THE EFFECT OF RECOMBINANT HUMAN LEUKEMIA INHIBITORY FACTOR (rhLIF) ON IN VITRO DEVELOPMENT OF MOUSE 2-CELL EMBRYOS AND THEIR ISOLATED BLASTOMERES

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ABSTRACT

In this study effect of recombinant human leukemia inhibitory factor on invitro development of 2 cells embryos and isolated blastomeres derived from mouse 2 cell embryos were investigated. Female ICR mice that were between 8 to 10 weeks old received intraperitoneal injection of 7.5 IU of PMSG for super ovulation followed by intraperitoneal administration of 7.5 IU of HCG 48 hours later. The mice were then mated to mature ICR male mice and were checked for vaginal plugs after 13-14 hours. Mice were killed 46-48 hours after HCG injection by cervical dislocation, their oviducts were removed and flushing 2 cell embryos were collected. The zona pellucida of 2 cell embryos were removed by Acid Tyrod solution and blastomeres separated with oocyte preparation pipette and then all embryos and blastomeres were cultured in Potassium Simplex Optimized Medium (KSOM) +Aminoacid (AA) different amounts of rhLIF (500IU/ml, 1000IU/ml and 1500IU/ml). Some embryos and individual blastomere also were cultured without rhLIF as control group. All samples were cultured in an incubator at 37^0C with 0.05 CO_2 for 120 hours. The rate of embryo and individual blastomeres which reached to 2 cell, 4 cell, 8 cell and 9-16 cell were the same in all groups. However in further developmental stages, morula and blastocyst between experimental and control groups were significantly different. Therefore it may be concluded that: cultivation of isolated blastomers up to the blastocyst stage with rhLIF has stimulatory effect on the preimplantation stage (morula and blastocyst) but it has no stimulatory and inhibitory effects when was added to culture media at the early cleavage stage.

Keywords: rhLIF, Isolated blastomere, 2 Cell embryos and cleavage

INTRODUCTION

The developmental capacity of isolated blastomere from mammalian embryos has been studied extensively and has proved of great value in investigation of cellular and regulatory aspects of development in mouse (1, 2), rat (3), rabbit (4, 5), pig (6) and cattle (7). In human isolated blastomeres resulting from cleavage stage of embryo has been employed for assessment of the developmental potential of the parent embryo (8), to gain insight into regulatory mechanism in preimplantation development (9), and to increase available embryos that have been used for treatment of infertile couples (10).

Blastocyst and normal offsprings have been obtained upon transfer of isolated blastomeres derived from embryos of mice (2), rabbits (5), sheeps (11) and swins (12). However in vitro developments of isolated blastomeres are limited by suboptimal culture system (2, 5, 11, 12). For enhancement of in vitro developmental capacity of isolated blastomere several investigations including application of lamb serum (13) and fibronectin have been reported (14). High rate of development of blastocysts were achieved when the culture dishes were coated with fibronectin (14) or when the medium was supplemented with lamb serum (13). Leukemia inhibitory factor (LIF) which was first identified in 1986 is a pleiotropic cytokine of interleukin-6 family, that affects many different cell types and its activities is not restricted to cells of one line age (15). LIF is a 45-56 KD glycoprotein (16) secreted by endometrial glandular epithelium (17), ampullary portion of fallopian tube (18) and even embryo (19). This factor exerts a vast variety and paradoxical functions such as induction of the differentiation and suppression of clonogencity of the murine monocyte leukemia cell line M1 (20), induction of acute phase of protein synthesis (21), suppression of differentiation of the normal embryonic stem cells (22), stimulation of calcium

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release from bones (23) and establishment of cholinergic phenotypes in rat sympathetic neuron (24). Furthermore, female mice lacking a functional leukemia inhibitory factor gene are sterile because of the blastocyst formation and hatching in mouse (26) and bovine embryos (27) and subsequent implantation rate in sheep (28). The effects of LIF on mouse isolated blastomeres have not yet been reported. The purpose of this study was to investigate the effect of different concentration of rhLIF on in vitro development of mouse 2 cell embryos and to evaluate the potential of the single mouse blastomeres which were isolated from 2-cell embryos in KSOM+AminoAcid and different concentration of rhLIF for in vitro developement.

MARERIALS AND METHODS

Ovulation induction

Female mice ICR between 8 to 10 weeks old were administered intra peritoneally 7.5 IU of the pregnant mare serum gonadotropin (PMSG) for superovulation followed by intraperitoneal administration of 7.5 IU human chorionic gonadotropin (HCG) 48 hours later. The mice were then mated to mature male mice between 10 and 12 weeks old and checked for vaginal plug 13-14 hours later. Mice were then killed by cervical dislocation 46-48 hours after HCG injection. After disinfection with 70% alcohol and opening the abdomen wall, the Y shaped uterus, ovaries (below the kidney, lights yellowish) and oviducts (whitish color) were identified. The oviducts were excised as follow: clamping coronus, dissecting the peritoneum and fat between ovary and tube and then cutting the whole oviduct from the proximal end. After washing and flushing the oviduct from the ampullary portion of oviduct, the flashed 2-cells embryo could be selected and collected under 100X microscopy. The total number of 2-cell embryos witch were obtained from 72 stimulated donors were 1028 of which 359 embryos were used for isolation and out of 712 blastomeres, 663 were obtained as intact and cultured as following groups and totally 49 blastomeres were damaged.

1: KSOM+AA 2: KSOM+AA+LIF 500IU/ml AA
3: KSOM+AA+LIF 1000IU/ml AA
4: KSOM+AA+LIF 1500IU/ml

Twenty one 2-cells embryos out of 669 cells were excluded and the remaining were cultured in a similar fashion as described above.

Generation of isolated blastomeres

Zona pellucida were removed by exposure of 2 cell embryos for a few minutes in 0.5 ml of Acids Tyrod Solution (PH=2.2). Individual blastomeres were isolated by repeated pipetting of the zona pellucida free embryos in magnesium and calcium free PBS (6) with oocyte preparation pipette (REF 441281 Rheinbach). Out of 712 blastomeres derived from 2 cells embryos, 49 blastomeres were damaged and excluded and the remaining 663 blastomeres were allocated to different treatment groups for further experiments.

In vitro culture procedures

Individual blastomeres and embryos were cultured in vitro in a humidified atmosphere with 0.05 CO2 at 37^0 C for 120 hours. The medium was KSOM by following contents:

KCl(Sigma)186.4 mg/100ml, NaCl(Sigma) 555.2 mg/100ml, $CaCl_2,2H_2O$(Wako) 251.4 mg/100ml, KH_2PO_4 (Sigma) 47.6 mg/100ml, $NaHCO_3$ (Sigma) 219 mg/100ml, $MgSO_4,7H_2O$ (Sigma) 49.3 mg/100ml, Glucose(Sigma) 2.2 mg/100ml, EDTA-2Na (Sigma)100 mg/100ml, Na Pyruvate (Sigma) 2.2 mg/100ml , Glutamine (Sigma) 14.6 mg/100ml, Antibiotic Antimycotic (Gibco BRL) 10000IU/100ml, MEM essential amino acid solution (Sigma) 1ml/100ml, MEM non essential amino acid solution (Sigma) 0.5 ml/100ml and 1mg/ml Bovin Serum Albumin(Sigma). Embryos or blastomeres were cultured individually in 35 micro liter droplets under mineral oil in Petri dishes. The micro drop media of all embryos were replaced every day. Embryonic development was evaluated by morphological observation under an inverted microscope every day by the reported method (29). The grading system was according to following category.

Grade 1: Embryo with blastomeres of equal size and no cytoplasmic fragmentation. Grade 2: Embryo with blastomeres of equal size and minor cytoplasmic fragmentation covering ≤10% of the embryo surface. Grade 3: Embryo with blastomeres of distinctly unequal size and variable fragmentation. Grade 4: Embryo with blastomeres of equal or unequal size and moderate-to-significant cytoplasmic fragmentation covering >10% of the embryo surface. Grade 5: Embryo with few blastomeres of any size and severe cytoplasmic fragmentation covering ≥50% of the embryo surface. In the present study grades 1 and 2 were considered as included samples and all other embryos of grades 3, 4 and 5 were excluded. The number and Percentage of embryos and isolated blastomeres reaching to 2 cells, 4 cells, 8 cells, 9-16 cells, and morula and blastocyst stage were recorded.

Recombinant Human Leukemia Inhibitory Factor (rhLIF)

One of the three concentrations (500 IU/ml, 1000 IU/ml and 1500 IU/ml) of rhLIF (Sigma) was added to KSOM+AA. rhLIF was chosen for this investigation because it had previously been

shown to be active in pig (6), murine (30) and mouse (31) embryos.

Statistically Analyses

Further evaluations of development of isolated blastomeres were made on 2-cells embryos in different media (KSOM+AA), (KSOM+AA+ rhLIF 500 IU/ml), (KSOM +AA + rhLIF 1000 IU/ml), (KSOM+AA+ rhLIF 1500 IU/ml). The statistical analysis was performed by using the SPSS statistical package. The X^2 and logistic regression model tests were used for statistical analysis. A p-value of 0.05 was considered statistically significant.

RESULTS

General Observation

A total of 1028 of 2 cell embryos were collected from 72 stimulated donors (14.21+2.2 per donor) of which, 359 embryos were used for isolation of blastomere. From a total of 712 blastomeres, 47 embryos were damaged and excluded and the remaining 665 were obtained as intact and cultured in different groups.

The effect of different concentration of rhLIF on the in vitro development of 2-cell embryos and the percentage and number of 2 cell embryos reaching to stage of 4, 8, 9-16 cells, morula and blastocyst are present in table 1.

The effect of different concentration of human leukemia inhibitory factor on in vitro development of isolated blastomeres, the percentage and number of isolated blastomere reaching the stage of 2 cells, 4 cells, 8 cells, 9-16 cells, morulae & blastocysts are present in table 2.

DISCUSSION

Results of the present study indicate the capacity of the mouse embryo maintained in isolated blastomeres derived from mouse 2-cell embryos for rapid cell division (cleavage) and these blastomeres were able to develop in vitro into apparently normal looking blastocysts. The results of our study coincide with the result of the previous study (4). At least up to 16 cell stages of individual blastomeres were able to develop into apparently normal looking blastocysts. Blastocysts from blastomeres of the parent 2-cells embryos consistently possessed an inner cell mass showing that at this stage of development the mouse blastomere still have the inherent potential to contribute equally to trophoectoderm and inner cell mass (4). It is known that the proper embryo of mouse should be derived from the inner cell mass at the early cleavage stage (32, 33) and also normal appearing blastocyst have been obtained following nuclear transfer of ICM cells whereas no development was obtained when TE cells were used as donors in nuclear transfer (34, 35). In contrast, some investigations showed that not only inner cell mass nuclei but also trophoectoderm nuclei of mouse blastocysts have developmental totipotency (36). Leukemia inhibitory factor (LIF) plays an important role in the embryo development (26, 27 and 28) but there is controversy as to the effect of rhLIF on the different developmental stages of the embryo development. It has been demonstrated (37) that rhLIF enhances blastocysts formation and decrease embryo fragmentation in the 2-cells stage. In contrast, in another report (38) it has been shown that rhLIF in standard medium does not enhances the development of early embryos. In this investigation it was demonstrated that addition of rhLIF to culture medium increased mouse morula and blastocyst formation. These data are consistent with previous reports (26, 27 and 28) that rhLIF improve the development of preimplantation embryo. Furthermore 500 IU/ml is the lowest concentration of rhLIF that provided the optimal enhancement similar to 1000 IU/ml and 1500 IU/ml concentrations which was in agreement with the previous study (30). LIF has no stimulatory and no inhibitory effect on the development of early cleavage stage (2 cells, 4 cells, 8 cells and 9-16 cells) of mouse embryos.

The main reason for these effects may be due to the differences in requirements during different embryo developmental stages since as the embryo grows the requirement for growth factor and nutrients are increased. To our knowledge this is the first report on in vitro culture of mouse isolated blastomeres up to the blastocyst stage in KSOM+AA+rhLIF. In our study it was demonstrated that rhLIF enhances morula and blastocyst formation when it was added to KSOM+AA at morula stage and also, there was no stimulatory and inhibitory effects of rhLIF when it was added to the KSOM+AA at early cleavage stage (2 cells, 4 cells, 8 cells and 9-16 cells stage) of blastomere derived embryo.

CONCLUSION

According to results of this study rhLIF has positive effects on preimplantation embryos development (morula and blastocyst) and has no significant influence on the early embryo development (2 cells, 4 cells and 9-16 cells) in KSOM medium. Furthermore, rhLIF has the same effects on the preimplantation stage (morula and blastocyst) of the isolated blastomeres of 2 cells embryos. For further studies of LIF effects on the isolated blastomeres in culture system, the use of LIF knock-out animals is recommended.

Table 1. Development of 2 cells embryos in 4 groups

	2cell	*Replicant*	*4cell*	*8cell*	*9-16cell*	*morula*	*b-cyst*
KSOM+AA Group I	165	10	158 (95.75%)	150 (90.90%)	129 (78.18%)	82 (49.69%)	65 (39.39%)
KSOM+AA+ LIF500IU/ml GroupII	161	10	149 (92.54%)	138 (85.71%)	129 (80.12%)	100 (62.11%)	94 (58.38%)
KSOM+AA+ LIF 1000IU/ml Group III	159	10	151 (94.96%)	142 (89.30%)	123 (77.35%)	105 (66.03%)	95 (59.74%)
Ksom+AA+ LIF 1500IU/ml Group IV	163	10	153 (93.86%)	143 (87.73%)	130 (79.75%)	104 (63.80%)	95 (58.28%)
P value Group II, III, IV versus Group I	---	--	0.624	0.503	0.92	0.012	<0.0001

The percentage of 2 cells embryos reaching to stages of 4, 8, 9-16 cells in all groups were not significantly different (P>0.05). Morula and blastocyst formation in groups II, III and IV were higher than group I (P≤0.05). The higher proportion of blastocyst was obtained by KSOM+AA+ rhLIF 1000 IU/ml and higher proportion of blastocysts were obtained in KSOM+AA+1000 IU/ml but the formation of morulae and blastocysts in groups II, III, IV were not significantly different (P>0.05)

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Table 2. Development of isolated blastomere between 4 groups.

	Blastomeres	*Replicant*	*2cell*	*4cell*	*8cell*	*9-16cell*	*morula*	*b-cyst*
KSOM+AA Group I	166	10	122 (73.49%)	99 (59.63%)	88 (53.01%)	79 (47.59%)	56 (33.73%)	41 (24.69%)
KSOM+AA+ LIF 500IU/ml GroupII	171	10	119 (69.59%)	106 (61.98%)	95 (55.55%)	90 (52.63%)	82 (47.95%)	69 (40.58%)
KSOM+AA+ LIF 1000IU/ml Group III	161	10	115 (71.42%)	104 (64.59%)	90 (55.90%)	84 (52.17%)	78 (48.44%)	70 (43.47%)
KSOM+AA+ LIF 1500IU/ml Group IV	165	10	118 (71.51%)	103 (62.42%)	94 (56.96%)	87 (52.72%)	79 (47.87%)	71 (43.03%)
P value Group II, III,IV versus Group I	---	--	0.890	0.83	0.904	0.748	0.016	0.001

The percentage of the isolated blastomere reaching the stage of 2 cells, 4 cells, 8 cells, 9-16 cells in all groups were not significantly differences (P>0.05). Morula and blastocyst formation in groups II, III, IV were higher than group I (P≤0.05). The higher proportion of morula was obtained in KSOM+AA+ rhLIF 1500 IU/ml and higher proportion of blastocyst was obtained in KSOM+AA+ rhLIF 1000 IU/ml but the formation of morulae and blastocysts in groups II, III, IV were not significant differences (P>0.05).

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