The reversion to diploid cells from established triploid V79 cells

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Abstract. The triploid V79 cells are stable under usual culture conditions, and do not revert to being diploid. Here, the triploid–diploid transition of triploid V79 cells has been successfully induced in suspension culture in culture dishes with untreated surfaces. The diploid cells began to appear in a population of triploid V79 cells cultured under these conditions for 4 weeks. All of the triploid cells were transformed to diploid through subsequent monolayer culture for 5 weeks. It was confirmed that the revertant diploid cells had the same characteristics as original diploid V79 cells, with respect to DNA histograms, cell volume and chromosome number. Thus, it seems that suspension culturing is an important factor that induces the triploid–diploid transition.

INTRODUCTION

V79 Chinese hamster lung cells (V79 cells) have been found to possess an unusually stable diploid chromosome pattern during sub-culturing. They are polyploidized by demecolcine (Colcemid), some of the cells becoming tetraploid (Harris 1971). Chromosome instability at higher ploidy is well recognized, and in V79 cells in some cases, the terminal ploidy is pseudo triploid (Moore *et al.* 1968) or aneuploid (Graves & McMillan 1984). A triploid V79 cell line has been established from V79 cells highly polyploidized by treatment with K-252a (Fujikawa-Yamamoto *et al.* 2002a). These triploid cells have three homologous chromosome sets and twice the volume (Coulter volume) of the parental diploid cells (Miyagoshi *et al.* 2004). However, whether the triploid V79 cells could revert to the parent diploid V79 cells has not previously been examined. Whereas there are many reports on the polyploidization of V79 cells (Fujikawa-Yamamoto *et al.* 2000; Fujikawa-Yamamoto *et al.* 2002a; Miyagoshi *et al.* 2004), there is little evidence about the reversion to the diploid state. In fact, an established tetraploid Meth-A cell type did not revert to diploidy, even after temperature changes in culture conditions (Fujikawa-Yamamoto *et al.* 2001; Miyagoshi *et al.* 2003), hydrochloric acid treatment (Fujikawa-Yamamoto *et al.* 2003) or changes in the serum concentration of the culture medium (Fujikawa-Yamamoto *et al.* 2002b). Here in this study, we have observed the reversion of triploid V79 cells to diploid V79 cells and we have concluded that the transition was brought about by suspension culturing.

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MATERIALS AND METHODS

Cells

V79 cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C as a monolayer culture in Leibovitz's L15:Ham's F10 mixture (7 : 3) supplemented with 10% foetal bovine serum (Sigma, MO, USA), streptomycin (100 µg/ml, Nakarai Testique Co, Japan) and penicillin (50 units/ml, Nakarai Testique Co.). The diploid, triploid and revertant diploid cells were cultured under the same conditions, described previously.

Suspension and subsequent monolayer cultures

Exponentially growing V79 cells were plated into surface-untreated culture dishes (90 mm diameter, Eiken Kizai, Japan) at a density of about 2×10^5 cells per dish, and the medium was changed 1 week after seeding. The cells were cultured in suspension. After 1, 2, 4 and 6 weeks, only V79 cell clumps were gently placed onto surface-coated culture dishes (60 mm diameter Nunc, Naperville, IL, USA). Every 3 days, the cells were sub-cultured at a 1/4 dilution by two washes with divalent cation-free phosphate-buffered saline (PBS⁽⁻⁾) and subsequent trypsinization (0.2% trypsin and 30 mM ethylenediaminetetraacetic acid). The residual cells were prepared for flow cytometry (FCM).

Cell preparation for flow cytometry and cell counting

The cells were fixed with 20% ethanol, and were then incubated with 0.5 ml of 0.25% RNase (type II-A, Sigma, MO, USA) for 3 h at 4 °C. At this stage, cell number was counted using a haemocytometer. Then, cells were stained with propidium iodide (PI, 7.5×10^{-5} M) and were examined for red fluorescence by FCM. Under these staining conditions, signal resulting from residual double-stranded RNA is negligible and relative intensity of red fluorescence corresponds to the DNA content (Krishan 1975).

FCM measurements

Fluorescence from individual cells was measured using a FACSORT (Becton-Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA). Fluorescence of individual cells irradiated with a focused laser (wavelength of 488 nm) was detected using a photomultiplier tube. The relative intensity of red fluorescence (FL2A and FL2H) was measured, and DNA histograms were obtained.

Chromosome analysis

Exponentially growing diploid, triploid and revertant diploid cells were exposed to demecolcine at a concentration of 270 nM for 1 h. Cells were trypsinized, swollen with 75 mM KCl, fixed with fixing solution (CH₃OH:CH₃COOH = 7 : 3) and were dropped on glass slides. They were then stained with Giemsa solution to visualize chromosomes for assessment and photography. Chromosome numbers were counted from the photographs.

Cell volume distribution

The exponentially growing diploid, triploid and revertant diploid cells were trypsinized, fixed with 20% ethanol and were resuspended in PBS⁽⁻⁾. Distribution of cell-volume (Coulter volume) was measured using a Coulter Counter (ZM/256, Coulter Electronics, Fullerton, CA, USA). Standard spheres (9.8 µm diameter, Coulter sphere, Coulter Electronics) were used as control. Note that the Coulter volume depends on the material tested, because it is calculated based on the resistance of particles.

Observation of morphology

The exponentially growing diploid, triploid and revertant diploid cells were washed once with PBS⁽⁻⁾ and were fixed with methanol before staining with haematoxylin and eosin. Photographs were taken using a light microscope (BX50 and CK2, Olympus, Tokyo, Japan) equipped with a digital camera system (DS4040, Olympus).

RESULTS

To examine the effects of suspension culture, diploid and triploid V79 cells were cultured in dishes with untreated surfaces for 1, 2, 4 and 6 weeks. Representative micrographs of them cultured in suspension for 6 weeks are shown in the upper panels of Fig. 1. The cells adhered to each other and formed globe-shaped clumps. The clumps of cells were re-cultured in surface-treated dishes (typical culture dishes). An example of cell proliferation from a massive clump was observed (lower panels Fig. 1), suggesting that the cells can grow after release from stressed culture conditions.

To examine DNA ploidy, changes in DNA histograms were measured for diploid and triploid cells in this suspension culture (Fig. 2). The DNA content of the main population was 2c and 3c at 2 and 3 weeks, respectively. A 2c peak appeared in the triploid cell population at 4 weeks (the arrow in Fig. 2(b)). The fraction of diploid cells became a major population by 6 weeks. Note

Figure 1. Micrographs of diploid (a, c) and triploid (b, d) V79 cells. Diploid and triploid cells formed cell clumps during suspension culture for 6 weeks (a, b). Clumps were placed into typical, surface-coated, culture dishes and were cultured for 2 days (c, d). In c and d, the cells were stained with Giemsa solution.

Figure 2. Changes in DNA fluorescence, histograms of diploid (a) and triploid (b) V79 cells in suspension culture for 6 weeks. Numerals in the histogram represent time in weeks, after suspension culture. The abscissa represents relative DNA content (C, complement). Longitudinal broken lines in the panel were drawn to facilitate understanding. Arrow indicates the 2c peak.

that there was considerable cell debris present, reflecting the presence of cell death in the suspension culture. These results indicate that the triploid cells reverted to diploid cells.

To examine the growth of diploid and triploid V79 cells after release from suspension culture, cell numbers were counted, corrected to take account of sub-culturing, and numbers were plotted against day after seeding into surface-untreated dishes (Fig. 3). The numbers of diploid and triploid cells did not increase during the suspension culture for 1–6 weeks, but did increase when cells were returned to monolayer culture. Doubling times for the population of diploid and triploid cells released from suspension culture were 20 and 21–23 h, respectively, suggesting that the cells were not damaged by the stress of suspension culture. Note that proliferation capability of reverted cells was similar to that of controls.

Figure 3. Growth curves of diploid (a) and triploid (b) V79 cells after release from suspension culture conditions. Exponentially growing diploid and triploid cells were cultured as suspension cultures. At 0 (○), 1 (●), 2 (□), 4 (■) and 6 (△) weeks after suspension culture, cells were released from the stress of suspension culture. Solid lines were drawn to facilitate understanding. Td is the doubling time of the cell population.

Figure 4 shows DNA histograms of cells 5 weeks after release from suspension culture (which had been performed for the previous 6 weeks). The population that consisted of triploid V79 cells now showed a diploid pattern, suggesting that all of the triploid cells had transformed to diploid cells. Note that only the clumps of triploid cells were returned to the surface-coated dishes to grow as monolayer cultures. To test the integrity of diploid, triploid and revertant diploid cells, chromosome distributions were examined (Fig. 5). Chromosomes of diploid, triploid and revertant diploid cells numbered 22, 33 and 22, proving that these cells were diploid, triploid and revertant diploids, respectively. To examine the morphological characteristics of the diploid, triploid and revertant diploid cells, phase-contrast micrographs and the cell distributions in terms of volume (Coulter volume) were measured (Fig. 6). Morphology of the revertant diploid cells resembled that of original diploid cells. Relative cell volumes of the diploid, triploid and revertant diploid cells were 1: 2: 1, suggesting that the revertant diploid cells have the same cell volume as the original diploid cells.

DISCUSSION

Polyploidization of mammalian cells occurs in various organs, particularly in aged or partially hepatectomized liver (Fogt & Nanji 1966; Mossin *et al.* 1994; Zong *et al.* 1994; Seglen 1997); however, there have been no reports on revertant diploid cells. Harris (1971) and Graves & McMillan (1984) established tetraploid V79 Chinese hamster cells by cloning after demecolcine treatment; however, DNA degradation occurred within 2 months in tetraploid cells.

In the present study, we have demonstrated that suspension culturing for several weeks is effective for inducing the triploid–diploid transition. Cell proliferation starts from cell clumps

Figure 4. DNA fluorescence histograms of diploid (a) and triploid (b) V79 cells treated by 6 weeks in suspension culture and a subsequent 5 weeks of monolayer culture. Paired numerals in the histogram represent the time in weeks, of suspension culture and in monolayer culture, respectively. The abscissa represents relative DNA content (C, complement). Longitudinal broken lines in the panel were drawn to facilitate understanding.

Figure 5. Micrographs and histograms of chromosome number of diploid (a), triploid (b) and revertant diploid (c) V79 cells. Exponentially growing cells were exposed to demecolcine for 1 h. Chromosomes were stained with Giemsa solution. The chromosomes of about 100 cells were counted from enlarged photographs.

in monolayer cultures. It seems here that the triploid–diploid transition occurred in clumped triploid cells. Moscona (1961) reported that cells dissociated with trypsin reform a structure that is similar to that found in the original tissue or organ if the cells are incubated under appropriate conditions. The reversion of triploid V79 cells to diploid cells may be explained by the previously

Figure 6. Micrographs and cell volume distribution of diploid (a), triploid (b) and the revertant diploid cells (c). Exponentially growing diploid, triploid, and the revertant diploid V79 cells were prepared for measurements. Cells were stained with haematoxylin and eosin. Longitudinal lines and a scale bar were drawn to facilitate understanding. C shows standard spheres 9.8 μm in diameter.

mentioned histogenesis or organogenesis phenomenon. It is of interest that the cell population doubling time was almost the same for both the diploid and triploid cells after suspension culture. These results were consistent with the doubling times that we have reported previously (Miyagoshi *et al.* 2004). Thus, it seems that suspension culture may not affect the doubling time, which provides an index of proliferating activity.

Although DNA degradation in polyploid Chinese hamster cells has been reported previously (Harris 1971; Graves & McMillan 1984), mechanism of the loss of chromosomes has not been elucidated. The triploid cells reverted to diploidy after suspension culture for 6 weeks, followed by subsequent monolayer culture for 5 weeks. In the triploid–diploid transition, a haploid set of DNA was lost abruptly, not gradually, from the triploid cells. This suggests that DNA is grouped as units of a haploid set of chromosomes (Fujikawa-Yamamoto *et al.* 2002a). The number of chromosomes in the recurrent diploid cells was 22, suggesting that integrity of the chromosome number was maintained. We have previously reported that triploid V79 cells had a tetraploid cell volume (Fujikawa-Yamamoto *et al.* 2002a). This means that they were established through the tetraploid–triploid transition without an additional cell division. The recurrent diploid cells, however, showed a diploid cell volume. It seems that the triploid–diploid transition was accompanied by an abnormal cell division that divided cells in proportion to the DNA content. The triploid–diploid transition may generate haploid cells and diploid cells with sex chromosomes such as YY, although it is uncertain whether haploid cells can survive in this culture medium.

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