Apoptotic Cell Death of High Polyploid Cells in a Cultured Sarcoma Cell Line


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ABSTRACT. It is well known that DNA-ploidy is useful independent prognosticator of malignancy. However, the biological significance of polyploid cells and the relation between polyploidy and prognosis is not well understood. We analyzed DNA ploidy by flow cytometry in Meth-A cells (a cultured sarcoma cell line) after treatment with K252a, a protein kinase inhibitor, and showed induction of polyploidization. Apoptotic cell death of the high polyploid cells was verified by flow cytometry, morphological observation and gel analysis of DNA integrity. Expression of tumor-suppressor nuclear protein p53 investigated by immunohistochemistry was increased 10-fold or more in cells with 16C (C=haploid DNA content) relative to cells with 2C, suggesting that the overexpression of p53 was involved in the apoptosis. These results may be of clinical relevance since it has been known that both DNA ploidy and p53 expression have prognostic significance.

The numerical chromosome aberrations of cells differing from the euploid complement through the loss or gain of one or more chromosomes in mitosis are involved in aneuploidy formation (1). The aneuploid condition is considered to be a common cause of spontaneous abortions, congenital malformations, and genetic disorders (2). Tumors with aneuploidy were found to be correlated to poor prognosis, indicating a high degree of tumor malignancy (3).

In contrast, polyploidization has been suggested to be a result of escaping the mitotic block, bypassing cellular division and entering into a new cycle of DNA replication (4). In malignancy, polyploid nuclei have long been known to exist in a wide range of tumors (5–7). Tumors with euploidy were found to be correlated to good prognosis, indicating a low degree of malignancy (8). Polyploid cells are not specific to cancer, since they are also found in terminally differentiated cells such as cardiomyocytes (9) and neurons (10). However, the biological significance of polyploid cells and relation between polyploidy and prognosis is not well understood.

Apoptosis, or 'programmed cell death' as reported by Kerr in 1965 (11), is a widespread process that occurs during embryogenesis (12); at the end of the life span of differentiated cells (13); and under many other physiological (14) and pathological conditions (15). It is a highly controlled process involving gene expression, protein synthesis, and the activation of specific enzymes (16, 17), and can be induced by various external or internal signals (18, 19). Recently, it has been proposed that tumor progression not only involves cell proliferation but also aberrant cell survival resulting from inappropriate suppression of apoptosis (20). It has also been suggested that apoptosis is a mechanism of cellular defense against disease (21). Therefore, the evaluation of apoptosis-inducibility is important when planning strategies of cancer therapy.

We have been interested in the relationship between polyploidization and apoptosis, since this would help determine the role of polyploid cells in the tumor population and explain the cause of the good prognosis in patients with tumor consisting of more polyploid cells. In the present study, we show both K252a, which inhibits protein kinase by competing with the binding of ATP to the kinase catalytic domain (22), and colcemid, a potent inhibitor of mitotic spindle which functions by antagonizing tubulin polymerization and inducing the disassembly of microtubules into monomers (23), induce polyploidization of Meth-A cells in vitro and in vivo, and demonstrated that the fate of high polyploid cells is apoptotic cell death and overexpression of p53 was involved in the apoptosis of these cells.
MATERIALS AND METHODS

Materials
K252a was purchased from Funakoshi Co., Japan, dissolved in dimethyl sulfoxide and stored in the dark at −20°C. Colcemid was purchased from Sigma Chemical Co., dissolved in phosphate-buffered saline (PBS) and stored in the dark at 4°C. The mouse monoclonal antibody against p53 protein used in this study was purchased from Wako Pure Chemical Ind. Co., Ltd., Tokyo. All chemicals were of reagent grade and purchased from Sigma Chemical Co.

Cell lines and culture
Meth-A cells (3-methylcholanthrene-induced sarcoma cell line, syngeneic to BALB/c mice) were grown in a mixture of Ham’s F-10 and L-15 (ratio, 3:7) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories), 50 units/ml of penicillin and 50 μg/ml of streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The viability of cells used in these experiments was consistently more than 95% when evaluated by the trypan blue exclusion method.

Animals
Pathogen-free male BALB/c mice, 7 weeks old, were purchased from SLC, Japan (Shizuoka). The mice were housed in animal quarters with controlled temperature (22-26°C), humidity (50-60%), and lighting (12 hour cycle). Five animals were used per experimental condition. All experiments were repeated at least twice.

Measurement of cellular parameters
The number and volume distribution of Meth-A cells were determined using a Coulter counter model ZM and a Channelizer model 256 (Coulter Electronics). The analyzer was calibrated using 9.61 μm styrene beads.

Flow cytometric measurements
Flow cytometry was employed to determine the DNA content of Meth-A cells. After fixation in 70% ethanol, the cells were treated exhaustively with pancreatic RNase A and stained with propidium iodide (PI, 10 μg/ml in PBS). Fluorescence from individual cells was measured with a Cytofluorograf System 50H (Ortho Instruments) equipped with a 4W argon ion laser. The fluorescence of individual cells irradiated with a focused laser light at a wavelength of 488 nm (200 mW laser power) was detected by a photomultiplier tube. The relative intensities of red fluorescence were measured and DNA histograms obtained.

Flow cytometry was also employed to assay the relative p53 protein content of Meth-A cells. The p53 protein content was detected by indirect immunofluorescence using specific monoclonal antibody and general methodology (24). Briefly, the cells were washed, fixed with 1% paraformaldehyde for 30 minutes at room temperature and treated with 0.3% Triton X-100 for 10 minutes at 37°C. The cells were then incubated with the primary mouse monoclonal antibody against p53 protein for 24 hours at 4°C. The cells were washed in PBS containing 1% bovine serum albumin (BSA) and stained with the secondary, rabbit anti-mouse IgG FITC-conjugated antibody. The cells were then washed again, resuspended in PBS containing RNase A and PI, and analyzed by flow cytometry.

In vivo experiments
Meth-A cells (1 x 10⁶) were i.p. inoculated to the BALB/c mice. Mice were checked daily for survival.

For analysis of morphological changes, 1 x 10⁶ Meth-A cells were s.c. inoculated at the flank of the BALB/c mice. When the emerging tumors consisting of Meth-A cells had reached approximately 5 mm in perpendicular diameter, colcemid was s.c. injected adjacent to the tumors once a day for 5 days at the dose 50 μg/mouse/injection. On the 6th day, the tumors were taken and processed for morphological observation. Mice remained healthy and no mortality was observed after such treatment.

Analysis of morphological changes
For observation of nuclear fragmentation, the Meth-A cells were fixed in methanol. The cell nuclei were stained with a solution containing 0.05 μg/ml of 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) at room temperature for 30 minutes, and observed for nuclear fragmentation under a fluorescent microscope.

For immunohistochemistry of p53 protein of Meth-A cells, the deparaffinized and dehydrated slides were boiled in 10 mM citrate buffer (pH 6.0) for 10 minutes in an autoclave at 121°C and then stained using general methodology (25). Briefly, incubation was with the primary mouse monoclonal antibody against p53 protein for 24 hours at 4°C and staining was with the secondary, rabbit anti-mouse IgG FITC-conjugated antibody. Finally, the slides were stained with PI and observed under a fluorescent microscope.

DNA gel electrophoresis
The pooled Meth-A cells were pelleted by centrifugation. The cell pellets were washed in PBS and then analyzed as described (26). Briefly, the resulting cell pellets were resuspended (5 x 10⁶ cells) in 200 μl of TBE (45 mM Tris-borate buffer, 1 mM EDTA, pH 8.0) containing 100 μg proteinase K, 100 μg RNase A and 2 mg sodium dodecyl sulfate (SDS), and incubated at 37°C for 30 minutes. DNA was then extracted with a solution of NaCl (6 M NaCl, 13 mM EDTA, 0.5% sodium-N-lauroylsarcosinate, 10 mg/ml glycogen and 26 mM Tris-HCl pH 8.0), and after that, with 100% isopropanol. The DNA samples were analyzed with a 2% agarose gel.
RESULTS

K252a inhibits proliferation and induces volume enlargement of Meth-A cells

Exponentially growing Meth-A cells in random culture were treated with 2 μM of K252a and then cultivated for 48 hours. Cells were counted 0, 12, 24, 36 and 48 hours after K252a addition. Fig. 1A shows the proliferation curves of the cells as a function of time of K252a treatment. The cell proliferation was almost completely arrested over time after incubation in the presence of 2 μM of K252a. At 48 hours, the cell number was slightly less than that at 0 hour, suggesting that a part of the cells died.

The effect of K252a on cell volume distribution was determined using a Coulter counter. Fig. 1B shows the cell volume distribution in control and K252a-treated cells. In cells treated for 36 hours with 2 μM of K252a, cell volume rose with a peak appearing at channel No.160-200 indicating increased cell size population, as compared to control cells which peaked at No. 20-40.

K252a induces polyploidization and apoptosis of Meth-A cells

To investigate the mechanism whereby K252a arrests cell proliferation and induces volume enlargement of Meth-A cells, we examined the effect of K252a on the cell cycle. The cells were stained with PI and subjected to flow cytometry. The distribution of DNA content was then examined. Histograms are shown in Fig. 2. The first, second, third and fourth peaks were produced by cells with 2C, 4C, 8C and 16C respectively. The hypodiploid population was composed of apoptotic bodies (27). With continued incubation after exposure to 2 μM of K252a for 36 hours (Fig. 2B), cells with progressively greater DNA content were observed and the main peak shifted from 2C in the control (Fig. 2A) to 8C. A peak composed of cells with 16C was clearly seen. At the same time, the hypodiploid population markedly increased. The simultaneous analysis of these cells by gel electrophoresis confirmed the presence of the ladder pattern on the gel after treatment with 2 μM of K252a for 36 hours. These results show that K252a induces simultaneously polyploidization and apoptosis.

Morphological changes of Meth-A cells treated with K252a

Figure 3 shows the morphological changes of those cells subjected to flow cytometry. Meth-A cells were stained with DAPI and the nuclear morphology was examined. After exposure to 2 μM of K252a for 36 hours, giant high polyploid cells that were much larger than control cells (Fig. 3A) in both whole cell and nuclear size were apparent. Fig. 3B shows a representative photograph of a giant high polyploid cell. Under the same treatment conditions, giant high polyploid cells containing remarkable apoptotic bodies characterized by nuclear fragmentation appeared, indicating apoptotic cell death of these cells (Fig. 3C).

Fig. 1. Inhibition of proliferation and induction of volume enlargement by K252a in Meth-A cells. Meth-A cells were treated with 0 (a) or 2 μM of K252a for 36 hours (b). The cell numbers (A) and volumes (B) were determined by a Coulter counter.
Overexpression of p53 protein in high polyploid Meth-A cells
To confirm the correlation between the high polyploid (>8C) cells and expression of p53 protein, flow cytometry was used to determine the quantitative changes. The Meth-A cells were simultaneously stained with PI and monoclonal antibody against p53 protein, and subjected to flow cytometry. The cells with 2C, 4C, 8C and 16C were gated respectively, and the expressions of p53 protein in cells with 2C, 4C, 8C and 16C displayed. As shown in Fig. 4, p53 protein was present but at a lower concentration in control cells with both 2C and 4C. After exposure to 2 μM of K252a for 36 hours, expression of p53 protein in cells with 2C was observed similar to control cells. However, expression of p53 protein in some cells with 4C was enhanced. The enhanced expression of p53 protein was more apparent in cells with progressively greater 8C and 16C. In cells with 16C, expression of p53 protein was markedly increased, to 10-fold or more that of the cells with 2C. These results support the conclusion that the fate of high polyploid cells is apoptotic cell death.

Inductions of high polyploid cells, apoptosis and expression of p53 protein in vivo
The results that indicate apoptotic cell death of high polyploid cells in vitro prompted us to examine whether or not a similar phenomenon occurs in vivo. Sections from the tumors consisting of Meth-A cells and treated locally by colcemid in vivo were simultaneously stained with PI and monoclonal antibody against p53 protein. Fig. 5 shows representative photographs of control cells (Fig. 5A), colcemid-induced giant high polyploid cells (Fig. 5B) and apoptotic cell death of high polyploid cells (Fig. 5C). Corresponding to the results observed by flow cytometry, giant high polyploid cells show strong nuclear immunostaining of p53 protein (Fig. 5B and 5C).

Survival of mice that received an injection i.p. of Meth-A cells
To determine the viability (or mitotic capacity) of K252a-induced high polyploid cells, cells of the control (A) and cells exposed to 2 μM of K252a for 36 hours (B) were injected i.p. into mice, respectively. Fig. 6 shows the survival rate of the mice. In group A, mice started to die from day 10 after an injection i.p. of Meth-A cells and by day 17, all mice were dead. In contrast, the mice in B group did not start to die until day 25, 16 days later than the mice in A group, with 80% still alive at day 60. Moreover, at day 60 the living mice were sacrificed and dissected, with no abnormal findings.
Apoptotic Cell Death of High Polyploid Cells

**DISCUSSION**

Clinically, the morphological diagnosis leading to a prognosis of malignancy is based on the pathologist's degree of personal experience. Therefore, accuracy varies among pathologists as well as by intratumorous variation. As an objective criterion, cytometric ploidy analysis has gained considerable attention in diagnostic tumor pathology in an attempt to make up for deficiencies in morphological prognoses. A great number of researchers have reported that DNA-ploidy is useful as an independent prognosticator of malignancy (28). However, we found that the definition of DNA-ploidy, that is, of aneuploidy from polyploidy, is indistinct. We emphasize the difference between aneuploidy and polyploidy through mitotic capacity, i.e. able to mitosis in aneuploidy and unable to mitosis in polyploidy. Little is known about the biological and clinical significances of polyploid tumor cells, which differ from aneuploid tumor cells demonstrated to correlate with advanced stage, a loss of tumor differentiation and metastases (29). Based upon this background we investigated whether or not the fate of polyploid cells is apoptotic cell death, to obtain evidence that tumors with polyploidy correlate to good prognosis indicating a low degree of malignancy.

In this study, we found that treatment by 2 μM of K252a for 36 hours resulted in proliferation arrest and volume increase of Meth-A cells. Flow cytometric analysis revealed that proliferation arrest and volume increase were the result of polyploidization induction. At the same time, DNA histograms obtained from flow cytometry showed a marked increase in the ratio of the hypodiploid population. This result suggests a close relationship between polyploidization and apoptosis. To confirm this, the morphological change of nuclei was observed by DAPI staining. The results showed that K252a induces giant high polyploid cells containing apoptotic bodies, indicating the apoptotic cell death of these cells.

Furthermore, to determine the viability (or mitotic capacity) of K252a-induced high polyploid cells after K252a was released, survival of mice bearing high polyploid cells was observed. The result that 80% of mice remained healthy and autopsy support the in vitro findings. From these findings we concluded that the fate of high polyploid cells is apoptotic cell death. This conclu-

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**Fig. 4.** Overexpression of p53 protein in high polyploid Meth-A cells. Meth-A cells were treated with 0 (A, B and C) or 2 μM(D, E, F and G) of K252a for 36 hours, simultaneously stained with PI and monoclonal antibody against p53 protein, and subjected to flow cytometry. The cells with 2C (B and D), 4C (C and E), 8C (F) and 16C (G) were gated respectively, and the expression of p53 protein in cells with 2C, 4C, 8C and 16C displayed. As a negative control for the immunofluorescence, normal mouse serum was employed (A).
tion is consistent with previous studies. Usui et al. were unable to establish a polyploid clone and suggested that cells with tetraploid or more nuclei lose their reproductive integrity (4). Ishida et al. proposed that cells continue through the cell cycle, becoming polyploid and losing viability (30). Lanks and Lehman demonstrated that giant nuclear cells undergo to eventual death resulting from continued DNA synthesis that is not coordinated with nuclear division (31). In fact, it has been known that after treatment of malignant tumors with ionizing radiation, tumor cells show polyploidization (32).

p53's multifaceted activities in G1, M and S phases of the cell cycle by concentrating on p21, one of its important effectors, and on apoptosis have been reported (33). Overexpression of p53 protein should arrest cells in the G1/S or G2/M phases of the cell cycle. p53 protein overexpression also induces apoptosis. To investigate the mechanism of induction of apoptosis, we determined the expression of p53 protein and found that in cells with 16C, the expression was 10-fold or more that in the cells with 2C, suggesting the overexpression of p53 protein was at least in part involved in the induction of apoptosis. A similar result was obtained in vivo. Therefore, we propose that overexpression of p53 protein results in apoptotic cell death of high polyploid cells.

In conclusion, we demonstrated that the fate of high polyploid cells is apoptotic cell death and the apoptosis may be due to overexpression of p53 protein. We expect the results obtained in this study will be used in the clinical diagnosis of tumor.

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REFERENCES

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