Culture of Clonal Lines in Porcine Fetal Fibroblast Cells*

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돼지 태아섬유아세포 Clonal Lines의 배양*

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ABSTRACT

This study was performed to establish the effective culture condition for the establishment of clonal lines from porcine fetal fibroblast cells. Fibroblasts derived from a pig fetus (Day 50) were cultured and passaged two times before use. A single cell was seeded in 96-well plates, cultured in medium supplemented with different concentrations of FBS, catalase or β-mercaptoethanol (βME), and classified by cell size and morphology. Cells were passaged two times into 4-well dish before freezing. The establishment efficiencies were not different among different concentrations of FBS (0.3 to 5.1%). However, population doubling time (PDT) was significantly decreased by increasing the FBS concentration ($P<0.05$). The establishment efficiency of βME-added group (10.4%) was significantly higher than those of catalase-added and control groups (3.5%, and 3.5%, respectively, $P<0.05$), and PDT was significantly decreased (23.6 vs 28.1, and 25.5 h, respectively, $P<0.05$). However, catalase did not show a positive effect on the establishment efficiency. Cell size and morphology did not affect the establishment efficiency and PDT of clonal lines. The result of present study shows that the establishment efficiency of clonal cell lines can be enhanced by the culture in media supplemented with 30% FBS and βME.

(Key words : Clonal cell lines, Population doubling, Serum concentration, Catalase, β-Mercaptoethanol)

I. INTRODUCTION

Since the production of a cloned lamb from somatic cells (Wilmut et al., 1997), various species of animals have been cloned (Kato et al., 1998; Wakayama et al., 1998; Baguisi et al., 1999; Wells et al., 1999; Polejaeva et al., 2000; Shin et al., 2002). The production of transgenic pigs with site specific alterations would have great benefits in agricultural and human therapeutic applications. Recently, these possibilities have more risen by the production of α-1,3-galactosyltransferase knockout pigs (Dai et al., 2002; Lai et al., 2002). In the production of transgenic or knockout animals, nuclear transfer using the cultured cells may be a preferable method.

One of the major procedure for the production of these animals involves the establishment of clonal cell lines, which is very efficiently transfected or knockouted cells. However, it is very difficult to establish the clonal lines derived from a single cell, it's efficiency is known as less than 5%. The low efficiency may be due to differences in the originated tissues, *in vitro* culture conditions, and culture period of cells as well as the cytogenetic properties of cells including the size, morphology, doubling time, cell cycle, chromosome constitutions, DNA methylation and so on.

Especially, a factor that affect cytogenetic properties or gene expression may be oxidative stress of cells by reactive oxygen species (ROS). Oxidative damage to nucleic acid polymers can

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disrupt transcription, translation and DNA replication, and can cause mutations and cell death (Kim et al., 1997; Winn and Wells, 1995; Liu and Wells, 1995). On the other hand, oxygen-induced growth inhibition has been examined using cultured cells isolated from a variety of animal species, such as human embryonic fibroblasts (Balin et al., 1976; Honda and Matsuo, 1980), human epidermal keratinocytes (Horikoshi et al., 1986), pig aortic endothelial cells (Junod et al., 1987), rat lung fibroblasts (Tzaki et al., 1988), mouse embryonic fibroblasts (Bradley et al., 1978), and Chinese hamster ovary (CHO) cells (Gille et al., 1992). However, aerobic cells have antioxidant defenses including superoxide dismutase (SOD) that remove O_2^- and produce H_2O_2 (Allen et al., 1998), catalase that remove H_2O_2, and glutathione (GSH) that act to maintain the cellular redox state (Chance et al., 1979). In cells, β-mercaptoethanol (βME) promotes the uptake of cysteine (Ishii et al., 1981), which enhances GSH synthesis. It has been suggested that GSH protects cells from oxidative stress and enhances the growth and colony formation *in vitro* culture of varies types of cells (Meister, 1983; Gardner and Reed, 1994; Takahashi et al., 1996). Therefore, addition of these antioxidants in culture medium may enhance not only the stability of cytogenetic properties but also the viability and proliferating potential of cells.

This study was performed to evaluate the effects of concentrations of FBS and catalase/βME in culture medium, and cell size and morphology of cultured cells on the establishment of clonal lines from porcine fetal fibroblast cells.

Ⅱ. MATERIALS AND METHODS

1. Preparation of porcine fetal fibroblast cells

A porcine fetus was obtained from a pregnant (Day 50) gilt. After through washing in sterilized physiological saline solution, the head and inner organ were removed and then chopped into small pieces. The tissues were enzymatically digested with 0.05% trypsin-EDTA in PBS for 30 min at 37℃ with occasional stirring. The digestion procedure was repeated two times, and the digested tissues were allowed to settle for 5 min and the supernatant containing disaggregated cells were transferred into a 15 ml conical tube. Cells were collected by centrifugation at 500 ×g for 5 min and washed once in culture medium. The cell pellet was diluted with Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% FBS and 50 ㎍/ml gentamicin(Sigma, St. Louis, MO, USA), and cultured at 37℃, 5% CO_2 in air. At about 90% confluency, the cells were passaged with a 1:2 division, and subsequently regrown cells were passaged two times before being frozen in FBS with 10% dimethylsulfoxide and stored in liquid nitrogen.

2. Culture of clonal cell lines

Thawed fetal fibroblast cells were cultured in 96-well plate at one cell per well. After 7~14 days, cell colonies were transferred into 4-well dish and expanded with selection through an estimated total of 18~24 population doublings. Established cell lines were cryopreserved in small aliquots.

3. Determination of population doublings

Population doubling time (PDT) was estimated by counting cell number at that time of passage. Population doubling was calculated using the $\log 10\ (N/N_0) \times 3.33$ formula (where N is the number of cells harvested and N_0 is the number of cells plated).

4. Experimental Designs

To examine the establishment efficiency of clonal cell lines, each cell were cultured by different conditions. In experiment 1, each cells were cultured in DMEM supplemented with 10, 30, or 50% FBS. In experiment 2, each cells were cultured in DMEM supplemented with 30% FBS and 100 ng/ml catalase or 100 nM βME. In experiment 3, fetal fibroblast cells with different size (<16 ㎛, 16~20 ㎛, and >20 ㎛) were cultured in DMEM supplemented with 30% FBS. In experiment 4, fetal fibroblast cells with different morphology (smooth and rough) were cultured in DMEM supplemented with 30 % FBS.

5. Statistical analysis

Percentage of adhesion, passage, establishment efficiency of cells were analyzed by Duncan's multiple range test using the General Linear Models procedure in the Statistical Analysis System.

Ⅲ. RESULTS

1. Effect of serum concentration

When each cells were cultured in DMEM supplemented with 10, 30, and 50% FBS, establishment efficiency was 0.3, 5.1, and 2.1%, with PDT of 36.7, 29.4, and 26.3 h, respectively (Table 1). The establishment efficiency was not different among treatments. However, PDT was significantly decreased by increasing the FBS concentration in culture medium ($P<0.05$).

2. Effect of catalase and βME

As shown in table 2, the establishment efficiency of clonal lines of βME-added group (10.4%) was significantly higher than those of catalase-added and control groups (3.5%, and 3.5%, respectively, $P<0.05$), and PDT was significantly decreased (23.6 vs 28.1, and 25.5 h, $P<0.05$). However, catalase did not have a positive effect on the establishment efficiency.

3. Effect of cell size

The establishment efficiency and PDT of cell lines in small (<16 ㎛), medium (16~20 ㎛), and large (>20 ㎛) groups was 5.3 to 8.2% and 23.7 to 27.9 h with no significant difference (Table 3).

4. Effect of morphology

Table 1. Effect of serum concentrations on the establishment efficiency of clonal cell lines

Serum con.(%)	No. of seeded cells	No. of cell line (%)				PDT* (h)
		Adhesion	Passage 1	Passage 2	Established	
10	363	187(51.5)	17(4.7)	4(1.1)[a]	1(0.3)	36.7[a]
30	354	207(58.5)	58(16.4)	25(7.0)[b]	18(5.1)	29.4[ab]
50	341	189(55.4)	33(9.7)	9(2.6)[ab]	7(2.1)	26.3[b]

* PDT : population doubling time.

a,b Values with different superscripts in the same column differ ($P<0.05$).

Table 2. Effects of catalase and β-mercaptoethanol on the establishment efficiency of clonal cell lines

Treatment*	No. of seeded cells	No. of cell line (%)				PDT* (h)
		Adhesion	Passage 1	Passage 2	Established	
Control	285	176(61.8)	44(15.4)[a]	17(6.0)[a]	10(3.5)[a]	25.5[a]
Catalase	286	162(56.6)	46(16.1)[a]	16(5.6)[a]	10(3.5)[a]	28.1[b]
βME	288	177(61.5)	95(33.0)[b]	47(16.3)[b]	30(10.4)[b]	23.6[c]

* Catalase (100 ng/ml), βME : β-mercaptoethanol (100 nM).

** PDT : population doubling time.

a,b,c Values with different superscripts in the same column differ ($P<0.05$).

Table 3. Effect of cell size on the establishment efficiency of clonal cell lines

Cell size (㎛)	No. of seeded cells	No. of cell line (%)				PDT* (h)
		Adhesion	Passage 1	Passage 2	Established	
Small(<16)	114	79(69.3)	29(25.4)[a]	13(11.4)	6(5.3)	23.7
Medium(16~20)	193	123(63.7)	52(26.9)[a]	20(10.4)	16(8.3)	27.9
Large(>20)	39	17(43.6)	5(12.8)[b]	4(10.3)	3(7.7)	25.8

* PDT : population doubling time.

a,b Values with different superscripts differ ($P<0.05$).

Table 4. Effect of cell morphology on the establishment efficiency of clonal cell line

Cell morph.	No. of seeded cells	No. of cell line (%)				PDT* (h)
		Adhesion	Passage 1	Passage 2	Established	
Smooth	194	108(55.7)[a]	43(22.2)[a]	21(19.4)	15(7.7)	23.5
Rough	152	111(73.0)[b]	43(28.3)[b]	16(14.4)	10(6.6)	24.0

* PDT : population doubling time.

[a,b] Values with different superscripts in the same column differ ($P<0.05$).

The rates of adhesion in rough (73.0%) cells were significantly higher than that of smooth (55.8%) cells ($P<0.05$), but there were no significant difference in establishment efficiency (6.6~7.7%) and PDT (23.5~24.0 h) between both cell types (Table 4).

Ⅳ. DISCUSSION

Generally serum is a partially undefined material that contains growth and attachment factors, and may show considerable variation in the ability to support growth of particular cells. Rossman and Goncharova (1998) reported that the level of oxidants in cells cultivated in the presence of low concentrations of serum (<0.25%) was higher compared to control cells (5%), and suggested that the increases in the spontaneous mutation rates under low serum conditions may be partly a result of oxidative stress due to a lack of serum antioxidants. In this study, the establishment efficiency of clonal lines by different FBS concentrations was not different, but PDT was significantly decreased by increasing the FBS concentration in culture medium ($P<0.05$).

In living organisms, it is necessary to limit the DNA damage caused by reactive species with antioxidants to survive. Damage to the genetic material induced by ROS has been studied from various aspects for many years. These include chemical analysis of DNA composition or DNA metabolites in body fluids (Rhaese and Freese, 1968; Ames and Gold, 1991), the ability to initiate transformation (Larson et al., 1992), changes in chromosome structure (Phillips et al., 1984), unusual expression of genes (Suzuki and Hei, 1996), changes of cell phenotype, and analysis of DNA sequences (McBride et al., 1991).

Catalase is one of the antioxidative enzymes that removes H_2O_2 from cells (Chance, 1947). Rossman and Goncharova (1998) reported that spontaneous mutation rates under low serum conditions could be reduced by the addition of catalase (100 ng/ml) to the medium, but it's growth rates did not affected. Balin et al. (2002) reported that there were no significant differences in the growth of cells established from fetal and postnatal donors even though the enzymic antioxidant defences were relatively lower in fetal cells. In this study, the addition of catalase in culture medium did not show a positive effect on the establishment efficiency of clonal lines compared to control. However, the establishment efficiency of clonal lines of βME-added group was significantly higher than those of catalase-added and control groups (see table 2). These results could be explained by previous reports that differences in levels of resistance to the toxic effects of oxygen observed in cells isolated from different species appear to depend more on GSH concentration than on levels of enzymic defenses (Yuan et al., 1995).

βME is one of the most effective and frequently used thiol compounds, which is known to enhance the growth or colony formation of various types of cells *in vitro*. Addition of βME promotes the uptake cystine in cells (Ishii et al., 1981), thereby promoting their proliferation and GSH levels. GSH synthesis is highly dependent on the availability of cysteine in the medium (Ishii et al., 1981; Rathbun and Murray, 1991), and it is known that the roles of GSH is to maintain the redox state in cells, protecting them against harmful effects caused by oxidative injuries. For example, it has been demonstrated that chronic stimulation of GSH synthesis increases the proliferating capacity of cells in culture while chronic inhibition of GSH decreases the proliferating life of cells (Honda and Matsuo, 1988). Futhermore, βME promotes not only embryo development, but also intracellular GSH synthesis (Takahashi et al., 1993).

Therefore, in cloning, the use of cells that were established in culture medium supplemented with βME may be also effective on enhancement of developmental rates and viability of cloned embryos.

The result of present study shows that the establishment efficiency of clonal cell lines can be enhanced by the culture in media supplemented with 30% FBS and 100 nM βME and PDT was decreased by increasing the establishment efficiency of clonal cell lines. However, catalase supplementation, and cell size and morphology did not affect the establishment efficiency of clonal cell lines.

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