DNA Loss and Related Alterations in Long-term Cultures of Diploid, Tetraploid and Octaploid Meth-A Cells

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Summary The DNA content of polyploid cells sometimes decreases during subculturing. The mechanism of DNA reduction is not yet known. Precise measurements of DNA decay and related alterations in polyploid cells will be required to understand the mechanism. Diploid, tetraploid and octaploid Meth-A cells were continuously cultured for 244 d and the cellular DNA content was measured from the DNA histograms. The DNA content decays gradually with day t as expressed by $f(t) = I_p \exp\{-G(t)\}$, where I_p is the initial ploidy, $G(t) = at/\{\exp(-bt) + ct\}$, and a , b and c are the following parameters: $a=0.026$, $b=0.01$ and $c=0.0175$ (for tetraploid cells) or $c=0.01$ (for octaploid cells). The DNA content of diploid Meth-A cells was constant within the experimental errors. The DNA loss of polyploid cells was confirmed by a decrease in chromosome number. The cellular morphology changed in diploid and octaploid Meth-A cells, but not in tetraploid cells, suggesting that DNA loss and morphology alteration are independent. The doubling time shortened with culture time in the tetraploid and octaploid cells. The findings suggested that chromosomes are not independent in polyploid cells.

Key words DNA loss, Ploidy degradation, Tetraploid Meth-A cells, Octaploid Meth-A cells, Flow cytometry.

The DNA content of mammalian diploid cells is well preserved during subculturing; however, that of polyploid cells is not. The DNA content of polyploid cells sometimes decreases gradually, and occasionally it decreases abruptly by half. A few studies of DNA degradation in polyploid cells have been reported. Moor, *et al.* (1968) concluded that near triploid is the terminal ploidy of near-tetraploid Ehrlich's ascites tumor cells based on 3 years of observation. Harris (1971) showed that the chromosome number was constant in diploid cells but decreased with subculturing in tetraploid and octaploid pig kidney cells. Graves and McMillan (1984) reported that tetraploid DON and BI Chinese hamster cells were karyotypically stable but the octaploid cells were not. In spite of these long-term studies, the mechanism of DNA content reduction is not yet known.

Meth-A cells always include a small population of large cells that are produced from diploid cells by spontaneous polyploidization and are eventually removed by apoptosis (Fujikawa-Yamamoto *et al.* 1997). Meth-A cells may be particularly susceptible to polyploid transformation. Tetraploid Meth-A cell lines have been reproducibly established from diploid Meth-A cells highly polyploidized by demecolcine (Fujikawa-Yamamoto *et al.* 2001). Octaploid Meth-A cells were also produced from tetraploid cells highly polyploidized by demecolcine (Fujikawa-Yamamoto *et al.* 2003). The diploid, tetraploid and octaploid Meth-A cells showed marked differences in the expression of cell surface hydrocarbon chains, suggesting that the ploidy transformation is accompanied by functional alterations (Fujikawa-Yamamoto and Sakuma 2003). The diploid, tetraploid and octaploid Meth-A cell lines may provide a cell system for investigating polyploid cells.

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In this study, changes in DNA histograms, chromosome numbers, growth morphology and doubling time of diploid, tetraploid and octaploid Meth-A cells were measured for 244 d to elucidate the features of DNA loss in polyploid Meth-A cells. A possible explanation for the observations will be mentioned

Materials and methods

Cells

Meth-A cells (a methylcholanthrene-induced mouse abdominal dropsy sarcoma cell line) were maintained in a humidified atmosphere of 5% CO₂ at 37°C as suspension cultures in a Leibovitz's L15 : Ham's F10 mixture (7 : 3) supplemented with 10% fetal bovine serum (CELLect GOLD, ICN Biomedicals, Aurora, OH, USA), streptomycin (100 µg/ml) and penicillin (50 units/ml). The tetraploid and octaploid Meth-A cells were cultured under the same conditions described above.

Cell culturing for 244 d

Tetraploid and octaploid Meth-A cells stored at -135°C in the early stage of establishment were thawed and cultured. Diploid Meth-A cells stored at -135°C were also thawed and cultured. The diploid, tetraploid and octaploid Meth-A cells were subcultured almost every day by 1 : 2 or 1 : 4 dilution in culture flasks (25 cm², Corning Costar Co., Acton, MA, USA). The cell number was counted using a hemocytometer at the time of subculturing. The cell density was maintained at more than 2 × 10⁵ cells/flask. The DNA histograms of the cell populations were measured by flow cytometry (FCM). For controls, diploid, tetraploid and octaploid Meth-A cells stored at -135°C were used immediately after they were thawed and cultured.

Cell preparation for flow cytometry (FCM) and cell counting

The cells were fixed with 20% ethanol, and then incubated with 0.25% RNase (Type II-A, Sigma, St. Louis, MO, USA) for 3 h at 4°C. The cells were counted using a hemocytometer. Immediately before the measurements, the cells were stained with propidium iodide (PI, 7.5 × 10⁻⁵ M) and examined for red fluorescence by FCM. Under these staining conditions, the signal due to residual double-stranded RNA is negligible and the relative intensity of the red fluorescence corresponds to the DNA content (Krishan 1975).

Flow cytometry (FCM)

The fluorescence from individual cells was measured using a FACSORT (Becton Dickinson Immunocytometry Systems, Franklin Lake, ND, USA). The fluorescence of individual cells irradiated with a focused laser light at a wavelength of 488 nm was detected using a photomultiplier tube. The relative intensity of red fluorescence (FL2A) was measured and DNA histograms were obtained. The coefficient of variation (CV) of DNA histograms was obtained using computer software (ModFit, Becton Dickinson Immunocytometry Systems).

Chromosome number

Exponentially growing diploid, tetraploid and octaploid Meth-A cells were plated in culture dishes (60 mm diameter, Nalge Nunc International, IL, USA) at a density of about 5 × 10⁵ cells/dish. Twelve hours thereafter, the cells were exposed to demecolcine at a concentration of 270 nM for 1 h. The cells were swelled by 75 mM KCl, fixed with a fixing solution (CH₃OH : CH₃COOH = 7 : 3) and dropped onto glass slides. The cells were stained with Giemsa solution and then the chromosomes were photographed. The chromosome number was counted from the photographs and histograms of the chromosome number (chromosome distribution) were obtained.

Cell morphology

The morphology of exponentially growing diploid, tetraploid and octaploid Meth-A cells in culture flasks was photographed under a low-phase contrast microscope (CK2, Olympus, Tokyo, Japan) equipped with a digital camera system (DS4040, Olympus). The images were entered into a personal computer and printed out at the magnification stated in the text.

Simulation curves

The simulation curves of $f(t)$ were obtained using computer software (Mathematica, Wolfram Research Inc.).

Results

To examine DNA loss in polyploid cells, diploid, tetraploid and octaploid Meth-A cells were continuously cultured for 244 d. Representative DNA histograms of the cells are shown in Fig. 1. At 0 d, the G_1 peaks of tetraploid and octaploid Meth-A cells were positioned near the G_2 peaks of diploid and tetraploid Meth-A cells, respectively, meaning that these cells had normal DNA content. At 240 d, the positions of the G_1 peaks of tetraploid and octaploid Meth-A cells were shifted to the late S phase regions of diploid and tetraploid cells, respectively, suggesting that DNA had been lost in the polyploid cells.

To examine the changes in DNA content of polyploid Meth-A cells, the positions of the G_1 peaks of tetraploid and octaploid Meth-A cells relative to those of diploid cells were plotted against the days in culture (Fig. 2). Because the electric origin of DNA histograms may drift from measurement to measurement, the positions of the G_1 and G_2/M peaks of diploid cells were regarded as

Fig. 1. Changes in DNA fluorescence histograms of diploid (D), tetraploid (T) and octaploid (O) Meth-A cells during 240 d of culture. Tetraploid and octaploid Meth-A cells stored in the early stage of establishment were re-cultured from freezing. The measurements were made starting 3 d after the re-culturing. The diploid, tetraploid and octaploid Meth-A cells were subcultured almost every day in culture flasks. The abscissa represents the relative intensity of red fluorescence. The histograms at a day were obtained under the same FCM conditions. Arrows in 135, 180 and 240 d indicate peaks newly appeared.

Fig. 2. Time-course of relative DNA content of octaploid (O), tetraploid (T) and diploid (D) Meth-A cells. The DNA content of tetraploid and octaploid cells was determined from the G_1 peak position in comparison with that of diploid cells. The abscissa represents days in culture. The solid lines represent the experimental equations: $y = 7.9 \exp[-0.026t / \{\exp(-0.01t) + 0.0175\}]$ for octaploid cells, $y = 4 \exp[-0.026t / \{\exp(-0.01t) + 0.0175\}]$ for tetraploid cells and $y = 2$ for diploid cells, where t is days in culture. The dotted lines ($y = 6.091$ for octaploid and $y = 3.447$ for tetraploid) were drawn to facilitate understanding of the terminal DNA content calculated using the equation.

those corresponding to 2C and 4C DNA content, respectively, where C means the complement of haploid chromosomes. It should be emphasized that the peak position is shifted by many experimental conditions, such as the cell density, the PI concentration in the sample solution, the temperature of the sample solution, the flow rate of the sheath fluid, the DNA content of cells, the pH of the sample solution and the optical and electrical conditions of the FCM machine. Although we measured DNA histograms at the same time with the same electrical conditions of the FCM machine, the resultant peak positions were fairly shifted each day, and the plots were considerably dispersed.

The DNA content of tetraploid and octaploid Meth-A cells decreased rapidly in the early stage of culture, and then reached a plateau where the DNA level was higher than that of diploid cells. The DNA content at time t (days), $f(t)$, could be described as $f(t) = I_p \exp\{-G(t)\}$, where $I_p = 4$ (for tetraploid cells) or 7.9 (for octaploid cells), $G(t) = at / \{\exp(-bt) + ct\}$, and a , b and c are the following parameters: $a = 0.026$, $b = 0.01$ and $c = 0.0175$ (for tetraploid cells) or $c = 0.01$ (for octaploid cells). Diploidy was clearly not the final ploidy. The meaning of the parameters a , b , and c will be discussed in the Discussion.

To examine whether or not diploid cells lost DNA, diploid Meth-A cells stored at -135°C were thawed and placed in culture and their DNA histograms were compared with those of diploid Meth-A cells that had been cultured for over 240 d (Fig. 3). Though the G_1 peak position of Meth-A cells that had been cultured for over 240 d was slightly shifted right compared to that of the control, the amount of DNA loss was within experimental error. It was concluded that the DNA content of diploid Meth-A cells could be regarded as a constant within experimental error.

To confirm that tetraploid and octaploid Meth-A cells lost chromosomes during cell culturing, the chromosome number was counted. Figure 4 shows the distribution of chromosome number for diploid, tetraploid and octaploid Meth-A cells. The modes of the chromosome number in the long-term cultured tetraploid and octaploid Meth-A cells were 78 and 127, respectively, and were smaller than those in the control cultures (88 and 158, respectively), suggesting that the cells lost a part of the chromosomes, or that the chromosomes combined with each other.

To examine whether or not the cellular morphology was altered in the long-term culture, the morphology of growing cells was compared at 0 and >240 d (Fig. 5). A distinct alteration in cell

morphology was observed in diploid and octaploid Meth-A cells, but not in the tetraploid cells. The morphology of diploid and octaploid Meth-A cells changed from a suspended sphere-like shape to a monolayered amoeba-like shape. The suspended, sphere-like and cell-cell aggregating characteristics of tetraploid Meth-A cells did not change throughout the cell culturing. Subtle distinctions among the culture flasks were also observed in the octaploid Meth-A cells. The morphology alteration that arose during long-term culture was clearly independent of the DNA loss, because the alteration occurred in diploid cells, whose loss of DNA content was negligibly small.

To examine whether or not the cell proliferation was altered in the long-term culture, the doubling time was measured by determining the cell-counts in subcultures of diploid, tetraploid and octaploid Meth-A (Table 1). The doubling time of diploid cells was not altered

Fig. 3. The DNA fluorescence histograms of diploid Meth-A cells at 0 (upper) and over 240 (lower) days of culture. Diploid Meth-A cells stored at -135°C were recultured and used as the 0d sample. The measurements were made using the same FCM gain. The abscissa represents the relative intensity of red fluorescence.

Fig. 4. Histograms of chromosome number of diploid (D), tetraploid (T) and octaploid (O) Meth-A cells at 0 days (left panels) and in over 240 d (right panels) of culture. Exponentially growing diploid, tetraploid and octaploid Meth-A cells were exposed to 270 nM demecolcine for 1 h. The chromosomes were stained with Giemsa solution. The chromosomes of about 100 cells were enumerated from enlarged photographs.

Fig. 5. Phase-contrast micrographs of exponentially growing diploid (A and D), tetraploid (B and E) and octaploid (C, F and G) Meth-A cells at 0 d (A, B and C) and over 240 d (D, E, F and G) of culture. The photographs in F and G are from different flasks.

Table 1. Changes in doubling times (h) of diploid, tetraploid and octaploid Meth-A cells*

Period (d)	0–20	35–55	80–100	125–145	160–180
Diploid	22.0	22.8	22.2	21.4	22.0
Tetraploid	25.2	24.0	23.5	20.4	20.4
Octaploid	28.8	26.4	25.5	23.4	21.6

* The doubling times were calculated from changes in the cell number of cell populations. Note that the terms “tetraploid” and “octaploid” have been used irrespectively of the DNA content of cell cultures.

within the experimental error, whereas those of tetraploid and octaploid cells decreased. The decrease in the doubling time, however, may not have been related to the DNA loss in the polyploid cells, because the doubling time was shorter than that of diploid cells.

Discussion

The DNA content of tetraploid and octaploid Meth-A cells at time t (day), $f(t)$, could be described within experimental error as $f(t) = I_p \exp\{-G(t)\}$, where $I_p = 4$ (for tetraploid cells) or $= 7.9$ (for octaploid cells), $G(t) = at / \{\exp(-bt) + ct\}$, and a , b and c are the following parameters: $a = 0.026$, $b = 0.01$ and $c = 0.0175$ (for tetraploid cells) or $c = 0.01$ (for octaploid cells). I_p is the initial ploidy, since $f(0) = I_p$. The rate of DNA reduction, $-df(t)/dt$, is aI_p at $t = 0$ and 0 at infinity, irrespectively of b and c . The parameter a may represent the probability of DNA loss in the polyploid Meth-A cells at the start of the culture.

In order to examine the characteristics of these parameters, simulation curves of $f(t)$ were

drawn by varying the parameters (Fig. 6). With the decrease of parameter a , the decay rate increases. As parameter b increases, the decay becomes more rapid in the early stage of culture and becomes more sigmoidal. When parameter c increases, the terminal ploidy decreases. At present, the meaning of these parameters is not clear. Furthermore, more suitable functions might be found. The value of parameter $a=0.026$, however, suggests that octaploid and tetraploid Meth-A cells, having about 160 and 80 chromosomes, will lose 4 and 2 chromosomes per cell division in the early stage of culture, respectively.

One interesting question is whether specific chromosomes were destined to be lost. To examine whether or not the polyploid cells lost the same kinds of chromosomes in a cell population, the CV values of DNA histograms were calculated. Although CV values of DNA histograms will be affected by many experimental conditions both of the samples and the FCM machine, they are the only available indicator of homogeneity of cellular DNA content (ploidy) in a cell population. We detected small CV values during the culture period examined (Fig. 7). The CV values for tetraploid and octaploid Meth-A cells increased gradually until about 180 d (from 4 to 5.2 % (tetraploid cells) and 7.0% (octaploid cells)) and then regressed to the initial level by 240 d. In contrast, the CV values for diploid Meth-A cells did not change with time. It seems that the DNA homogeneity of populations of tetraploid and octaploid Meth-A cells decreases with culture time. In the early stage of culture, however, the CV values retained near the initial level, suggesting that the same amount of DNA was lost in most cells in the population. It is probable that the chromosomes to be lost are previously determined in each cell in a population. Unfortunately, we could not determine what kinds of chromosomes were lost at first, because the karyotype of Meth-A cells is not stable like the karyotypes of many mammalian cell lines.

It is interesting that a clear G_1 peak of intact diploid cells appeared in the DNA histograms at 135, 180 and 240 d in a population of cells whose ploidy was about 3.5 (indicated by arrows in Fig. 1). No sub- G_1 peaks were observed in the octaploid cell population, at least for 240 d (data not shown). These phenomena mean that a hypotetraploid-diploid transition (reverse transformation) occurred in hypotetraploid Meth-A cells and that an intact set of diploid chromosomes is preserved in the process of ploidy degradation of tetraploid Meth-A cells, but not of octaploid cells. From these experimental results, one can make the important prediction that chromosomes are not independent in polyploid cells, but are present in units of the diploid set. A model that has been proposed for the triploid V79 cells may be applicable (Fujikawa-Yamamoto *et al.* 2002).

Fig. 6. Simulation curves of $f(t)=8.0 \exp[-at/\{\exp(-bt)+ct\}]$ changing a (A), b (B) and c (C). The fixed values of the parameters are $a=0.026$, $b=0.01$ and $c=0.01$. The abscissa represents t . In A, parameter a is changed as 0.0 (1), 0.002 (2), 0.003 (3), 0.004 (4), 0.005 (5), 0.006 (6), 0.007 (7), 0.008 (8) and 0.026 (9). In B and C, parameters b and c are respectively varied as 0.0 (1), 0.004 (2), 0.006 (3), 0.008 (4), 0.01 (5), 0.012 (6), 0.014 (7), 0.016 (8) and 0.1 (9).

Fig. 7. The coefficient of variation (CV) of DNA histograms of diploid (D), tetraploid (T) and octaploid (O) Meth-A cells during 244 d of continuous culture. The CV values were obtained using computer software (ModFit). The values above 8% were cut off. Solid lines were drawn to facilitate understanding.

It should be emphasized that the present results about DNA loss apply only to variation in a long-term culture of a particular type of proliferating cells, Meth-A cells.

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