ANIMAL EXPERIMENTATION

Autocrine Effects of Transforming Growth Factor- α on the Development of Preimplantation Mouse Embryos

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Purpose: We wished to explore the role of transforming growth factor (TGF)- α in mouse embryonic development.

Methods: We examined the gene expression of TGF- α and epidermal growth factor receptor (EGFR) in mouse blastocysts by the reverse transcription-polymerase chain reaction and evaluated the effects of TGF- α on the development of preimplantation mouse embryos using TGF- α antisense oligodeoxynucleotide. Mouse teratocarcinoma F9 cells were also a subject of this study.

Results: Gene transcripts of TGF- α and EGFR were present in both blastocysts and F9 cells. TGF- α significantly stimulated the rate of blastocoel expansion in early-cavitating blastocysts and the proliferation of F9 cells. Northern blot analysis showed that TGF- α gene expression in F9 cells was markedly suppressed in the presence of TGF- α antisense oligodeoxynucleotide. TGF- α antisense oligonucleotide significantly reduced the rate of blastocoel expansion and the growth of F9 cells. The inhibitory effects of TGF- α antisense oligonucleotide on blastocysts and F9 cells were reversed by the addition of TGF- α .

Conclusions: The present observations suggest that TGF- α acts as an autocrine factor in the development of preimplantation mouse embryos.

KEY WORDS: transforming growth factor- α ; epidermal growth factor receptor; preimplantation embryo; teratocarcinoma F9 cell; antisense oligodeoxynucleotide.

INTRODUCTION

Transforming growth factor (TGF)- α is a 50-amino acid polypeptide derived from a 160-amino acid transmembrane precursor by specific proteolytic cleavage (1,2). TGF- α shares significant structural and functional homology with epidermal growth factor (EGF) and binds to EGF receptors (EGFRs), promoting intracellular phosphorylation and stimulation of the phosphatidylinositol pathway, leading to DNA synthesis and cellular division (3–5). A variety of normal and transformed cells expresses the TGF- α gene. The coexpression of TGF- α and EGFRs, at either the mRNA or the protein level, is often observed in normal and malignant cells, suggesting that the TGF- α /EGFR autocrine mechanism commonly operates in various types of cells (6).

Several growth factors have been implicated to have essential roles during embryonic development (7). During the preimplantation period, TGF- α or EGF stimulates embryonic development and increases cell proliferation of mouse blastocysts (8). TGF- α /EGF also accelerates the rate of blastocoel expansion in the preimplantation mouse embryo (9). Expression of TGF- α , but not EGF, and EGFRs has been demonstrated in the preimplantation mouse embryos (10–12). TGF- α is also produced by the maternal reproductive tract, which stimulates embryonic development (13). These data suggest a possible autocrine/paracrine regulatory mechanism of embryonic development by the TGF- α /EGFR system.

Antisense oligodeoxyribonucleotides have been shown to be valuable molecular tools for modulating

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gene expression in preimplantation embryos (14–18). In this study, we explored the role of TGF- α as an autocrine factor in mouse embryonic development using TGF- α antisense oligodeoxynucleotide.

MATERIALS AND METHODS

Growth Factors and Other Reagents

Recombinant human TGF- α was purchased from Becton Dickinson Labware (Bedford, MA). Monoclonal anti-TGF- α antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other culture-grade reagents were obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan) or Sigma Chemical Co. (St. Louis, MO).

Oligodeoxynucleotides

The designs of oligonucleotides used in this study, with the sequence position, are given below. Single-stranded phosphorothioate-derived oligodeoxynucleotides were designed to overlap the translation initiation codon of the mouse TGF- α mRNA (19). The selected antisense oligonucleotide corresponded to positions 1–15 of the TGF- α cDNA, and the corresponding complementary (sense) oligomer was used as a control for nonspecific effects. The oligonucleotides were synthesized by Nippon Gene Co., Ltd. (Toyama, Japan): TGF- α antisense, 5'-CGGGGACCATCTTCC-3', and sense; 5'-GGAAGATGGTCCCCG-3'.

Embryo Collection and Culture

Female B6C3F₁ mice were injected with 7.5 IU of pregnant mare serum gonadotropin (Peamex, Sankyo Zouki Co., Ltd., Tokyo) and 7.5 IU of human chorionic gonadotropin (hCG; Pregnyl; N.V. Organon, Tokyo) 48 hr apart. Immediately after the hCG injection, female mice were placed with male mice. Two-cell embryos were collected from the oviducts of the mated female mice 42 hr after hCG injection. Embryos were cultured to early blastocysts in Biggers–Whitten–Whittingham (BWW) medium containing bovine serum albumin (BSA; 0.1 mg/ml) at 37°C in an atmosphere of 5% CO₂ for 48 hr. Early blastocysts were then placed in medium containing TGF- α (0.05–1 ng/ml) and/or anti-TGF- α antibody (0.1 μ g/ml) and cultured for 6 hr to assess the rate of blastocoel expansion. In antisense experiments, early blastocysts were placed in 50- μ l drops of BWW medium under oil containing

either TGF- α antisense oligodeoxynucleotide at 10 μ M or sense oligonucleotide at the same concentration for the control.

Mouse teratocarcinoma F9 cells (F9 JCRB0721; Health Science Research Resources Bank, Osaka), which have characteristics similar to those of inner cell mass (ICM) cells, were seeded at $2\text{--}5 \times 10^5$ cells/ml in Dulbecco's modified Eagle's medium (DMEM) with 2% fetal bovine serum. To examine the effects of TGF- α on F9 cell proliferation, TGF- α (0.1–100 ng/ml), anti-TGF- α antibody (1 μ g/ml), or TGF- α antisense oligonucleotide (0.1–30 μ M) was added to the medium, and F9 cells were cultured for 48 hr.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from blastocysts (50 per group) and F9 cells by the guanidinium thiocyanate method as described by Chomczynski and Sacchi (20). The detailed procedures followed the guidelines provided by the manufacturer (Isogen, Nippon Gene). Reverse transcription–polymerase chain reaction (RT-PCR) was performed according to the methods in the manufacturer's manual (GeneAmp RNA PCR Core Kit; Perkin Elmer, NJ). Reverse transcription of RNA to cDNA was performed with 2.5 U/ μ l of MuLV reverse transcriptase. Thirty cycles of PCR amplification were carried out in 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl₂, and 1 U Taq DNA polymerase. Each cycle consisted of 0.5 min at 94°C, 0.5 min at 60°C, and 1.5 min at 72°C. The specific primer pairs were derived from published sequences for mouse TGF- α (19), EGFRS (21), and β -actin (22) cDNA sequences and were as follows: TGF- α —5' primer = 5'-CAGCTCGCTCTGCTAGCGCT-3'; 3' primer, 5'-TGGGCTTCTCATGTCTGCAG-3'; PCR-amplified cDNA = 409 bp; EGFR—5' primer, 5'-GGAGGAAAGAAAGTCTGCC-3'; 3' primer, 5'-CTGATGATCTGCAGGTTCTC-3'; PCR-amplified cDNA, 248 bp; β -actin—5' primer, 5'-TCGTGGGCGCCCTAGGCAC-3'; 3' primer, 5'-TGGCCTTAGGGTTCAGGGGG-3'; PCR-amplified cDNA, 243 bp. PCR products were resolved on 2% agarose gels along with a low molecular weight DNA marker (X174 digested with *Hae*III).

Measurement of the Rate of Blastocoel Expansion

Early-cavitating blastocysts were placed in culture medium containing TGF- α , anti-TGF- α antibody, or

oligodeoxynucleotides. Assuming that the blastocoel is prolate spheroid in shape, its volume was calculated by measurement of the area of the blastocoel cavity using an NIH Image analyzer. The embryos were then cultured for 6 hr and the rate of increase in blastocoel volume was measured.

Northern Blot Analysis

Cells were collected, and RNA was extracted as described above. Total RNA was size-fractionated by electrophoresis on 1% formaldehyde-agarose gels and transferred to nitrocellulose membrane. The membranes were baked at 80°C for 2 hr. Prehybridization was conducted for 6 hr at 42°C in buffer comprised of 50% formamide, 5 $\times$ SSPE (0.75 M NaCl, 50 mM sodium dihydrogenphosphate, 5 mM EDTA), 5 $\times$ Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% BSA, 0.1% Ficoll 400), 0.5% sodium dodecyl sulfate (SDS), and 0.2 mg/ml salmon sperm DNA. Hybridizations were conducted for 16 hr at 42°C in buffer that contained a mouse TGF- α cDNA probe (gift from Dr. J. C. Pascall) labeled with [α -³²P]CTP using a multiprime DNA labeling kit (Amasham Life Science, Tokyo). Thereafter, the blots were washed twice with 2 $\times$ SSPE and 0.1% SDS for 15 min at room temperature, once with 1 $\times$ SSPE and 0.1% SDS for 20 min at 65°C, and once with 0.1 $\times$ SSPE and 0.1% SDS for 20 min at 65°C. Autoradiography of the membranes was performed at -70°C using Kodak X-Omat AR film. The presence of equal amounts of total RNA in each lane was verified by visualization of ethidium bromide-stained 28S and 18S ribosomal RNA subunits and by analysis of β -actin mRNA, using a complementary DNA probe (Oncogene Science, Inc., MA) radiolabeled with [α -³²P]CTP. The autoradiographic bands were quantified using an NIH Image program. To compare TGF- α mRNA levels in F9 cells that had been exposed to antisense oligonucleotide, relative densities of TGF- α -specific mRNA signals were normalized with β -actin.

Statistical Analysis

Data are expressed as mean $\pm$ standard error of the mean (SE). Results were evaluated by the nonparametric Mann-Whitney test, with *P* less than 0.05 considered statistically significant.

RESULTS

Expression of the TGF- α and EGFR Genes in Mouse Blastocysts and F9 Cells

RT-PCR was performed to detect TGF- α and EGFR gene transcripts in blastocysts and F9 cells. The results showed that the predicted fragments of 409 bp for TGF- α and 248 bp for EGFR were amplified from total RNA isolated from blastocysts (Fig. 1A). Expression of TGF- α and EGFR mRNA was also detected in F9 cells (Fig. 1B).

Effects of TGF- α on Blastocoel Expansion and Growth of F9 Cells

The mean blastocoel volume in the control group increased from 25 ± 2 to 73 ± 3 pl after 6 hr of incubation; the rate of increase in blastocoel volume was $182 \pm 16\%$ (Fig. 2). Treatment of early-cavitating blastocysts with 0.1 ng/ml TGF- α significantly stimulated the rate of blastocoel expansion ($222 \pm 15\%$). The increase in the rate of TGF- α -mediated blastocoel expansion was abolished by the addition of 0.1 μ g/ml anti-TGF- α antibody ($168 \pm 20\%$). Anti-TGF- α antibody alone showed an inhibitory effect on the rate of expansion ($133 \pm 15\%$).

As F9 cells were also found to transcribe the EGFR gene, the effect of TGF- α on the growth of F9 cells was examined. The number of cells was recorded after 48 hr of culture. As shown in Fig. 3, the addition of TGF- α to the culture medium induced a significant increase in cell growth, and the maximum stimulation of twofold growth was obtained at a concentration of 10 ng/ml. The addition of anti-TGF- α antibody (1 μ g/ml) reduced the proliferation of F9 cells.

TGF- α Gene Expression in the Presence of TGF- α Antisense Oligodeoxynucleotide

Northern blot analysis was performed to confirm the effects of TGF- α antisense oligodeoxynucleotide on the reduction of TGF- α mRNA transcripts in F9 cells. As shown in Fig. 4A, transcripts of the expected size (4.5 kb) for TGF- α mRNA were detected. TGF- α antisense oligonucleotide markedly decreased the TGF- α mRNA level (Fig. 4B), suggesting that the antisense oligonucleotide bound precisely to TGF- α mRNA in F9 cells.

Fig. 1. Expression of the TGF- α and EGFR gene in mouse blastocysts and teratocarcinoma F9 cells. RT-PCR was performed as described under Materials and Methods. (A) Mouse blastocysts; (B) F9 cells. Lane 1 *Hae*III-digested DNA size markers; lane 2 TGF- α (409 bp); lane 3 EGFR (248 bp); lane 4 β -actin (243 bp).

Fig. 2. The effect of TGF- α on blastocoel expansion. Early blastocysts were cultured in BWB medium containing TGF- α (0.05–1 ng/ml) and/or anti-TGF- α antibody (0.1 μ g/ml) for 6 hr to assess the rate of blastocoel expansion. TGF- α (0.1 ng/ml) was used in combination with anti-TGF- α antibody (0.1 μ g/ml). C, control. Values are expressed as mean $\pm$ SE. * P < 0.05 vs control.

Effects of TGF- α Antisense Oligodeoxynucleotide on Growth of F9 Cells and Blastocoel Expansion

F9 cells were incubated with TGF- α antisense or sense oligonucleotides (0.1–30 μ M) for 48 hr. A 10 μ M concentration of antisense oligonucleotide significantly reduced cell growth (Fig. 5A). Cell toxicity of oligonucleotides was seen at a concentration of 30 μ M. The addition of 10 ng/ml TGF- α neutralized the

inhibitory effect of 10 μ M antisense oligonucleotide on cell proliferation (Fig. 5B).

Early blastocysts incubated with the TGF- α antisense oligonucleotide at a concentration of 10 μ M significantly decreased the rate of blastocoel expansion compared to the control blastocysts (111 ± 15 vs $155 \pm 12\%$) (Fig. 6). Sense oligonucleotide at the same concentration showed no detectable effect on the rate of expansion ($183 \pm 27\%$). The addition of 0.1

Fig. 3. The effect of TGF- α on the proliferation of F9 cells. F9 cells (2.4×10^5 /ml) were seeded in DMEM supplemented with TGF- α (0.1–100 ng/ml) or anti-TGF- α antibody (1 μ g/ml), and cell counts were performed after 48 hr of incubation. C, control. Values are expressed as mean $\pm$ SE. * P < 0.01 and ** P < 0.05 vs control.

Fig. 4. Reduction of TGF- α transcript in F9 cells treated with TGF- α antisense oligodeoxynucleotide. (A) Northern blot analysis of TGF- α mRNA in F9 cells. (B) Hybridization signals quantified by densitometry. Relative densities of TGF- α -specific mRNA signals were normalized with the corresponding β -actin. Each bar represents the mean $\pm$ SE. ANS, treated with TGF- α antisense oligonucleotide (10 μ M); SNS, treated with sense oligonucleotide (10 μ M); C, untreated.

ng/ml TGF- α reversed the inhibitory effect of antisense oligonucleotide on blastocoel expansion ($195 \pm 10\%$).

DISCUSSION

The results of the RT-PCR experiment showed that transcripts of the TGF- α and EGFR genes were coexpressed in mouse blastocysts and teratocarcinoma F9 cells. The rate of blastocoel expansion and the proliferation of F9 cells were enhanced by TGF- α . The autocrine loops of TGF- α and EGFR in blastocysts and F9

Fig. 5. The effect of TGF- α antisense oligodeoxynucleotide on the proliferation of F9 cells. (A) F9 cells (5.1×10^5 /ml) were seeded and cultured with TGF- α antisense or sense oligonucleotide (0.1–30 μ M) for 48 hr. (B) F9 cells (4.1×10^5 /ml) were seeded and cultured with TGF- α antisense oligonucleotide (10 μ M) and/or exogenous TGF- α (10 ng/ml) for 48 hr. ANS, treated with TGF- α antisense oligonucleotide; SNS, treated with sense oligonucleotide; C, control. Values are expressed as mean $\pm$ SE. * $P < 0.05$ vs control.

cells were effectively blocked using TGF- α antisense oligodeoxynucleotide. TGF- α antisense oligonucleotide, which was shown to suppress expression of specific gene function, significantly reduced the rate of blastocoel expansion and the proliferation of F9 cells. The addition of TGF- α reversed the inhibitory effect

Fig. 6. The effect of TGF- α antisense oligodeoxynucleotide on blastocoel expansion. Early blastocysts were cultured in BWB medium containing TGF- α antisense (10 μ M) or sense (10 μ M) oligonucleotide. ANS, treated with TGF- α antisense oligonucleotide; ANS + TGF- α , treated with TGF- α antisense and exogenous TGF- α (0.1 ng/ml); SNS, treated with sense oligonucleotide; C, control. Values are expressed as mean $\pm$ SE. * P < 0.05 vs control, ANS + TGF- α , and SNS.

of the antisense oligonucleotide on blastocoel expansion and growth of F9 cells. These results may suggest that TGF- α is an autocrine growth factor in the regulation of mouse embryonic development.

To evaluate the effects of TGF- α on the development of preimplantation embryo, we used F9 teratocarcinoma stem cells. The characteristics of F9 cells resemble those of ICM cells of mouse blastocysts (23), but F9 cells differ from ICM cells in their lower rate of spontaneous differentiation and limited differentiation potential, which enabled us to evaluate the effects of TGF- α on ICM cell function of mouse blastocysts.

TGF- α transcripts are detected in the unfertilized mouse oocyte and then rapidly destroyed at the two-cell stage, but later resynthesized by the preimplantation conceptus (10,24). EGFR mRNA and protein are present in the unfertilized oocyte (11) and throughout the development of the mouse embryo (25,26). Our observations in the present study are consistent with the previous findings. TGF- α -like activity has been found in the culture medium of both preimplantation mouse (27) and human (28) embryos.

TGF- α and EGF possess similar biological activities, and they bind to the same tyrosine kinase receptors that are expressed on the cell surface (29). We recently found that the EGF gene was not expressed in mouse blastocysts (12). EGFRs are present on the apical surface of the trophectoderm (TE), although the majority of the receptors is located on the basolateral surface. In addition, EGFRs are present on all surfaces of the

ICM. The majority of embryonic TGF- α is present in the ICM and polar TE (30). These results support the hypothesis that TGF- α , which is endogenously produced and secreted into the blastocoel, could act and stimulate functions of the ICM and TE. The effects of TGF- α on embryonic development in vitro have been studied extensively. TGF- α has stimulatory effects on DNA and protein synthesis in preimplantation embryos (12,31), on the rate of development of singly cultured embryos (8), and on the rate of blastocoel expansion (9). The formation of blastocoels depends on the fluid-transporting activities of the differentiated trophectodermal cells of the blastocyst (32), which requires an active Na⁺/H⁺ exchanger (33).

Single-stranded antisense phosphorothioate oligodeoxyribonucleotides appear to provide an attractive approach to inhibiting the translation of a specific gene in several cell systems in vivo and in vitro (34). Selective ablation of the targeted gene product in preimplantation embryos can be achieved by the addition of antisense oligonucleotides to the culture medium. Developmental arrest of mouse two-cell embryos at the morula stage and a significant reduction in the rate of embryonic cleavage division were observed in the presence of antisense to the *c-myc* (15) and *Ped* (17) genes, respectively. Ablation of the action of either insulin-like growth-factor II (14) or EGFRs (16) by the appropriate antisense oligonucleotide inhibits development to the blastocyst stage. The TNF- α p60 receptor antisense protected rat blastocysts from the inhibitory effect of TNF- α on embryonic cell proliferation (19). In this study, we found for the first time that TGF- α antisense oligonucleotide suppressed TGF- α -mediated stimulation of the rate of blastocoel expansion. This inhibitory effect was reversed by the addition of TGF- α . These results suggest that TGF- α is a possible autocrine growth factor for preimplantation embryos.

Expression of EGF, TGF- α , and EGFRs in human fallopian tubes has been reported (35). Expression of EGF and TGF- α mRNA in fallopian tissue is cycle-dependent and is influenced by ovarian steroids (13,36). Expression of EGF and TGF- α has also been demonstrated in mouse uterine epithelial cells (37,38). These findings suggest that TGF- α may influence the growth and differentiation of embryonic cells in both an autocrine and a paracrine fashion.

In conclusion, the stimulatory effect of TGF- α on mouse embryonic cell function and the expression of TGF- α and EGFR gene in mouse blastocysts led 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 also suggest that endogenous TGF- α may be required for development and differentiation of preimplantation embryos.

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