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Maintenance and Differentiation of Pluripotential Embryonic Cell Lines from Mouse Blastocysts

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BCF_1 생쥐 배반포기 유래 배아간세포 작성에 관한 연구

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요 약

생쥐 배반포기 내부세포괴를 체외에서 분리 배양하여 분화가 억제된 내부세포괴 유래 증식세포를 미분화 상태에서 무한히 증식할 수 있는 전능성을 지닌 배아간세포 (embryonic stem cell: ES cell)로 확립하고자 본 연구를 실시하였다. BCF_1 생쥐 배반포를 10% FCS, 0.1mM nonessential amino acid, 0.1mM sodium pyruvate, 0.1mM 2-mercaptoethanol과 1,000U/ml LIF (세포분화억제인자)가 첨가된 DMEM 기초배양액에 mitomycin-C를 처리한 STO 단층배양세포에서 배양하여 분화가 억제된 내부세포괴 유래의 배아간세포를 분리하였다. 배반포를 STO 단층배양세포에서 4일간 배양하여 영양아세포의 접착 신장 이탈에 의해 내부세포괴를 분리, trypsin-EDTA 용액으로 처리하여 해리된 내부세포괴 세포를 신선한 STO 단층배양세포에서 약 5일 간격으로 반복하여 계대배양을 실시하였다. 5차 계대배양후 뚜렷한 분화 양상없이 배양된 미분화 세포군에 대한 alkaline phosphatase (AP)염색과 체외분화능 검색을 실시한 결과 적색의 미분화 AP 양성반응이 확인되었으며 체외에서 배분화 형성이 유도됨에 따라 배양된 배아간세포주의 다능성 배아간세포 특성을 확인할 수 있었다.

I. INTRODUCTION

Murine embryonic stem (ES) cells are immortal totipotent cells lines which have been isolated *in vitro* from morulae (Eistetter, 1989), inner cell mass of blastocyst (Evans and Kaufman, 1981) or embryonic ectoderm (Wells et al., 1991). ES cells maintained and manipulated *in vitro* are able to contribute to normal embryonic and fetal development. ES cells have the potential to form all kinds of tissue in chimeric mice after being injected into blastocysts (Evans and Kaufman, 1981; Martin, 1981; Axelrod, 1984; Bradley et al., 1984; Rossant and Papaioannou, 1984). Thus ES cells can be used to study embryonic growth control and differentiation in culture (Doetschman et al., 1985). Furthermore, ES cells have provided the means to perform the precise genetic manipulations *in vitro* followed by reconstitution of the germ-line *in vivo* from ES cell-derived chimeras. Genes are introduced into the ES cells in vitro by DNA transfection or viral infection, and the desired ES clones bearing the introduced genes are propagated and used to generate chimeras. Manipulated cell lines can be stored indefinitely as frozen stocks, providing an unlimited source of the selected genotype for future use. Therefore, ES cells provide an im-

portant method for the introduction of both dominant (Williams et al., 1988a) and recessive (Zijlstra et al., 1989; Schwartzberg et al. , 1989) genetic mutations into mice.

Maintenance of the stem cell phenotype *in vitro* requires the presence of a feeder-layer of fibroblasts (Evans and Kaufman, 1981; Martin, 1981; Martin and Evans, 1975) and of differentiation inhibitory sources such as buffalo-rat liver (BRL) and leukemia inhibitory factor (LIF) (Hooper et al., 1987; Smith and Hooper, 1987; Williams et al., 1988). Without feeder cells, once ES cell lines have been established in culture system with medium conditioned by certain tumor cell lines, such as BRL or 5637 cells. This indicates that there is a soluble differentiation-inhibiting activity (DIA) which prevents the differentiation of established ES cell lines (Smith and Hooper, 1987). It was discovered that LIF shared a number of features in common with DIA (Williams et al., 1988b; Smith et al., 1988; Gough and Williams, 1989). Considerably, ES cells maintained in LIF retained the ability to form germ-line chimeric mice indication that LIF is sufficient to maintain the developmental potential of ES cell lines previously isolated and maintained on feeders (Williams et al., 1988b). However, the ability of LIF to replace feeder cells during the isolation of ES cell lines has not been tested.

The present experiments showed the isolation and some preliminary characterization of cell lines derived from BCF_1 hybrid mice blastocysts. Especially, the characterization of developmental potential of isolated ES-like cell lines was evaluated with respect to *in vitro* differentiation and specific activity of alkaline phosphatase. In addition, parameters affecting the isolation of further embryonic stem cell lines were optimized.

Ⅱ. MATERIALS AND METHODS

1. Embryos

F_1 female mice (C57BL/6 ×CBA) were produced by crossing CBA male and C57BL/6 female mice (obtained from The Jackson Laboratory). BCF_1 mice were caged in pairs and examined for mating plugs each morning. Blastocysts were recovered by flushing the uterine horns on 3.5 days (the day of finding vaginal plug is termed day 1/2).

2. Preparation of feeder layers

A continuous mouse fibroblast cell line (STO; American Type Culture Collection, Rockville, MD) was routinely cultured as described by Robertson (1987) in 25 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) supplemented with 10% FBS (GIBCO), penicillin (100 IU/ml) and streptomycin (50μg/ml). STO feeder cells harvested by trypsinization were treated with 10μg/ml mitomycin-C (Sigma, USA) in DMEM containing 10% FBS for 2hr to block mitotic activity of the fibroblasts, and were then added to four-well tissue culture dishes at an approximate concentration of 2.5×10^4cell/cm^2 cells. The mitomycin C-treated feeder cells were maintained in DMEM supplemented with 10% FBS, 0.1 mM 2-mercaptoethanol (Sigma, USA) in a humidified atmosphere, with 5% CO_2 in air at 37℃.

3. Culture condition and isolation of cell lines

All culture was carried out in DMEM (high glucose) supplemented with 10% FCS (GIBCO), 0.1 mM nonessential amino acid (Flow Lab., USA), 0.1 mM sodium pyruvate (Flow Lab.), 0.1 mM 2-mercaptoethanol (Sigma) and 1,000 U/ml LIF (AMRAD, ESGRO, Australia). Medium here means ES culture medium. All tis-

sue culture dishes were pretreated with 0.1% gelatin in PBS for more than 2 hr at room temperature. Expanded blastocysts were explanted onto mitotically-inactivated STO feeder layers and cultured for 4 days. Full-grown ICMs were picked off from the feeder layer and disaggregated by a short - term trypsin treatment (0.25% trypsin / 0.04% EDTA for 2~3min) using a micropipette. The resulting dissociated clumps were seeded on a new STO feeder layer. Seven to ten days later, colonies composed of cells with ES-like morphology were selected for further passage. Single colonies were dissociated as described above and reseeded. Subsequently, non-differentiating clonal lines were partly frozen, partly passaged. The cultures were examined daily and the whole culture medium containing 1,000U/ml LIF was replaced every second day throughout the experiment. All cultures described above were carried out in a humidified atmosphere with 5% CO_2 in air at 37℃.

4. Analysis of stem and differentiated cell markers

1) Alkaline phosphatase staining

Histochemical staining for alkaline phosphatase activity was done as described by Donovan et al. (1986). Cultures containing colonies were fixed in 4% formaldehyde in PBS for 10~15 min. After washing with distilled water, the fixed cultures were incubated for 15min in distilled water containing 1mg/ml fast red TR salt and 40㎕/ml napthol AS-MX phosphate (Sigma). Alkaline phosphatase is expressed in the ES cells but not their differentiated derivatives. ES colonies were classified as stem or differentiated colonies according to colony morphology and staining for alkaline phosphatase.

2) *In vitro* differentiation of isolated cell lines

To stimulate organized differentiation, embryo-derived cells with ES-like morphology were disaggregated by trypsinization and then seeded onto tissue culture plates which had been coated with a layer of 0.5% agarose as described by Magrane (1982) for stimulation of formation of embryoid bodies by human teratocarcinoma cell cultures. To enhance cell differentiation, both mercaptoethanol and FCS were omitted from the medium.

Ⅲ. RESULTS

1. Isolation of embryo-derived cell lines with ES-like morphology

Blastocysts were explanted into STO feeder layer in ES cell culture medium containing 1,000 U/ml LIF. Within 48hr the trophectoderm had attached to feeder layer. After a further 4 days the ICM had formed a distinct clump of cells on the trophectoderm cells. These clumps were picked out using a glass capillary, trypsinized to dissociate the clump, and plated into 4 well dishes on feeder layer. The primary outgrowths were disaggregated 4 days after explantation and passaged to fresh feeder layers. Progressively growing colonies were formed which grew as a monolayer with very distinct colony boundaries. To prevent differentiation of the cells, approximately 1/2 of the ES culture medium was replaced by culture medium containing LIF preincubated for 12hr. Colonies of ES-like cells were observed 5 days after the 1st passage. These colonies were repeatedly passaged at approximately 5 day intervals. Figure 1 shows a cell type which is more stable and able to grow in larger colonies. Two cell lines have been maintained in continuous culture for more than 5 passages without change of cell phenotype. However, when the cells were permitted to reach high density,

Fig. 1. Morphologies of colonies resulting from disaggregated primary outgrowths of inner cell masses : Phase contrast microscopy of typical ES-like cell colonies after second passage.

differentiation of these cells occurred spontaneously. Therefore, overtly differentiated cells failed to reattach after passage, leading to regeneration of undifferentiated cultures (Figure 3).

The cells of growing colony shown in Figure 2

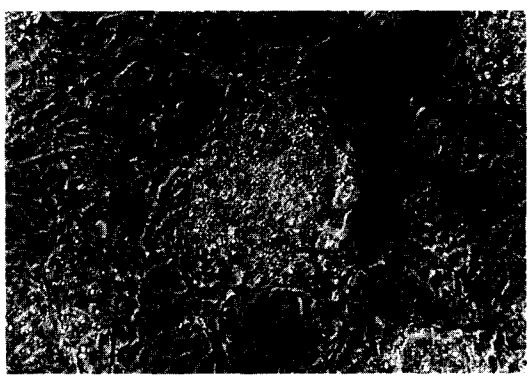

Fig. 2. A colony of ES-like cells, cultured for 5 passages on STO feeders, showing the typical undifferentiated cell morphology. The colony was derived from a expanded BCF1 blastocyst. × 200.

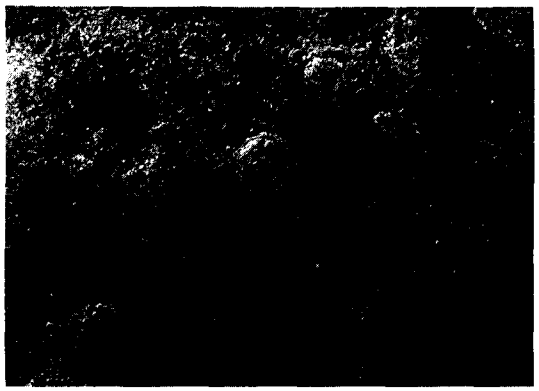

Fig. 3. ES-like cells culture in which the conditions are suboptimal. Culture in which the majority of cells have differentiated, with few stem cell colonies remaining. Colonies of differentiated cells (D) are visible, and other ES colonies are flattened out (Di), which is the first overt sign of their starting to differentiate.

are epithelioid with large clear nuclei and relatively sparse cytoplasm. Some differences in the appearance of these cells have been noticed between different isolates, and these are mainly related to cell size. In Figure 2, a colony of smaller cells are a more rarely isolated form.

In an attempt to improve the isolation procedures, parallel experiments were performed. Parameters investigated were examined in the type of culture systems, with feeder cells only, feeder cells plus LIF medium, or LIF only medium during isolation of cell lines. For three types of cell culture system, the use of STO feeder cells plus LIF addition medium resulted in a two to three fold increase in the frequency of isolated cell lines. The result of the isolation experiments are summarized in Table 1.

2. Analysis of stem and differentiated cell markers

Table 1. Passages of plated ICM, which isolated from BCF_1 mouse, on different types of culture condition: survial profile of embryo-derived cell lines during plating and repeated passage

Passage number	Types of culture conditions		
	STO[a]	LIF[b]	STO : LIF[c]
O[d]	56	44	71
First	11	21	13
Second	7	10	12
Third	4	1	7
Forth			5
Fifth			5
N			

[a] Blastocysts were cultured in DMEM + 10% FCS onto STO feeder layer.

[b] Culture medium supplemented with LIF in the absence of feeders.

[c] Culture condition as in (a), supplemented with LIF.

[d] Values for 0 passage are numbers of ICMs plated for the different culture conditions.

N = more than 6 passages

LIF = 1,000 unit / mℓ (10ng / mℓ)

1) Alkaline phosphatase staining

Mouse embryonic stem cells as well as cells of the ICM are known to show a high specific activity in AP. This activity declines during progressive differentiation resulting in low AP activities in somatic differentiated cells. Therefore, specific activity of AP served as a biochemical marker with regard to the differentiation status of embryonic stem cells. ES cell colonies were classified as stem or differentiated colonies according to colony morphology and staining for AP. Determination of AP yielded a high specific activity in the cultured cell lines for 5 passages and a low activity in STO fibrolasts which were used as feeder cells (Figure 4). On the other hand, AP staining was lost upon differentiation or senescence of colonies. Also, all differentiated embryo-derived cell cultures were negative for AP staining. AP is a convenient marker for undifferentiated embryonic cells in culture when used in conjunction with cell morphology analysis.

2) *In vitro* differentiation into embryoid bodies

Induction of *in vitro* differentiation was performed by growing large populations of undifferentiated cells in suspension. As a test of differentiation potential (Martin and Evans, 1975), cells from the cell line which had been maintained for 30 days were induced to form aggregates by seeding onto a non-adhensive substratum in culture medium without LIF. After several days, an outer smooth layer of cuboidal epithelial cells appeared at one end of the aggregates. Within 7 days the aggregates formed simple embryoid bodies and after further days, complex cystic embryoid bodies had formed (Figure 5). At the first steps, the embryoid body had a monolayer wall; after continued culturing, there appeared an embryoid body with a multilayer wall (Figure 5B). It can be clearly seen that they are composed of endoderm-like cells, which form a membrane-like structure.

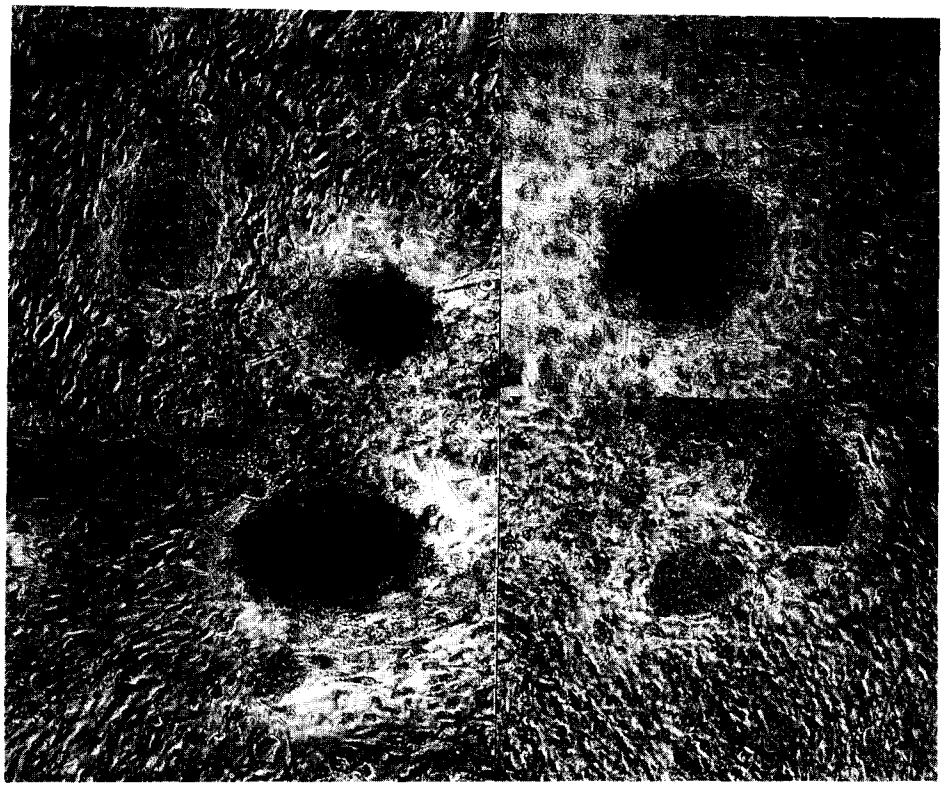

Fig. 4. These ES-like cells were positive for AP staining, a biochemical marker for undifferentiated ES cells in culture. Colonies of ES-like cells derived from BCF_1 bastocysts were cultured for 5 passages on STO feeder cells in the presence of soluble LIF.

Ⅳ. DISCUSSION

Establishment of mouse ES cell lines from various inbred and mutant strains would enable wider utilization of ES cells in manipulation of the early mouse embryo and mouse germ-line. So far, however, almost all of the ES cell lines have been derived from 129 strain mice (Gossler et al., 1986; Robertson et al., 1986; Hooper et al., 1987; Pease et al., 1990; McMahon and Bradley, 1990), because there are many delicate differences in the early embryonic cells among mouse strains. By improvement of methods for establishment and maintenance of ES cell lines efficiently could be derived from explanted blastocysts of the hybrid BCF_1 mouse, that an optimal approach was the combination of LIF added medium and of mitotically inactivated STO cells as feeder layer during the initial proliferation of the ICM of outgrowing blastocysts.

To investigate the consequence of removal of LIF from the cell culture medium ES-like cells were passaged in the medium of removal LIF. After several days, stem cell colonies were identified as compact colonies of stem cells which expressed AP, which is expressed by ES cells but not their differentiated derivatives (Figure

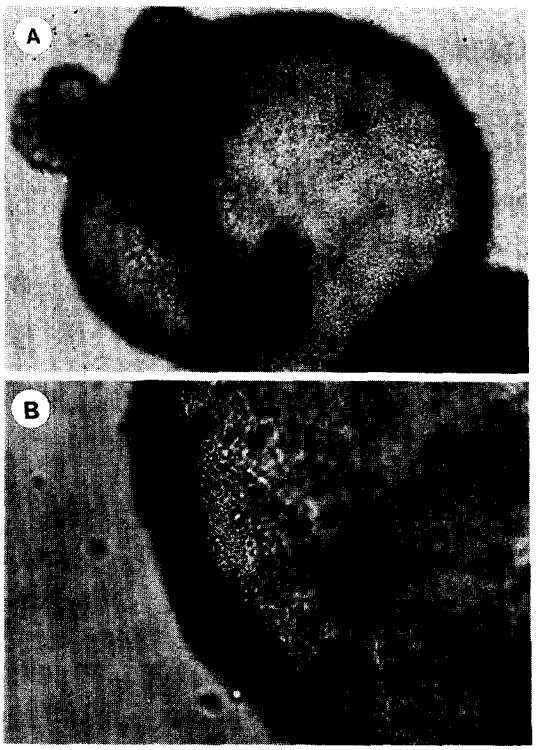

Fig. 5. (A) Morphology of a cystic embryoid body arisen 14 days after placement of ES-like cells in suspension culture. The embryoid body is composed of a multilayer wall, with the external layer consisting of endoderm-like cells and the internal layer of mesenchyme-like cells. × 200. (B) Magnification of a multilayer wall. × 320.

4). However, the absence of LIF in the cell culture medium resulted in an increase in the number of cell colonies containing large and flat differentiated. In the absence of LIF over 80% of the colonies were differentiated as assessed by cell morphology and lack of the AP activity. The undifferentiated and pluripotent status of the cell lines was confirmed by *in vitro* differentiation and specific activity of AP as follows; (1) to define the characteristics of ES-like cells, the cells were tested for the presence of AP, a biochemical marker characteristic of mouse ES cells. The resulting determination of AP in cultured cell line yielded a high specific activity and (2) when cultured in suspension, the majority of aggregates were capable of forming simple embryoid bodies, and then showed the capacity for forming cystic multilayer embryoid bodies. Subsequently, extensive differentiation occurred when the embryoid bodies were permitted to attach to the substratum, by replating onto tissue culture dishes. Cells migrated and multiplied to form dense culture, with several types visible, including epithelium, endoderm, muscle and neural cells. These differentiated cells are representative derivatives of all three embryonic germ layers, and suggest that the stem cell-like culture represents a primary ectodermal lineage of the pre-somite embryo. The data from AP activity and *in vitro* differentiation analysis in these experiments are in agreement to those from previous isolated mouse (Evans and Kaufman, 1983; Robertson et al., 1983; Rastan and Robertson, 1985; Doetschman et al., 1985) and hamster ES cell lines (Doetschman et al., 1988).

The developmental potential of isolated ES-like cells in these experiments was investigated after aggregate these cells to zona removed morulae. Some of the aggregated embryos reached the fully blastocysts in *in vitro* culture and subsequently transferred to foster mothers, obtained 7 offspring (not shown data). However, analysis of the biochemical markers and coat color did not demonstrate the presence of chimaeras among offsprings. These preliminary results suggest that their pluripotential capacities are restricted and that the cause of the reduced pluripotency of cell lines is unclear.

In these experiments, the optimal culture conditions for the formation of BCF_1 mouse ES-like cells and their potential was established. We are

currently attempting to establish whether these cultures represent primary ectodermal lineages which would be of particular relevance to developmental and transgenic studies. It seems that further works are necessary to the verification of the totipotency of ES-like cells by producing live offspring upon injection of ES-like cells into blastocysts and transfer of mutant gene to ES cells as a vector for genetic manipulation.

V. SUMMARY

The present study was designed to demonstrate that ES cell lines efficiently could be isolated from explanted blastocysts of hybrid BCF1 mouse when grown on STO feeder layer derived from mouse fibroblasts in culture medium supplemented with leukemia inhibitory factor (LIF). The expanded blastocysts were attached to mitomycin C-inactivated STO feeder layer and were cultured for 4 days. Four days later the ICM was disaggregated by a short term trypsin treatment (0.25% trypsin / 0.04% EDTA for 2~3 min). The resulting cell suspension was seeded on a new STO feeder layer and covered with DMEM supplemented with 10% FCS, 0.1 mM nonessential amino acid, 0.1 mM sodium pyruvate, 0.1 mM mercaptoethanol and 1,000 U/ml LIF. Colonies of ES-like cells were observed after the second passage. These colonies were repeatedly passaged at approximately 5 day intervals. In this study, five ES-like cell lines were isolated by directly explanting blastocysts, but three lines were lost after the 5th passage, possibly due to toxic effects of a new FCS batch. The characterization of developmental potential of isolated cell lines was performed with respect to *in vitro* differentiation and specific activity of alkaline phosphatase (AP). When cells were cultured in suspension, the aggregates of cell lines were capable of forming simple embryoid bodies (EB), and showed the capacity for forming cystic multilayer EBs. In addition, the cell lines were positive for AP staining, a biochemical marker characteristic of mouse ES cells.

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