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Animal Experimentation

Influence of Transforming Growth Factor-α on Expression of Matrix Metalloproteinase-2, Matrix Metalloproteinase-9, and Epidermal Growth Factor Receptor Gene in the Mouse Blastocysts

Jeong Hee Kim,[1,2] Seok Ho Hong,[1,3] Hee Young Nah,[1] Ji Yun Lee,[1,3] Hee Dong Chae,[1] Chung Hoon Kim,[1] Byung Moon Kang,[1] and In Ha Bae[2,4]

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***Purpose*:** This study was carried out to investigate the influence of transforming growth factor-α (TGF-α) on the expression of mRNA for matrix metalloproteinase-2 (MMP-2), MMP-9, and epidermal growth factor receptor (EGFR) in mouse blastocysts and the effect on the production and activation of MMP-2 and MMP- 9 during blastocyst outgrowth.

***Methods*:** Two-cell mouse embryos were cultured for 96 h in the presence or absence of various concentrations of TGF-α. Reverse transcription–polymerase chain reaction (RT-PCR) was used to examine the expression of mRNA for MMP-2, MMP-9, and EGFR in in vitro cultured blastocysts. To investigate the effect on the production and activation of MMP-2 and MMP-9 during blastocyst outgrowth, the conditioned medium collected after 3 and 5 days of embryo culture were assayed for MMP activity by gelatin zymography.

***Results*:** The relative mRNA levels of MMP-2 and MMP-9 in blastocysts treated with TGF-α were higher than that of the control in a concentration-dependent manner. The relative mRNA level of EGFR in blastocysts treated with TGF-α was higher than that of the control. In conditioned medium collected after 3 days of embryo culture, TGF-α induced the gelatinase activities of proMMP-9 in all groups and activated MMP-2 in the 10 and 100 ng/mL TGF-α treated groups. In conditioned medium collected after 5 days, TGF-α induced the gelatinase activities of proMMP-9 in all groups and activated MMP-9 in the TGF-α treated group. TGF-α also induced the gelatinase activities of activated MMP-2 in the 1 and 10 ng/mL TGF-α treated groups and the control.

***Conclusions*:** These results suggest that the addition of TGF-α to in vitro culture medium is proper to create a favorable environment for preimplantation embryo development and implantation.

KEY WORDS: Matrix metalloproteinase-2; matrix metalloproteinase-9; mouse blastocyst; transforming growth factor-α; zymography.

INTRODUCTION

The implantation of the mammalian blastocyst requires extensively regulated tissue remodeling between maternal and fetal tissues. The embryonic trophoblast cells must cross the basement membranes of the uterine epithelium, connective tissue, and blood

[1] Department of Obstetrics and Gynecology, College of Medicine, Asan Medical Center, University of Ulsan, Seoul, South Korea.
[2] Department of Biology, College of Natural Sciences, Sungshin Women's University, 249-1 3ka Dongsun-dong, Seongbuk-ku, Seoul 136-742, South Korea.
[3] Department of Life Science, College of Natural Sciences, Hanyang University, Seoul, South Korea.
[4] To whom correspondence should be addressed; e-mail: ihbae@cc.sungshin.ac.kr.

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vessels to ensure successful implantation. Tissue remodeling is requisite to uterine preparation, embryo invading of the epithelial basement membrane, and subsequent preparation of the endometrial stroma. Convincing evidence has been presented to support the hypothesis that matrix metalloproteinases (MMPs) are essential for the breakdown of extracellular matrix (ECM) components during this process (1,2).

The MMPs are a multigene family of zinc-dependent proteinases divided into three subclasses based on substrate specificity: collagenases, stromelysins, and gelatinases (3,4). MMPs are secreted from the cell in latent zymogen form and activated by proteolytic processing and their substrates are the major component of the ECM. The latency is attributable to a conserved cysteine that binds to zinc at the active center of the molecule. In particular, MMP-2 and MMP-9 degrade denatured collagen (gelatin) and type IV collagen, a major component of basement membranes. It has been demonstrated that the invasiveness exhibited in vitro by the rodent and human trophoblasts depends on the production of MMP-9. The expression of this protein also coincides with the maximal invasive potential of the trophoblasts in vivo, which is exhibited in mice on Day 7.5 of gestation and in humans during the first trimester (2,5–7). Both MMP-2 and MMP-9 were secreted by human trophoblasts isolated from first trimester placentas. However, trophoblasts from third trimester placentas secreted primarily MMP-9; MMP-2 was secreted in minimal amounts (7). MMP-2 may play a role in several stages of implantation by promoting embryo hatching and controlled degradation of uterine ECM during trophoblast invasion (8). Several different mechanisms exist in order to exert stringent control over the degradation of the ECM by MMPs. These include control over transcription, translation, and secretion of MMPs. Although mechanisms regulating MMP gene expression need further elucidation, mounting evidence suggests that hormones, growth factors, and cytokines influence MMP levels during implantation. Although in vivo functional roles for cytokines have yet to be elucidated, leukemia inhibitory factor (LIF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and interleukin-1β (IL-1β) regulate MMP levels in cultured trophoblasts or blastocysts during outgrowth (9–11).

The epidermal growth factor receptor (EGFR) and its ligands are potential components of embryo–maternal cross-talk. Embryonic EGFR plays an essential role during implantation depending on the genetic background. Drastic negative effects on the inner cell mass, on placenta formation, and on viability of the offspring have been reported for EGFR knockout mice (12,13). Among the members of the EGF-like growth factor family, TGF-α has been implicated in implantation due to its expression in the uterus and embryo. Regulated developmental expression of TGF-α mRNA has been detected in the maternal decidua of the rat, in the decidua of the pregnant mouse, in murine placenta and embryos and in the developing mouse fetus, and human endometrium, decidua, and trophoblast (14–19). TGF-α enhanced mouse blastocyst attachment and trophoblast spreading and increased trophoblast surface in vitro (20,21).

To investigate whether there is a relationship between TGF-α and MMPs expression by blastocysts, we examined the expression of mRNA for MMP-2, MMP-9, and EGFR in the blastocysts cultured in various concentrations of TGF-α medium by reverse transcription–polymerase chain reaction (RT-PCR). We also investigated the effects of TGF-α on MMPs enzymatic activity during blastocyst outgrowth by gelatin zymography.

MATERIALS AND METHODS

Embryo Collection and Culture

ICR mice were bred in (Department of Laboratory Animal Research, Asan Institute for Life Sciences) and maintained on a 12L:12D cycle. Female mice (8–10-week-old) were induced to superovulate by injections of 5 IU pregnant mare serum gonadotropin (PMSG, Sigma, St. Louis, MO) and 5 IU human chorionic gonadotropin (hCG, Sigma) 48 h later. The female mice were then caged with male mice (12–16-week-old) and mating was confirmed next morning by visible vaginal plug. Two-cell embryos were collected from the oviducts 48 h after hCG injection and cultured in groups of 30–50 in microdrops (50 μL) of human tubal fluid (HTF) medium (22) containing 5% Serum Substitute Supplement (Irvine, CA) under autoclaved mineral oil (Sigma) in a humidified atmosphere of 95% O_2/5% CO_2 at 37°C for 96 h in the presence or absence of various concentrations of TGF-α (Sigma).

Evaluation of Embryo Development

Embryos were scored at 72 h for progression blastocyst and hatching blastocyst stage. The degree of blastocyst outgrowth was monitored at 96 h by visual

inspection. Embryos collected at the two-cell stage were routinely cultured in vitro to the blastocyst stage instead of being flushed from the uterus at the blastocyst stage, since a much higher yield of embryos is obtained this way. The in vitro culture period does not affect the process of development (23). Blastocysts were examined for their trophoblast outgrowth under a light microscope. Trophoblast outgrowth was identified as a lawn of cells extending out from the blastocyst. Their appearance was usually accompanied by a loss of the smooth, round blastocyst morphology (24).

RT-PCR

Total RNA was extracted from blastocysts (40 per group) cultured for 96 h after their colleciton by the guanidium thiocyanate method with some modification as described by Chomczynski and Sacchi (25). RNA was reverse transcribed (RT) by oligo (dT) priming and avian myeloblastosis virus (AMV) reverse transcriptase (Takara, Japan) and the cDNA derived from equivalent amounts of total RNA from four embryos was used in PCR to specifically amplify cDNAs of interest. The mRNA for β-actin might be expected to occur in all cells and has been given a positive RT-PCR product (16). The sequences of oligonucleotide primers for MMP-2, MMP-9, EGFR, and β-actin are given in Table I. The RT product was amplified in a PCR reaction mixture (40 μL) containing 4 μL of reverse transcribed cDNA, 1.25 mM $MgCl_2$, 0.25 mM dNTP, 10 pmol each of antisence and sense primers, 1.8 units of Taq DNA polymerase (Takara), and PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl). The PCR reaction was conducted in a Thermal Cycler (Crocodile III, Oncor, MD). After preheating at 94°C for 5 min, we performed the following sequential steps for 35 cycles (β-actin, EGFR: 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s; MMP-2, MMP-9: 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min). The final incubation was performed at 72°C for 10 min. The amount of template cDNA used in each reaction was normalized to the amount of β-actin mRNA. The amplified products were electrophoresed on 1.5% (w/v) agarose gels and visualized by ethidium bromide staining for quantitative identification of specific transcripts. Gel images were scanned with the Gel Documentation System (Gel Doc 1000; Bio-Rad, Hercules, CA), and relative densities were analyzed using the Multi-analyst finger printing program (version 1.1).

Zymography

Two-cell embryos were collected from the oviducts 48 h after hCG injection and cultured in groups of 20 in microdrops (50 μL) of HTF medium containing 0.4% BSA (Sigma) in the presence or absence of various concentrations of TGF-α. The conditioned medium was collected after 3 days of embryo culture (120 h after hCG) and assayed for proteinase activity. To prepare serum-free conditioned medium for SDS-substrate gel analysis, Dulbecco's modified eagle's medium (DMEM, Sigma) containing 5% fetal bovine serum (FBS, Gibco BRL) was replaced for 24 h to allow the embryos to attach to the culture dish and undergo trophoblast outgrowth. The culture medium was then replaced with HTF containing 0.4% BSA in the presence or absence of various concentrations of TGF-α. The conditioned medium was collected 24 h later and assayed (11). MMP activity in conditioned medium was determined by gelatin zymography with some modification (29). Conditioned medium was mixed with substrate gel sample buffer (10% SDS, 4% sucrose, 50% glycerol, 0.1% bromophenol blue, and 0.25 M Tris-HCl pH 6.8) and loaded into wells of a 4% acrylamide Laemmli

Table I. Primers Used for RT-PCR and the Size of Their Amplification Products

Gene	Primer	Primer sequence	Position on cDNA	Fragment size (bp)
β-actin[a]	Sence	5′-GTGGGCCGCTCTAGGCACCAA-3′	25–45	539
	Antisence	5′-CTCTTTGATGTCACGCACGATTTC-3′	541–564	
MMP-9[b]	Sence	5′-AGGCCTCTACAGAGTCTTTG-3′	1581–1600	824
	Antisence	5′-CAGTCCAACAAGAAAGGACG-3′	2386–2405	
MMP-2[b]	Sence	5′-GGCCATGCCATGGGGCTGGA-3′	1259–1278	761
	Antisence	5′-CCAGTCTGATTTGATGCTTC-3′	2001–2020	
EGFR[c]	Sence	5′-GGAGGAAAAGAAAGTCTGCC-3′	134–153	304
	Antisence	5′-CCCATAGTTGGATAGGATGG-3′	418–437	

[a] Rappolee *et al.* (26).
[b] Mark *et al.* (27).
[c] Paria *et al.* (28).

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

Concentration of TGF-α (ng/mL)	No. of embryos[a]	After 72 h		After 96 h
		Blastocysts (%)	Hatching blastocysts (%)	Outgrowth blastocysts (%)
0	158	93.0 ± 1.9	74.7 ± 2.0	11.4 ± 1.5
1	158	95.6 ± 1.5	75.9 ± 3.4	16.5 ± 2.6
10	163	98.2 ± 0.6	83.4 ± 2.3[b]	21.5 ± 4.9[b]
100	154	97.0 ± 2.8	87.4 ± 2.7[b]	22.1 ± 5.7[b]

Note. Results are mean ±SE.
[a] Four sets of replication.
[b] $p < 0.05$ vs. control.

stacking gel on a Mini-Slab gel apparatus (Hoefer mini gel, USA). Gelatin (Sigma) at a final concentration of 1 mg/mL was incorporated into the running gel containing 8% acrylamide; a stacking gel containing 4% acrylamide was layered. After electrophoresis, gels were soaked for 30 min in 2.5% Triton X-100 to remove SDS and reactivate the gelatinases and then overnight at 37°C in 50 mM Tris-HCl (pH 8.0) containing 5 mM $CaCl_2$ with or without 5 mM ethylenediaminetetraacetic acid (EDTA, Sigma), 5 mM 1,10-phenanthroline (Sigma), and 5 mM phenylmethylsulfonylfluoride (PMSF, Sigma) respectively. Gels were stained in 0.5% Coomassie Blue R-250 (Sigma) and destained to reveal clear regions indicative of proteolysis. Confirming that the gelatinase activities of blastocyst outgrowths were MMPs was shown by a complete inhibition of activity on gels developing in the presence of 5 mM 1,10-phenanthroline, 5 mM EDTA respectively but not in the presence of 5 mM PMSF, a serine proteinase inhibitor (30,31). The position of the two bands on the gels ($Mr = 105 \times 10^3$ and $Mr = 95 \times 10^3$) identified the activities as MMP-9. Gelatinase activity at $Mr = 95 \times 10^3$ represents the activated form of murine MMP-9 (11). The position of the band on the gel ($Mr = 62 \times 10^3$) identified the activities as activated MMP-2 (32).

Statistical Analysis

Data are expressed as mean ± standard error (SE) of the mean. Results were evaluated by chi-square test, with p less than 0.05 considered statistically significant.

RESULTS

The percentage of blastocysts formation in all the TGF-α treatment group was not significantly different from that for the control. The percentage of hatching blastocysts was significantly higher in the TGF-α treatment group at 10 ng/mL (83.4% ± 2.3%) and 100 ng/mL (87.4% ± 2.7%) compared to the control (74.7% ± 2.0%) ($p < 0.05$, Table II). The percentage of outgrowth blastocysts was significantly higher in the TGF-α treatment group at 10 ng/mL (21.5% ± 4.9%) and 100 ng/mL (22.1% ± 5.7%) compared to the control (11.4% ± 1.5%) ($p < 0.05$, Table II).

Figure 1 shows a typical gel electrophoresis of RT-PCR products for MMP-2, MMP-9, and EGFR

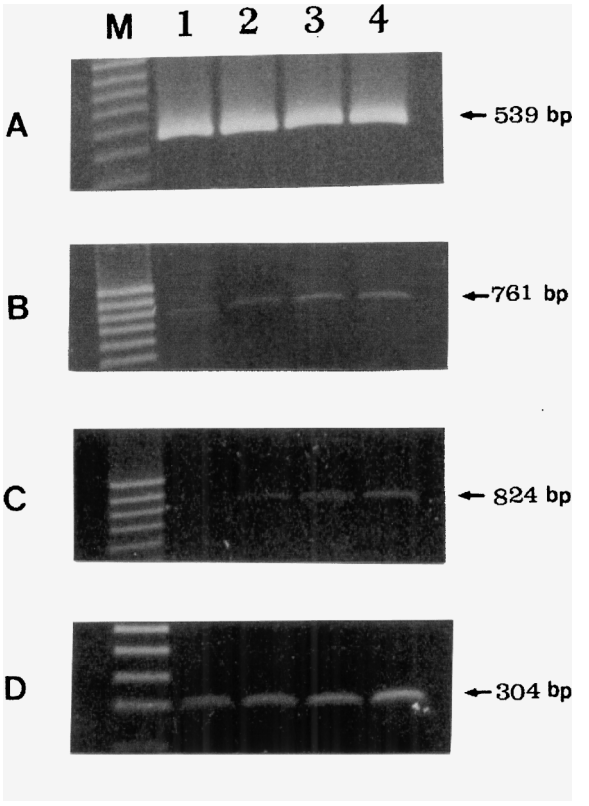

Fig. 1. Expression of mRNA for β-actin, MMP-2, MMP-9, and EGFR in mouse blastocysts. RT-PCR was performed as described under Materials and Methods. (A) β-actin; (B) MMP-2; (C) MMP-9; (D) EGFR. Lanes are **M** = 100 bp DNA ladder; **1** = control; **2** = 1 ng/mL TGF-α; **3** = 10 ng/mL TGF-α; **4** = 100 ng/mL TGF-α.

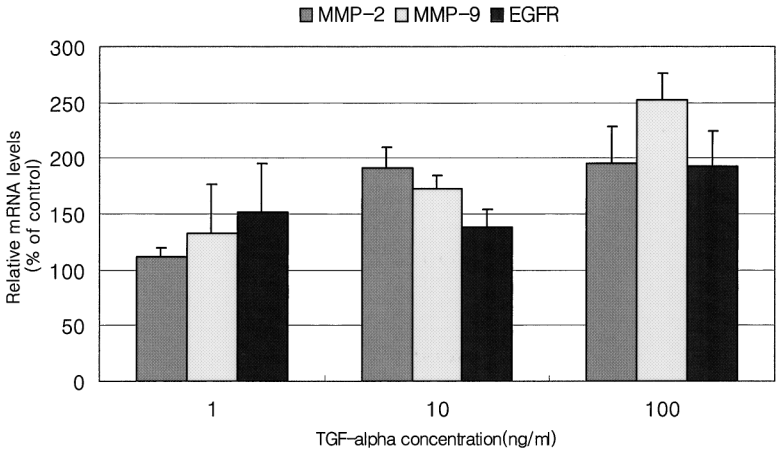

Fig. 2. Effect of TGF-α on the expression of mRNA for MMP-2, MMP-9, and EGFR in mouse blastocysts. β-actin mRNA was used as an internal control. The relative mRNA levels of MMP-2, MMP-9, and EGFR were calculated by normalizing the PCR products for MMP-2, MMP-9, and EGFR against the β-actin expression. Results are the mean ±SE from three individual experiments and are represented as a percentage of the control value.

from blastocysts. The relative mRNA levels of MMP-2 and MMP-9 in blastocysts treated with TGF-α were higher than that of the control in a concentration-dependent manner. The relative mRNA level of EGFR in blastocysts treated with TGF-α was higher than that of the control (Fig. 2).

Zymographic analysis of gelatin-degrading activities in conditioned medium from blastocyst is shown in Fig. 3. The major activity was identified as MMP-9 and MMP-2 by virtue of its size (MMP-9: major band at $Mr = 105 \times 10^3$, 95×10^3; MMP-2: major band at $Mr = 62 \times 10^3$). In conditioned medium collected after 3 days of embryo culture, TGF-α induced the gelatinase activities of proMMP-9 in all groups and activated MMP-2 in the 10 and 100 ng/mL TGF-α treated groups. In conditioned medium collected after 5 days, TGF-α induced the gelatinase activities of proMMP-9 in all groups and activated MMP-9 in the TGF-α treated group. TGF-α also induced the gelatinase activities of activated MMP-2 in the 1 and 10 ng/mL TGF-α treated groups and the control. All areas of lysis on gelatin gels were blocked by 1,10-phenanthroline and EDTA but not by PMSF, a serine proteinase inhibitor, indicating that the observed gelatinases were metalloproteinases.

DISCUSSION

The expression of several growth factors and their receptors in the preimplantation embryo and the maternal reproductive tract suggests that such factors may influence embryonic and uterine development and functions in an autocrine/paracrine manner (16,33). More recent studies suggest roles for the EGF family of growth factors in preimplantation embryo development and its implantation in the uterus. Members of EGF family are structurally related and they can interact with the receptor subtypes of the *erbB* gene family, which is composed of four receptor tyrosine kinases: ErbB1 (EGFR), ErbB2, ErbB3, and ErbB4. After ligand binding, these erbB receptor tyrosine kinase family form hetero- or homodimers that enable cells to respond to a variety of physiological stimuli (34–36). EGFR mRNA and protein have been identified throughout the development of the mouse embryo from the unfertilized oocyte to the postimplantation embryo (37).

Among the EGF family, TGF-α is a 50 amino acid polypeptide derived from a 160 amino acid transmembrane precursor by specific proteolytic cleavage (38). 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 and are expressed in large amounts in the mouse uterus during early pregnancy (15,16,39) Harada *et al.* (40) explored the effect of TGF-α on preimplantation embryo development by measuring the rate of in vitro development, but no stimulatory effects were detected. Our results for effects on the rate of blastocyst formation from the two-cell stage embryos in TGF-α supplemented media were also not significant.

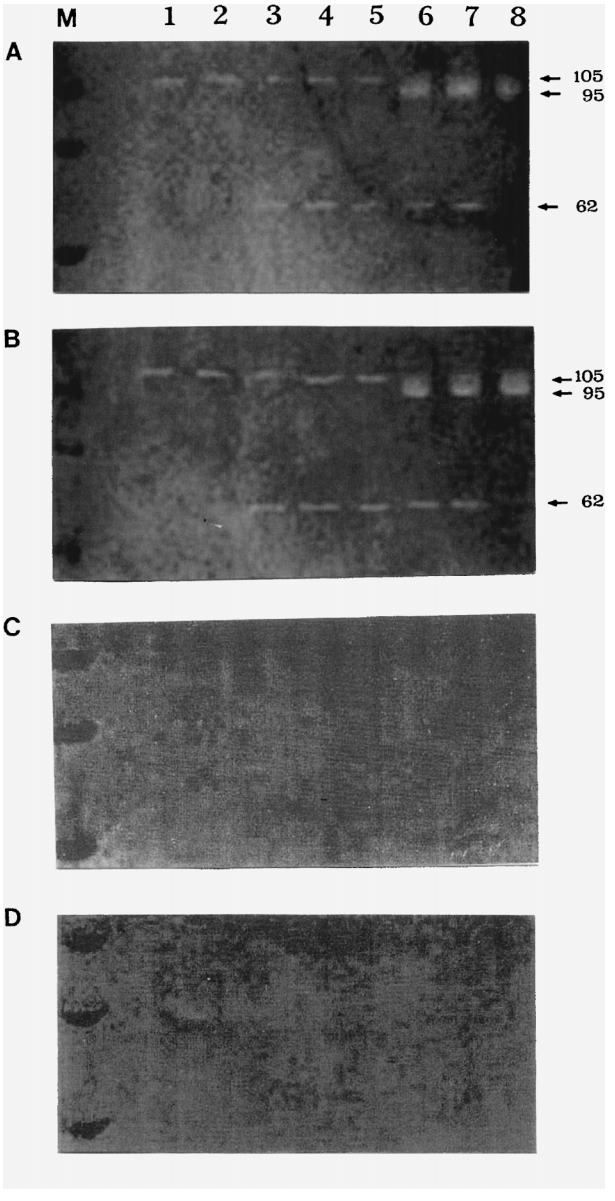

Fig. 3. Effect of TGF-α on blastocyst-derived MMP activities. (A)–(D) Gelatin zymography of conditioned medium collected after 3 and 5 days of embryo culture. (B) Gel was incubated throughout in the presence of 5 mM PMSF. (C) Gel was incubated throughout in the presence of 5 mM EDTA. (D) Gel was incubated throughout in the presence of 5 mM 1,10-phenanthroline. Inhibition of the gelatinase activities by EDTA and 1,10-phenanthroline confirmed that the proteins were MMPs. M = MW markers; Lanes 1, 5 = control; lanes 2, 6 = 1 ng/mL TGF-α; lanes 3, 7 = 10 ng/mL TGF-α; lanes 4, 8 = 100 ng/mL TGF-α; lanes 1–4 = conditioned medium collected after 3 days; lanes 5–8 = conditioned medium collected after 5 days. Molecular weight markers (kDa) are shown on the left. Gelatinolytic activities corresponding to proMMP-9 (105 kDa), activated MMP-9 (95 kDa), and activated MMP-2 (62 kDa) are indicated by the arrows.

The percentage of hatching blastocysts was significantly higher in the TGF-α treatment group at 10 and 100 ng/mL compared to that in the control. Several authors have shown the stimulatory effect of TGF-α on mouse blastocyst outgrowth in vitro (20,41). In the present study, the percentage of outgrowth blastocysts was significantly higher in the TGF-α treatment group at 10 and 100 ng/mL compared to that in the control. The mechanisms by which mitogenic and differentiating effects of TGF-α on preimplantation embryos are mediated are unclear.

For trophoblast cells to invade the uterine stroma, they have to degrade the components of the uterine epithelium basement membrane. Thus, enzymes capable of degrading these components, such as the MMPs, and plasminogen activators (PAs) are necessary for invasion. The most significant proteinases produced by the implanting embryo are uPA, stromelysin, MMP-2, and MMP-9 (42,43). Tissue invasion can be modulated at several levels. These include production of latent proteinases, activation of proMMPs via the uPAs/plasmin/MMP cascade, and the synthesis of specific inhibitors. All of these regulatory steps have been shown in a variety of cells to be positively and negatively influenced at the transcriptional and posttranscriptional levels by growth factors and cytokines. In previous work, both LIF and EGF induce uPA and MMP-9 production during trophoblast outgrowth. EGF induced MMP-9 but without leading to the appearance of activated forms of MMP-9. TGF-α has 40% sequence homology with EGF and possesses similar biological activities, because it binds to the same cell surface tyrosine kinase receptor (44,45).

In RT-PCR experiments, the relative mRNA level of EGFR in embryos treated with TGF-α was higher than that of the control. The relative mRNA levels of MMP-2 and MMP-9 in blastocysts treated with TGF-α were higher than that of the control in a concentration-dependent manner. This suggests that TGF-α might enhance the expression of EGFR, MMP-2, and MMP-9 mRNA and act directly on embryos, maybe in a paracrine manner.

In zymographic analysis, in conditioned medium collected after 3 days of embryo culture, TGF-α induced gelatinase activities of proMMP-9 in all groups and activated MMP-2 in the 10 and 100 ng/mL TGF-α treated groups. In conditioned medium collected after 5 days, TGF-α induced gelatinase activities of proMMP-9 in all groups and activated MMP-9 in the 1, 10, and 100 ng/mL TGF-α treated groups. TGF-α also induced gelatinase activities of activated MMP-2 in

the 1 and 10 ng/mL TGF-α treated group and the control. TGF-α was more effective than EGF in induction of gelatinase activities of the activated form of MMP-9. This is not surprising, since EGF and TGF-α bind differently to EGFR (46).

Analysis of the mechanism of implantation is very important because, in clinical practice, the success rate of pregnancy in in vitro fertilization and embryo transfer (IVF-ET) is still low. For obvious practical and ethical reasons, studies involving the use of peri-implantation embryos or trophoblast cannot be carried out in humans. Our study suggests that TGF-α may be important in embryo development at later stages and implantation, and therefore simple culture media may be inadequate for maintenance of embryo development at later stages and exogenous TGF-α supplementation may be beneficial. For this reason, the study of these growth factors on human preimplantation embryos and implantation may result in improved culture media for in vitro culture.

In conclusion, TGF-α might have a positive effect on embryonic development at later stages and implantation by production and activation of MMP-2 and MMP-9 in the embryo itself at the specific concentration. The exact mechanism of TGF-α associated with embryonic development, implantation, and MMPs expression remains unclear. Therefore, further investigations are needed to study the mechanism and functional role of TGF-α in the preimplantation embryo development and the implantation process.

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