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Establishment of two chicken embryonic cell lines in a newly developed nutrient medium

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SUMMARY

Two cell lines, named KCEK and KCEL, were established from chicken embryonic kidney and lung. The basal culture medium was newly developed and the cell growth medium consisted of K1999 supplemented with 10% heat inactivated chicken serum. Both cells were well adapted to grow in vitro and more than 50 passages have been made so far. Once the cell lines were established the cells were easily adapted to grow in other growth media supplemented with fetal calf serum. Neither tumor formation in chicks nor P52 avian leucosis common antigen was detected in these cells. However, the oncogene analysis on these cells has not been performed yet. Both cells were permissive hosts for the Aujeszky's disease virus, Newcastle disease virus, and vesicular stomatitis virus.

KEY WORDS: Chicken embryonic cell line, Novel cell culture medium

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Chicken cell lines of non-tumor origin or viral antigen-free are still exceptional. Majority of avian cell lines reported so far is originated from tumor organs (Akiyama and Kato, 1974; Baba *et al.,* 1978; Calnek *et al.,* 1978; Dienglewicz and Parcells, 1999; Morgan and Parsons, 1986; Kawaguchi *et al.,* 1987) and/or transformed cells (Chang and Delany, 2004; Danforth *et al.,* 1994; Desjardins *et al,* 1986; *Kaaden et al.,* 1982; Kitamoto *et al.,* 1980; Beug e*t al.,* 1979) by oncogenic virus transfection (Abujoub and Coussens, 1995; Ikuta *et al.,* 1987; Zinkewich-Peotti *et al.,* 1988; Hill *et al.,* 1985). At present, the exceptional cases reported are one called virus-free cell line (Ogura and Fujiwara, 1987) and another named DF-1 (Himly *et al.,* 1998). The present author developed a basal nutrient medium (BM1999) for chicken cell culture in 1999 (PCT/JP2006/310852) after many trials and errors. During the study on the BM1999 two cell lines originated from chicken embryo (kidney and lung) were established.

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The cell growth medium (GM) consisted of BM1999 supplemented with 10% chicken serum (CS), substituted to fetal calf serum (FCS) after the establishment of cell lines. The most of primary cultures were successfully passaged more than 20 times when cultures were incubated at 38-39°C in stationary condition.

The composition of BM1999 is shown in Table 1. All ingredients are dissolved in double distilled water and sterilized by filtration with 220 mm pore membrane (Millipore Co.,). It is stored at 4-8°C in dark and used within 6 months after the preparation. GM employed for the establishment of cell lines was consisted of 9 parts of BM1999 and 1 part of home-prepared CS. CS was separated from the blood harvested from young broilers by cardiac puncture. CS was clarified, filtered, heated at 55°C for 30 minutes, and stored at 4°C. Antibiotics, ampicillin Na, streptomycin sulfate, and kanamycin sulfate, were added at 100 mg per ml of GM and or cell maintenance medium (MM), BM1999 without supplement of serum.

The kidney and lung were collected from a chicken embryo one day before hatching of conventional egg, white leghorn breed (Saitama experimental animal supply center). The tissue was cut into small fragments and treated with 0.1% col-

TABLE 1 - *Basal nutrient medium 1999 for chicken cell culture.*

Ingredients	*(mg per 1 liter)*
Sodium chloride	6,200
Potassium chloride	400
Calcium chloride dihydrate	200
Magnesium sulfate	90
Sodium dihydrogenphosphate anhydrous	140
Sodium bicarbonate	2,000
Iron (III) nitrate enneahydrate	0.1
HEPES	250
Glucose	1,000
Galactose	1,000
Fractose	1,000
N-acehyl-D(+)-glucosamine	300
Sodium pyruvate	200
Succinic acid	100
L-Alanine	300
L-Arginine hydrochloride	80
L-Cysteine hydrochloride	70
L-Glutamine	600
Glycine	230
L-Histidine hydrochloride	40
L-Isoleucine	100
L-Leucine	100
L-Lysine hydrochloride	240
L-Methionine	40
L-Ornithine hydrochloride	200
L-Phenylalanine	60
L-Proline	100
L-Serine	64
L-Threonine	490
L-Tryptophan	16
L-Tyrosine, disodium	90
L-Valine	10
Choline bitartrate	10
Folic acid	4
Nicotinamide	4
Calcium pantothenate	4
Pyridoxal hydrochloride	4
Riboflavin	0.4
Thiamine hydrochloride	4
i-inositol	10
Phenol red	5

lagenase solution (cell culture grade, WAKO, Japan) for cell dispersion. The cells were suspended in GM
containing c. a half million viable cells per ml, dispensed to flasks (Nunclon delta surface, Sweden), and stationary incubated at 38-39°C. Within seven days incubation, more than 60% confluent monolayer were formed. These cultures were successively passaged in flasks for three times. In this stage only EDTA solution (0.05% EDTA in PBS), without trypsin, was used for cell dispersion.

Since cells were stable to grow in vitro, cloning was made by a limited-dilution precedure, seeding a small number of viable cell suspensions into 24-well type microplates (Nunclon). Clones actively grown in the plates were further passaged in flasks. The cells established from kidney were named KCEK and the cells from lung were named KCEL respectively.

After 15th passage level, both KCEK and KCEL were adapted to grow in the RPMI-1640, slightly modified to contain extra sugars (2 g of glucose, 0.5 g of galactose, and 0.5 g of fructose per 1,000 ml) supplemented with 10% FCS. These cells have been well preserved for more than 2 years by a manner generally employed.

Cellular morphology was studied on slip cultures. Giemsa stainings were made after methanol fixation. KCEK cells show a typical epithelial shape and KCEL cells show a pleomorphic epithelioid morphology. Both cells are distinguished by microscopical observation according to their morphology in the early incubation.

Chromosome analysis was done in the usual manner. Cells at log phase growth were incubated for 8 hours in the presence of colchicine (0.02 mg/ml), allowed to swell in 70 mM KCl for 15minutes, fixed in cold methanol-acetic acid (3:1), centrifuged, and resuspended in 40% acetic acid solution. The cells were spread on warm slides and air-dried. After Giemsa staining, the preparations were analyzed and photographed under a light microscope. Fifty metaphase spreads were counted per cell line. Karyotyping estimated for both cells was quasidiploid and major chromosome number was of 2N=76. Sex chromosomes were hardly identified since the size of chromosome was so small. KCEL was estimated to be alveolar macrophages (or secondary alveoli in other name) since the cells were positive

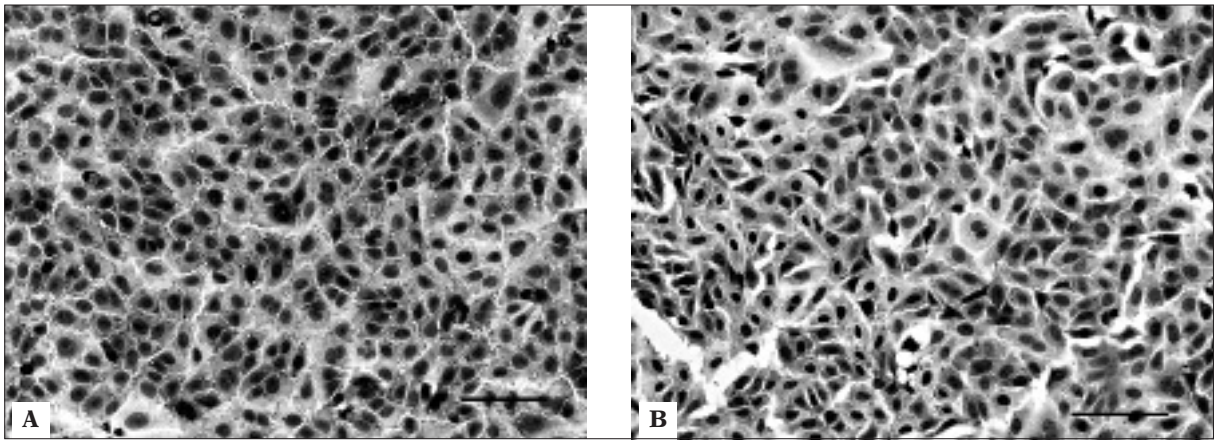

FIGURE 1 - *Morphology of KCEK and KCEL cells. (A) KCEK cells, passage 44th level, showing an epithelial morphology. Methanol fixed and Giemsa stained. Bar = 100 mm. (B) KCEL cells, passage 49st level, showing epithelioid morphology. Methanol fixed and Giemsa stained. Bar = 100 mm.*

for acid phosphatase and weakly positive for nonspecific esterase. Both enzymatic reactions were examined by a commercially available kit (Muto Chem. Japan). None of these enzymes were demonstrated in KCEK cells.

Tumorgenicity in vivo and p52 antigen tests were examined as follows. The cell suspension in PBS, containing at ca. two million viable cells per ml, were subcutaneously injected to 10 of one-day old chicks at 1 ml per chick respectively. Clinical observation was made for 3 months, and then autopsy and pathological examinations were performed as usual.

The cellular extract prepared from c. one million cells was tested for p52 antigen, know as a common avian leucosis, by the kit (IDEXX Lab. USA) according to the manufacturer's instruction. Neither tumorgenicity in 1-day-old chicks nor p52 common avian leucosis antigen was demonstrated for both cells.

A possibility to certify viral contaminations was tested as follows. The cellular lysate was respectively prepared from both KCEK and KCEL cells, c. 2 million cells each. After clarification they were inoculated to monolayer cell cultures of KSEK6 (Kadoi, 1992), KMP (Kadoi *et al.,* 1997), and KDK-1 cells (Kadoi *et al.,* 1992). These cells were previously confirmed in our hands to be highly sensitive for a variety of viruses. The cultures were maintained at 34°C for 10 days. None of these cultures, KSEK6, KMP, and KDK-1, proved any CPE agents in the first inoculation. However, three serial blind passages were performed for a confirmation. None of viral contaminations was demonstrated after all.

Virus susceptibility was tested as follows. Three strains of viruses, Aujeszky's disease virus (ADV) (Fukusho *et al.,* 1981), Newcastle disease virus (NDV) (Nobuto, 1968), and vesicular stomatitis virus (VSV) (Karstad and Hanson, 1958), were tested for viral susceptibility. All these viruses were known to grow in variety of cells. They were adapted to grow in KSEK6 in advance. Confluent monolayer of both KCEK and KCEL cells were respectively infected with these viruses at MOI =0.05 in the first infection. When infected cells showed clear CPE, second and third virus passages were serially made to inoculate at 1/100 dilution of earlier passages to confirm the replication of infective progenies. The infectivity of third passages was measured by inoculating 10-fold-dilution of virus in KSEK6 cells grown microplates and expressed in $TCID_{50}$ per ml according to the CPE occurrence. Both cells were permissive for all viruses tested. Infective progeny virus produced in both cells was at least $10^{7.50}$ $TCID_{50}$ per ml for ADV and VSV, and at $10^{5.50}$ $TCID_{50}$ per ml for NDV.

Adaptation to other media was tested. Trials were also made on the two cell lines on incubation temperatures at 37-39°C, the application of other cell culture media commercially available as Eagle MEM (containing 0.1% glucose) (MEM) and RPMI-1640 (containing 0.1% glucose) (RPMI). KCEK cells were easily adapted to grow in both MEM and or RPMI supplemented with 10%

FCS. However, KCEL tended to grow better in GM mentioned above. Their growth at 38-39°C was superior than at 37°C.

The cell lines of either tumor origin or transformed by oncovirus transfection are not suitable host cells for biological products although some of such cells has been used for virus assay system when their susceptibility is high enough for practical application. A large number of SPF chicken hatching eggs have been utilized worldwide, not only for vaccine production and also diagnostic procedures. The egg supply demands major financial support. In these circumstances established chicken cell lines free from viral contamination or not tumor origins are highly expected. Both KCEK and KCEL have not been analyzed for oncogenes. Therefore at present it is limitedly expressed that two cell lines were established from chicken embryo and the newly developed BM1999 was beneficial for in vitro culture of chicken cells. These cells are novel candidate tools in biosciences including virology. In the author's limited experience (unpublished data), both cells were good feeder cells for the hybridomas during cloning procedures in monoclonal antibody preparation.

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