Elucidation of proliferative capability of mononuclear tetraploid cells, emerging spontaneously from diploid cells, using image cytometry and fluorescence *in situ* hybridization

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Abstract

Objectives: Proliferation of tetraploid cells (TCs) emerging from diploid cells is considered to be a critical event toward tumorigenesis, or cancer progression. Recently, several studies have reported that binuclear TCs emerging from normal cells are capable of mitosis, however, it has not been confirmed directly whether mononuclear TCs emerging from normal cells could proliferate, even cancer cells. The aim of this study is to detect mononuclear TCs *in vitro*, spontaneously emerging from diploid cells and to elucidate their proliferative capability directly. For this purpose, we have developed a novel method.

Materials and methods: In this study, two completely disomic cell lines were used, TIG-7, a fibroblast cell line and CAL-51, a breast cancer cell line. Cells were cultured on microscope slides and their DNA content was determined using an image cytometer. On the same slides, chromosome numbers were scored using centromere fluorescence *in situ* hybridization (FISH). For evaluating proliferative capability of TCs, bromodeoxyuridine (BrdUrd) incorporation and colony-forming ability were examined.

Results: Using our method, spontaneous emergence of mononuclear TCs was detected in both TIG-7 and CAL-51. Colonies of TIG-7 TCs were not observed, but were observed of CAL-51 TCs.

Conclusions: Our method enables detection of mononuclear TCs and elucidation of their proliferative capability, directly; this evidence reveals that mononuclear TIG-7 TCs do not proliferate but that mononuclear CAL-51 TCs are able to.

Introduction

A tetraploid cell (TC) is defined as being a cell containing four complete sets of chromosomes. In both animal and plant evolution, TCs have contributed to genome evolution (1). Several studies have suggested that increasing G1/G0 TCs is a trigger for tissue transformation and tumour formation. Shackney in 1989, proposed that emergence of G1/G0 TCs was an early step in tumorigenesis (2). Increasing G1/G0 TCs in a population of diploid cells (DCs) is observed in early stages of tumorigenesis in uterine cervical cancer (3) and in Barrett's oesophagus, levels of G1 TCs are also elevated (4); in breast cancers, TCs are observed in early stages of tumour progression (5). Several studies have suggested that tetraploidization is an intermediate stage in the sequence leading to aneuploidy (6–8).

TCs are occasionally observed in normal human tissues in non-malignant condition TCs emerging from DCs (9–11). These TCs have generally been proposed to be arrested by the apparent tetraploidy checkpoint of the cell cycle (12–15). Dysfunction of this checkpoint and proliferation of TCs have both been considered to be a crucial steps towards tumorigenesis (8,14).

Recently, several studies have shown that binuclear TCs (that is, TCs with two diploid nuclei) may have mitotic capability, and as a result, two daughter mononuclear TCs have emerged (16–20). These studies have questioned the existence of a tetraploid checkpoint. Up to now, no direct evidence concerning whether mitosis

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and proliferation of mononuclear TCs is able to occur or not. To the best of our knowledge, even in cancer cells, proliferation of mononuclear TCs emerging spontaneously from DCs has not been directly observed, thus, accordingly, we developed a method to directly observe TCs, and to elucidate proliferation of mononuclear TCs emerging spontaneously from DCs.

To observe emergence and proliferation of TCs emerging spontaneously from DCs, we have performed image cytometry combined with fluorescence staining for enumeration of chromosomes, evaluation of DNA synthesis (bromodeoxyuridine, BrdUrd), and expression of cell cycle-associated protein cyclin B1. Image cytometry methods are powerful and are capable of identifying small numbers of distinct cells among large numbers of cells (21). Using repeated staining and washing, a multi-parameter study of single cells on glass slides can be conducted, including individual DNA content measurement, immunocytochemistry, BrdUrd assay and fluorescence *in situ* hybridization (FISH). In the DNA diploid G2/tetraploid G1 region of a DNA histogram, G1/G0 TCs and G2/M DCs coexist; FISH can be used to distinguish these two types of cells. Presence of colony formation, providing evidence of clonal proliferation of an individual cell, can also be assessed directly with this method. We believe that our report is the first direct observation of such emergence of mononuclear TCs and determination of their proliferation, *in vitro*. A number of reports has indiscriminately named both mononuclear TCs and binuclear TCs as "tetraploid cells", but here we exclude binuclear TCs from the definition of tetraploid cells, to avoid confusion.

The aim of this study was to detect TCs emerging from DCs and to demonstrate their proliferative capability *in vitro*, and to this end a normal fibroblast cell line, TIG-7 and a complete disomic breast cancer cell line, CAL-51, reported to have tetraploid population (22) were tested.

Materials and methods

Cells

Two complete disomic cell lines, TIG-7 R073 (foetal lung fibroblast cell line, purchased from JCRB, Osaka, Japan) (23) and CAL-51 (breast cancer cell line, purchased from DSMZ, Braunschweig, Germany) were used in this study (22).

Cell culture

TIG-7 and CAL-51 were cultured on glass slides in Dulbecco's modified Eagle's Medium (DMEM) supple-

mented with 10% calf serum, in an incubator at 5% CO₂ at 37 °C. Cells were then fixed in 100% ethanol for 1 h at -20 °C and dried for one day.

PI staining

Propidium iodide (PI) staining was performed as previously described (24). Intracellular RNA was digested with 1 mg/ml ribonuclease A from bovine pancreas (Sigma-Aldrich, St Louis, MO, USA), and nuclei were stained with 50 µg/ml PI (Calbiochem, San Diego, CA, USA).

Fluorescence in situ hybridization

Two-colour FISH was applied as has previously been described (25). We used chromosome enumeration DNA FISH probes for centromeres of chromosome 7 (green fluorescence) and 11 (red fluorescence; CEPs 7 and 11, Abbott/Vysis, Abbott Park, IL, USA) to determine numbers of chromosomes 7 and 11, respectively.

Fluorescence immunocytochemistry for cyclin B1

Fluorescence immunocytochemistry was performed to quantify cyclin B1 expression in individual cells. After DNA content was measured with the aid of an image cytometer (CELAVIEW-RS100; Olympus, Tokyo, Japan), samples were washed in PBS to remove PI. Slides were then immersed in 10% goat serum for 5 min and subsequently incubated overnight at 4 °C with primary mouse anti-cyclin B1 monoclonal antibody, 1:200 dilution (Calbiochem). Then they were washed in PBS and incubated in goat anti-mouse IgG secondary antibody conjugated with Alexa 488, 1:200 (Alexa Fluor 488; Life Technologies, Carlsbad, CA, USA), for 1 h at room temperature (RT). Finally, cells were counter-stained with PI once more.

BrdUrd assay

Cells were incubated in medium containing 10 µM BrdUrd (Exalpha Biologicals, Cambridge, UK) for 1 h at 37 °C; they were fixed in 100% ethanol and dried overnight. Prior to immunocytochemical staining of BrdUrd, FISH was performed as previously described, and cell figures were captured using the image cytometer. BrdUrd was then detected with anti-BrdUrd antibody, 1:200 dilution (Exalpha Biologicals). After being treated with 10% goat serum to prevent non-specific reaction, cells were incubated in primary antibody overnight at 4 °C. Slides were then washed and cells were incubated in goat IgG secondary antibody, conjugated

with Alexa 488 (1:200) for 1 h, RT. Following washing in PBS, cells were counterstained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Roche, Basel, Switzerland).

Image cytometry

In 4.0×2.6 mm areas of microscope slides, polarizing microscope images and nuclei stained with PI were captured using the image cytometer. Total PI intensity, maximum PI intensity, nuclear area, morphological nuclear circularity, and X/Y position of cells were measured. Doublet or triplet cells were excluded by using parameters of circularity and nuclear area, in the analysing software (CELAVIEW-RS100; Olympus). Furthermore, we manually checked figures to exclude doublet and triplet cells.

According to measured nuclear DNA content, cells were classified into five fractions: those in DNA G1 diploid, DNA S diploid, DNA G2 diploid/G1 tetraploid, DNA S tetraploid, and DNA G2 tetraploid/G1 octaploid regions of the DNA histogram. To distinguish cells in which two ploidy levels occupied the same peak, we used the two-colour centromere FISH method. Any cell with two spots of CEPs in the DNA G2 diploid/G1 tetraploid region, we defined as G2/M DC; one with

four spots of CEPs in DNA G2 diploid/G1 tetraploid region as G1/G0 TC; one with four spots of CEPs in DNA G2 tetraploid/G1 octaploid region as G2/M TC, and one with two spots of CEPs in DNA G1 diploid region as G1/G0 DC. As FISH spots were occasionally overlapping or missing, cells with three spots of CEP 7 and four spots of CEP 11 or four spots of CEP 7 and three spots of CEP 11 were counted as TCs. After nuclear DNA content was measured, immunocytochemistry for cyclin B1 expression and detection of BrdUrd and FISH spots were performed for chromosomes 7 and 11, respectively. For analysis of cyclin B1 expression and BrdUrd incorporated nuclei, fluorescence intensity was measured using the image cytometer. For FISH analysis, figures of nuclei subjected to FISH were captured with the image cytometer. DNA content, cyclin B1 expression, FISH spots and BrdUrd incorporated into nuclei were integrated to individual cells.

Colour-coding of nuclei

For identifying TC colonies, cells were manually colour-coded with software Photoshop CS5 (Adobe Systems, Inc., San Jose, CA, USA) according to DNA content. Colour patterns of TC colonies were different from those of DC colonies, making it easy to identify them.

Figure 1. Propidium iodide (PI) staining and fluorescence *in situ* hybridization (FISH) of TIG-7 and CAL-51 cells in each region of DNA histogram. After each cell was stained by PI and measured DNA contents by an image cytometer (CELAVIEW-RS100), FISH was performed on the same cell using centromere probes. Colors of DNA histograms and PI stained cell image: sky blue, DNA G1 diploid region; green, DNA S diploid region; red, DNA G2 diploid/G1 tetraploid region; purple, DNA S tetraploid region; yellow, DNA G2 tetraploid/G1 octaploid region. DC, diploid cell; TC, tetraploid cell; FISH: green, centromeres of chromosome 7; red, centromeres of chromosome 11.

Statistical analysis

Chi-square test and Fisher's exact tests using R version 2.15 (R Foundation for Statistical Computing, Vienna, Austria) were performed.

Results

Image cytometry in this study enabled us to obtain values for several parameters including nuclear DNA content, number of CEP spots, cyclin B1 expression, and BrdUrd content, in individual cells. Cell morphology was also examined and noted.

Detection of TCs by image cytometry combined with FISH

For detecting TCs, we performed image cytometry combined with FISH in the TIG-7 and CAL-51 cells, cultured for 7 days. In each cell line, cells with two spots of CEPs for chromosome 7 and 11 were present in DNA G1 diploid, DNA S diploid, and DNA G2 diploid/G1 tetraploid regions of the DNA histogram. These cells were G1/G0 DC, S DC and G2/M DC, respectively. Cells with four spots of CEPs were present in DNA G2 diploid/G1 tetraploid, DNA S tetraploid and DNA G2 tetraploid/G1 octaploid regions of the DNA histogram. These cells were G1/G0 TC, S TC and G2/M TC, respectively. In CAL-51, cells with eight spots of CEPs in the octaploid region (G1/G0 octaploid cells) were observed (Fig. 1).

In DNA G2 diploid/G1 tetraploid region of the DNA histogram, G2/M DCs coexisted with G1/G0 TCs. For confirmation that cells with four spots of CEPs were G1/G0 TCs, expression of cyclin B1 was evaluated in both cell lines. None of the cells, of either line, with four spots of CEPs in DNA G2 diploid/G1 tetraploid regions expressed cyclin B1 (Fig. 2).

Enumeration of TCs at different stages of cell culture

Two days after culture was initiated, TCs were detected in 3.7% (13 of 354 observed cells) of TIG-7 and 2.5% (12 of 486 observed cells) of CAL-51 cells. On day 7, TCs were detected in 6.1% (83 of 1362 observed cells) TIG-7 and 3.6% (107 of 2980 observed cells) CAL-51 cells. A total of 0.4% of TIG-7 on day 7, 0.4% of CAL-51 cells on day 2 and 0.1% of CAL-51 cells on day 7 were G1/G0 octaploid cells (cells with eight spots of

Figure 2. Confirmation of the existence of G1/G0 tetraploid cells in DNA G2 diploid/G1 tetraploid region in DNA histogram using FISH probes (centromere 7 and 11) in TIG-7 and CAL-51. *Fisher's exact test. Cell with two spots of CEPs, DNA G2 diploid/G1 tetraploid cell with two centromere FISH spots of chromosome 7 and 11; Cell with four spots of CEPs, DNA G2 diploid/G1 tetraploid cell with four centromere FISH spots of chromosome 7 and 11. Note: In DNA G2 diploid/G1 tetraploid cells, none of cells with four centromere FISH spots shows cyclin B1 positive.

Table 1. Enumeration of diploid and tetraploid cells at different stages of cell culture. The cells cultured in 4.0×2.6 mm surface area on slide glass were analyzed

	Diploid cells	Tetraploid cells	<i>P</i>
TIG-7			
Day 2	341 (96.3%)	13 (3.7%)	>0.05*
Day 7	1274 (93.5%)	83 (6.1%)	
CAL-51			
Day 2	472 (97.1%)	12 (2.5%)	>0.05*
Day 7	2870 (96.3%)	107 (3.6%)	

*The ratio of tetraploid cells in day 7 compared with the ratio in day 2 by Chi-squared test ($P > 0.05$).

CEPs); in each cell line, remaining cells were DCs. In each cell line, numbers of TCs increased during culture; however, proportions of TCs did not increase significantly (Table 1). On day 2, not all of phases of TCs (G1/G0 TCs, S TCs, and G2/M TCs) were observed, whereas on day 7, all phases of TCs were observed in each cell line, as shown in Fig. 3.

Evaluation of TC proliferation

For evaluating proliferation of TCs in each cell line, the BrdUrd incorporation study was performed, and a search

Figure 3. The ratio of G1/G0, S and G2/M tetraploid cells (TCs) at day 2 and day 7.

was conducted for TC colonies. TCs incorporating BrdUrd were detected in both TIG-7 TCs and CAL-51 TCs (Fig. 4).

Of the TIG-7s, no TC colony was detected. TCs were occasionally detected without colony formation when approximately 1200 cells were examined during culture up to day 14. Solitary TCs or two twin-like TCs resembling each other, were observed. We repeated the investigation 8 times using cells with different passage

Figure 4. Detection of diploid cells and tetraploid cells incorporated BrdUrd. Cells incorporated BrdUrd were detected fluorescent immunocytochemistry. Diploid cells and tetraploid cells incorporated BrdUrd in each cell line. (green arrow: BrdUrd positive diploid cells, purple arrow: BrdUrd positive tetraploid cells). PI, propidium iodide; FISH, fluorescence *in situ* hybridization. FISH: green, centromeres of chromosome 7; red, centromeres of chromosome 11.

Figure 5. Nuclei color-coded for detecting tetraploid cell colonies, polarizing microscope image + PI image, color-coded image, and FISH image in TIG-7 and CAL-51. The color is decided according to the DNA contents. In TIG-7, tetraploid cell (TC) colony do not arise in 1200 cells and solitary TCs or twin-like TCs are detected. In CAL-51, three TC colonies are detected in 1500 cells. (TC colony: yellow arrow). In polarizing microscope + PI image, color-coded image, and FISH image, white arrows indicate TCs in TIG-7, sky blue arrowheads indicate diploid cells and pink arrowheads indicate TCs in CAL-51. PI, propidium iodide; FISH, fluorescence *in situ* hybridization. FISH: green, centromeres of chromosome 7; red, centromeres of chromosome 11.

numbers, but still observed no TC colonies in TIG-7 (Fig. 5).

In contrast, of CAL-51s, 3 TC colonies were observed in approximately 1500 cells during culture on glass slides up to day 14. We confirmed that cells composing TC colonies, detected by their colour pattern, corresponded to spots of CEPs. In polarizing microscope images, larger nuclei and extensive cytoplasm were observed for cells of the TC colonies, comparing to those of DC colonies.

Confirmation of TCs from DCs in CAL-51

With the aim of confirming that TCs emerged from DCs in CAL-51, a colony was formed from single cell. On the periphery of the colony, TCs were noted to have emerged from DCs (Fig. 6).

Discussion

In the present study, we confirmed that of TIG-7 cells, mononuclear TCs arising spontaneously did not proliferate, whereas mononuclear TCs arising from CAL-51 did proliferate. Although mitosis of TCs was observed, this may have been an isolated incident and further mitosis did not occur. Thus, observation of mitosis was insufficient for elucidation of proliferation. We believe that colony formation of TCs is direct evidence of their proliferation and this is the first report regarding observation of proliferation of mononuclear TCs emerging from DCs spontaneously, confirmed directly by using image cytometry.

To avoid confusion, we excluded binuclear TCs in this study. However, as we could not distinguish binuclear cells and mononuclear cells by automatic measure-

Figure 6. The colony derived from single-cell in CAL-51. Tetraploid cells with four spots of centromere FISH probes emerged in the peripheral zone of the colony (white arrow). FISH: green, centromeres of chromosome 7; red, centromeres of chromosome 11. PI, propidium iodide; FISH, fluorescence *in situ* hybridization.

ment, we confirmed that detected TCs were mononuclear cells, by polarizing microscope images.

To distinguish TCs and determine cell cycle phases, we scored spots of CEPs after measuring DNA content, in each cell line. To confirm that cells with four spots of CEPs in the DNA G2 diploid/G1 tetraploid region were G1/G0 TCs, we assessed expression of cyclin B1. Cells in G2 phase express cyclin B1 (26). Cells with four spots of CEPs in DNA G2 diploid/G1 tetraploid regions did not express cyclin B1 in either cell line. The present study confirmed that cells with four spots of CEPs in DNA G2 diploid/G1 tetraploid regions were G1/G0

TCs. Thus, the present method allowed successful detection and characterization of TCs.

By our observations, TCs in TIG-7 were often solitary or occasionally twinned. Twin mononuclear TCs emerge from binuclear TCs by mitosis in normal cells/normal tissues (16,17,19,20). As we did not detect colony formation of mononuclear TCs in TIG-7, these TCs were regarded as cells devoid of proliferative capability. In fibroblasts, polyploidization by endoreduplication occurs occasionally (27,28). In this process, non-proliferating S TCs and G2/M TCs can arise and non-proliferating cells can incorporate BrdUrd. In our TIG-7, TCs incorporating BrdUrd detected could be associated with the process, as mentioned above. Thus, it is next to impossible to determine proliferation of TCs by detection of S or G2/M TCs or BrdUrd assay only.

p53 had been considered to be a key signal molecule of tetraploid checkpoints (8). However, recently, it has been reported that p53 can work as an inhibitor to multipolar abnormal mitosis of TCs, rather than as a tetraploid checkpoint (19,20,29–31); in CAL-51, p53 is reported to be intact (32). Thus, p53 may be related to proliferation of TCs without changing aneuploid cells in CAL-51. There were differences between TIG-7 and CAL-51 cells, causing proliferation of mononuclear TCs, but we were unable to specifically identify these differences.

Here, we observed more than 1200 cultured cells for up to 14 days. Proliferation of TCs may begin *in vivo* after a long periods of time; however, temporal observation of cell growth is difficult by the present method as cells ultimately reach confluence in their plates. We also did not confirm that G1/G0 TCs carried four complete sets of chromosomes due to difficulty experienced in accurately counting numbers of each chromosome in TCs. For this reason, we considered that cells with four spots of CEPs were TCs.

In conclusion, by observation of colony formation, we confirmed that mononuclear TCs arising spontaneously in TIG-7 did not proliferate, whereas mononuclear TCs arising in CAL-51 cells proliferated. Our methods and cell lines we have described may be suitable for study of proliferation of TCs by direct observation.

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