Overcoming the Mouse 2-Cell Block by Co-Culture with Established Cell Lines

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Abstract. To overcome the mouse 2-cell block, embryos resulting from *in vitro* fertilization (IVF) were co-cultured with established cell lines, namely, CHO-K1 (derived from Chinese hamster ovary), CHL/IU (derived from Chinese hamster lung), and HEPM (derived from human embryonic palatal mesenchyme). Differences of embryonal development were observed in the different established cell line co-cultures. CHO-K1 were the best feeder cells for overcoming the block, while the CHL/IU exhibited this ability to a much lesser degree. HEPM was not able to overcome the block. Observations of the structure of these established cell lines, revealed variations in the morphology and density of the feeder cells. A large number of villi were observed on the surfaces of CHO-K1 and CHL/IU cells, and the cell density of CHO-K1 was much higher than that of CHL/IU. On the other hand, the surfaces of HEPM cells exhibited no villi or other structures. These findings suggest that the morphology and density of the feeder cells play a role in overcoming the 2-cell block.

Key words: Two-cell block, Co-culture, Feeder cell, Established cell lines.

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The development of early stage embryos in several mammalian species is known to be blocked in *in vitro* cultures [1-5]. Mouse and rat embryos exhibit a block at the 2-cell stage, hamster embryos at both the 2- and 4-cell stage, pig embryos at the 8-cell stage, and sheep and cow embryos at the 16-cell stage. One of the methods employed to overcome the block and improve development, has been the co-culture of embryos with feeder cells. The feeder cells used were of various origins, including oviduct, uterus, ovary, kidney, spleen, and chick embryo [6-10]. The feeder cells used have not always been from primary cultures, nor have they been limited to the same species as the embryos, nor have they always been derived from the genital tract. Ouhibi *et al.* [9] also used the established cell lines, including MDBK (derived from bovine kidney) and Vero (derived from Green monkey kidney) as feeder

cells. They found a high rate of blastocyst formation with MDBK, however Vero did not overcome the 2-cell block. The commercially available established cell lines have various benefits—they are easily maintained, and they allow numerous passages without alteration.

In the present investigation, we studied the co-culture of mouse embryos resulting from IVF with 3 established cell lines.

Materials and Methods

Eight- to 12-week-old ICR mice (CLEA Japan, Tokyo), whose embryos are known to exhibit the 2-cell block *in vitro*, were used. As feeder cells, we used the commercially available established cell lines CHO-K1 (ATCC CCL 61), CHL/IU (JCRB 0030), and HEPM (ATCC CRL 1486), which we purchased from Dainippon Pharmaceutical Co., Ltd., Osaka. Cell suspensions containing $5\sim6\times10^4$ cells were seeded in 1.0 ml portions in the wells of

4-well plastic dishes (Nunc, Roskilde, Denmark) 48 h prior to the co-culture. Dulbecco's phosphate-buffered saline solution (Flow Laboratories, USA), supplemented with 10% (v/v) fetal calf serum (FCS; Filtron, Altona, Victoria, Australia), was used as medium. Unfertilized eggs were collected from mice which had been superovulated with a 5 i.u. injection of pregnant mare serum gonadotropin (PMSG; Serotropin, Teikoku Hormone Manufacturing Co., Ltd., Tokyo), and a 5 i.u. injection of human chorionic gonadotropin (hCG; Gonatropin, Teikoku Hormone Manufacturing Co., Ltd.), given 48 h later. Seventeen h after the hCG injection, unfertilized eggs were collected from the ampullae of the mice. One h before insemination, sperm was collected from the caudal epididymis of male mice of the same strain and the same age as the females. To induce capacitation, the sperm was cultured at 37 C in a 5% CO₂ and air mixture until insemination. The IVF procedures were performed in a micro drop of HTF medium [11] (Irvine Scientific, Santa Ana, CA, USA), supplemented with 10% FCS, under paraffin oil (E. Merck, Darmstadt, Germany). Six to 7 h after insemination, the fertilized eggs within the micro drops were transferred to the wells of the 4-well plastic dishes in which the feeder cells were already seeded. The embryos were allowed to lie on the feeder cells; co-culture was commenced at 37 C in a 5% CO₂ and air mixture. The number of fertilized eggs in each well was about 10 to 20. Fertilized eggs in the control group were cultured without feeder cells. The numbers of fertilized eggs used were 106 (Control group), 221 (CHO-K1 group), 123 (CHL/IU group) and 95 (HEPM group), and they were derived from 4, 9, 5 and 5 dams, respectively. Co-cultures were continued for 5 days and embryo morphology was observed

microscopically every day. To observe the structure of the feeder cells, scanning electron micrographs were taken at approximately the time that co-culture commenced.

Results

The results are summarized in Table 1. Few

Fig. 1. Scanning electron micrographs of the established cell lines, CHO-K1, CHL/IU, and HEPM which were used as feeder cells for co-culture with ICR mouse embryos. These photographs were taken at approximately the time that co-culture was commenced. A) CHO-K1 (Chinese hamster ovary), magnification $\times 1500$. A large number of villi can be seen on the surfaces of these cells, and the cell density is high. B) CHL/IU (Chinese hamster lung), magnification $\times 1500$. A large number of villi can be seen, however, the cell density is not as high as that of CHO-K1. C) HEPM (human embryonic palatal mesenchyme), magnification $\times 1000$. There are no villi or any other complicated structures on the surfaces of these cells.

Table 1. *In vitro* development of ICR mouse eggs co-cultured with various established cell lines.

Established cell lines	No. of eggs	Development stage for 5 days			
		2-Cell	4-Cell	Beyond 4-cell	Blastocyst
Control (None)	106	80 (75%)	1 (1%)	0	—
CHO-K1	221	182 (82%)	98 (44%)	91 (41%)	6 (3%)
CHL/IU	123	89 (72%)	8 (7%)	6 (5%)	0
HEPM	95	65 (68%)	0	—	—

differences were observed between feeder cells and the control with regard to the rate of development from 1-cell to 2-cell stage. The rates of development from the 2-cell to the 4-cell stage were 1% in the control, 44% in CHO-K1, and 7% in CHL/IU. In the HEPM group, however, no embryos developed to the 4-cell stage. The rates of development beyond the 4-cell stage were 41% in CHO-K1 and 5% in CHL/IU. Development to the blastocyst stage was observed only in CHO-K1, the rate being 3%.

Scanning electron micrographs of CHO-K1, CHL/IU, and HEPM are shown in Fig. 1. On the surfaces of CHO-K1 and CHL/IU cells, a large number of villi were observed, and the cell density of CHO-K1 was much higher than that of CHL/IU. On the other hand, on the surfaces of HEPM cells, there were no villi or any other complicated structures.

Discussion

We assessed established cell lines, namely, CHO-K1, CHL/IU and HEPM as feeder cells in the present study. The results of our experiments indicate that established cell lines can be used to overcome the block in embryonic development. We found that CHO-K1 were the best feeder cells for overcoming the block followed by CHL/IU. HEPM cells could not overcome the block.

It has been speculated that feeder cells release embryo trophic factors into the medium, or that they detoxify some factors present in the medium or derived from the embryo. Recently, activin was identified as a factor that overcomes the 2-cell block and stimulates early embryonic development of mice *in vitro* [12]. The presence of activin was

demonstrated in oviductal epithelial cells [13]. Such an embryo trophic factor may be released from feeder cells. EDTA which chelates Ca^{2+} or Mg^{2+} is known to play a beneficial role in embryonic development [14], and chemically defined medium which contains low Na^+ , K^+ , Cl^- or glucose to improve the embryonic development [15]. These findings suggest that feeder cells lower the concentration of these ions and/or glucose. In addition, the addition of superoxide dismutase (SOD), a potent scavenger of superoxide radicals, to the culture medium, markedly reduced *in vitro* 2-cell block in the mouse [16]. These findings suggest that the 2-cell block phenomenon in mouse embryos is related to oxygen toxicity, and that SOD play a crucial role for protecting the embryos from oxygen radicals. The consumption of oxygen around the embryos by the feeder cells may detoxify oxygen. The differences in among established cell lines as feeder cells may be due to their characteristics such as metabolism or growth rate.

Tokunaga *et al.* [8] found that the effect of co-culture with feeder cells was influenced by the concentration and the number of passages of the feeder cells, and conditioned medium obtained by fibroblast cells was not effective for the development of embryos. They speculated that overcoming of *in vitro* 2-cell block by co-culture was due to direct contact or a diffusible factor. By scanning electron microscopy, a large number of villi were observed on the surfaces of CHO-K1 and CHL/IU cells, and the cell density of CHO-K1 was much higher than that of CHL/IU. On the other hand, the surfaces of HEPM cells, exhibited no villi or other structures. These findings suggest that the morphology and density of the feeder cells play a role in overcoming the 2-cell block.

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