Developmental potential of isolated blastomeres from early mouse embryos in the presence and absence of LIF and GM-CSF

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Received: 16 September 2007 / Accepted: 22 November 2007 / Published online: 18 January 2008
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Abstract

Purpose The aim of this study was to investigate the developmental potential of isolated blastomeres in the presence and absence of leukemia inhibitory factor (LIF) and granulocyte–macrophage colony stimulating factor (GM-CSF).

Methods The blastomeres of two (1/2) and eight cells (1/8) embryos were isolated and cultured in T6 medium in the presence and absence of LIF (1,000 IU/ml) and or GM-CSF (2 ng/ml) up to 120 h. The diameter and cell number of blastocysts were measured.

Results The developmental rates of 1/2 isolated blastomeres developed to blastocysts stages in the presence and absence of LIF and GM-CSF were 45.80, 35.10 and 48.66, 41.66, respectively. The diameter of blastocysts was higher in GM-CSF group and total cell number of blastocyst in both treated groups was higher than control ($P < 0.05$). No 1/8 blastomeres developed to morula and blastocyst stages.

Conclusions LIF and GM-CSF could improve the development of 1/2 isolated blastomeres.

Keywords Granulocyte–macrophage colony stimulating factor · Leukemia inhibitory factor · Isolated blastomere

Introduction

The developmental capacity of isolated blastomeres from several species of mammalian embryos has been studied in different types of studies [1–5]. Isolated blastomeres derived from mammalian embryos have been used for particular purposes such as multiplication of genotypes of valuable animals, DNA probe for sex determination or screening for the transmission of genetic disease. The efficiency of pre-implantation genetic diagnosis (PGD) could be improved by increasing the amount of material for testing by proliferation of the single biopsied blastomeres in culture [6].

The investigations on development of mammalian blastomere have shown that single blastomeres derived from 2- to 8-cell embryos have the capacity to give rise to normal embryo but the in vitro developmental potential of isolated blastomeres is limited by non optimal culture system [5, 7].

By several investigators an alternative procedure was introduced to improve the rate of in vitro embryo development to blastocysts stage using supplementation of medium with growth factors and cytokines including leukemia inhibitory factor (LIF) [8–11] and granulocyte–macrophage colony stimulating factor (GM-CSF) [12–16].

LIF and GM-CSF are expressed and secreted by the female reproductive tract of mammals and affect the development of pre-implantation embryos [17–20]. Several cell lineages in the reproductive tract and gestational tissues synthesize LIF and GM-CSF under direction by ovarian steroid hormones [18].

Leukemia inhibitory factor (LIF) is the main cytokine having a role in implantation, embryo development and facilitating endometrial receptivity [20, 21]. It is expressed at high level in the mouse uterus near the time of ovulation and implantation [21, 22].

Capsule GM-CSF improved the developmental potential and the quality of isolated blastomeres derived from mouse two cells embryo.

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The GM-CSF is a lymphohemopoietic factor synthesized in uterus of non pregnant mouse (peak in estrus) and maintained at comparable or moderately higher level during the pre-implantation period of pregnancy [23]. Epithelium of uterine and oviduct is the major site of GM-CSF secretion [24] and its targets in this site are myeloid leukocytes, pre-implantation embryo and trophoblast cells [25, 26].

It has been shown that GM-CSF and LIF are embryo trophic in mammals and several studies have shown the supplementation of embryo culture media with LIF increase blastocyst hatching and the implantation rate and also decrease embryo degeneration [8–16].

The embryos should retain its developmental abilities after removing some cells. However, it is not clear if there is any difference between developmental potential of isolated blastomere derived from different stages of pre-implantation embryos. Also, the effect of LIF and GM-CSF on the development of mouse isolated blastomeres has not been investigated so far. Therefore, the purpose of this study was to investigate and compare the developmental potential of isolated blastomeres from two and eight cell embryos in the presence and absence of LIF and GM-CSF.

Materials and methods

Collection of two and eight cell mouse embryos

The NMRI mice were cared for and used according to the guide for the care and use of laboratory animals of the university and housed under a 12 h light:12 h dark regimen. A total of 40 adult female mice with 6–10 weeks old were superovulated using 10 IU injection of pregnant mare serum gonadotropin (PMSG, Folligon; Intervet) followed with another injection of 10 IU human chorionic gonadotropin (hCG, Sereno, Switzerland) hormone 48 h later. Following hCG injection, one or two females were caged with NMRI male mice (8–10 weeks old). The presence of vaginal plug was examined in the following morning. To collect two cell and eight cell embryos, the pregnant females were sacrificed by cervical dislocation at 48 and 72 h post hCG injection respectively.

After removing the fallopian tube, the embryos were flushed in the T6 medium supplemented with 5 mg/ml bovine serum albumin (BSA, Sigma, Germany). Then they were washed and placed into drops of T6 culture medium under mineral oil (Sigma, Germany) at 37°C with 5% CO₂. Morphologically intact embryos were used for the experiments.

Blastomeres isolation

The blastomeres were isolated mechanically (by gentle pipetting with a flame-polished micropipette). Blastomeres

derived from two cell embryos designated 1/2 and those from eight cell embryos as 1/8 blastomeres. The blastomeres were washed in culture medium and then were placed in 20 µl droplets under mineral oil.

In vitro culture and evaluation of the development of isolated blastomeres

The isolated blastomeres from two and eight cell embryo were cultured in T6 medium supplemented with 5 mg/ml BSA with or without recombinant mouse recombinant LIF (1,500 IU, Sigma, USA) [9] or recombinant GM-CSF (2 ng/ml, R & D systems) [16].

The blastomeres were cultured in vitro in a humidified atmosphere with 5% CO₂ in air at 37°C. The single isolated blastomeres were cultured to 96 h after isolation to obtain a similar end point in all groups. The development of blastomeres was assessed daily under inverted microscope.

After culturing, the diameter of blastocysts was measured with a calibrated eyepiece. Then the blastocysts were fixed and stained to count the number of cells. For each group, between 5 and 10 replicate experiments were performed.

Blastocyst staining

For blastocyst staining, the embryos were incubated in 500 µl solution of BSA free T6 with 1% triton x-100 and 100 µg/ml propidium iodide up to 10 s or until trophoctoderm visibly changed color to red and shrank slightly, as monitored visually using a dissecting microscope. Blastocysts were transferred immediately into 500 µl of fixative solution containing 100% ethanol with 25 µg/ml bisbenzimidazole stored at 4°C overnight. The stained embryos were put into glycerol and mounted on the glass microscope slide [27]. Then cell counting was performed under fluorescent microscope in 2 or 3 planes.

Statistical analysis

Differences between the developmental rates, blastocysts formation and survival rates in all groups were analyzed by the Chi-square test. The blastocysts cell number and diameters were analyzed using one-way ANOVA test. Significance was assigned at $P < 0.05$.

Results

The morphology of cultured isolated blastomeres derived from early mouse embryo is shown in Fig. 1a–c.

Fig. 1 Four cell embryo (a), morula (b) and blastocyst (c) developed from 1/2 isolated blastomeres after 48, 72 and 120 h culturing respectively. Blastocysts of in vitro produce embryo derived from 1/2 isolated blastomeres stained with propidium iodide and bisbenzimidide (d). Two morphological abnormal embryo derived from isolated blastomeres (e and f) original magnification for all figures magnification $\times 400$

Development of isolated blastomeres in the presences of LIF

The results of the effect of LIF on the development of 1/2 and 1/8 isolated blastomeres are shown in Table 1. The rates of blastomeres (1/2) reached the 2 cell, 4 cell, 8 cell, morula and blastocysts stages in the control were 61.1, 52.4, 48.1, 40.7, 35.1%, and in LIF treated group were 66.60, 61.10, 59, 52, 45.8% respectively. The degeneration rate of blastomeres after 120 h culturing was 50.60 and 41.60 in control and LIF groups. There was a significant difference between developmental rates of blastomeres 48 h after culture in the presence of LIF with control group ($P < 0.001$).

The developmental rate of 1/8 isolated blastomeres to 2 cell, 4 cell and 8 cell embryos in control and LIF treated groups were 12.10, 7.31, 2.43% and 13.80, 5.70, 0%, respectively. No blastomere developed to morula and blastocyst stages. The degeneration rates of blastomeres after 120 h culturing were 78 and 80.50 in control and LIF groups.

Development of isolated blastomeres in the presences of GM-CSF

The developmental rates of isolated blastomeres (1/2) reached the 2 cell, 4 cell, 8 cell, morula and blastocysts stages in control were 60.7, 53.57, 51.19, 50.59, 41.66% and in GM-CSF treated group were 63.3, 59.33, 54, 53.33, 48.66%, respectively (Table 2). The developmental rate after 24 and 48 h post culturing was higher in GM-CSF group than their control ($P < 0.05$). The degeneration rates of blastomeres were 42.855% and 42.66% in control and GM-CSF groups.

The developmental rate of 1/8 isolated blastomeres reached to 2 cell, 4 cell and 8 cell embryos in control and GM-CSF treated groups were 13.3, 10, 6.66% and 8.57, 2.85, 2.85%, respectively and no blastomere developed to morula and blastocyst stages. The degeneration rates of blastomeres were 70 and 77.14% in control and GM-CSF groups.

Table 1 Developmental rates of isolated blastomeres from 2 and 8 cell mouse embryos in the presence and absence of LIF

Groups	LIF	No of isolated blastomeres (Repeat)	No. of 2 cell (%) 24 h	No. of 4 cell (%) 48 h	No. of 8 cell (%) 72 h	No. of morula (%) 96 h	No. of blastocyst (%) 120 h	No. of degenerated (%) 120 h
2 cell (1/2)	–	162 (13)	99 (61.10)	85 (52.40)	78 (48.10)	66 (40.70)	57 (35.10)	82 (50.60)
2 cell (1/2)	+	144 (14)	96 (66.60)	88 (61.10)*	85 (59)	75 (52)	66 (45.80)	60 (41.60)
8 cell (1/8)	–	41 (6)	5 (12.10)	3 (7.31)	1 (2.43)	–	–	32 (78)
8 cell (1/8)	+	36 (7)	5 (13.80)	2 (5.70)	–	–	–	29 (80.50)

*There were significant differences with control ($P < 0.001$)

Table 2 Developmental rates of isolated blastomeres from 2 and 8 cell mouse embryos in the presence and absence of GM-CSF

Groups	GM-CSF	No of isolated blastomeres (Repeat)	No. of 2 cell (%) 24 h	No. of 4 cell (%) 48 h	No. of 8 cell (%) 72 h	No. of morula (%) 96 h	No. of blastocyst (%) 120 h	No. of degenerated (%) 120 h
2 cell (1/2)	–	168 (14)	102 (60.70)	90 (53.57)	86 (51.19)	85 (50.59)	70 (41.66)	72 (42.85)
2 cell (1/2)	+	150 (12)	95 (63.30)*	89 (59.33)*	81 (54)	80 (53.33)	73 (48.66)	64 (42.66)
8 cell (1/8)	–	30 (7)	4 (13.30)	3 (10)	2 (6.66)	–	–	21 (70)
8 cell (1/8)	+	35 (7)	3 (8.57)	1 (2.85)	1 (2.85)	–	–	27 (77.14)

*There were significant differences with control ($P<0.05$).

The diameter and cell number of blastocysts in LLF and GM-CSF treated embryo

As shown in Table 3, the diameter of blastocysts derived from 2 cell embryos in the control, LIF and GM-CSF treated groups were 81 ± 3.02 , 86 ± 0.74 and 93.89 ± 4.93 micrometer, respectively. It was higher in GM-CSF group (Fig. 1d) than their control and LIF group ($P<0.05$).

The cell number of blastocysts in previous groups were 44.7 ± 2.84 , 53.4 ± 1.82 and 52.8 ± 1.79 , respectively. There were significant differences between LIF and GM-CSF treated groups with control ($P<0.05$). Also some abnormal morphological embryos were seen in all groups of study (Fig. 1e and f).

Discussion

In the present study, it was shown that under our experimental conditions using LIF and GM-CSF in culture media between 60.7 and 63.3% of 1/2 isolated blastomeres were cleaved and of these 41.66–48.66% reached the blastocysts stage. This data reconfirmed that the 1/2 isolated blastomeres could develop to blastocyst in vitro whereas in comparison with the intact mouse two cell embryo, the rate of blastocyst formation was very low [16, 28]. Also, embryos derived from isolated blastomeres

required more time to develop (1–2 days delay in growth was observed).

One explanation for the low embryo developmental rate could be the lower total cell number in embryos derived from isolated blastomeres than the intact embryo. The cell number of blastocysts derived from 1/2 isolated blastomeres in the presence of LIF and GM-CSF were higher than control ($P<0.05$). On the other hand, the results obtained in this in vitro study showed that LIF and GM-CSF act as a growth factor or a proliferative factor causing an increase in the cell number of blastocysts obtained from treated groups than control. Although in agreement with this conclusion, a similar effect of LIF [20] and GM-CSF [14, 29, 30] on the cell number of intact pre implantation embryos was shown. Desai et al. showed GM-CSF enhanced cell survival and prevented apoptosis of mouse embryo but did not influence overall cell number in developing blastocysts [31].

However it, has been shown that GM-CSF is a mitogen for hematopoietic and several non- hematopoietic cells types such as osteoblast, smooth muscle, endothelial and epithelial cells [32, 33]. GM-CSF has shown to stimulate the rate of DNA synthesis [34].

The similar studies in mice and human have shown that single blastomeres isolated from cleavage stage embryos can proliferate in vitro yielding multiple progeny cells but the isolation procedure had negative influence on the further development of isolated blastomere [3, 4]. This negative effect may be due to high incidence of nuclear anomalies [6]. However by scanning electron microscopic, the study showed that in vitro manipulation of mouse embryos had no effect on the surface characteristics of the plasma membrane of early cleavage stage embryos obtained from isolated blastomeres [35].

Furthermore, our study showed that the supplementation of culture media with LIF and GM-CSF could not improve the rate of blastocyst formation whereas in previous investigations, it was shown that the supplementation of culture media could increase the blastocysts formation [9–11, 14, 15, 36]. It seems that the number of cells at the time

Table 3 Diameter and cell number of blastocyst derived from isolated blastomeres of two cell embryos

Groups	Blastocysts diameter Mean $\pm$ SD	Cell no. of blastocysts Mean $\pm$ SD
Control	81 ± 3.02	44.7 ± 2.84
LIF	86.5 ± 0.74	$53.4\pm1.82^*$
GM-CSF	$93.89\pm4.93^*$	$52.8\pm1.79^*$

*There were significant differences with control ($P<0.05$).

of blastulation is very important; therefore, insufficient cells in embryos produced from isolated blastomeres may be an explanation for the low developmental rates in isolated blastomeres. Besides, the results suggest that LIF and GM-CSF may have different effects on embryo development in different in vitro systems. Their effects on embryo development may be cell stage and concentration dependent and species specific.

The results of our study suggest that under our experimental condition, the development of 1/2 blastomeres to blastocyst stage was superior to that of 1/8 blastomeres. This finding is in agreement with reports of other studies which found a progressive loss of developmental capacity of isolated blastomeres with increasing cell stage of the parent embryo in mice [37, 38]. The similar results obtained by Eckert et al., who showed that the porcine 1/4 blastomeres have a higher developmental potential to the blastocyst stage than 1/8 blastomeres, and 1/8-derived blastocysts had fewer total cell numbers than that of 1/4 derived blastocysts [5]. These differences may be due to negative effects of isolation procedure on 8 cell embryos.

At the 8-cell stage, intercellular junctions between blastomeres were tighter than the two cell stage embryo; therefore, mechanical blastomere isolation procedure may have led to damage to blastomeres. Also, it seems that 1/2 blastomeres are more totipotent than 1/8 blastomeres. Thus, the cells of the early mammalian embryo are not all functionally equivalent.

In conclusion, the developmental potential of isolated blastomeres from two cell embryos was better than 8 cell embryos and GM-CSF improved their quality.

Acknowledgment The authors would like to thank Miss Ebrahimi for photographic art work and Mr Pourbyranvand for technical assistance.

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