
30	60	32/38 (84.2)[d]	30 (93.8)

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

[a-d]Means in the column without common superscripts are significantly different ($P<0.05$).

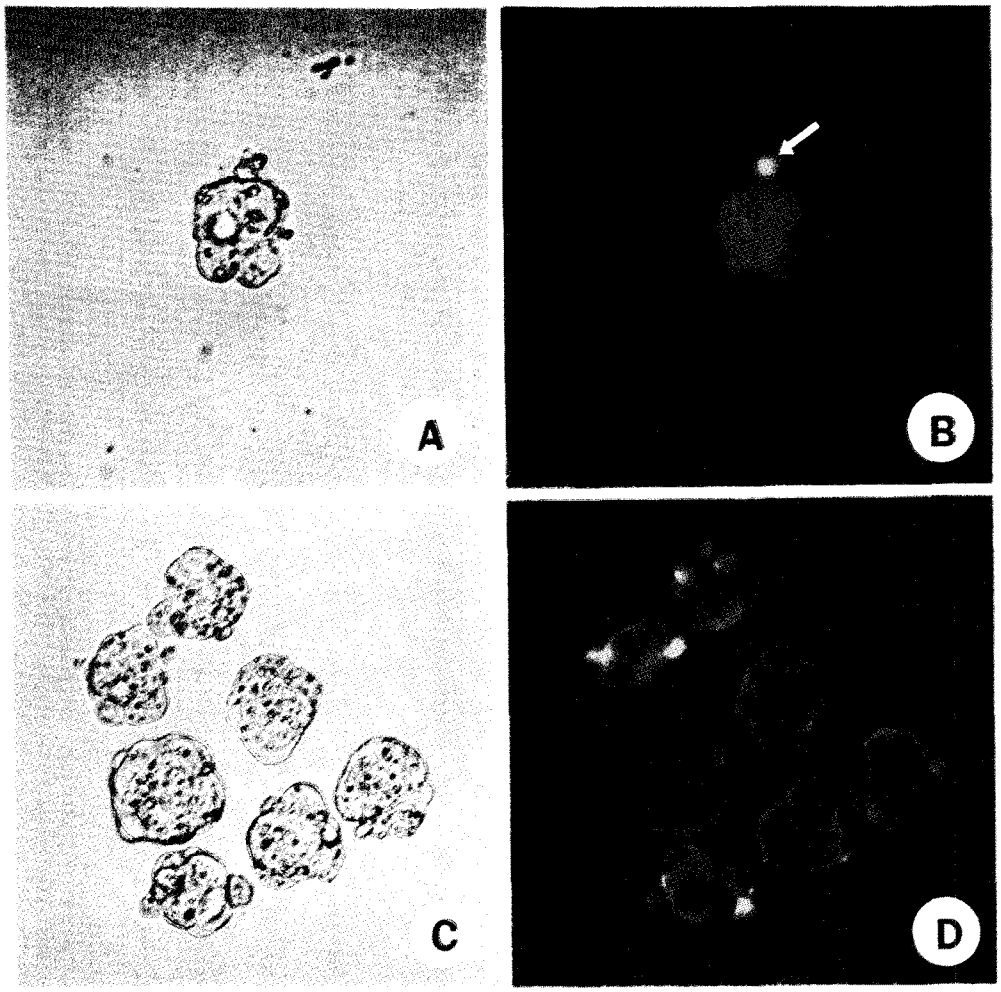

Fig. 1. Live/dead staining (L/D) and indirect immunofluorescence (IIF) of ICMs obtained from mouse IVF/IVC blastocysts by immunosurgery. (B) L/D with calcein (live-green) and ethidium homodimer (dead-red), and corresponding phase micrograph (A). It shows that trophectoderm cells remainded in state of dead on ICMs post immunosurgery (arrow, B). (D) IIF with the binding between EGF and EGF-R, and corresponding phase micrograph (C). All ICMs show that EGF-Rs are expressed on cell surface. ×300.

and complement for immunosurgically isolating ICMs was presented in Table 2. As shown in Table 2, when blastocysts were exposed to antiserum for 15 - 30 min. and then transferred them to complement for 15 - 60 min., recovery rates of isolated ICMs were 8.0 - 84.2%. Especially, the best recovery (84.2%) of ICMs was obtained when exposure time to antiserum and complement was 30 min. and 60 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 (93.8 - 100.0%) of isolated ICMs were not damaged if separated from TE cell (Table 2 and Fig. 1B).

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

Expression of EGF-R on ICMs recovered from blastocysts post immunosurgery was examined. As indicated in Table 3, fluorescence emission of EGF treatment group was 89.3% when compared with control group (0.0 %). In

Table 3. Expression of EGF-R on ICM of mouse IVF/IVC blastocyst

Treatment*	No. of blastocysts	No. (%) of isolated ICM	Rate (%) of fluorescent emission
Control	25	18 (72.0)	0 (0.0)
EGF (+)	35	28 (80.0)	25 (89.3)

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

our previous study (Kim *et al.*, 1997), we demonstrated that stimulating effect of EGF with the development level was significantly increased after 4-cell stage and that ICM proportion also showed the increased pattern with the developmental level in the EGF treatment group although there is not significantly different. Also, we indicated that the expression of EGF-R by indirect immunofluorescence (IIF) was detected after 4-cell stage. In this study, we detected the expression of EGF-R on ICM cell obtained from mouse IVF/IVC blastocyst by IIF (Fig. 1D). This result is consistent with study described by Wiley *et al.* (1992) and Dardik *et al.* (immunogold EM methods, 1992). In addition, Brison and Schultz (1996) indicated the quantitative information on relative levels of mRNA of EGF-R in the ICM cells and TE cells by using reverse transcription-polymerase chain reaction. Possibly, exogenous EGF and EGF-R, ligand-receptor pair, are likely to play a role in ICM since our previous results (Kim *et al.*, 1997) showed that EGF could stimulate the cell number of ICM cell as well as the that of TE cell. Therefore, in basis of these results, we indicate that EGF-R expression on the ICMs can stimulate the higher usablity of exogenous EGF to improve the preimplantation embryo development *in vitro*.

SUMMARY

This study was undertaken to examine the expression of EGF-R protein on ICM obtained from mouse IVF/IVC blastocyst by immunosurgery and indirect immunofluorescence (IIF). ICM cells used for this experiment were obtained from immunosurgery of mouse blastocysts produced at 96 h after IVF, and recovered ICMs were assayed for cell viability and expression of EGF-R. The results obtained in this experiment were summarized as follows: when blastocysts were exposed to rabbit anti-mouse serum (antiserum) for 15 - 30 min. and then transferred them to guinea pig serum (complement) for 15 - 60 min., recovery rates of isolated ICMs were 8.0 - 84.2%. Especially, the best recovery (84.2%) of ICMs was obtained when exposure time to antiserum and complement was 30 min. and 60 min., respectively. In addition, when viability of isolated ICMs after immunosurgery was assessed by live/dead staining method, in all groups viability (93.8 - 100.0 %) of isolated ICMs were not damaged if separated from TE cell. Also, we detected the expression of EGF-R on ICM cell by IIF. Therefore, these results suggest that EGF-R expression on the ICMs can stimulate the higher usablity of exogenous EGF to improve the preimplantation embryo development *in vitro*.

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