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

Ⅰ. Additive Effect of EGF and Expression of EGF-R on Mouse IVF Embryo Development

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체외생산된 생쥐배에 대한 EGF와 EGF-R 발현에 관한 연구
Ⅰ. 체외수정된 생쥐배 발달에 대한 EGF 첨가제 효과와 EGF-R 발현

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= 국문초록 =

본 연구는 EGF가 체외수정된 생쥐배의 착상전 발달 및 inner cell mass (ICM) 과 trophectoderm (TE) 세포수에 미치는 영향을 조사하고, 그와 더불어 간접 면역형광방법을 이용하여 배 발달 단계에 따른 EGF-receptor (EGF-R) 단백의 발현유무를 조사하기 위해 실시하였다. 그 결과를 요약하면 다음과 같다. 2-세포기 배의 group (5/25 μl) 배양은 단독 배양보다 양호한 배 발달을 유도 할 수 있었으며, 단독 배양에서의 저조한 배 발달은 EGF를 첨가함으로서 개선시킬 수 있었다. 특히, 10 ng/ml의 EGF가 첨가된 단독배양군 (62.4%) 은 단독대조군 (47.9%) 에 비하여 유의하게 높은 배 발달을 나타냈다. 또한, ICM과 TE세포수 공히 EGF 첨가에 의해 증가되는 것을 differential labelling기법으로 확인 할 수 있었다. 발달단계에 미치는 EGF의 효과를 검토하였던 바 4-세포기 이후의 배 발달에 유의하게 영향을 미치는 것을 알 수 있었고, 특히 배반포기배의 ICM 도 동시에 증가되는 것을 알 수 있었다. 한편, 간접 면역형광에 의한 EGF-R의 발현유무를 조사한 결과, 4-세포기 이후에 EGF-R가 발현되는 것을 확인할 수 있었다. 따라서, EGF는 착상전 생쥐배의 4-세포기 이후에 발현되는 EGF-R에 반응하여 배 발달을 유기하며, 또한 배반포기배의 ICM과 TE세포수의 증가에 영향을 미치는 것을 알 수 있었다.

INTRODUCTION

Despite the successful *in vitro* culture of preimplantation embryos from a number of mammalian species, such embryos are at a developmental disadvantage when compared to their *in vivo* counterparts (Heyner *et al.*, 1993). This disadvantage can be overcome to some extent by adding growth factors (Yang *et al.*, 1993). In fact, various growth factors are produced by the preimplantation embryo and/or reproductive tract (Rappolee *et al.*, 1988; Zhang *et al.*, 1994; Gandolfi, 1994; Harvey *et al.*, 1995). Also, control of growth and differentiation during mammalian embryogenesis may be regulated by those growth factors (Rappolee *et al.*, 1988). Among the growth factors, EGF is one of the most biologically potent mitogen (Carpenter and Cohen, 1979).

It stimulates protein synthesis (Wood and Kaye, 1989) and blastocyst rate of preimplantation embryos (Paria and Dey, 1990; Yang *et al.*, 1993) by binding on EGF-R which is expressed on cell membrane after 4-cell stage (Wiley *et al.*, 1992). Also, It was reported that the effect of EGF was confined to TE cell (Wood and Kaye, 1989). Paria and Dey (1990) observed a cooperative interaction among preimplantation mouse embryos. They demonstrated that embryos cultured in groups showed better development to the blastocyst stage and higher cell number than embryos cultured singly. In addition, inferior development of singly cultured embryos should be overcome by addition of EGF, TGF-α or TGF-β in culture medium. Also, they indicated that EGF stimulated hatching for embryos cultured in groups and singly from the 8-cell stage. To confirm the viability of preimplantation embryos treated with EGF, cell number and the allocation of ICM and TE cells may be a valid indicator in later preimplantation stage (Papaioannou and Ebert, 1988; Iwasaki *et al.*, 1994).

The objective of this study was to determine the effect of EGF on the preimplantation development of mouse IVF embryos and their ICM and TE cell number. And also, we examined the expression of EGF-R protein on embryonic development by indirect immunofluorescence.

MATERIALS & METHODS

1. Mouse IVF

Oocytes were recovered from four to six week old F1 hybrid female mice (C57BL/6 x CBA) which had been superovulated by intraperitoneal injections of 7.5 IU pregnant mare's serum gonadotrophin (Sigma) followed 50 hr later by 7.5 IU human chorionic gonadotrophin (hCG; Sigma). At 13.5 hr post hCG injection, the mice were killed by cervical dislocation and their oviducts were removed in M2 containing 4 mg/ml BSA and then cumulus-oocyte complexes (COCs) were transferred into 50 μl drop of M16 for culture medium until sperm insemination. Spermatozoa for mouse IVF were obtained from the dissected caudae epididymis of mature F1 hybrid mice (C57BL/6 x CBA) from eight to ten week old age. At 14 hr post hCG injection, sperm of about final concentration 1×10^6 cells/ml were introduced into the 50 μl drop containing COCs and sperm and eggs were incubated for 5 hr at 37℃. And then eggs were collected and transferred into fresh culture drops. Fertilization was assessed by 2-cell cleavage on day 1 after IVF

2. Embryo culture experiments

Cleaved embryos were washed with Ham's medium containing 4 mg/ml BSA (control medium) and cultured in singly or group in 25 μl drop of control medium with or without EGF. The cell number of blastocysts was determined by differential labelling method.

Experiment I: **Effect of EGF on blastocyst development and cell number following culture of 2-cell embryos in singly or group in 25 μl of medium**

Two-cell embryos were cultured in singly or group in Ham's medium supplemented with EGF at concentrations of 0, 1, 10, 100 ng/ml for 72hr. In the case of group culture, 5 embryos were cultured in each drop.

Experiment II: **Effect of EGF according to the development level of mouse embryos produced *in vitro***

Two-cell, four-cell, morula embryos were recovered at day 1, day 2 and day 3 after IVF in the same embryo group and they were cultured group (5 embryos/25 μl drop) in Ham's medium without or with (10 ng/ml) EGF for 72, 48 and 24 hr, respectively. Also, to examine the effect of EGF on hatching of blastocyst, some blastocysts of each treatment group were further cultured for 48 hr (day 6

after IVF).

3. Detection of EGF-R on embryonic stage by indirect immunofluorescence

To detect EGF-R on embryonic stage, embryos were treated with mouse EGF (Sigma), rabbit anti-mouse EGF (Sigma) and/or FITC-conjugated goat anti-rabbit IgG (sigma). Preimplantation embryos at 2-cell, 4-cell, 8-cell, morula, and blastocyst stages were recovered on days 1-4 after IVF. Embryo zona was removed in 0.5% pronase (Sigma) solution and returned in M16 medium for at least 30 min. prior to assay. Assays were performed at 4℃. All antibody solutions were centrifuged immediately prior to use. Living embryos were processed through the following steps; (1) 200 ng/ml EGF in PBS, 1hr; (2) washing sufficiently; (3) antibody to EGF diluted 1:50 with PBS, 2hr; (4) washing completely; (5) FITC-conjugated goat anti-rabbit IgG diluted 1:50 with PBS, 4hr. And then they were washed completely, transferred to a drop of PBS on a slide glass and observed immediately with an inverted phase-contrast microscope fitted with epifluorescence illumination.

4. Differential labelling of ICM and TE nuclei

ICM and TE nuclei of blastocysts were differentially labelled by using a method of Hardy *et al.* (1989) with some modifications. The protocol was as follows; embryo zona was removed in 0.5% pronase (Sigma) solution and allowed to recover for 10 min. in TL-Hepes. Embryos were incubated on ice for 10～15 min. in 10 mM trinitrobenzene sulfonic acid (Sigma) containing 4 mg/ml PVP (Sigma) in TL-Hepes. After washing completely, embryos were incubated in 0.1 mg/ml anti-DNP-BSA (ICN Immunobiological.) in TL-Hepes for 10 min. at 37℃. After washing sufficiently in TL-Hepes, the embryos were incubated in 0.01 mg/ml propidium iodide (PI) and 10% (v/v) guinea pig complement (Sigma) in TL-Hepes for 15～30 min. at 37℃. After 15 min., they were transferred into 0.05 mM bisbenzimide in absolute alcohol. After overnight storage at 4℃, the embryos were washed in absolute alcohol for at least 1 hr, and mounted in glycerol under a coverslip on a slide glass. Labelled nuclei were observed under fluorescence microscope fitted witrh ultra violet excitation filter and TE nuclei labelled with PI and bisbenzimide appeared pink or red, ICM nuclei labelled with bisbenzimide appeared blue or unlabelled.

5. Statistical analysis

Difference in development rate and number of cells between treatment groups was compared using the Chi-square and Student's t-test, respectively.

RESULTS

To determine the effect of EGF on blastocyst development and cell number following culture of 2-cell embryos in singly or group, they were treated with EGF at concentrations of 0, 1, 10, 100 ng/ml. As shown in Table 1, group culture showed more improved development rate to blastocyst than singly culture. Also, inferior development of singly cultured embryos showed the increased pattern by the addition of EGF. Especially, 2-cell embryos cultured singly in 10 ng/ml of EGF (62.4%) indicated significant difference in development to blastocyst compared with contol group (47.9%). The cell number of ICM and TE by differential labelling showed the increased pattern in EGF treatment group compared with control, although there are not significantly different among the group. To examine the effect of EGF according to the development level, 2-cell, 4-cell and morula embryos were treated with 10 ng/ml of EGF. As indicated in Table 2, the stimulating effect of EGF on the development level was significantly increased after 4-cell stage ($p<0.05$). Also, to determine the

effect of EGF on zona hatching of blastocyst, some blastocysts of the each treatment group were further cultured for 48 hr. As shown in Table 2, hatching of blastocysts by the addition of EGF also showed the increased pattern with the development level. Especially, ICM proportion showed the increased pattern with the developmental level in the EGF treatment group. In addition, expression of EGF-R by indirect immunofluorescence presented aft-

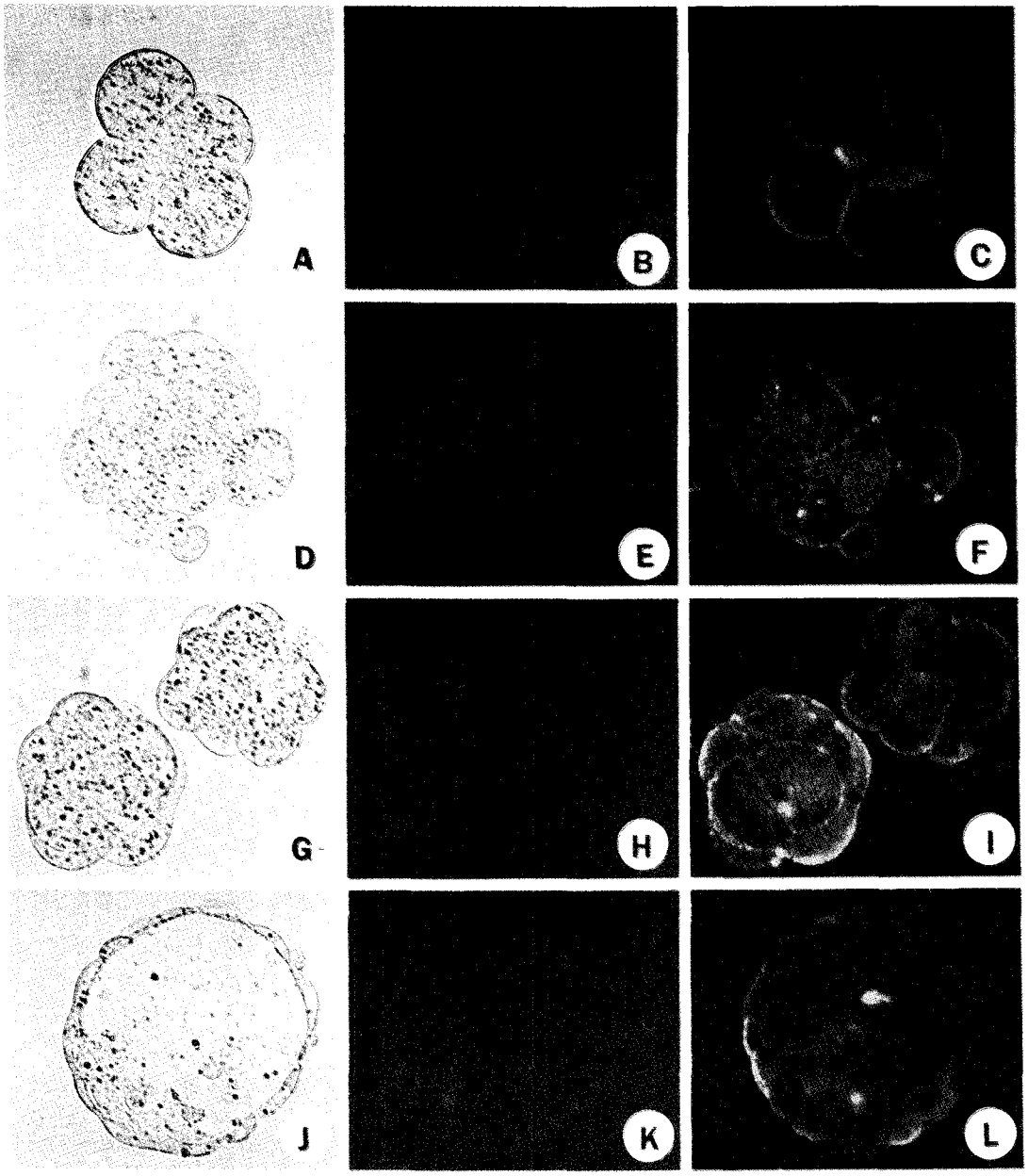

Fig. 1. Indirect immunofluorescence (IIF) assays to demonstrate the staining results according to binding between EGF and EGF-R. (C, F, I and L) IIF; (A, D, G and J) corresponding phase micrographs; (B, E, H and K) control treatment. (**A, B** and **C**) Four-cell embryo at 46h after IVF; (**D, E** and **F**) 8-cell embryo at 56h after IVF; (**G, H** and **I**) morulae at 70h after IVF; (**J, K** and **L**) blastocyst at 94h after IVF. ×300 magnification.

er 4-cell stage (Fig. 1).

DISCUSSION

This study indicates the important role of EGF and EGF-R in the preimplantation embryo development. In our result (Table 1), although the effect of EGF was obtained only when 2-cell embryos were cultured in singly which is treated with EGF, total cell number was not significantly different among the treatment group. However, there are significantly different in 2-cell embryos cultured in singly by the addition of 10 ng/ml EGF compared with control. In count of cell numbers of the ICM and TE of blastocyst, the increased pattern of ICM and TE cells showed in the EGF treatment group. The results obtained in these experiment demonstrated that there are cooperative interaction among the murine preimplantation embryo development as described in Paria and Dey (1990). It means that stimulatory effect of growth factor on embryo development disappeared when embryos were cultured in group due to autocrine effect of growth factors secreted by the embryos themselves. Also, when 2-cell embryos were cultured in singly or group with EGF at concentrations of 0, 1, 10, 100 ng/ml for 72 hr, 100 ng/ml of EGF did not further improve embryo development and cell number compared with that at 10 ng/ml. This concentration (10 ng/ml) is the same as the 3.3 nM-EGF which is the maximum concentration of stimulating the protein synthesis of mouse morula (Wood and Kaye, 1989). EGF clearly stimulate the proliferation of epidermal and epithelial tissue in mammals, including other cell types (Carpenter and Cohen, 1979). In addition, cellular metabolic effects of EGF include stimulation of ion influxes (Rosengurt, 1975), glucose transport (Barnes and Colowick, 1976), glycolysis (Diamond *et al.*, 1978) and synthesis of DNA, RNA and proteins (Carpenter and Cohen, 1979; Wood and Kaye, 1989). Also, Paria and Dey (1990) have shown that embryos bind ^{125}I-EGF as early as the compacted 8- to 16-cell stage. We also investigated the effect of EGF with the development stage (Table 2). In this experiment, 2-cell, 4-cell and morula embryos recovered with the development time and they were treated with 10 ng/ml of EGF. In the results, EGF significantly stimulated the embryonic development after 4-cell stage. Also, to determine the effect of EGF on the zona hatch-

Table 1. Effects of EGF on blastocyst development and cell number following culture of mouse IVF 2-cell embryos in 25 μl medium

Culture	EGF (ng/ml)	No.	Blastocysts at day4 after IVF			
			Rate (%)	Cell number (Mean±SEM)		
				ICM	TE	Total
Singly	0	117	56 (47.9)[a]	6.8±1.8	26.8±1.6	34.8±5.7
	1	117	63 (53.8)[a,b]	10.5±1.5	27.6±1.4	38.3±4.7
	10	109	68 (62.4)[a]	11.2±1.6	33.4±1.6	46.5±3.2
	100	109	65 (59.6)[a,b]	8.2±2.7	30.9±1.7	39.2±2.8
#Group	0	98	63 (64.3)	12.4±1.6	29.3±1.4	43.1±3.1
	1	98	64 (65.3)	11.5±1.2	30.2±1.1	42.9±3.4
	10	96	72 (75.0)	14.2±1.1	32.0±1.2	46.5±3.2
	100	98	61 (62.2)	14.4±1.2	31.7±1.5	47.7±4.5

Group culture: 5 embryos/drop

[a,b]Means in the same column (singly) without common superscripts are significantly different ($p<0.05$).

ing of blastocyst, some blastocysts of the each treatment group were further cultured for 48 hr (until day 6 after IVF). However, increased pattern of hatching blastocyst showed in the EGF treatment group although there is not significantly different. On the other hand, when metabolic fate of cell-bound EGF was investigated by fluorescence microscopy of fluorescein conjugated EGF (Haigler *et al.*, 1978) and quantitative electron microscopic autoradiography of cell-bound ^{125}I-EGF (Gordon *et al.*, 1978), cell bound is internalized within endocytotic vesicles which subsequently fuse with lysosomes. Also, membrane-bound ^{125}I-EGF is internalized by the cell in a time- and temperature-dependent fashion (Gordon *et al.*, 1978). Therefore, experiment on the expression of EGF-R is very difficult. In fact, study on the EGF-R number per embryo is not carried out yet. Wiley *et al* (1992) indicated that EGF-Rs are expressed at increasing levels on mouse preimplantation embryos. Also, they presented that mRNA and protein of EGF-R are increased after 4-cell stage by studying with reverse transcription-polymerase chain reaction and indirect immunofluorescence. In our results, we confirmed that EGF-R presents after 4-cell stage, although low level of expression of EGF-R detects in fluorescence level at late 2-cell stage (30 hr after IVF) (Data not shown). On the other hand, Adamson and Meek (1984) demonstrated that EGF-Rs are expressed in the TE cells. Also, Wood and Kaye (1989) indicated that EGF stimulates the TE cells (but not the ICM cells) to synthesize one third more protein in the morula to blastocyst stage. But, our result is similar to that of Wiley *et al.* (1992). They found a low level of expression of EGF-R protein on ICM cells by using immunosurgery and indirect immunofluorescence. Although we did not study on the expression of EGF-R of pure ICM, proportion of ICM cell in the EGF treatment group was higher than that of TE cell (Table 2). Further studies will be taken about the effect of EGF on the ICM.

SUMMARY

The objective of this study was to determine the effect of EGF on the preimplantation development of mouse IVF embryos and their ICM and TE cell number. And also, we examined the expression of EGF-R protein on embryonic development by indirect immunofluorescence. The results obtained in these experiments were summarized as follows; Group culture (5 embryos/ 25 μl) showed more improved development rate to blastocyst than singly culture. This inferior development of singly cultured 2-cell embryos improved by the addition of EGF. Especially, 2-cell embryos cultured singly in 10 ng/ml of EGF (62.4%) indicated significant difference in development to blastocyst compared with contol

Table 2. Effects of EGF (10ng/ml) on the development stage of mouse IVF embryo

Embryo stage	No. of embryo	Blastocysts at day 4 after IVF				Rage (%) of hatching blastocysts at day 6 after IVF
		Rate (%)	Cell number			
			ICM		Total (M±SEM)	
			M±SEM	Proportion(%)		
control	81	61 (75.3)[a]	9.9±1.5	26.4	37.5±3.4	21/31 (67.7)
2-cell	82	65 (79.3)[a,b]	11.6±1.0	27.8	41.7±2.1	24/35 (68.6)
4-cell	86	77 (89.5)[b]	13.3±4.4	30.2	44.0±3.1	35/47 (74.5)
morula	90	83 (92.2)[b]	13.4±0.8	32.8	40.9±1.5	39/53 (73.6)

[a,b]Means in the same column without common superscripts are significantly different ($p<0.05$).

group (47.9%). Also, cell number of ICM and TE by differential labelling showed the increased pattern in the EGF treatment group. The stimulating effect of EGF with the development level was significantly increased after 4-cell stage ($p<0.05$). ICM proportion also showed the increased pattern with the developmental level in the EGF treatment group. In addition, expression of EGF-R by indirect immunofluorescence detected after 4-cell stage. Therefore, EGF could stimulate preimplantation mouse embryo development by binding with expressed EGF-R after 4-cell stage and produce the more increased ICM and TE cell number of blastocyst.

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