Effects of Epidermal Growth Factor on Preimplantation Mouse Embryos

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Purpose: Our purpose was to clarify the effects of epidermal growth factor (EGF) on mouse preimplantation embryos.

Methods: We examined the effect of EGF on two-cell and four-cell stage mouse embryos cultured in vitro. In preimplantation embryos, we analyzed the binding of ¹²⁵I-EGF by autoradiography and EGF receptor mRNA by the reverse transcription-polymerase chain reaction.

Results: At more than 0.005 ng/ml, EGF relieved the two-cell block and regulated the differentiation of morula-stage embryos. These effects were negated by antiserum. EGF did not exhibit any marked effect on embryos between the four-cell and the morula stages. Specific binding for EGF and EGF receptor mRNA was detected during and after the morula stage.

Conclusions: The effects of EGF on preimplantation mouse embryos differed according to the stage of development, promoting cleavage before the four-cell stage and regulating differentiation after the morula stage. This regulatory action is thought to be transmitted to the cells via EGF receptors.

KEY WORDS: epidermal growth factor; preimplantation mouse embryo; epidermal growth factor receptor; development.

INTRODUCTION

It has been hypothesized that secretions of the oviduct and uterus stimulate the proliferation of cells in preimplantation mammalian embryos and promote the early events in differentiation that lead to successful implantation (1–4). The milieu of the preimplantation embryo contains many kinds of growth factors and cytokines that act in an autocrine, paracrine, and juxtacrine manner (5–10).

EGF plays a key role in the early pregnancy of mammals, including maturation of the oocyte (11),

preimplantation embryo development (12–18), and infiltration of the trophoblast into the endometrium (19–22). Some studies suggest that EGF receptors (EGFRs) are present in the preimplantation embryos of humans (23), cattle (24), and mice (25). EGF reportedly enhances development when added to the medium used to culture preimplantation murine embryos. This effect is neutralized by anti-EGF antibody (11–14). However, other authors reported that EGF inhibits or modulates development of mouse preimplantation embryos (15,16). Thus EGF is a well-studied growth factor known to influence embryonic development, but the effects on embryos reported for them are not consistent. In addition, none of these studies demonstrates the possibility of the stage-specific effects that EGF may have on preimplantation embryos. This remains unclear.

In evaluating the stage-specific effect of EGF on development of preimplantation mouse embryos, we examined its effect on development from the two-cell to the four-cell stage and from the four-cell stage to the expanded blastocyst. We also evaluated the presence of EGFRs in preimplantation embryos at various stages of development using a radioreceptor assay and the presence of EGFR mRNA by use of the reverse transcription-polymerase chain reaction (RT-PCR) method.

MATERIALS AND METHODS

Collection and Culture of Embryos

Female mice of the ICR strain (8 to 9 weeks old) were subjected to superovulation induced by the intraperitoneal injection of 7.5 IU of pregnant mare serum (PMS), followed 48 hr later by the intraperitoneal injection of 7.5 IU of human chorionic gonadotropin (hCG; Sigma Chemical Co., St. Louis, MO). These

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mice were then mated with males of the same strain. Detection of a vaginal plug the next morning served as an indicator of pregnancy. The pregnant females were killed by cervical dislocation 42 hr later for two-cell stage embryos or 56 hr later for four-cell stage embryos. Their oviducts were removed and placed in human tubal fluid (HTF) buffered with 1 mM HEPES (Irvine Scientific, Santa Ana, CA). Embryos were collected by flushing the oviducts with approximately 0.3 ml of HEPES-buffered HTF and were pooled in 0.4 ml of HTF (Irvine Scientific). Embryos that appeared morphologically normal were randomly distributed into the experimental groups. They were cultured, in groups of seven to nine embryos, in 60 μ l of HTF containing 0.3% bovine serum albumin (BSA; Sigma) under mineral oil (Sigma) in the presence or absence of various concentrations of EGF (Boehringer Mannheim Kontroll-Labor Biochemie, Penzberg, Germany). In some cases, anti-EGF antiserum (Boehringer Mannheim), 100 nl/ml, was simultaneously added to the medium containing EGF, to determine whether the manifested effects of EGF would be canceled. Embryo cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. Each experiment was performed 10 times to obtain reliable data. The exact composition of the medium used (HTF) is shown in Table I (26).

Criteria for Scoring Embryos

Our criteria for scoring the embryos were as follows.

Two-cell stage embryo:	An embryo in which two blastomeres evenly cleaved are observed and fragmentation is not observed.

Four-cell stage embryo:	An embryo in which four blastomeres evenly cleaved are observed and fragmentation is not observed.
Morula:	An embryo that is at the stage following compaction but that does not begin to form a blastocoele.
Early blastocyst:	An embryo in which blastocyst formation is observed, but the blastocoele is not expanded.
Expanded blastocyst:	An embryo in which blastocyst formation is observed, and the blastocoele is completely expanded.

EGF-Binding Analysis

Preimplantation embryos at the two-cell, four-cell, morula, and early blastocyst stages were recovered in HTF medium by flushing the oviducts. The zona pellucida was left intact. They were then incubated for 30 min at 37°C in an atmosphere of 5% CO₂ in air with 20 pM ¹²⁵I-EGF (sp act = 5.4–7.4 MBq/ μ g; Amersham Life Science, Buckinghamshire, England) in HTF containing 0.3% BSA in the presence or absence of a 1000-fold excess of unlabeled EGF. After incubation, they were washed several times in cold (5°C) phosphate-buffered saline (PBS) and fixed in cold 4% paraformaldehyde in PBS for 15 min. They were then mounted on poly-L-lysine-coated glass slides. The slides were dehydrated in an ascending series of alcohols, dipped into KONICA NR-M2 emulsion, exposed for 3 weeks, developed, and examined.

Preparation of Total RNA from Mouse Embryos

RNA was extracted by means of the acidic guanidine thiocyanate/phenol/chloroform method, as described by Chomczynski and Sacchi, with minor modifications (27). Briefly, washed embryos were collected in batches of 50 in about 10 μ l of HTF and added to 100 μ l of 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.1% sodium sarcosyl, and 1% β -mercaptoethanol, with 5 μ g of *Escherichia coli* tRNA as carrier. Eleven microliters of 2 M sodium acetate (pH 4.8) was added and the mixture was extracted twice with phenol/chloroform and once with chloroform. RNA was then precipitated with same volume of isopropanol and washed twice with 70% ethanol. The final pellet was vacuum-dried and dissolved in 10 μ l of H₂O.

Table I. The Exact Composition of HTF (26)

Sodium chloride	101.6 mM
Potassium chloride	4.69 mM
Magnesium sulfate, anhydrous	0.20 mM
Potassium phosphate, monobasic	0.37 mM
Calcium chloride, anhydrous	2.04 mM
Sodium bicarbonate	25.0 mM
Glucose	2.78 mM
Sodium pyruvate	0.33 mM
Sodium lactate	21.4 mM
Penicillin-G	100 U/ml
Streptomycin sulfate	50 mg/L
Phenol red	0.01 g/L
Osmolarity	280
pH	7.3–7.5
Endotoxin levels	Not detectable

Detection of EGFR mRNA by RT-PCR

Primers for PCR were designed based on the published sequence of mouse EGFR cDNA (28). The sequence of 5' primer was 5'-TATGTGCAAAG-GAATTACGACC-3' (nucleotide numbers 364–385), and that of 3' primer was 5'-CAGCTTGGATCA-CATTGGGG-3' (nucleotide numbers 740–719). The 5' sequence was separated from the 3' sequence by introns of the mouse EGFR gene. The expected size of the PCR product was 377 bp. After heat denaturation of 10 μ l of total RNA solution for 1 min at 95°C, followed by cooling on ice for 2 min, RT was performed for 60 min at 37°C in a total volume of 20 μ l that contained PCR buffer, MMLV reverse transcriptase, 10 U/ μ l (Life Science Inc.), RNase inhibitor, 1 U/ μ l (Nippon Gene Inc., Toyama, Japan), a 500 μ M concentration of each dNTPs, and 10 pmol of 3' primer. All RT reaction solution was subjected to PCR in a volume of 30 μ l. The mixture contained PCR buffer, 30 pmol each of 3' primer and 5' primer, a 200 μ M concentration of each dNTP, and 5 U of Taq polymerase (Takara Shuzou, Tokyo, Japan). The mixture was cycled 40 times at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec. The products of RT-PCR were separated by 1.5% agarose gel electrophoresis and transferred to a nitrocellulose membrane. Southern blot hybridization with a cRNA probe was performed by the manufacturer's protocol. A 293-bp (nucleotide numbers 423–715) RT-PCR product of EGFR mRNA amplified from mouse uterus total RNA was subcloned into the pCRII vector (Invitrogen, San Diego, CA) and sequenced. After digesting plasmid with *Pst*I (Nippon Gene Inc), the cRNA probe was synthesized by use of T7 RNA polymerase (Nippon Gene Inc., Toyama, Japan) and [³²P]UTP following digestion of template DNA with DNase.

Statistical Methods

All statistical analyses were performed with Fisher's exact probability method to compare the growth rates of embryos. Statistical significance was determined by values of $P < 0.05$.

RESULTS

Effect of EGF on the Two-Cell block

To confirm the effect of EGF on the two-cell block, we collected embryos at the two-cell stage, 42 hr after

Fig. 1. Development of embryos cultured for 24 hr from the two-cell stage in the absence (control) or presence of EGF. Bars show the percentage of four-cell stage embryos developed/total embryos. Numbers are indicated on each bar. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ (Fisher's test) compared with the control.

the injection of hCG, and cultured then for 24 hr in a medium containing various concentrations of EGF. As shown in Fig. 1, the two-cell block-relieving effect of EGF was observed at concentrations of 0.005 to 0.05 ng/ml. The percentage of embryos that developed to the four-cell stage was higher following incubation with EGF at concentrations of 0.005 to 0.05 ng/ml vs higher concentrations, but not to a significant extent. To determine whether the effect at this stage was specific for EGF, anti-EGF antiserum (100 nl/ml) was added to a medium in which EGF was also present. As shown in Fig. 2, the effect of EGF on enhancing embryonic development to the four-cell stage was neutralized by coincubation with anti-EGF antiserum.

Fig. 2. Development of embryos cultured for 24 hr from the two-cell stage when anti-EGF antiserum, 100 nl/ml, was added to the medium in which EGF, 0.05 ng/ml, was also present. Bars show the percentage of four-cell stage embryos developed/total embryos. Numbers are indicated on each bar. *** $P < 0.005$ (Fisher's test) compared with the control.

Anti-EGF antiserum alone did not influence cell cleavage.

Effect of EGF After the Four-Cell Stage

To confirm the effect of EGF after the four-cell stage, we next collected embryos at the four-cell stage 56 hr after the injection of hCG and cultured them for 48 hr in a medium containing various concentrations of EGF. Figure 3 shows the percentage of the embryos cultured for 24 hr that developed to the morula stage and the percentage of the embryos cultured for 48 hr that developed to the expanded blastocyst stage. About 90% of the embryos cultured for 24 hr reached the morula stage. No group showed a statistically significant difference in growth rate compared with the control. When these embryos were cultured for 48 hr, development of four-cell embryos was significantly inhibited in cultures supplemented with EGF in a concentration-dependent manner. The difference was statistically significant at a EGF concentration of 0.01 ng/ml. A plateau was reached at 0.05 ng/ml. To determine the specificity of the effect, anti-EGF antiserum (100 nl/ml) was added to the medium. As shown in Fig. 4, the regulatory effects of EGF on embryos cultured for 48 hr were completely canceled by the addition of anti EGF antiserum. Anti-EGF antiserum alone did not influence embryo development from the four-cell stage.

Furthermore, we tried to clarify the effect of EGF on embryos from the four-cell stage to the morula

Fig. 3. Development of embryos cultured from the four-cell stage in the absence (control) or presence of EGF. White bars show the percentage of morula-stage embryos developed/total embryos cultured for 24 hr; black bars show the percentage of expanded blastocyst-stage embryos developed/total embryos cultured for 48 hr. Numbers are indicated on each bar. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ (Fisher's test) compared with the control.

Fig. 4. Development of embryos cultured for 48 hr from the four-cell stage when anti-EGF antiserum, 100 nl/ml, added to the medium in which EGF, 0.05 ng/ml, was also present. Bars show the percentage of expanded blastocyst stage embryos developed/total embryos. Numbers are indicated on each bar. *** $P < 0.005$ (Fisher's test) compared with the control.

stage and from the morula stage to the expanded blastocyst stage distinctively. The following experiment is shown schematically in Fig. 5A. Four-cell embryos in the four groups were cultured for a total of 48 hr. All had been washed, transferred to a medium in which EGF was either present at 0.05 ng/ml or absent, and cultured for 24 hr, at which time most of the embryos had reached the morula stage. Embryos in group A were not exposed to EGF during the 48-hr period of culture, those in group B were exposed to EGF only during the first 24 hr, those in group C were exposed to EGF only during the latter 24 hr, and those in group D were exposed to EGF for 48 hr. Figure 5B shows the percentage of embryos cultured for 24 hr that developed to the morula stage and the percentage of embryos cultured for 48 hr that developed to the expanded blastocyst stage in each group. The growth rates in groups B, C, and D are compared with that in group A. No group showed a statistically significant difference in growth rate when cultured for 24 hr. However, when they were cultured for 48 hr, a significant decrease in growth was observed in embryos in groups C and D compared with group A. There was no significant difference between groups A and B. The data shown in Fig. 5B indicate that EGF regulates the growth of embryos after the morula stage but that it has no marked effect during the period between the four-cell and the morula stages.

In our experiments described above, shown in Figs. 1–5, a few embryos that had been cultured for 24 or

Fig. 5. Effects of exposure to EGF for a limited period on development from the four-cell stage. (A) Scheme for the experiments. Recovered four-cell embryos were cultured for 48 hr while exposed to EGF, 0.05 ng/ml, for a limited period. Black zones indicate the period during which the embryos were cultured in the presence of EGF; white zones indicate the period of the absence of EGF. Numbers in parentheses indicate the estimated time from fertilization. (B) Development of embryos cultured from the four-cell stage with exposure to EGF, 0.05 ng/ml, for a limited period. White bars show the percentage of morula-stage embryos developed/total embryos cultured for 24 hr; black bars show the percentage of expanded blastocyst-stage embryos developed/total embryos cultured for 48 hr. Numbers are indicated on each bar. * $P < 0.05$ (Fisher's test) compared with group A.

48 hr developed beyond the expected stage. They were handled as the expected-stage embryos.

Specific Binding of EGF of Mouse Preimplantation Embryos

Under the conditions of our analysis of EGF binding, there was no significant evidence of specific binding

of ^{125}I -EGF to embryos at the two-cell or four-cell stage (Figs. 6a–d), but high-intensity signals of ^{125}I -EGF binding to embryos were detected at the morula and early blastocyst stages (Figs. 6e and g). The binding was specific, because ^{125}I -EGF was competitively displaced by unlabeled EGF (Figs. 6f and h). At the early blastocyst stage, the signal of specific binding was found not only on the trophectoderm, but also on cells that were destined to form the inner cell mass (ICM).

Determination by RT-PCR of EGF Receptor mRNA in Preimplantation Embryos

As shown in Fig. 7, the expected 377-bp amplification product for EGFR mRNA was detected at all stages analyzed, as well as in the mouse uterus total RNA used as a positive control. No product was detected in *E. coli* tRNA or in embryo culture supernatant.

DISCUSSION

Some studies of cultured mouse embryos exhibit the two-cell block, a phenomenon in which the progression of cell cleavage is arrested following the two-cell stage in vitro (28). If EGF were able to relieve the two-cell block, we would expect it to affect the culture of embryos from the two-cell stage to the blastocyst stage. Although several reports suggest that EGF promotes embryonic development prior to implantation, thus reflecting a two-cell block-relieving effect, EGF may exert other effects during and after the four-cell stage. We initially confirmed the two-cell block-relieving effect of EGF. We then collected four-cell embryos, cultured them, and examined the effects of EGF on their growth. EGF exhibited no effect on the growth of these embryos from the four-cell stage to the morula stage. However, EGF inhibited the growth rate during and after the morula stage in a concentration-dependent manner. Cancellation of this effect by anti-EGF antiserum confirmed that these effects were specific to EGF. These observations indicate that EGF acts as a growth-promoting factor during and before the four-cell stage and, also, as a growth-suppressive factor during and after the morula stage. Its effect on embryonic development is considered to change at some point between the four-cell and the morula stages. We suggest the following chain of events. Mouse embryos are cleaved evenly from fertilization to the eight-cell stage as nonpolar groups of blastomeres.

Fig. 6. Autoradiograms of ¹²⁵I-EGF binding on preimplantation embryos observed microscopically. (a, b) The two-cell stage; (c, d) the four-cell stage; (e, f) the morula stage; (g, h) the early blastocyst stage. a, c, e, and g were exposed to ¹²⁵I-EGF alone; b, d, f, and h were exposed to ¹²⁵I-EGF plus an excess of nonradioactive EGF.

Fig. 7. EGFR gene expression in mouse preimplantation embryos at different stages detected by RT-PCR. Southern blot of 10 μ l of the RT-PCR reaction solution of 30 embryos in each lane. Embryo stages examined are shown along the abscissa. Mo, morula; Bl, early blastocyst; E, *E. coli* tRNA; U, mouse uterus total RNA (10 ng); Md, culture medium in which two-cell embryos were washed.

Compaction occurs at the eight-cell stage (29). Following compaction, the ability of the cells to differentiate is gradually restricted, and differentiation begins to take place in the cells that constitute the inner cell mass (ICM) and those that constitute the trophectoderm (29). The definition of "development" contains two elements: proliferation, or the enlargement of an organism by an increase in the number of cells through repeated cell division; and differentiation, the transformation of undifferentiated cells into cells with specific functions. Both elements are present in development of preimplantation embryos. EGF is considered to aid the development by promoting proliferation before the four-cell stage and exerting a regulatory effect on differentiation after the morula stage.

In the present study, these effects of EGF on the development of the mouse preimplantation embryo before the four-cell stage and after the morula stage were observed at concentrations less than 1 ng/ml. Previous reports evaluated its effects at higher concentrations, 1–10 ng/ml. Several authors report that EGF is expressed by the epithelium of the oviduct in pigs (30) and human (31). Dalton *et al.* (32) evaluated the synthesis of several growth factors and cytokines in the mouse oviduct using northern blotting and immunohistochemical techniques. They could not demonstrate the synthesis of EGF in the mouse oviduct (32). The concentration of EGF in mouse follicular fluid is about 1 ng/ml (33); in mouse serum it is about 0.5–1 ng/ml. The concentration of EGF to which mouse preimplantation embryos are exposed *in vivo* is considered to be less than 1 ng/ml.

We performed a radiobinding assay without removing the zona pellucida. If the signals of EGF were transmitted to the cell by receptors on the surface of the blastomere, EGF would be able to penetrate the zona pellucida. Observation of specific binding of EGF would be possible, and would be more physiologic, if the zona pellucida were preserved. Therefore, we chose to observe the specific binding of EGF without remov-

ing the zona pellucida. Specific binding of EGF and EGF-receptor mRNA was detected during and after the morula stage. This finding confirmed that the regulatory effects of EGF on embryos after the morula stage are transmitted to the cell via specific receptors. No clear specific binding of EGF was observed in the binding assay before the four-cell stage when EGF promoted cleavage. However, EGF-receptor mRNA was detected by the RT-PCR method at the two-cell stage. Wiley *et al.* detected mRNA for EGF receptor in preimplantation mouse embryos and, also, detected it in the two-cell stage. They suggested that such mRNA may have been derived from the maternal granulosa cells, which possess EGF receptors (33). In our efforts to detect EGF receptor mRNA, we washed the embryos with the culture medium, then used the culture medium in which the washed two-cell embryos had been suspended as a control. If even a trace of granulosa cells had entered the suspension after washing, we would have detected EGF receptor mRNA; however, the results were negative. Therefore, the EGF receptor mRNA detected in our study at the two-cell stage is considered to be derived from the embryos as in other stages. The effect of EGF on promoting cleavage seems likely to be transmitted into the cell even during and before the four-cell stage. However, as no clear receptor binding of EGF was demonstrated in the two-cell embryos, further investigation is needed.

CONCLUSIONS

We conclude that the effects of EGF on preimplantation mouse embryos differed according to the stage of development, promoting cleavage before the four-cell stage and playing a regulatory role in the differentiation after the morula stage. This regulatory action is thought to be transmitted into the cell via specific receptors.

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