ANIMAL EXPERIMENTATION

Expression of Transforming Growth Factor Alpha (TGF-α) Gene in Mouse Embryonic Development

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Purpose: *The expression of genes for TGF-α, epidermal growth factor (EGF), and the EGF receptor (EGFR) in mouse blastocysts was evaluated by the reverse transcription-polymerase chain reaction (RT-PCR). We evaluated the effects of TGF-α and EGF on the development of mouse embryo prior to implantation.*

Results: *The results revealed the presence of transcripts of TGF-α and EGFR. However, EGF mRNA was not observed in repeated experiments. None of these growth factors influenced the rate of development from the two-cell stage to the blastocyst stage when added to the culture medium. These effects were further examined on measuring the incorporation of tritiated thymidine and leucine, providing indices of the synthesis of DNA and protein, respectively. A concentration of only 0.1 ng/ml of TGF-α, which shares a cell surface receptor with EGF, stimulated the synthesis of both DNA and protein. EGF at a concentration of 10 ng/ml stimulated the synthesis of DNA and protein by blastocysts. To explore autocrine effects of TGF-α on the rate of blastocoel expansion, TGF-α antisense oligodeoxynucleotides was used to reduce expression of the TGF-α gene. TGF-α at a concentration of 0.1 ng/ml stimulates the rate of blastocoel expansion in early cavitating mouse blastocysts. In contrast, TGF-α antisense oligonucleotides significantly reduced the rate of expansion.*

Conclusions: *Our present observations suggest that TGF-α/EGF and the EGFR may be involved in regulating embryonic development. In particular, TGF-α may serve as an autocrine factor in the regulation of embryonic development.*

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KEY WORDS: antisense oligodeoxynucleotides; epidermal growth factor (EGF); EGF receptor; embryo; reverse transcription-polymerase chain reaction; transforming growth factor alpha.

INTRODUCTION

The expression of several growth factors (GFs) and their receptors in the preimplantation embryo and the maternal reproductive tract suggests that such factors may influence the growth and differentiation of embryonic cells in an autocrine/paracrine manner (1,2). The development of embryos in vitro prior to implantation was retarded in terms of the number of cells in the embryos (3), the synthesis of DNA and RNA (4), and a reduced rate of implantation following transfer to the surrogate mother (5). These findings suggest that GFs and cytokines may enhance the development of the embryo in vivo. GFs and cytokines produced by the embryo itself or by the maternal reproductive tract were shown to stimulate embryonic development in vitro (6–8). Additional information is needed about the mechanism by which the GFs regulate the growth and differentiation of the embryo prior to implantation. Such information might be useful in establishing optimal culture conditions for in vitro fertilization, in which human embryos are transferred into the uterine cavity at a suboptimal stage (two to four cells), instead of the blastcyst stage.

Our objective was to evaluate the role of transforming growth factor-α (TGF-α), epidermal growth factor (EGF), and the EGF receptor (EGFR) ligand–receptor system in the development of preimplantation embryos. The expression in the blastcysts of mRNA of TGF-α, EGF, and the receptor for EGF/TGF-α was

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evaluated by the reverse transcription-polymerase chain reaction (RT-PCR). The effects of TGF-α on preimplantation embryos were evaluated by sensitive measure of DNA and protein synthesis. To examine further autocrine effects of TGF-α on the rate of blastocoel expansion, TGF-α antisense oligodeoxynucleotides were used.

MATERIALS AND METHODS

Growth Factors and Other Reagents

Receptor-grade natural mouse EGF was purchased from Genzyme (MA). Recombinant human TGF-α was purchased from Collaborative Research Inc. (MA). Anti TGF-α antibody was purchased from Oncogene Science, Inc. (NY). All other culture-grade regents were obtained from Wako Pure Chemical (Tokyo) or Sigma (MO).

Embryo Collection and Culture

B6C3 F_1 mice were used in all experiments. Female mice were injected with 7.5 IU of pregnant mare serum gonadotropin (PMSG; Sankyo Zoki Co., Ltd., Tokyo) and 10 IU of human chorionic gonadotropin (hCG; Pregnyl; N.V. Organon, Tokyo) 48 hr apart. Immediately after the hCG injection, the female mice were placed with the male. Two-cell embryos were collected from the oviducts of the mated female mice 44 hr after hCG injection. Biggers–Whitten–Whittingham (BWW) medium was prepared as detailed previously (4). GFs were added to BWW medium containing bovine serum albumin, 1 mg/ml (BSA; Fraction V, Sigma). Cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air.

RT-PCR

Total RNA was extracted from blastocysts (20–50 per group) by the guanidinium thiocyanate method as described by Chomczynski and Sacchi (9). The detailed procedures followed the guidelines provided by the manufacturer (Isogen; Nippon Gene Co., Tokyo). To maximize isolation of RNA, 20 μg of yeast transfer RNA was added as a carrier. RNA from embryos was reverse transcribed into cDNA by use of a cDNA Cycle kit (Invitrogen, San Diego, CA). The reverse transcription of RNA to cDNA was performed with 5 U of AMV reverse transcriptase.

Amprification was carried out in 50 m*M* KCl, 10 m*M* Tris–HCl (pH 8.8), 1.5 m*M* $MgCl_2$, and 2 U Taq DNA polymerase (Nipppon Gene). Each cycle consisted of 1 min at 94°C, 1 min at 55°C, and 2 min at 75°C. The specific primer pairs were derived from published sequences for mouse β-actin, EGF, TGF-α, and EGFR cDNA sequences and were as follows: β-actin—5′ primer = 5′TCGTGGGCCGCTCTAGGCA C3′, 3′ primer = 5′TGGCCTTAGGGTTCAGGGG G3′ PCR amplified cDNA = 243 bp (1); EGF—5′ primer = 5′CCAGTTCAGTAGAAACTGGG3′, 3′ primer = 5′TGGTTTCTAATGATTTTCTCC3′ PCR amplified cDNA = 247 bp (10); TGF-α—5′ primer = 5′ACCTGCAGGTTTTTGGTGCAG3′, 3′ primer = 5′GCAGACGAGGGCACGGCACCA3′ PCR amplified cDNA = 239 bp (11); and EGFR—5′ primer = 5′GGAGGAAAAGAAAGTCTGCC3′, 3′ primer = 5′CCCATAGTTGGACAGGATGG3′ PCR amplified cDNA = 248 bp (12).

PCR products were resolved on 2% agarose gels along with a small molecular weight DNA marker (ϕ × 174 digested with *Hae*III).

For restriction enzyme analysis, amplified PCR products were treated with phenol–chloroform and resuspended in the appropriate buffer for restriction enzymes (Nippon Gene).

Thymidine and Leucine Incorporation

To quantify the synthesis of DNA and protein, the incorporation of thymidine and leucine was measured by the methods described previously (4,13). In brief, blastocysts that had been cultured in the medium supplemented with various growth factors were washed three times in BWW medium containing only BSA. Embryos were then transferred to a medium containing, ^{3}H-thymidine, 50 μCi/ml (sp/act, 25 Ci/mmol; Amersham Japan, Tokyo), or ^{3}H-leucine, 50 μCi/ml (sp/act, 5 Ci/mmol; Amersham Japan), and incubated for 4 hr. After being washed five times in cold PBS, three to five embryos were transferred to a 1.5-ml microcentrifuge tube. The addition of 200 μl of distilled water was followed by two cycles of freezing and thawing, then by precipitation with 20% trichloroacetic acid. The precipitate was dissolved in a tissue homogenizer (Soluene 350, Packard Japan). Liquid scintillation counting was carried out in a Beckman LS 5801. Incorporation of thymidine and leucine was calculated and is expressed as counts (dpm) per embryo.

Measurement of the Rate of Blastocoel Expansion

Early cavitating blastocysts cultured from the two-cell stage were placed in 1 ml of the culture medium containing TGF-α or oligonucleotides. The rate of increase in blastocoel volume was measured essentially as described previously (14,15). The volume of the cavity was determined by measuring the area of the blastocoel cavity on an Olympus inverted microscope at a 200-fold magnification; the focal plane was adjusted to measure the maximum diameter of the cavity. The embryos were then cultured at 37°C in 5% CO_2 in air and the size of the blastocoel cavity was measured 6 hr later.

Oligonucleotides

The designs of oligodeoxynucleotides used in this study showing sequence position by nucleotide are given below. The oligomers were designed to overlap the initiation codon of the rat TGF-α mRNA (11). TGF-α phosphorothioate oligonucleotide was synthesized by Nippon Gene.

Initiation codon oligonucleotides:

TGF-α antisense, 5′-CGGGGACCATCTTCC-3′; sense, 5′-GGAAGATGGTCCCCG-3′.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SE). Results were evaluated by the nonparametric Mann–Whitney test, with $P < 0.05$ considered statistically significant.

RESULTS

Expression of TGF-α and the EGFR Gene in the Mouse Blastocyst

Total RNA isolated from 20, 30, and 50 blastocysts was used in RT-PCR for β-actin expression as a control for RNA integrity. Using primers specific for β-actin, all samples gave an amplified product of the predicted size (243 bp) and approximately concentration dependent in intensity (Fig. 1A).

Results in Fig. 1B indicate that the EGFR gene is expressed in the blastocyst. After amplification by the first 30 cycles, only the PCR product for β-actin was visualized; nested PCR for another 30 cycles generated sufficient ethidium bromide fluorescence to show the predicted 248 bp of the EGFR PCR fragment. To verify that the correct target sequence had been amplified by the PCR reaction, PCR products were digested with *Pst*I. The 248-bp EGFR PCR fragment was digested to yield two fragments, 173 and 75 bp in length, as predicted from the mouse cDNA sequence. Expression of the TGF-α gene was also detected by repeated amplification (Fig. 1C). The fragment was cut by *Sph*I, generating two fragments with predicted lengths. In contrast, transcripts of the EGF gene were not amplified in repeated experiments.

Effects of TGF-α on the Development of Mouse Two-Cell Embryos

TGF-α and EGF were tested for potential mitogenic effects on mouse embryos. The number of blastocysts was recorded after 48 hr of culture in the medium, with GF added at a concentration of 0.1, 1, or 10 ng/ml. None of the GF-supplemented media enhanced the rate of development of blastocysts compared with the control (Table I).

DNA Synthesis by Mouse Blastocysts Grown in Medium Supplemented with TGF-α

The effects of various concentration of TGF-α or EGF on DNA synthesis in the embryos was examined, since they share a cognate cell surface receptor. EGF, at 10 ng/ml, produced a significant increase in thymidine incorporation. TGF-α, at 0.1 ng/ml, also stimulated thymidine incorporation. This concentration was 100 times lower than the concentration of EGF required (Fig. 2).

Effects of TGF-α on the Protein Synthesis by Blastocysts

We measured the incorporation of leucine in blastocysts grown from two cell embryos in a medium containing TGF-α or EGF. As in the measurement of DNA synthesis, EGF, 10 ng/ml, and TGF-α, 0.05 and 0.1 ng/ml, stimulated the incorporation of leucine (Fig. 3). These results suggest that a similar effect of EGF and TGF-α on embryonic cell function but that TGF-α may be more potent. To minimize the possibility that a contaminant present in the growth factor preparations was responsible for stimulating the protein synthesis, a neutralizing antibody directed against TGF-α to block specific action. The positive effects of TGF-α were abolished by the addition of 0.2 μg/ml anti TGF-α antibody.

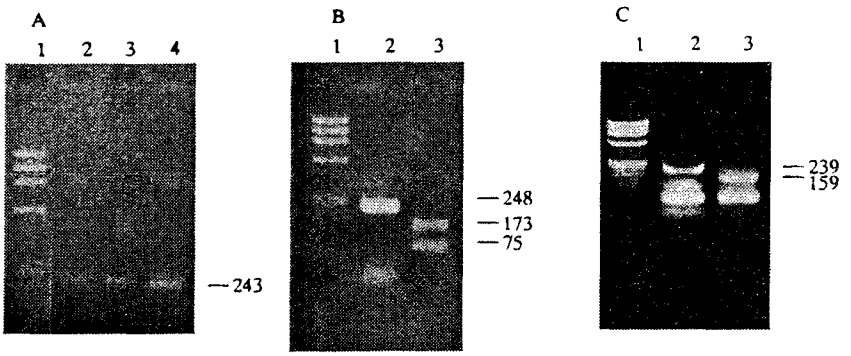

Fig. 1. Expression of mRNA for β-actin, the EGFR, and TGF-α by mouse blastocysts. RT-PCR was performed as described under Materials and Methods. (A) Transcripts for β-actin generated from 20, 30, and 50 blastocysts by the first 30 cycles in lanes 2, 3, and 4, respectively. (B) Undigested (lane 2) and *Pst*I-digested (lane 3) transcripts for EGFR were electrophoresed through agarose gel. (C) Undigested (lane 2) and *Sph*I-digested transcripts for TGF-α are shown. *Hae*III-digested ϕ × 174 DNA size markers were used in the left lane.

Effects of TGF-α Antisense Oligonucleotides on Blastocoel Expansion

Treatment of early cavitating blastocysts with 0.1 ng/ml TGF-α stimulated the rate of blastocoel expansion (Fig. 4). The preimplantation embryos tolerated the oligonucleotides well, and toxicity with control sense oligonucleotides was seen only above 50 μ*M*. Exposure to 10 μ*M* antisense oligonucleotides resulted in a significant decrease in the rate of blastocoel expansion. Sense oligonucleotides at the same concentration had no detectable effect on the rate of expansion (Fig. 4).

DISCUSSION

The results of RT-PCR showed that transcripts of the TGF-α and EGFR genes, but not of the EGF gene, were expressed by blastocysts. We stimulated mouse preimplantation embryos to increase the synthesis of DNA and protein by exposing them to TGF-α and EGF. TGF-α and EGF probably possess similar biological activities, because they bind to the same cell surface tyrosine kinase receptor (16). However, the results of this study suggested that TGF-α may be more potent than EGF. Autocrine effects of TGF-α on the rate of blastocoel expansion were examined using TGF-α antisense oligodeoxynucleotides. TGF-α antisense oligodeoxynucleotides, which were used to reduce the expression of specific gene, significantly reduced the rate of expansion. The results suggest that TGF-α may be an important autocrine factor in the regulation of embryonic development.

Table I. Development of Blastocysts Following Culture of Two-Cell Embryos in a Medium Containing TGF-α or EGF

Treatment (ng/ml)	No. of 2-cell embryos cultured	No. of blastocysts (%)
Control	335	299 (89)
TFG-α		
0.1	67	60 (90)
1	76	66 (87)
10	84	78 (93)
EGF		
0.1	65	55 (88)
1	111	96 (86)
10	131	116 (89)

In RT-PCR experiments, expression of β-actin mRNA was detected after the standard 30 cycles of PCR, while mRNA of TGF-α and EGFR was visualized by ethidium bromide staining only after an additional 30 cycles of amplification. These results were reproducible. TGF-α transcripts have been detected in the unfertilized mouse oocyte and are rapidly destroyed at the two-cell stage, but are later resynthesized by the preimplantation conceptus (1,17). In contrast, expression of the EGF gene is not observed until after implantation and the beginning of organogenesis (18–20). EGFR mRNA and protein have been identified throughout the development of the mouse embryo from the unfertilized oocyte (21) to the postimplantation embryo (22,23). Our observations are consistent with these reports.

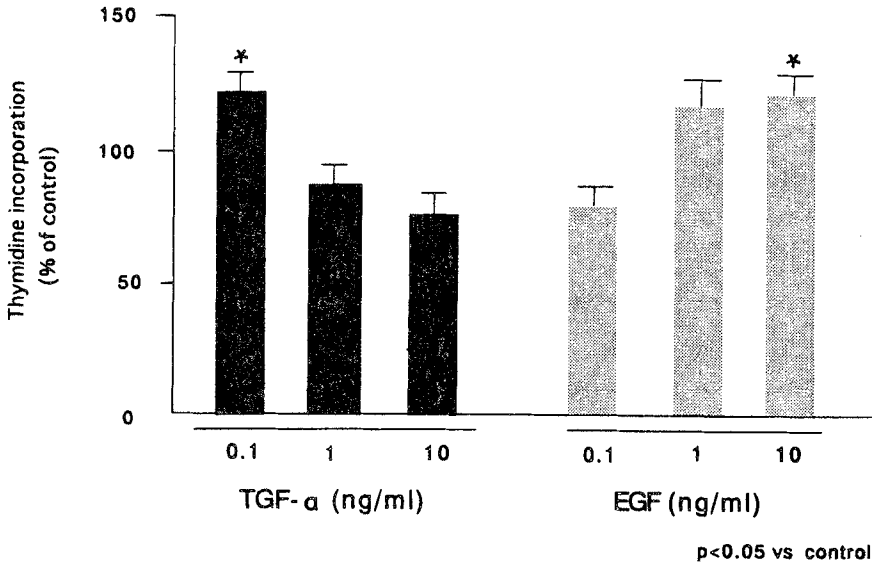

Fig. 2. Thymidine incorporation by blastocysts cultured in the medium containing various concentrations of TGF-α or EGF. Each sample consisted of three to five embryos. The asterisk indicates a significant difference compared to the control ($P < 0.05$). The data are expressed as mean ± SE.

The expression of EGF, TGF-α, and the EGFR has been reported in fallopian tubes of humans (8,24,25). It has been shown that the expression of EGF and TGF-α mRNA in fallopian tissue is cycle dependent and influenced by ovarian steroids (8,25). Expression of both GFs has also been demonstrated in mouse uterine epithelial cells (26,27). The results suggest that EGF and TGF-α are important autocrine/paracrine factors, being involved in a variety of tubal or uterine functions.

Several authors explored the effects of GFs on preimplantation embryo development by measuring the rate of in vitro development, the number of cells per blastocyst, or the number of implantations after transfer (28–30), but no stimulatory effects were detected. Our results for effects on the rate of blastocyst forma-

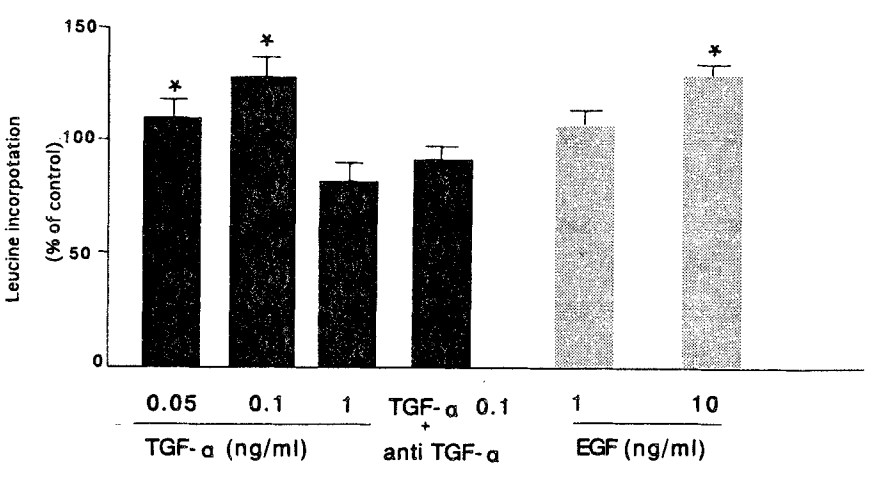

Fig. 3. Leucine incorporation by blastocysts cultured in a medium containing various concentrations of TGF-α or EGF. Anti TGF-α antibody was used for neutralizing the effects of TGF-α. Each sample consisted of three embryos. The asterisk indicates a significant difference from the control ($P < 0.05$). The data are expressed as mean ± SE.

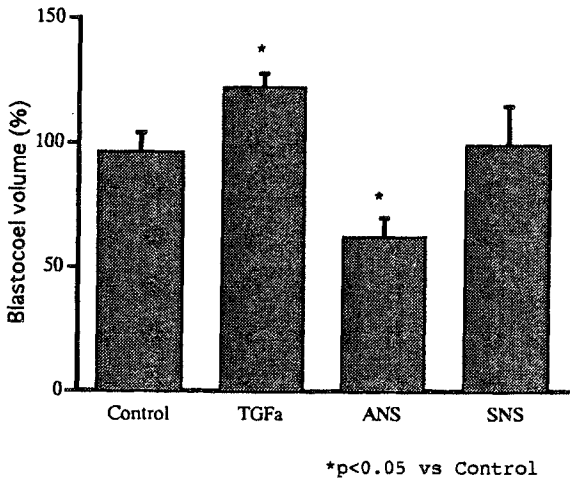

Fig. 4. Effect of TGF-α antisense oligonucleotide on blastocoel expansion. Early cavitating blastocysts were treated with 0.1 ng/ml TGF-α or antisense (ANS) or sense (SNS) oligonucleotide at a concentration of 10 μ*M*.

tion from two-cell stage embryos in GF-supplemented media were also not significant. The effects of TGF-α or EGF have been manifested as stimulation of protein synthesis (29), an increased rate of development of singly cultured embryos (6), and stimulation of the rate of blastocoel expansion (15). We used measurements of the incorporation of tritiated thymidine and leucine as indices of the synthesis of DNA and protein, respectively, because they can detect subtle differences in embryonic cell function (4,31). Our results showed that stimulation of the synthesis of DNA and of protein by TGF-α was detected at a lower concentrations (0.1 ng/ml) than was stimulation by EGF (10 ng/ml). Brucker *et al.* (32) reported that concentrations of TGF-α as low as 0.1 ng/ml, a level consistent with our observations, stimulated the breakdown of the enclosed mouse oocyte germinal vesicle. Although TGF-α and EGF exert similar effects in many systems, TGF-α appears to be more potent than EGF in terms of Ca^{2+} release (33,34), prostaglandin synthesis in vascular endothelial cells (35), and an increase in arterial blood flow (36) and in angiogenesis (37). However, differences in the binding of EGF and TGF-α to EGFR may alter the biological activities (38).

TGF-α is a 50-amino acid polypeptide derived from a 160-amino acid transmembrane precursor by specific proteolytic cleavage (39). Cultured preimplantation mouse embryos produced TGF-like bioactivity that promotes anchorage-independent growth (40). TGF-α activity is also produced by human embryos in culture at the time of transformation of morula to blastocyst (41). In light of these studies and present observations suggesting that TGF-α may have a role as an autocrine factor, we used TGF-α antisense oligonucleotide to reduce expression of the endogenous TGF-α gene. Antisense oligonucleotides have been used to reduce the expression and function of intracellulary expressed genes such as c-*myc* (42) and growth factors (43), and thus they are a proven method for decreasing gene expression in preimplantation mouse embryos (44). To examine the autocrine effects of TGF-α, we used TGF-α antisense oligonucleotide to supress TGF-α-mediated stimulation of the rate of blastocoel expansion. It has been reported that picomolar concentrations of exogenous EGF or TGF-α increase the rate of fluid uptake and of blastocoel expansion in the early cavitating blastocysts of preimplantation mouse embryos (15). In this study, TGF-α antisense oligonucleotides significantly reduced the rate of blastocoel expansion. These results suggest that TGF-α may be an important autocrine growth factor for preimplantation embryos.

In conclusion, EGF and TGF-α may be important in regulating the development of the mouse prior to implantation, acting via their common receptor. The stimulatory effects of TGF-α on embryonic cell function and expression of the TGF-α gene by mouse blastocysts lead to the hypothesis that TGF-α is an autocrine growth factor that may work in the local environment of the embryos. TGF-α antisense experiments suggest that TGF-α may be required for development and differentiation of the preimplantation embryo.

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