Estrous Cycle Independent Embryo Growth Promoting Activity of Mouse Uterus

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Abstract. To evaluate the intrauterine environment on embryo development, we examined the effects of co-culture with uterine epithelial cells and of uterine fluids on mouse embryo development *in vitro*. In the co-cultures of mouse uterine epithelial cells and 8-cell embryos, the rate of expanded blastocyst after 24 h of co-culture and the rates of hatching blastocysts 48 h and 72 h after the initiation of co-culture (29.4%, 56.7% and 78.9%, respectively) were significantly higher than those in the control (11.3%, 26.5% and 49.7%, respectively). The incorporation of L-[4,5-³H] leucine by the morulae after 24 h of co-culture was significantly higher than that of the control (an increase of 50.1%). The similar results were obtained in the cultures of 8-cell embryos in the medium added with uterine fluids. Moreover, the stimulating effect was exerted not only by uterine fluids from random stages of the estrous cycle but also by the fluids collected in three different stages, suggesting that the effect observed in this study might be independent of the estrous cycle. These results suggest that the intrauterine environment promotes the embryo development throughout the estrous cycle.

Key words: Mouse embryo, Co-culture, Uterine fluid, Estrous cycle independency.

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Human *in vitro* fertilization and embryo transfer (IVF-ET) has been in clinical use for more than 10 years. The rate of pregnancy, however, is generally low. It is necessary for improving the rate of pregnancy to increase our understanding the embryo and the uterus. As far as the timing and the method of ET are concerned, the present methods may not be based on such an understanding. At present, a majority of clinics perform intrauterine ET using the embryos in relatively early stage such as 2-cell or 4-cell, to make the *in vitro* culture period as short as possible. Some studies have also shown that pregnancy was achieved by intrauterine transfer of pronuclear stage embryos [1]. These studies suggest that even embryos in such an early stage can grow in the uterus, but the biological basis of ET at such a

stage is still obscure.

In this study, to evaluate the intrauterine environment on embryo development, we examined the effects of co-culture with uterine epithelial cells and of uterine fluids on mouse embryo development *in vitro*.

Materials and Methods

Mouse embryo recovery and culture conditions

Five units each of pregnant mare serum gonadotropin (PMSG; Teikoku Zoki Co., Tokyo, Japan) and human chorionic gonadotropin (hCG, Teikoku Zoki Co.) were intraperitoneally administered to 4-week-old female ICR mice at 48-h intervals to induce superovulation, and then the animals were housed with male mice of the same

strain overnight. Vaginal plug formation was confirmed in the next morning (Day 1 of pregnancy) and the mice with vaginal plugs were used for the experiments. Embryos were recovered from oviducts or uteri with phosphate buffered saline (PBS). The standard culture medium was Biggers-Whitten-Whittingham's (BWW) medium [2] and the standard culture condition was 5% CO₂ in air at 37C.

Culture of mouse uterine epithelial cells

According to the method of Tomooka *et al.* [3], the uteri of 3-to 4-week-old female ICR mice were excised and cut longitudinally to expose the endometrial surface. The uterine pieces were transferred to a disposable sterile 50-ml polypropylene tube and rinsed with the culture medium. The uterine pieces were incubated in 10 ml of a solution of 0.5% bovine pancreatic trypsin (Sigma Co., St. Louis, U.S.A.) in Ca- and Mg- free PBS. Tissues were first incubated at 4C for 60 min and then at 37C for another 40 min. After incubation, the tube was gently shaken, dissociating the epithelium from the uterine pieces. Sheet-like epithelial fragments in the supernatant were collected with a pipet. The epithelial fragments were subjected to Percoll gradient centrifugation for selection of epithelial tissues [4]. The culture medium was a 1: 1 mixture of Ham's F-12 and DMEM (Sigma Co., St. Louis, U.S.A.), which contained 10% fetal calf serum (Gibco, New York, U.S.A.). The concentration of Ca²⁺ in the medium was adjusted to 0.1 mM. A suspension of 4–5 × 10⁵ cells per ml was prepared, and 1 ml of the suspension were sprayed on collagen-coated 24-well multiplates (Corning, New York, U.S.A.). The culture condition was 5% CO₂ in air at 37C.

The cells became almost confluent after 3 to 5 days of culture. Observation with a phase-contrast microscope revealed that most of the cells showed an epitheloid pattern.

Incorporation of L-[4, 5-³H] leucine by mouse embryos

The incorporation of L-[4, 5-³H] leucine (specific activity, 5.0 TBq/m mol; Amersham Japan, Tokyo, Japan) was determined as an index of the amount of protein synthesized by mouse embryos. After embryos were cultured with BWW solution containing 1.85 MBq of L-[4, 5-³H] leucine per ml for 2 h at 37C, they were washed 3 times with

BWW solution containing a 1,000-fold amount of cold L-leucine (Nacalai Tesque Inc., Kyoto, Japan). Groups of 5 embryos were fused with 1N NaOH, which were then neutralized with 0.5% HCl. Ice-cooled 20% trichloroacetic acid (TCA; Nacalai Tesque Inc., Kyoto, Japan) was added to each group, and the radioactivity of the TCA insoluble fraction was determined with a liquid scintillation counter.

Collection of mouse uterine fluids

Eight to 10-week-old female ICR mice were used as fluid donors. According to the method of O'Neill and Quinn [5], the uteri were flushed with 50 µl of cold PBS from oviductal ends by using a 32-G injection needle, taking care not to introduce any contaminating fluid or cellular debris into the flushings, which were kept on ice during the collection period. Fluid from 30–40 animals was pooled and centrifugated at 70,000g for 60 min at 4C. The supernatant fluid was dialysed against pure water for 48 h (using the Visking dialysis tubing, size 36/32). Protein was quantitatively determined by the method of Lowry *et al.* [6], and the specimen was transferred into several glass tubes. The samples were then freeze-dried and stored at –76C until use.

Experiment 1: Co-culture of uterine epithelial cells and embryos

When the cultured uterine epithelial cells became confluent, the culture was washed with BWW solution 3 times, and the pronuclear stage embryos or 8-cell embryos, which were recovered 17 or 66 h after administration of hCG, were co-cultured in 1 ml of BWW solution containing 0.3% bovine serum albumin (BSA; Sigma Co., St. Louis, U.S.A.). The embryos were observed every 24 h with a phase-contrast microscope. The embryos cultured in BWW solution containing 0.3% BSA without epithelial cells served as the control.

After co-culture of the morulae recovered 72 h after administration of hCG with uterine epithelial cells for 24 h, the incorporation of L-[4, 5-³H] leucine by the embryos was determined by the method described above. The control of the experiment were the embryos which were cultured in BWW solution containing 0.3% BSA without epithelial cells for 24 h and used for the incorporation experiment. Moreover, the embryos which

were recovered 96 h after administration of hCG were immediately used for the incorporation experiment (*in vivo* group).

Experiment 2: Addition of uterine fluids from mice at random stages of the estrous cycle

Uterine fluids were collected from mice at random stages of the estrous cycle by the method described above. The amount of protein in the uterine fluids was adjusted to 1 mg/ml by adding BWB solution containing 0.3% BSA to glass tubes, and 100 μ l quantities were dropped into wells of a 4-well multidish (Nunc, Roskilde, Denmark). The pronuclear stage embryos or 8-cell embryos, which were recovered 17 or 66 h after administration of hCG, were cultured in the drops under mineral oil. The embryos were observed every 24 h with a phase-contrast microscope. The embryos cultured in BWB solution containing 0.4% BSA served as the control.

After the morulae recovered 72 h after administration of hCG were cultured in uterine-fluids-added BWB solution for 24 h, the incorporation of L-[4, 5-³H] leucine by the embryos was determined. The control of the experiment were the embryos which were cultured in BWB solution containing 0.4% BSA for 24 h and used for the incorporation experiment. Moreover, the embryos which were recovered 96 h after administration of hCG were immediately used for the incorporation experiment (*in vivo* group).

Experiment 3: Addition of uterine fluids from mice at different stages

Uterine fluids samples were taken at 3 different stages; (1) 20 h after administration of PMSG (5

units), (2) 20 h after administration of hCG (5 units) following PMSG (5 units) administration at 48-h earlier, and (3) on Day 4 of pregnancy following natural mating. After the morulae recovered 72 h after administration of hCG were cultured in BWB solution with one of these uterine fluid specimens for 24 h, the incorporation of L-[4, 5-³H] leucine by the embryos was determined. The embryos which were cultured in BWB solution containing 0.4% BSA for 24 h served as the control.

Results

Experiment 1: Co-culture of uterine epithelial cells and embryos

In the co-cultures of mouse uterine epithelial cells and 8-cell embryos, the rate of expanded blastocyst after 24 h of co-culture (Day 4) was 29.4%, which was significantly ($P<0.01$) higher than the 11.3% in the control, although there was no significant difference in the rate of blastocysts between the co-culture group and the control group on Day 4. The rates of hatching blastocysts 48 h (Day 5) and 72 h (Day 6) after the initiation of co-culture were 56.7% and 78.9%, respectively, which were significantly ($P<0.01$) higher than the 26.5% and 49.7%, respectively, in the control (Table 1). No effect of co-culture was observed on the development of pronuclear stage embryos (Table 2).

The incorporation of L-[4, 5-³H] leucine by the embryos after co-culture for 24 h was significantly ($P<0.01$) higher than that of the control (an

Table 1. *In vitro* development of 8-cell mouse embryos in co-culture with uterine epithelial cells

	No. of experiments	No. of embryos	No. (%) of embryos developed to			
			Blastocyst (Day 4) ^a	Expanded Blastocyst (Day 4)	Hatching Blastocyst (Day 5) ^b	Hatching Blastocyst (Day 6) ^c
Co-culture	6	194	105(54.1%)	57*(29.4%)	110*(56.7%)	153*(78.9%)
Control ^d	6	151	79(52.3%)	17 (11.3%)	40 (26.5%)	75 (49.7%)

Twenty five to 33 embryos were used per experiment.

^a Twenty four h after the initiation of co-culture.

^b Forty eight h after the initiation of co-culture.

^c Seventy two h after the initiation of co-culture.

^d Culture in BWB medium supplemented with 0.3% BSA.

* $P<0.01$, significantly different from the control (χ^2 test).

Table 2. *In vitro* development of pronuclear stage mouse embryos in co-culture with uterine epithelial cells

	No. of experiments	No. of embryos	No. (%) of embryos developed to	
			4-cell	Blastocyst
Co-culture	6	127	29 ^{NS} (22.8%)	5 ^{NS} (3.9%)
Control ^a	6	145	32 (22.1%)	4 (2.8%)

Twenty to 25 embryos were used per experiment. ^aCulture in BWW medium supplemented with 0.3% BSA. ^{NS}Not significantly different from the control (χ^2 test).

Table 3. Incorporation of L-[4, 5-³H] leucine by mouse embryos

Experimental groups		Incorporation (cpm/2 h/embryo) ^a
Experiment 1	co-culture	2909.6±123.3*
	control	1938.3±138.5
	<i>in vivo</i>	3696.1±129.2#
Experiment 2	uterine fluids ^b	2821.1±140.6*
	control	2152.6± 89.4
	<i>in vivo</i>	3763.3±134.6#
Experiment 3	uterine fluids; P	2865.6±138.6*
	uterine fluids; P-H	2800.3±170.1*
	uterine fluids; Day 4	2980.0±188.1*
	control	2083.8±116.1

^a Values are mean±s.e.m. for 9 replicates per experiment. ^b Collected from mice at random stages of the estrous cycle. P; 20 h after PMSG administration. P-H; 20 h after administration of hCG following PMSG administration at 48-h earlier. Day 4; on Day 4 of pregnancy following natural mating. *P<0.01, significantly different from the control (student's *t*-test). #P<0.01, significantly different from other groups (student's *t*-test).

increase of 50.1%), showing an increase in the amount of protein synthesized.

However, the level was still lower than that in *in vivo* group (P<0.01) (Table 3).

Experiment 2: Addition of uterine fluids from mice at random stages of the estrous cycle

In the cultures of 8-cell embryos in uterine-fluids-added medium, the rate of expanded blastocysts on Day 4 was 43.7%, which was significantly (P<0.05) higher than the 27.8% in the control, although there was no significant difference in the rate of blastocysts between the 2 groups on Day 4. The rates of hatching blastocysts on Day 5 and on Day 6 were 44.8% and 70.1%, respectively, which were significantly (P<0.01) higher than the 19.6% and 53.6%, respectively, in the control (Table 4). No effect of the addition of the uterine fluids was

observed on the development of pronuclear stage embryos (Table 5).

The incorporation of L-[4, 5-³H] leucine by the embryos after culture in uterine-fluids-added medium for 24 h was significantly (P<0.01) higher than that of the control (an increase of 31.0%), showing an increase in the amount of protein synthesized. However, the level was still lower than that in *in vivo* group (P<0.01) (Table 3).

Experiment 3: Addition of uterine fluids from mice at different stages

All of the cultures to which uterine fluids from different stages were added showed significantly (P<0.01) higher incorporation of L-[4, 5-³H] leucine than the control group, but there was no difference among the 3 groups (Table 3). No suppressive effect was observed in any culture to

Table 4. Effects of uterine fluids on *in vitro* development of 8-cell mouse embryos

	No. of experiments	No. of embryos	No. (%) of embryos developed to			
			Blastocyst (Day 4 ^a)	Expanded Blastocyst (Day 4)	Hatching Blastocyst (Day 5 ^b)	Hatching Blastocyst (Day 6 ^c)
Uterine fluids ^d added	4	87	72(82.8%)	38*(43.7%)	39*(44.8%)	61**(70.1%)
Control ^e	4	97	79(81.4%)	27 (27.8%)	19 (19.6%)	52 (53.6%)

Twenty to 25 embryos were used per experiment. ^aTwenty four h after the initiation of culture. ^bForty eight h after the initiation of culture. ^c Seventy two h after the initiation of culture. ^d Uterine fluids collected from mice at random stages of the estrous cycle. ^e Culture in BWB medium supplemented with 0.4% BSA. * P<0.05, significantly different from the control (χ^2 test). **P<0.01, significantly different from the control (χ^2 test).

Table 5. Effects of uterine fluids on *in vitro* development of pronuclear stage mouse embryos

	No. of experiments	No. of embryos	No. (%) of embryos developed to	
			4-cell	Blastocyst
Uterine fluids ^a added	4	82	28 ^{NS} (34.1%)	6 ^{NS} (7.3%)
Control ^b	4	78	28 (35.9%)	5 (6.4%)

Nineteen to 21 embryos were used per experiment. ^a Uterine fluids collected from mice at random stages of the estrous cycle. ^b Culture in BWB medium supplemented with 0.4% BSA. ^{NS} Not significantly different from the control (χ^2 test).

which uterine fluids were added.

Discussion

Co-culture of embryos and somatic cells is regarded as an *in vitro* model for determination of factors necessary for growth of embryos and of their interaction. Accordingly, attempts at co-culture between embryos in each stage and somatic cells of such organs as the oviduct and the uterus have been made in many species of mammals including the mouse. Some combinations are reported to stimulate the embryo growth [7]. Co-culture of embryos with uterine cells stimulated the growth of bovine morulae in some studies [8] and the growth of 8-cell mouse embryos in others [9]. Our results also showed that co-culture of embryos and uterine epithelial cells stimulated the growth of mouse embryos at least after 8-cell stage. The stimulating effect was observed as measured not only by morphological criteria such as the rates of blastocysts and hatching blastocysts but also biochemical indices such as

the amino acid incorporation. However, no stimulating effect was observed on pronuclear stage embryos. Rexroad and Powell [10] co-cultured one-cell ovine ova with oviductal cells and found the increased development of embryos. The same result was reached by Gandolfi and Moor [11]. Similarly, Noda *et al.* [12] reported that co-culture of pronuclear stage mouse embryos with hamster oviductal cells stimulated the embryo growth. Rexroad [7] stated that the type of cell that is optimal in co-culture with embryos may depend on the stage of development of the embryo. In the available literature, it seems that oviductal cells have been effective for 1-cell and 2-cell embryos and that uterine cells have been effective for morulae and blastocysts. Recently, we have reported that the first cell cycle must be protected from oxidative stress to overcome the blocking phenomenon that occurs in the following cell cycle [13, 14, 15]. The release of the antioxidant activity from somatic cells into the culture medium is considered to be highly likely in co-culture system. The difference of the biological functions between oviductal and uterine cells could be discussed from

this point of view.

Noda *et al.* [12] reported that the conditioned medium obtained by the culture of somatic cells exerted no such stimulating effect and reached the conclusion that the factor observed in co-culture was not a stable substance easily transferred from one culturing system to another such as a glycoprotein but was a substance of a very short life time that could exist only in a medium containing living cells. Anyway, there have been no reports on isolation of the embryo growth stimulating factor in any co-culture experiment.

Uterine fluids are believed to express the intrauterine environment directly surrounding embryos, they have been analyzed for a long time. The attempts at analysis are divided into chemical analysis and analysis using the embryo culture system. As for the first type of analysis, numerous reports have appeared over the last several years that deal with uterine secreted substances including steroids, prostaglandins, histamine and proteins [16, 17, 18]. Particularly, uterine secreted proteins are believed to be involved in the implantation and the interactions between the embryo and the uterus [19, 20]. However, there has not been good agreement about which proteins actually change in various species or what functions they subserve. As for the second type, some studies have shown that uterine fluids stimulate embryo growth under specific conditions [21, 22, 23, 24]. However, uterine fluids have also been reported to inhibit the incorporation of [³H]-uridine by mouse blastocysts [5, 25]. Hence, no definite opinion has been established on uterine fluids. Toole *et al.* [24] stated that substances in uterine secretions could have inhibitory and stimulatory influences on the early development of embryos. In our present experiments, however, the macromolecular components of uterine fluids exerted a certain stimulating effect on the *in vitro* growth of embryos at least after 8-cell stage, and the effect was observed morphologically and biochemically, showing a pattern similar to that observed in the co-culture experiments. Weitlauf [25] has shown that the inhibitory effect can be removed by dialysis. As far as the smaller molecular components, other analyses should be needed.

The most important point of this study is that the stimulating effect was exerted not only by uterine fluids in the specific stage but also by the

fluids collected in 3 different stages. These results suggest that the embryo stimulating effect of uterine fluids observed in this study may be independent of the estrous cycle. Although the nature of the stimulating effect remains unclear, it seems unlikely that uterus specific growth stimulating factors are involved, judging from the finding that a similar effect was observed throughout the estrous cycle. Several growth factor receptors such as insulin-like growth factor (IGF)-I-receptor and insulin-receptor are detected in mouse embryos from the 8-cell stage, although insulin or IGF-I mRNA are not found in preimplantation mouse embryos [26]. These growth factors might be involved in the stimulating activity of uterine fluids.

Thus, the intrauterine environment is considered to promote the embryo development throughout the estrous cycle. This estrous cycle independency may be the experimental confirmation of the fact that ET even in the non-physiological stage is providing pregnancies in human IVF-ET, although the rate is low. In mouse intrauterine ET, however, the combination of donor and recipient is believed to be the determinant of the rate of implantation. Some studies [27, 28] have shown that ET by an inappropriate combination yields only a limited rate of implantation while that by the appropriate combination yields a high rate. This estrous cycle dependency cannot be explained only by our present attempt at *in vitro* analysis of the intrauterine environment. To solve this contradiction, the fate of embryos transferred into the uterus by an inappropriate combination remains to be elucidated. The intrauterine environment should also be analyzed *in vivo* as well.

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