DNA-unstable decaploid mouse H1 (ES) cells established from DNA-stable pentaploid H1 (ES) cells polyploidized using demecolcine

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Abstract

Objectives: DNA content of diploid H1 (ES) cells (2H1 cells) has been shown to be stable in long-term culture; however, tetraploid and octaploid H1 (ES) cells (4H1 and 8H1 cells, respectively) were DNA-unstable. Pentaploid H1 (ES) cells (5H1 cells) established recently have been found to be DNA-stable; how, then is cell DNA stability determined? To discuss ploidy stability, decaploid H1 (ES) cells (10H1 cells) were established from 5H1 cells and examined for DNA stability.

Materials and methods: 5H1 cells were polyploidized using demecolcine (DC) and 10H1 cells were obtained by one-cell cloning.

Results: Number of chromosomes of 10H1 cells was 180 and durations of their G₁, S, and G₂/M phases were 3, 7 and 6 h respectively. Volume of 10H1 cells was double that of 5H1 cells and morphology of 10H1 cells was flagstone-like in shape. 10H1 cells exhibited alkaline phosphatase activity and their DNA content decayed in 91 days of culture. 10H1 cells injected into mouse abdomen formed solid tumours that contained several kinds of differentiated cells with lower DNA content, suggesting that 10H1 cells were pluripotent and DNA-unstable. Loss of DNA stability was explained using a hypothesis concerning DNA structure of polyploid cells as DNA reconstructed through ploidy doubling was arranged in mirror symmetry in a new configuration.

Conclusion: In the pentaploid–decaploid transition of H1 cells, cell cycle parameters and pluripotency

were retained, but morphology and DNA stability were altered.

Introduction

H1 (ES) cells, mouse germline-transmissible embryonic stem (ES) cells, were established from blastocysts of C3H/He mice, and it has been confirmed that these cells have the ability to differentiate into neural cells, epithelial cells, muscle cells, hair follicle cells and chondrocytes (1). Tetraploid H-1 (ES) cells (4H1 cells) have been established from diploid H1 (ES) cells (2H1 cells) through polyploidization, using demecolcine (DC) (2). Octaploid H1 (ES) cells (8H1 cells) were also established from 4H1 cells using DC (3). Pentaploid H1 (ES) cells (5H1 cells) were serendipitously established from an 8H1 cell (4). Pluripotency of 2H1, 4H1, 8H1 and 5H1 cells was demonstrated by positive expression of alkaline phosphatase or ability to form teratocarcinomas.

DC antagonizes tubulin polymerization and induces disassembly of microtubules into monomers. Chinese hamster V79 cells exposed to DC exhibit deformed cytoplasmic morphology from sphere- to amoeba-like in M phase (5), and polyploidization accompanied by various types of nuclear morphology (6). This drug inhibits formation of spindle fibre in M phase and polyploidizes cells, depending on the cell type. DC can polyploidize H1 cells as well as many other cells, including V79 and mouse Meth-A cells, but additional types of cells that can be polyploidized by DC remain unknown.

Polyploidization of mammalian cells occurs in various organs, particularly in aged or partially hepatectomized liver; however, causes and mechanisms involved are poorly understood (7–9). The DNA content of mammalian diploid cells is well preserved during subculturing; however, DNA content of polyploid cells sometimes decreases gradually and occasionally it decreases abruptly by half. Moor *et al.* (10) concluded that near-triploid is the terminal ploidy of near-tetraploid Ehrlich's ascites tumour cells.

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Figure 1. Growth curves (a) and DNA histograms (b–e) over the course of establishing 10H1 cells. Exponentially growing 5H1 cells were exposed to several concentrations of demecolcine (DC) and cell numbers were counted (a). Symbols $\circ$, $\triangle$, $\bullet$ and $\blacktriangle$ represent DC concentrations of 0, 27, 81 and 270 nm respectively. Panel B represents DNA histograms of 5H1 cells exposed to DC for 2 days, in which numerals on the upper left side represent DC concentration (nm). Exponentially growing 5H1 cells were exposed to 270 nm DC and then released from the drug (c). In panel c, the three hyphenated numerals in units of days represent duration of DC exposure, time after drug removal and time after first subculturing, in that order. Numerals on the upper right side represent time (days) after DC addition. Cell population of 2-2-20 was one-cell cloned, and seven decaploid clones were obtained (d and e). They were gathered into one cell line and used as decaploid H1 cell population. Notations of upper left in histograms of d and e represent clone name. In the DNA histograms, abscissa represent DNA content (C, complement). Longitudinal broken lines were drawn to facilitate comprehension.

Harris (11) has shown that chromosome number was constant in diploid cells but decreased with subculturing in tetraploid and octaploid pig kidney cells.

DNA content of tetraploid and octaploid Meth-A cells decayed gradually with culturing and reached a plateau phase (12), while several studies have reported DNA loss in polyploid cells. DNA content of triploid V79 cells was stable (13), except in one special case where the cells were suspension-cultured (14). Matveeva *et al.* (15) have created many types of tetraploid hybrid cells by cell fusion, and reported that chromosome loss in hybrid cells fell into three types: stable, bilateral loss, and unilateral segregation of chromosomes. It has been reported that mouse ES cell/fibroblast hybrid cells with near-tetraploid karyotype

yielded diploid/tetraploid chimaeras after injection into C57BL mouse blastocysts, suggesting that the DNA of tetraploid hybrid cells was stable following chimaera formation (16). In spite of these long-term studies, the mechanism of stability of DNA content still not yet known.

4H1 cells have been shown to lose DNA content gradually in long-term culture (17) or abruptly in DME medium (18). 8H1 cells also showed DNA decay where these octaploid cells changed to hexaploid teratocarcinoma cells (3). In 5H1 cells, recently established in the process of cloning 8H1 cells (4), DNA content was stable, unlike that of 4H1 cells and 8H1 cells. Such excellent stability of DNA content of 5H1 cells was explained using a hypothesis concerning DNA structure of polyploid cells (19). We

Figure 2. Growth curves (a), DNA histograms (b, e, h), volume distribution (c, f, i) and phase-contrast micrographs (d, g, j) of exponentially growing 2H1 (b–d), 5H1 (e–g) and 10H1 cells (h–j). In growth curves, symbols ○, ● and □ represent 2H1, 5H1 and 10H1 cells respectively. DNA histograms were used in cell cycle analysis. In panels b, e and h, synthesized (grey line) and observed (black line) histograms are superposed. In DNA histograms, abscissa represent DNA content (C, complement). Cell volume was measured using a Coulter counter. Vertical dotted lines and scale are drawn to facilitate comprehension. Phase-contrast micrographs shown as inserts are of low-magnification fields.

Table 1. Cell cycle parameters of 2H1, 5H1 and 10H1 cells

Phase	G ₁	S	G ₂ /M
2H1 cells (Td = 15.6 h)			
Fraction	0.21 (0.190)	0.52 (0.467)	0.27 (0.236)
Duration (h)	2.5	7.7	5.3
5H1 cells (Td = 15.6 h)			
Fraction	0.20 (0.184)	0.55 (0.499)	0.25 (0.226)
Duration (h)	2.4	8.2	5.0
10H1 cells (Td = 15.6 h)			
Fraction	0.23 (0.208)	0.47 (0.417)	0.30 (0.269)
Duration (h)	2.8	7.0	5.9

Td is the doubling time calculated from Fig. 2. Phase fractions were determined by omitting those for cells with other ploidies in the cell population. Numerals in parentheses represent the fraction of the total cell population. Phase duration was calculated using conventional equations (30) employing the doubling time instead of the cell cycle time.

were interested in whether DNA of decaploid H1 cells (10H1 cells), formed by double ploidy of DNA-stable 5H1 cells, was stable or not. In the study described here,

10H1 cells were established from 5H1 cells and examined for DNA stability.

Materials and methods

Cells

H-1 (ES) cells established from a blastocyst of a C3H/He mouse (1) were purchased from RIKEN Bio Resource Center (Institute of Physical and Chemical Research, Tsukuba, Japan) and were maintained in a humidified atmosphere of 5% CO₂ at 37 °C in Leibovitz's L15 : Ham's F10 mixture (7:3) (L15F10) medium supplemented with 10% foetal bovine serum (CELLect GOLD; ICN Biomedicals, Aurora, OH, USA), 2-mercaptoethanol (0.1 mM), streptomycin (50 µg/ml), penicillin (50 U/ml), and leukaemia inhibitory factor (LIF, 500 U/ml, ESGRO; Chemicon International Inc., Funakoshi, Japan). LIF was added to the medium to prevent differentiation of the ES cells (20). H-1 cells in L15F10 medium were designated H1 cells. Pentaploid H1 cells (4) were obtained from an

Figure 3. Chromosomes and pluripotency of 10H1 cells. Exponentially growing 10H1 cells were exposed to 270 nM DC for 3 h and stained with Giemsa solution, then chromosomes were counted. Panels a and d represent a chromosome micrograph and a chromosome number histogram of 10H1 cells respectively. Panel b is presented to show that 10H1 cells were mononucleate. Numerals of panels a and b represent chromosome number counted. Panel c shows nuclei of exponentially growing 10H1 cells. In panel d, open arrows denoted with 2H1 and 5H1 indicate 40 and 90, modes of chromosome numbers for 2H1 and 5H1 cells respectively (4). 2H1 (e), 5H1 (f), and 10H1 (g, h) cells growing exponentially were stained for alkaline phosphatase activity, in which activity-positive cells were stained red. Photograph 'h' is negative control in which cells were stained without substrate. Inserts at upper left and upper right show phase-contrast microphotographs and low-magnification microphotographs for main fields, respectively.

octaploid cell (3) induced from tetraploid H1 cells (2) established from diploid H1 cells. Population doubling level (PDL) of the early stage of establishment was in the order of 50 for both tetraploid and pentaploid cells used. Pentaploid H1 cells that had been stored at -135°C in the early stage of establishment (PDL~50) were thawed and used in experiments (4). Diploid, pentaploid and decaploid H1 (ES) cells were cultured under identical culture condi-

tions. The diploid, pentaploid and decaploid H1 (ES) cells were designated 2H1, 5H1 and 10H1 cells respectively. The 2H1 and 5H1 cells were used as controls.

Polyplodation using DC and cloning of decaploid cells

To determine appropriate DC dose for polyplodation, 5H1 cells were exposed to DC at concentrations of 0, 27,

Figure 4. DNA histograms of 2H1 (a, d), 5H1 (b, e) and 10H1 (c, f) cells at 21 (a–c) and 91 (d–f) days after start of culturing. Cells that had been frozen at -135°C at early stage (PDL $\approx$ 50) of establishment were used. In DNA histograms, abscissa represent DNA content (C, complement). Longitudinal broken lines were drawn to facilitate comprehension.

81 and 270 nM, and growth curves and DNA histograms were obtained, then 270 nM DC was used to polyploidize 5H1 cells.

5H1 cells exposed to 270 nM DC were released from the drug by washing twice in drug-free medium, and then were cultured again in DC-free medium. At various times, cells were subcultured and used to obtain DNA histograms. Population of 5H1 cells 20 days after first subculture at 2 days, after DC exposure for 2 days (2-2-20 in Fig. 1c) was a mixture of 5H1 cells and 10H1 cells, and from these they were single-cell cloned. Cells were seeded into 96-well dishes (96-well tissue culture clusters, 6.4 mm diameter; Costar Co., Cambridge, MA, USA), and cells were incubated in a CO_2 incubator. Seven clones that had decaploid DNA content were obtained (Fig. 1d,e) and gathered into one cell line. Hereafter, these cells obtained will be called 10H1 cells for convenience. At that time, expected number of cell divisions since first cloning was >40 .

Flow cytometry

Cells were fixed in 20% ethanol, incubated with 0.1% RNase (Type II-A; Sigma, St Louis, MO, USA) for 3 h at 4°C , and counted using a haemocytometer. Immediately before measurements, the cells were stained with propidium iodide (7.5×10^{-5} M) and examined for red fluores-

cence by flow cytometry (FCM). Under these staining conditions, the signal arising from residual double-stranded RNA is negligible and relative intensity of red fluorescence corresponds to DNA content (21). Fluorescence from individual cells was measured using a FACSORT (Becton Dickinson Immunocytometry Systems, Franklin Lake, ND, USA). Fluorescence of individual cells irradiated using focused laser light at wavelength of 488 nm was detected using a photomultiplier tube. Relative intensity of red fluorescence (FL2H) was measured and DNA histograms were obtained.

Cell cycle analysis

FCM data of FL2H through a logarithmic amplifier for 10 000 cells were inputted into CASL software (Mathematica; Wolfram Research Inc., Champaign, IL, USA) for cell cycle analysis of DNA histograms, on a log scale using transfer software 'FACS to ASCII' (freeware), and DNA histograms were decomposed into phase fractions based on DNA content (22). The algorithm of CASL is similar to Fried's method (23) except that normal distribution functions with the same half-width instead of the same coefficient of variation value are used as components. Doubling time of exponentially growing 2H1, 5H1 and 10H1 cells was used to calculate durations of G_1 , S and G_2/M phases.

Figure 5. DNA histograms (a, c), abdominal photograph (b) and a histological section (d). 10H1 cells used for DNA histogram a were injected into mouse abdomens at time = 0 days. At $t = 81$ days, solid tumours appeared in there (b). Tumours showed DNA histogram c, indicating that this was composed of a cell mixture containing hexaploid cells. Histological sections of such tumours showed many kinds of differentiated cells as well as undifferentiated cells (d). Inserts of panel a and c are DNA histograms of 2H1 and heart cells respectively. Abscissa of DNA histograms represent DNA content (C, complement). Insert of panel d is a macrograph of square region indicated in the main frame image.

Cell morphology and cell volume

The morphology of exponentially growing cells in culture flasks was photographed using a phase-contrast microscope (CK2; Olympus, Tokyo, Japan) equipped with a digital camera system (C4040; Olympus). Images were printed at magnifications stated in the text.

Exponentially growing cells were trypsinized, fixed in 20% ethanol and resuspended in divalent cation-free phosphate-buffered saline [PBS⁽⁻⁾]. Distribution of cell volumes was measured using a Coulter counter (ZM/256; Coulter Electronics, Fullerton, CA, USA).

Chromosome distribution

Exponentially growing cells were exposed to 270 nm DC for 3 h. Mitotic cells were collected, swollen with 75 mM KCl, fixed in fixing solution (methanol:acetic acid = 7:3) and dropped on glass slides. Chromosomes were stained with Giemsa solution (Sigma) and photographed; chromosome numbers were counted manually from the photographs.

Alkaline phosphatase activity

Exponentially growing cells in a Lab-Tek Chamber Slide (Nalge Nunc International, Naperville, IL, USA) were washed twice with PBS⁽⁻⁾ and fixed in fixing solution (methanol:formalin:acetic acid = 9000:1000:1). Cells in the Chamber Slide were stained for alkaline phosphatase activity using standard methods as recommended by the test kit instructions (Histofine; Nichirei Bio Science Co., Tokyo, Japan). Cells stained without substrates were used as negative control. Cells were photographed using a microscope (BX 60; Olympus) equipped with a digital camera system (C4040; Olympus). Images were entered into a personal computer and printed out at magnifications stated in the text.

Ninety day cell culture

10H1 cells stored at -135°C during early stages (PDL $\approx$ 50) of establishment were thawed and cultured. 2H1 and 5H1 cells were also thawed and cultured. Cells were trypsinized in 0.17% trypsin and 30 mM EDTA, and

Figure 6. Models of genome structures of diploid (a, f), tetraploid (b, g), octaploid (c, h), pentaploid (d, i) and decaploid cells (e, j). Small circles represent chromosomes and homologous chromosomes are represented by the same size and colour. Solid and broken lines of circles indicate male and female origins respectively. Homologous chromosomes array point symmetry (a), mirror symmetry (b, c, e), or asymmetry (d). In point-symmetric configuration, homologous chromosomes cannot come close to each other in folding structures (f). Therefore, somatic diploid cells are stable. In mirror-symmetric configuration (b, c, e), homologous chromosomes are close to each other (g, h, j) and DNA decays through bypassing DNA replication (g, h, j). With deviation from mirror symmetry, DNA decay ceases (k–m). In asymmetric configuration (d), no fitting of homologous chromosomes occurs, resulting in DNA-stable cells. Note that asymmetric DNA configuration of pentaploid cells (d) was rearranged to mirror-symmetric configuration in decaploid cells (e).

were subcultured almost every day in 1:2 or 1:4 dilutions, in culture flasks (25 cm²; Corning Inc., Corning, NY, USA). Cell density was maintained at approximately 5×10^6 cells/flask. DNA histograms of cell populations were obtained by FCM.

Tumour formation

Solid tumours formed by intraperitoneal injection of 10H1 cells into normal C3H/He male (5w) mice (Japan SLC Inc., Shizuoka, Japan) were sectioned and stained with haematoxylin and eosin (HE), the standard method. Their morphology was recorded using a microscope (BX 60, Olympus) equipped with a digital camera system; mouse abdomens were also photographed using a digital camera (μ 720, Olympus). Part of the solid tumour was cut, minced using scissors, filtered through 40 μ m nylon mesh and prepared for FCM. Part of the heart of the same animals was also prepared as controls.

Results

To examine effectual concentration of DC, 5H1 cells were exposed to various concentrations of it and their growth curves and DNA histograms were obtained (Fig. 1a,b). At 81 nM DC apoptosis was induced, thus 270 nM was used to polyploidize 5H1 cells. Polyploidization was induced by DC at 270 nM, the same concentration that has been used to polyploidize 2H1 and 4H1 cells (2,3) (Fig. 1b). To obtain decaploid cells, exponentially growing 5H1 cells were exposed to 270 nM DC for 1, 2, or 3 days, and then released from exposure (Fig. 1c). The DNA histogram of 5H1 cells 2 days after DC exposure (2-0-0 in Fig. 1c) had a 40C peak, suggesting that 5H1 cells were eicosaploidized. 5H1 cells 20 days after first subculture at 2 days after DC exposure for 2 days (2-2-20 in Fig. 1c) were of pentaploid–decaploid mixture, and these were then single-cell cloned. Seven clones of decaploid cells were obtained (Fig. 1d,e) and gathered into one

cell line; these were designated 10H1, cells and used in subsequent analyses.

To examine cell cycle parameters, doubling time and phase fraction of 10H1, cells were measured and compared to those of 2H1 and 5H1 cells (Fig. 2a,b,e,h and Table 1). Durations of G₁, S and G₂/M phases were 3, 7 and 6 h, respectively, essentially the same as those for 2H1 and 5H1 cells. To examine morphological characteristics, cell volume and morphology were measured (Fig. 2c,d,f,g,i,j). Modes of cell volume of 10H1 cells was double that of 5H1 cells, suggesting that cell cycle progression without cell division had occurred in the formation process of 10H1 cells (Fig. 2i). Morphology of growing 10H1 cells was flagstone-like in shape, differing from the spherical cluster shape of parent 5H1 cells, suggesting that expression of genes controlling cell morphology was altered in pentaploid–decaploid transition (Fig. 2j).

To examine nuclear characteristics, 10H1 cells were stained with Giemsa solution. Chromosomes of 10H1 cells were acrocentric, as were those of 2H1 and 5H1 cells (Fig. 3a); mode of their chromosome numbers was 170–180, double that of 5H1 cells, indicating that 10H1 cells have double ploidy of 5H1 cells (Fig. 3d). Nuclei of 10H1 cells had deformed mononuclei (Fig. 3b,c).

To examine pluripotent potential, 10H1 cells were stained to exhibit alkaline phosphatase activity (Fig. 3e–h). 10H1 cells were positive for alkaline phosphatase expression, suggesting that pluripotent potential was preserved during the pentaploid–decaploid transition (Fig. 3g).

To examine stability of DNA content, 2H1, 5H1 and 10H1 cells stored at -135°C at the early stage (PDL $\approx$ 50) of establishment were placed in medium and cultured continuously for 91 days (Fig. 4). DNA content of 10H1 cells decreased through both gradual and abrupt decay (Fig. 4f); however, DNA content of 2H1 and 5H1 cells was maintained over this period (Fig. 4d,e respectively), suggesting that DNA-stable 5H1 cells were transformed to DNA-unstable 10H1 cells.

To examine pluripotency and DNA stability *in vivo*, 10H1 cells were injected intraperitoneally into male mice (Fig. 5a). Around 81 days later, solid tumours were formed in the abdomens of the mice (Fig. 5b). DNA content of tumours was hexaploid, suggesting that DNA ploidy of 10H1 cells declined in tumour formation (Fig. 5c).

Tumours contained many kinds of differentiated cells as well as undifferentiated cells, suggesting that pluripotency of 10H1 cells was retained irrespective of DNA decay (Fig. 5d).

To verify that they had been established, 10H1 cells at early stage (PDL $\approx$ 50) of culture were stored at -135°C

for 1 month and then placed in culture. Cells at steady state population growth showed cell cycle parameters, morphology and DNA content equivalent to those of the cells before freezing, suggesting that 10H1 cells had thus been established (data not shown).

Discussion

DNA content of 5H1 cells was stable over long-term culture (4), but 4H1 (17) and 8H1 (3) cells decayed. 10H1 cells induced from 5H1 cells lost DNA in long-term culture. How then is cell DNA stability determined? What is the difference between DNA-stable cells and DNA-unstable cells? To explain the phenomenon that DNA content of 10H1 cells is unstable, a hypothesis that genome structure is fractal has been used (19). This hypothesis was constructed, mainly based on the reports of Myhra and Brogger (24), Bak *et al.* (25), Wollenberg *et al.* (26) and Nagele *et al.* (27).

Figure 6 provides schema of DNA configurations of diploid (a, f), tetraploid (b, g), octaploid (c, h), pentaploid (d, i) and decaploid cells (e, j). Homologous chromosomes are arranged point-symmetrically in diploid cells (a); thus, chromosome pairing that can induce chromosome loss is prevented. Tetraploid and octaploid cells that are made from diploid and tetraploid cells, respectively, result in mirror-symmetric DNA configurations (b, c) in which chromosome loss by homologous chromosome pairing occurs (g, h) and cease with loss of symmetry (k, l). Pentaploid cells are asymmetric in chromosome configuration (d); hence, they are stable (i). DNA configuration of decaploid cells results in mirror symmetry (e, j); therefore, they are unstable.

According to this hypothesis, even-ploidy cells (double ploidy cells), such as 4H1 and 8H1 cells, degrade in terms of ploidy and reach a semi-stable hypoploid state, while in odd-ploidy cells, such as 5H1, DNA decay is spatio-forbidden. Furthermore, any double ploidy cells that are constructed through polyploidization of DNA-stable cells (such as 10H1 cells), must have mirror-symmetric DNA configuration, and the DNA configuration allows DNA decay. This prospect may be in accordance with results that 10H1 cells were DNA-unstable.

Results for a series of polyploid H1 cells, 4H1, 5H1, 8H1 and 10H1 cells, may suggest basic characteristics of polyploid cells induced by DC exposure (2–4). Cell cycle parameters and pluripotency were retained; on the other hand, cell morphology and DNA stability were variable. DNA-stable 2H1 and 5H1 cells have growth morphology of spherical clusters, and DNA-unstable 4H1, 8H1 and 10H1 cells had flagstone-like shape. DNA-stable diploid Meth-A cells were spherical, but DNA-unstable tetraploid and octaploid Meth-A cells, double ploidy of diploid and

tetraploid Meth-A cells, respectively, exhibited flagstone-like shapes (28,29). DNA-stable diploid and triploid V79 cells were both spindle shaped (13). It seems that morphological alterations correlated with DNA stability.

It should be emphasized that these results pertaining to cellular responses of 10H1 cells can be applied only to particular types of proliferating cells cultured in L15F10 medium under specific conditions.

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