Expression of Inhibin/Activin Subunits and Their Receptors and Binding Proteins in Human Preimplantation Embryos

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Submitted: July 27, 1998
Accepted: August 31, 1998

Purpose: Our purpose was to study the role of inhibin/activin during embryogenesis.

Methods: Transcripts of inhibin/activin subunits (α , β_A , β_B), activin receptors (types I and II), and follistatin were detected by a reverse transcriptase-polymerase chain reaction in human reproductive cells and preembryos cultured alone or co-cultured with human endometrial cells.

Results: Transcripts of α , β_A , β_B subunits were all detected in granulosa luteal cells, but only β_A units were detected in endometrial stromal and decidualized cells. In human preimplantation embryos, none of these subunits were detected in embryos from the four-cell to the morula stage and only β_A subunits were detectable in blastocyst embryos. Activin receptors were detectable in all of the studied embryos and cells. Transcripts of β_A , activin receptors, and follistatin were differentially expressed in human preimplantation embryos cultured in vitro and their expressions were significantly enhanced with the presence of endometrial stromal cells.

Conclusions: Our data suggest that there is a possible endometrium-embryo interaction via endometrial activins and preimplantation embryo receptors and that the embryonic expressions of these activins, their receptors, and binding proteins are dependent on embryonic stage.

KEY WORDS: activin; activin receptors; semiquantitative RT-PCR; gene expression; embryo-endometrium interaction.

INTRODUCTION

Inhibin and activin, members of the transforming growth factor (TGF)- β family, are gonadal glycoprotein hormones that regulate the secretion of follicle-stimulating hormone by the pituitary (1,2) and, thus modulate the secretion of sex steroids from the ovaries.

Inhibins are heterodimers consisting of one α and β_A , subunit (inhibin A) or one β_B subunit (inhibin B). Activins are either homodimers of two β_A subunits (activin A), or two β_B subunits (activin B), or heterodimers of one β_A and one β_B subunit (activin AB) (3). Activins and inhibins have diverse roles in the growth and differentiation of various cells including tissues of granulosa (4,5), decidual, and gonadotropic cells (6) and are believed to be involved in folliculogenesis, embryogenesis, and the physiology of pregnancy. Activin executes its bioactivity by interacting with activin type-I and type-II receptors (Act-IR and Act-IIR) (7,8). Its bioactivity is modulated by follistatin, an activin-binding protein (9,10). In animal studies, both inhibins and activins have been demonstrated to be effective stimulators of oocyte maturation (8) and potent inducers of mesoderm formation (11–13). However, their bioactivity in human oocytes and preimplantation embryos has not yet been clarified. In humans, inhibins/activins modify steroid and hormonal production in granulosa luteal (14) and placental cells in vitro. In addition, plasma immunoactive inhibin B has recently been shown to be a marker of granulosa cell maturation and a predictor of pregnancy outcome after in vitro fertilization (IVF)-embryo transfer (ET) (15). Thus, it is possible that inhibins/activins play a role in human oocyte maturation and early embryo development. Therefore, we conducted this study to observe the impact of activins/inhibins on human embryo development and to reveal the possible interaction between embryo and endometrium during implantation.

MATERIALS AND METHODS

Human Embryos, Endometrial Stromal Cells, and Granulosa Luteal Cells

Multiple embryos were harvested from patients undergoing IVF-ET. The best-quality embryos were

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selected for fresh ET and/or for frozen storage 3 days after harvest. The majority of the deselected embryos were poor in quality. They were slow growing ($\leq$ four-cell stage on day 3), highly fragmented ($>20\%$), degenerated, vacuolated, or arrested. In this study, deselected day-3 embryos were donated by patients after consent was obtained. This study was approved by the Committee on Human Rights in Research of The New York Hospital–Cornell Medical Center Institutional Review Board following the guidelines established by the Helsinki Declaration of 1989 on human experimentation (IRB-approved protocol 0995-9970).

Ten specimens were obtained from patients who have been subjected to biopsy for a variety of medical reasons. Endometrial stromal cells were separated from epithelial glands by digesting the tissue fragments (late secretory endometrium) with 0.02–0.05% collagenase II, as described previously (16). These isolated cells were cultured in Ham's F10 medium with penicillin sodium sulfate (50 U/ml; Gibco BRL), streptomycin (50 U/ml; Gibco BRL), plus 15% human serum in flasks and incubated at 37°C in a 5% CO₂ humidified air incubator. The medium was changed every other day for a week. The cells (5×10^5) were replated after treatment with 0.2 μ M ethylenediaminetetraacetic acid (EDTA; Sigma, St. Louis, MO) in Ca²⁺-, Mg²⁺-free Hank's balanced salt solution (HBSS; Gibco BRL) in tissue culture flasks (25 cm²). The replated cells were treated either with or without medroxyprogesterone (MPA; 0.2 μ M) for a week (16) before gene detection by reverse transcriptase (RT)–polymerase chain reaction (PCR) was performed.

The aspirated follicular fluid from four IVF-ET patients was used to isolate the granulosa luteal cells. After the removal of the aspirated oocytes, granulosa luteal cells were separated from the red blood cells through 50% Percoll (diluted with HBSS) (17). The isolated granulosa luteal cells (2×10^5) were used to study the gene expression by RT-PCR.

Co-culture of Human Embryos and Human Endometrial Stromal Cells

The endometrial cells isolated from endometrial biopsies were co-cultured with human embryos to observe the impact of endometrial cells on embryonic gene expressions. The endometrial stromal cells (5×10^3 cells per well) were cultured in 96-flat-well microliter tissue culture plates. Sixteen day-3 donated human embryos (ranging from two- to eight-cell stages) obtained from 11 patients were cultured alone

($n = 4$) or co-cultured ($n = 12$) with endometrial stromal cells in Ham's F10 medium plus 15% human serum for an additional 3 days. Of 12 co-cultured embryos, 3 remained at the two- to three-cell stage, 3 at the four-cell stage, 1 at the six- to eight-cell stage, 1 at the morula stage, and 4 at the blastocyst-stage after culture. Whereas, of the four cultured alone embryos, one was at the three-cell stage, one at the four-cell stage, one at the six- to eight-cell stage, and one at the morula stage. Whole embryos, used to study the gene expression by RT-PCR and the expressions of the co-cultured embryos ($n = 12$) at various embryonic stages, were further compared to study the temporal patterns of gene expressions in human preimplantation embryos.

Isolation of Blastomeres from Donated Human Embryos

To study the consistency of gene expressions among sibling blastomeres, the blastomeres from four donated human embryos (from four patients) at the four-, seven-, eight-, and nine-cell stages were isolated by digestion with protease (250 U/ml; Sigma, St. Louis, MO) for approximately 1 min. The isolated blastomeres were then washed well with phosphate-buffered saline (PBS) before detection of gene expression by RT-PCR.

RNA Extraction, RT-PCR, and Semiquantitative Comparison of Amplified Products

Recently, a semiquantitative RT-PCR detection method was developed in our laboratory to determine simultaneously multiple gene expressions in individual human preimplantation embryos (18). In brief, RNA of individual embryos was extracted by phenol–chloroform–isoamylalcohol (25:24:1) and further purified by deoxyribonuclease treatment, phenol–chloroform–isoamylalcohol extraction, and ethanol precipitation. The purified RNA was used for cDNA synthesis by M-MLV RT and random hexanucleotide or an oligo(dT) primer. Subsequently, the synthesized cDNA was amplified by PCR with Taq DNA polymerase and specific primers (Table II). The amplified products were then separated on 3% agarose gels and visualized by ethidium bromide staining for qualitative identification of specific transcripts. The intensity of the ethidium bromide fluorescence was measured with a photographic imaging system for semiquantitative analysis. The intensity of a specific stained product was related to the number of templates, shown by the

linear increase in the products amplified with rabbit globin mRNA templates from 0.125 to 2 pg/tube (Fig. 1). To ensure the consistency of the assay, external standards (with rabbit globin mRNA templates from 0.125 to 2 pg/tube) and external controls (with 1 pg/tube of rabbit globin mRNA templates) were performed along with the samples to monitor the inter- and intracoefficient variations. The recovery rate of extraction was also monitored by comparison of amplified products of external controls (1 pg/tube of rabbit globin mRNA) with or without the normal procedure of RNA extraction. Our data consistently showed that our recovery rate was greater than 90% and inter- and intracoefficient variation were 10% and 7%, respectively.

Statistical Analysis

Comparison of gene expression was analyzed by nonparametric alternatives of the Kruskal–Wallis

Fig. 1. Linear increase in the amplified products with the number of templates. Rabbit globin mRNA templates from 0.125 to 2 pg/tube were used to amplify as described in the text. The upper panel shows the identification of amplified products on 3% agarose gels. Lane M shows ladder markers and lanes 1 to 6 represent the amplified products of rabbit globin mRNA templates, ranging from 0.125, 0.250, 0.5, 1.00, 2.0, and 4.0 pg, respectively. The lower panel shows the linear relationship between the stained products and the amount of templates used. The intensity of ethidium bromide-stained products increased linearly according to the amount of templates used.

analysis of variance test. A *P* value of less than 0.05 defined the level of statistical significance.

RESULTS

Expression of α , β_A , and β_B Subunits; Activin Receptors (Type I and Type II); and Follistatin in Human Preimplantation Embryos and Reproductive Cells

Thirteen day-3 donated human embryos (ranging from the two- to the eight-cell stages) from 12 patients (shown in Table I) were cultured in Ham's F10 medium plus 15% human serum for an additional 3 days. The embryonic stage on day 6 was recorded and individual embryos were then subjected to detection of gene expression by RT-PCR. Meanwhile, pooled granulosa luteal cells from all aspirated follicles of the same patient as well as the endometrial stromal cells treated with or without progestin in vitro were also used to detect their gene expression.

The amplified products were identified by separation on 3% agarose gels (Fig. 2) and by restriction enzyme digestion analysis (data not shown). Table I shows the results of gene expression in human preimplantation embryos, granulosa–luteal cells, and endometrial stromal cells.

Expression of Activin-Related Genes in Co-cultured Embryos

To study the interaction between embryos and endometrium, the gene expression of human preimplantation embryos cultured with or without human endometrial stromal cells was detected by semiquantitative RT-PCR for comparison. Another 16 preembryos were cultured with or without endometrial stromal cells. The presence of human endometrial stromal cells significantly enhances the expression of β_A (12.5 ± 8.3 vs. 479 ± 255 ; $P < 0.01$), activin IIR (850 ± 65 vs. 1375 ± 272 ; $P < 0.005$), and follistatin (144 ± 87 vs. 291 ± 118 ; $P < 0.05$), but did not affect the expression of activin IR (70.3 ± 65.0 vs. 104 ± 61) (Fig. 3). The gene expression of embryos after co-culture was further subgrouped according to their embryonic stage to study the temporal patterns of gene expression in vitro (Fig. 4). The expression of β_A subunit, activin IR, and follistatin was barely detectable in embryos before the morula stage, but significantly increased at the blastocyst stage. On the other

Table I. Gene Expression of α , β_A , and β_B Subunits; Activin-IR and -IIR; and Follistatin in Human Reproductive Cells and Preimplantation Embryos

	α subunit (n) ^a	β_A subunit (n)	β_B subunit (n)	Activin-IR (n)	Activin-IIR (n)	Follistatin (n)
4- to 6- cell preembryo	-(5) ^b	-(5)	-(5)	+(5)	+(5)	-(5)
7-cell to morula preembryo	-(4)	-(4)	-(4)	+(4)	+(4)	+(4)
Blastocyst	-(4)	+(4)	-(4)	+(4)	+(4)	+(4)
Granulosa luteal cells	+(4)	+(4)	+(4)	+(4)	+(4)	+(4)
Endometrial stromal cells	-(10)	+(10)	-(10)	+(10)	+(10)	+(10)
P-treated endometrial stromal cells	-(10)	+(10)	-(10)	+(10)	+(10)	+(10)

^a Number of samples.^b +/-, positive/negative gene expression.

hand, the expression of activin IIR increased steadily from the two-cell to the blastocyst stage.

Single-Cell RT-PCR and Detection of Gene Expression in Individual Blastomeres

Embryos at the four-, seven-, eight-, and nine-cell stages were used to isolate individual blastomeres by protease treatment. Two sibling blastomeres were isolated from each studied embryo to detect the consistency of their gene expression. It appeared that our semiquantitative RT-PCR was sensitive enough to detect simultaneously three genes in a single blastomere. Judging from the mean and standard deviation of the gene expression in sibling blastomeres, we found that the expression of sibling blastomeres was relatively consistent and there was increased gene expression in blastomeres obtained from advanced stages (Fig. 5).

DISCUSSION

In this study, the role of activins/inhibins during human embryogenesis was studied. Transcripts of

inhibin/activin subunits, activin receptors (type I and type II), and follistatin in human granulosa luteal cells, endometrial stromal cells, and preimplantation embryos were detected by our semiquantitative RT-PCR method (18) to understand the possible interaction between the embryo and endometrium during implantation.

All inhibin/activin subunits, detected by RT-PCR and confirmed by restriction enzyme digestion mapping (data not shown), were highly expressed in the granulosa luteal cells, confirming previously published reports that these cells are able to synthesize and secrete both activins and inhibins (2,14). Whether the development of human oocytes is influenced by activin and inhibin from granulosa cells requires further study. In contrast, activin A mRNA is the only subunit detectable in fresh human endometrial stromal cells and in endometrial stromal cells decidualized by progesterone treatment *in vitro*. Transcripts of Act-IR, Act-IIR, and follistatin are also highly expressed in both granulosa luteal cells and endometrial stromal cells. Therefore, one could postulate that inhibins/activins in granulosa luteal cells and activin A in endometrial stromal cells may play a role in autocrine regulation of local biologi-

Table II. Primers and Conditions of Amplification

Gene	Primer	Amplicon primer sequence	Amplicon size (bp)	Annealing temperature (°C)	Cycle (N)
Inhibin α subunit	5'	5'-CCCGAGGAAGAGGAGGATGTC-3'	395	60	42
	3'	5'-CAGGTACAGAGGGGACAGC-3'			
Inhibin β_A subunit	5'	5'-GATGTACCAACTCTCAGCC-3'	393	55	40
	3'	5'-GAAGAGGCGGATGGTGACTT-3'			
Inhibin β_B subunit	5'	5'-GCGTTTCCGAAATCATCAGC-3'	337	55	40
	3'	5'-TGCACGTCTAGGTTGAGTCG-3'			
Activin-IR	5'	5'-TGGAGTGCTGCCAAGGGGACT-3'	252	58	40
	3'	5'-TTGTGGTGATGAGCCCTTC-3'			
Activin-IIR	5'	5'-GAAGACCCAGGGAAGTGGAT-3'	331	55	40
	3'	5'-CAGTCCTGTATAGCAGTTG-3'			
Follistatin	5'	5'-CCCAACTGCATCCCTGTAAA-3'	533	62	40
	3'	5'-CCAACCTTGAAATCCCATAAA-3'

Fig. 2. Identification of activin/inhibin subunits, activin receptors (type I, type II), and follistatin. Embryos at the four- to six-cell, seven-cell to morula, and blastocyst stages as well as granulosa luteal and endometrial stromal cells were used to detect simultaneously the transcripts of activin/inhibin subunits, activin receptors (type I and type II), and follistatin by RT-PCR. The amplified products were then separated on 3% agarose gels for identification.

cal function along with follistatin. Our data show that as human preembryos progress to the more advanced stages, more of the genes in question are transcribed. This is the first report of expression of activins and their receptors during early human embryogenesis. In human embryos at the four- to six-cell stage, only Act-IIR was transcribed; in the seven-cell to morula stages, Act-IR, Act-IIR, and follistatin were transcribed; while at the blastocyst stage, Act-IR, Act-IIR, follistatin, and activin A were all transcribed. Act-IIR appears to be the major activin receptor during early embryo development ($\leq$ six-cell stage). The availability of Act-IR after the seven-cell stage may change or diversify activin bioactivity by permitting the binding of activin A to both Act-IR and/or Act-IIR. Early embryos (i.e., four-cell to morula) are unable to synthesize activin and/or inhibin as no mRNA for these proteins was detected. Activins that bind to the Act-IR and Act-IIR on the preembryos are suspected to originate from surrounding cells such as the fallopian and/or endometrial cells. We postulate that the interaction of the preembryos with the endometrium may occur via activins produced by endometrial stromal cells binding to preimplantation embryo receptors. Our results obtained from human specimens were similar to those from the mouse. In mice, it was also found that β_A was expressed exclusively in maternal decidual tissues while activin IIR was exclusively detected in the embryos. In contrast, follistatin transcripts were present in both the decidua and the embryos during the early postimplantation stages (19,20).

Embryo viability has been proven to be improved in the presence of human endometrial stromal cells

Fig. 3. Presence of human endometrial stromal cells enhance the expression of activin-related genes in human preimplantation embryos. Gene expression of embryos cultured with (■) or without (□) human endometrial stromal cells was detected by semiquantitative RT-PCR. The expression of the studied genes was enhanced by the presence of endometrial stromal cells.

Fig. 4. Temporal patterns of gene expression in human preimplantation embryos. The gene expression of embryos after co-culture was subgrouped according to embryonic stage to study the temporal pattern of gene expression in vitro. The expression of β_A subunit, activin-IR, and follistatin was barely detectable in embryos before the morula stage, but significantly increased at the blastocyst stage. On the other hand, the expression of activin-IIR increased steadily from the two-cell to the blastocyst stage.

(21). Human endometrial stromal cells not only can secrete growth factors to nourish the embryos (21) but also can enhance embryonic expression of growth factor receptors (22), which is a potential marker for embryo quality (23). In this study, the presence of human endometrial stromal cells significantly enhanced the embryonic expression of β_A , and moderately enhanced the expression of activin IIR and follistatin, but did not affect the expression of activin IR at all. Whether the increase in these gene expressions is also a prerequisite for the stimulation of embryo development, or vice versa, requires further study.

In mice, it was found that the expression of activin subunits and follistatin was developmentally regulated, whereas the expression of active receptors was constitutional (24,25). In humans, we found that the expression of β_A subunits, activin IR, and follistatin was barely detectable in preimplantation embryos before the morula stage but significantly increased at the blastocyst stage. Therefore, the expression of β_A subunits, activin IR, and follistatin was dependent on embryonic stage. On the other hand, the expression of activin IIR

increased steadily from the two-cell to the blastocyst stage. Thus, the expression of activin IIR was constitutional and the presence of activin IIR in embryos before the six- to eight-cell stages (i.e., time of initiation of human genome transcription) was maternal in origin. However, it should be emphasized that these temporal changes observed in vitro should be taken in the context that embryo quality and in vitro laboratory conditions may have profound impacts on gene expression.

The preliminary results obtained from the few deselected poor-quality human embryos may shed light on the conclusions that (a) activins/inhibins synthesized by granulosa luteal cells and activin A synthesized by endometrial stromal cells may have autocrine regulation of these cell functions by interacting with their local receptors; (b) activin A, detected in embryos at the blastocyst stage, may play an important role in implantation and early embryo development; (c) the interaction of embryo and endometrium may occur via activin produced by endometrial stromal cells and activin receptors on the preimplantation embryo; and (d) the embryonic expression of activin and their recep-

Fig. 5. Detection of gene expression in human individual blastomeres at various embryonic stages. Individual blastomeres at the four-, seven-, eight-, and nine-cell stages were isolated for detection of the studied gene expression. The upper panel shows the identification of gene products by 3% agarose gel, and the lower panel shows the semiquantitative comparison of gene expression in blastomeres isolated from embryos at various embryonic stages. Each bar depicts the mean and standard deviation between two sibling blastomeres isolated from the same embryo.

tors is stage dependent and is affected by the embryo culture environment.

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