Opposite effects of microtubule-stabilizing and microtubule-destabilizing drugs on biogenesis of mitochondria in mammalian cells

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SUMMARY

Distribution of mitochondria as well as other intracellular organelles in mammalian cells is regulated by interphase microtubules. Here, we demonstrate a role of microtubules in the mitochondrial biogenesis using various microtubule-active drugs and human osteosarcoma cell line 143B cells and rat liver-derived RL-34 cells. Depolymerization of microtubules by nocodazole or colchicine, as well as 2-methoxyestradiol, a natural estrogen metabolite, arrested asynchronously cultured cells in G_2/M phase of cell cycle and at the same time inhibited the mitochondrial mass increase and mtDNA replication. These drugs also inhibited the mitochondrial mass increase in the cells that were synchronized in cell cycle, which should occur during G_1 to G_2 phase progression in normal conditions. However, stabilization of microtubules by taxol did not affect the proliferation of mitochondria during the cell cycle, yet a prolonged incubation of cells with taxol induced an abnormal accumulation of mitochondria in cells arrested in G_2/M phase of cell cycle. Taxol-induced accumulation of mitochondria was not only demonstrated by mitochondria-specific fluorescent dyes but also evidenced by the examination of cells transfected with yellow fluorescent protein fused with mitochondrial targeting sequence from subunit VIII of human cytochrome c oxidase (pEYFP) and by enhanced mtDNA replication. Two subpopulations of mitochondria were detected in taxol-treated cells: mitochondria with high $\Delta\Psi_m$, detectable either by Mito Tracker Red CMXRos or by Green FM, and those with low $\Delta\Psi_m$, detectable only by Green FM. However, taxol-induced increases in the mitochondrial mass and in the level of acetylated α-tubulin were abrogated by a co-treatment with taxol and nocodazole or taxol and colchicine. These data strongly suggest that interphase microtubules may be essential for the regulation of mitochondrial biogenesis in mammalian cells.

Key words: Mitochondrial biogenesis, Microtubules, Microtubule-active drugs, Fluorescent dyes, Flow cytometry, Taxol

INTRODUCTION

Biogenesis of mitochondria proceeds in two main steps as proposed by Attardi and Schatz (Attardi and Schatz, 1998): (1) formation of a mitochondrial mass depending mainly upon nuclear transcripts of mitochondrial proteins; and (2) energetic differentiation that can be inhibited by the depletion of mitochondrial DNA (mtDNA). It is established that mitochondria are formed by the growth and division of pre-existing ones based on a body of experimental data using a variety of cells, including yeast cells (Haslam et al., 1973; Linnane et al., 1976), HeLa cells (Storrie and Attardi, 1973; Posakony et al., 1977), *Neurospora crassa* (Luck, 1963; Luck, 1965) and rat liver (Bergeron and Droz, 1969).

Elements regulating the coordination of mitochondrial and cytoplasmic events leading to the growth and division of mammalian cells are largely unknown. Mitochondrial proliferation and degradation have been suggested to depend upon functional states of the organelles or energetic states of the cell (Luzikov, 1999). Disorganization of these processes is often associated with abnormal accumulation of mitochondria in various models of cell death. For example, an abnormal proliferation of mitochondria was reported in herbimycin A-treated human colon carcinoma cell line (Mancini et al., 1997) and in aphidicolin-treated CHO cells (Camilleri-Broet et al., 1998).

Early studies on mitochondrial biogenesis using mainly the serial sectioning technique for electron microscopy and three-dimensional reconstitution of mitochondria have disclosed that the number, form and volume of mitochondria are closely related to the cell cycle phase as well as functional states of the cell (Hoffman and Avers, 1973; Osafune, 1973; Posakony et al., 1977). For example, giant mitochondria are formed temporarily by the fusion of smaller mitochondria before the cell division (Osafune, 1973). Using electron microscopic techniques and synchronized HeLa cells, Posakony et al. have shown that increases in the total volume of mitochondria per cell occur during interphase, and that the ratio of mitochondrial to cellular volume is constant during cell cycle (Posakony et al., 1977). As pointed out by James and Bohman, however, a

simple count of the number of mitochondria per cell is often misleading, because of the plasticity of mitochondria, variability in their sizes and the extent to which they fuse or fragment (James and Bohman, 1981). However, use of fluorescent dyes combined with flow cytometric techniques has enabled us to monitor the biosynthesis of mitochondria as a function of cell cycle. Detailed analyses on mitochondrial biogenesis using rhodamine 123 have revealed almost linear synthesis of mitochondria occurring over the cell cycle of human leukemia cell line (James and Bohman, 1981). Leprat et al. have found that synthesis of mitochondria occurs essentially in the G_1 phase of cell cycle but also starts in late S phase (Leprat et al., 1990).

Microtubules (MTs) seem to be the major component of cytoskeletal systems that are responsible for the regulation of the distribution of mitochondria in mammalian cells (Heggeness et al., 1978), in contrast to yeast cells, where actin microfilaments play a major role in this matter (see review by Yaffe, 1999). Discovery of Kif1B and Kif5B proteins belonging to the kinesin protein family, which are responsible for the movement of mitochondria along MTs (Nangaku et al., 1994; Tanaka et al., 1999), has accelerated the research on factors controlling the distribution of mitochondria in the cell. MT-dependent clustering of mitochondria around the nucleus has been shown in various cells under various experimental conditions (Smirnowa et al., 1998; De Vos et al., 1998; Li et al., 1998; Tanaka et al., 1998). Furthermore, MTs regulate the distribution of intracellular organelles besides mitochondria: endoplasmic reticulum (ER) (Terasaki and Reese, 1994), Golgi apparatus (Thyberg and Moskalewski, 1985), peroxisomes (Wiemer et al., 1997) and lysosomes (Collot et al., 1984). Overexpression of tau, a mictotubule-associated protein, not only causes aberrant distribution of mitochondria via the inhibition of kinesin-dependent movement of the organelles along MTs but also affects morphology and distribution of ER in the cell (Ebneth et al., 1998). Chemical modifications of microtubule dynamics by microtubule-active drugs (MADs) have been widely used as an experimental approach to explore the role of MTs in various cellular functions (Gurland and Gundersen, 1995; Wiemer et al., 1997; Ebneth et al., 1998; Jordan and Wilson, 1998).

In the present study, we demonstrate evidence that interphase MTs are involved in mitochondrial biogenesis, besides the regulation of mitochondrial distribution in the cell. Chemical depolymerization of MTs invariably leads to the inhibition of mitochodnrial volume and mass increases during interphase of cell cycle. Stabilization of MTs by taxol does not affect mitochondrial proliferation during cell cycle, but causes the accumulation of both ‘active’ and ‘non-active’ mitochondria.

MATERIALS AND METHODS

Cell culture

143B (TK[-]) human osteosarcoma cell (ρ^+) (ATCC CRL-8303) and rat liver epithelial cell line RL-34 cells (JCRBB 02247) were grown at 37ºC in a humidified atmosphere with 5% CO_2 in Dulbecco’s modified Eagles medium (Nissui, Tokyo, Japan) containing 1 mM pyruvate supplemented with 10% fetal bovine serum and 50 μg/ml kanamycin. 143B cells were kindly provided by Dr M. Tanaka (Department of Gene Therapy, Gifu International Institute of Biotechnology, Japan).

Synchronization of cells in cell cycle and treatment with chemicals

Cells were cultured in the presence of various drugs specified below: taxol as a mictotubule-stabilizing reagent (Sigma, St Louis, MO); nocodazole and colchicine (Sigma), as microtubule-destabilizing reagents; and 2-methoxyestradiol (Sigma). All reagents were prepared as 1000×-concentrated stock solutions dissolved in DMSO and stored at –20ºC. Cells were synchronized in the G_1/S phase of cell cycle by the double hydroxyurea block. Cells were plated on 60 mm plastic culture dishes at a density of 0.5×10^5 per dish, and cultured for 48 hours and then treated with 0.1 mM hydroxyurea (the first exposure for 24 hours, intermission for 8 hours followed by the second exposure for 16 hours). Cells were then washed three times with a complete medium for the release from the hydroxyurea block, and then cultured in the presence and absence of MADs.

Measurement of the mitochondrial membrane potential ($\Delta\Psi_m$), volume and mass

For the detection of the changes in $\Delta\Psi_m$, cells growing on 60 mm culture dishes were stained with Mito Tracker Orange CMTMRos (CMTMRos) (the final concentration, 100 nM) (Molecular Probes, Eugene, OR) for 30 minutes at 37ºC in a humidified 5% CO_2 atmosphere. After staining with the dye, cells were collected by trypsinization and submitted to FACS analysis. Volume of mitochondria per cell was assessed by treating cells with carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) (Sigma) or valinomycin (Sigma) for 5 minutes followed by staining with CMTMRos. It has been reported that $\Delta\Psi_m$ per cell measured in the presence of uncouplers such as CCCP reflects the total volume of mitochondria per cell and are in parallel with the mass of mitochondria per cell measured by 10-*n*-nonyl acridine orange (NAO) or JC-1 (Leprat et al., 1990; Vander Heiden et al., 1997).

Mitochondrial mass per cell was measured by flow cytometry using Mito Tracker Green FM (Green FM) (Molecular Probes) or NAO (Molecular Probes) as described by Mancini et al. (Mancini et al., 1997). Cells were collected by trypsinization, suspended in 0.5 ml PBS and fixed with a fixative containing 2% glutaraldehyde and 2% formaldehyde in PBS for Green FM staining, or with cold 70% ethanol for NAO staining. Fixed cells were washed in PBS, suspended in PBS (3×10^5 cells/ml) and stained with 75 nM Green FM for 30 minutes, or with 10 μM NAO for 10 minutes at room temperature in the dark. After two washes with cold PBS, cells were immediately analyzed by flow cytometry.

Flow cytometry

Coulter Epics XL flow cytometer (Coulter, Miami, FL) was used for all experiments. The 525 nm filter was used for the observation of the green fluorescence and 585 nm for orange fluorescence.

Visualization of mitochondria and MTs by confocal microscopy

Staining of mitochondria

Cells growing on 10×10 mm glass coverslips under various experimental conditions were incubated with 100 nM Mito Tracker Red CMXRos (CMXRos) (Molecular Probes) for 30 minutes at 37ºC with 75 nM Mito Tracker Green FM (Green FM) (Molecular Probes) for 1 hour at room temperature in the dark in a atmosphere of 5% CO_2. In some experiments, the double staining with CMXRos and Green FM was also carried out: cells stained with CMXRos were fixed with a fixative containing 2% glutaraldehyde and 2% formaldehyde in PBS for 30 minutes at room temperature, and post-stained with Green FM.

Transient transfection of cells with pEYFP-Mito

For the visualization of the total mass of mitochondria per cell, cells were transfected with mitochondrial targeting sequence from subunit

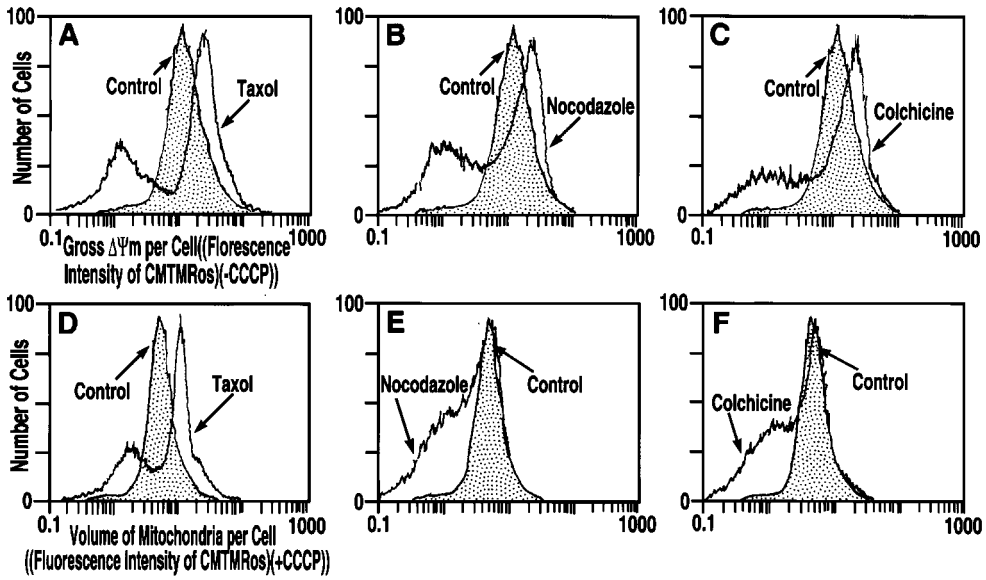

Fig. 1. Flow cytometric analysis on the effect of MADs on $\Delta\Psi_m$ and volume of mitochondria per cell in 143B cells. Cells were treated for 24 hours with 2.5 μM taxol (A,D), 10 μM nocodazole (B,E) or 5 μM colchicine (C,F), then stained with CMTMRos in the presence (D-F) or absence (A-C) of the pre-treatment with CCCP.

VIII of human cytochrome *c* oxidase fused with enhanced yellow fluorescent protein (pEYFP-Mito) (CLONTECH Laboratories, Palo Alto, CA). Transfections were performed using Lipofectamine Plus reagent (GIBCO BRL, Getherrburg, MD) according to manufacturer's instructions. After transfection cells were cultured for 24 hours and then treated with MADs for various lengths of time, and analyzed by confocal microscopy. Final pictures obtained were the results of merging optical sections taken at every 0.5 μm using Adobe Photoshop 5.0. Vertical sections were obtained by scanning cells at every 0.2 μm and were automatically merged by BioRad Lasersharp MRC 1024 scanning confocal microscope's software.

Detection of MTs

After cells were stained with CMXRos, as described above, they were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. Cells were then blocked by 1% BSA in PBS, and then incubated with monoclonal, anti-α tubulin antibodies (clone NO. B-

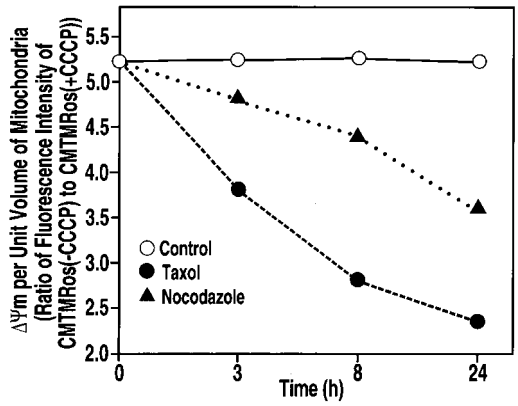

Fig. 2. Correlation between $\Delta\Psi_m$ per unit volume of mitochondria and the duration of the treatment with taxol or nocodazole. Experimental conditions are the same as those described in the legend for Fig. 1, except that cells were treated with taxol or nocodazole for 3, 8 and 24 hours.

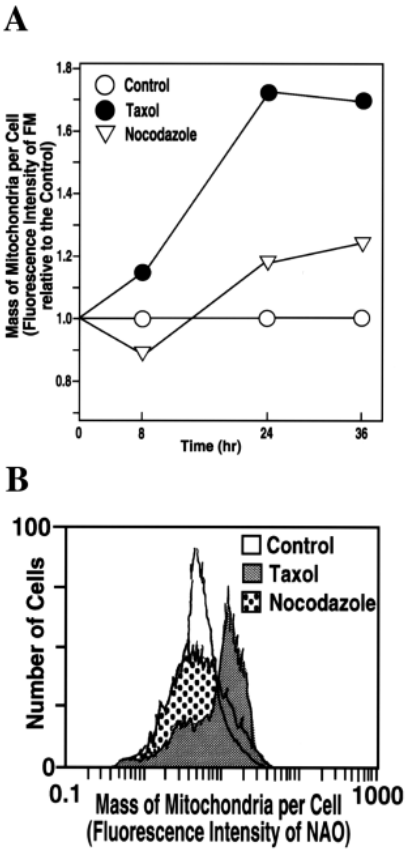

Fig. 3. Effects of taxol and nocodazole on the mass of mitochondria per cell in 143B cells. (A) Mass of mitochondria per cell detected by Green FM was plotted against the duration of time of the treatment with 2.5 μM taxol or10 μM nocodazole, taking the values of the control as 1.0. (B) Flow cytometry on the mass of mitochondria detected by NAO in the cells treated for 24 hours with 2.5 μM taxol or 10 μM nocodazole.

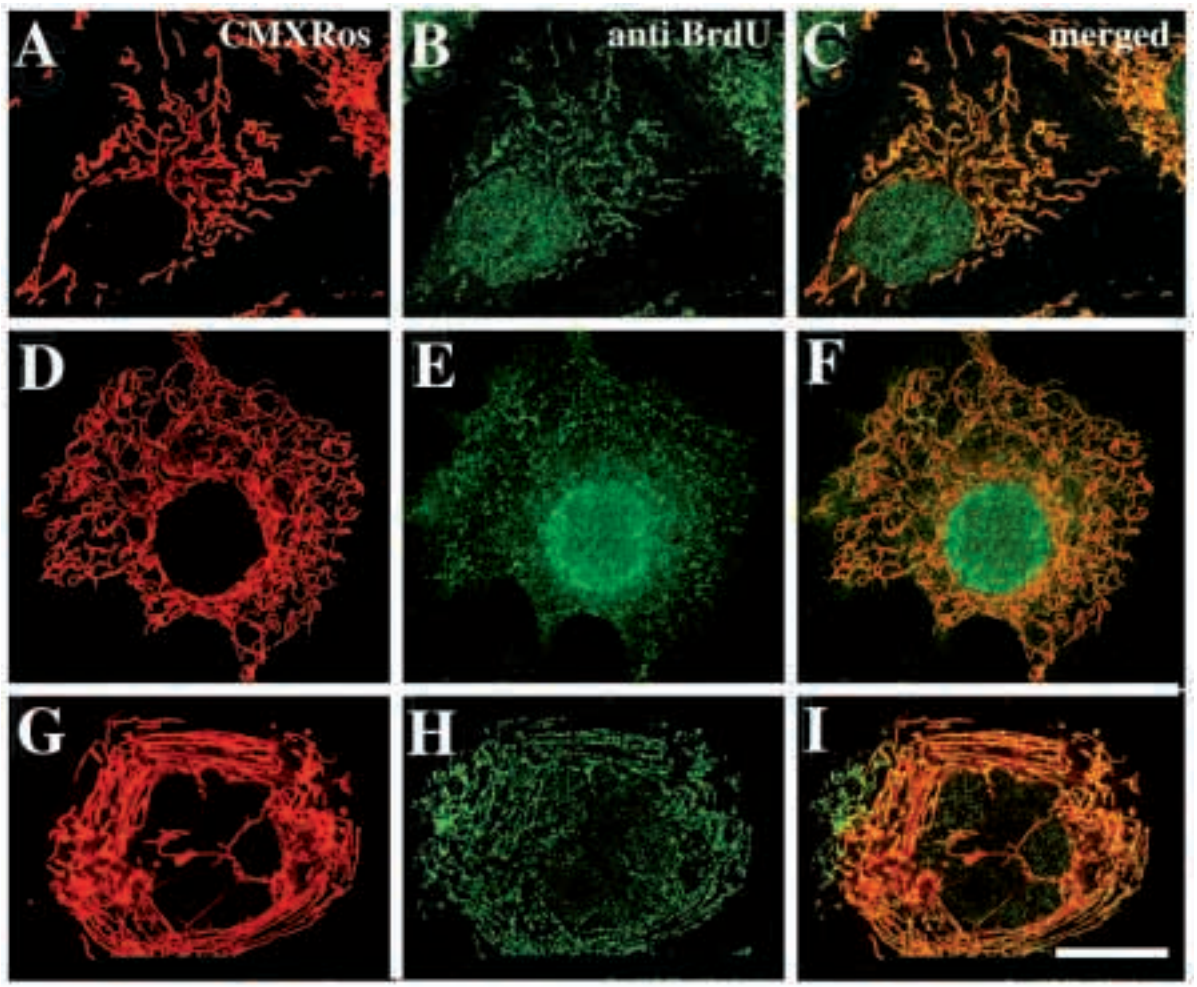

Fig. 4. Detection of mtDNA replication in nocodazole-and taxol-treated cells. Control (DMSO-treated) cells (A-C), those treated for 24 hours with 10 μM nocodazole (D-F) or 2.5 μM taxol (G-I) in the presence of 15 μM BrdU were stained with CMXRos (A,D,G), and then incubated with mouse anti-BrdU antibodies and stained with FITC-labeled goat anti-mouse IgG to detect newly replicated mtDNA (B,E,H). (C,F,I) Simultaneous visualization of mitochondria and mtDNA. Scale bar: 20 μm.

5-1-2, Sigma) diluted 250 times with a blocking buffer for 1 hour at room temperature, incubated with secondary antibodies: 100×-diluted FITC-labeled goat anti-mouse IgG (Molecular Probes) for 45 minutes at room temperature. Coverslips were placed on glass slides in a Perma Fluor mounting solution (Immunon, Pittsburgh, PA), and analyzed by a BioRad Lasersharp MRC 1024 scanning confocal microscope as described before (Karbowski et al., 1999).

In situ detection of mtDNA replication

Replication of mtDNA was detected in situ according to the method of Davis and Clayton (Davis and Clayton, 1996). 143B cells devoid of soluble thymidine kinase(TK[-]) grown on 10x10- mm glass coverslips were incubated with 15 μM 5-bromo-2-deoxy-uridine (BrdU) (Sigma) for 1-24 hours in the presence or absence of MADs. Cells were then stained with 100 nM CMXRos for 30 minutes, fixed in 2% glutaraldehyde/2% formaldehyde in PBS and permeabilized in acetone at –20ºC. After rehydration in PBS, DNA was denatured by 2N HCl at 37ºC. Coverslips were treated with 100 mM Tris-HCl, pH 8.2, washed in PBS and then blocked with Block Ace reagent (Dai Nihom Seiyaku, Osaka, Japan) for 30 minutes at room temperature. Cells were incubated with mouse anti-BrdU antibodies (clone OS 94.6, Oncogene, Boston) (diluted 50 times with Block Ace reagent) for 60 minutes, washed in PBS and stained with FITC-labeled goat anti-mouse IgG (Molecular Probes; diluted 100-fold) and analyzed by confocal microscopy.

Immunoblotting of α-tubulin and acetylated α-tubulin

Control and experimental cells growing on six-well-culture plates were washed twice with a microtubule-stabilizing buffer containing 80 mM Pipes, 1 mM $MgCl_2$, 2 mM EGTA, pH 6.9, supplemented with 1 mM phenylmethylsulfonyl fluoride and 20 μM leupeptin, and then lyzed with the same buffer supplemented with 1% sodium dodecyl sulfate (SDS). Aliquots were taken from the samples for the protein determination. The remaining samples were mixed with an equal volume of 2× SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 10% glycerol, 2% β-mercaptoethanol, 0.006% bromophenol blue) containing 3% SDS. After electrophoresis proteins were transferred onto the Protran nitrocellulose membranes (Schleicher & Schull, Dassel, Germany) and blocked with Block Ace reagent (Dai Nihon Seiyaku, Osaka, Japan). Blots were then reacted with either monoclonal anti-α-tubulin antibodies (clone No. B-5-1-2, Sigma) or monoclonal anti-acetylated tubulin antibodies (clone No. 6-11 B-1, Sigma). After staining with horseradish peroxidase-conjugated anti-mouse secondary antibodies (Promega Madison, WI), signals were detected by enhanced chemiluminescence using Amersham ECL kit (Amersham, UK) according to the manufacturer's instructions.

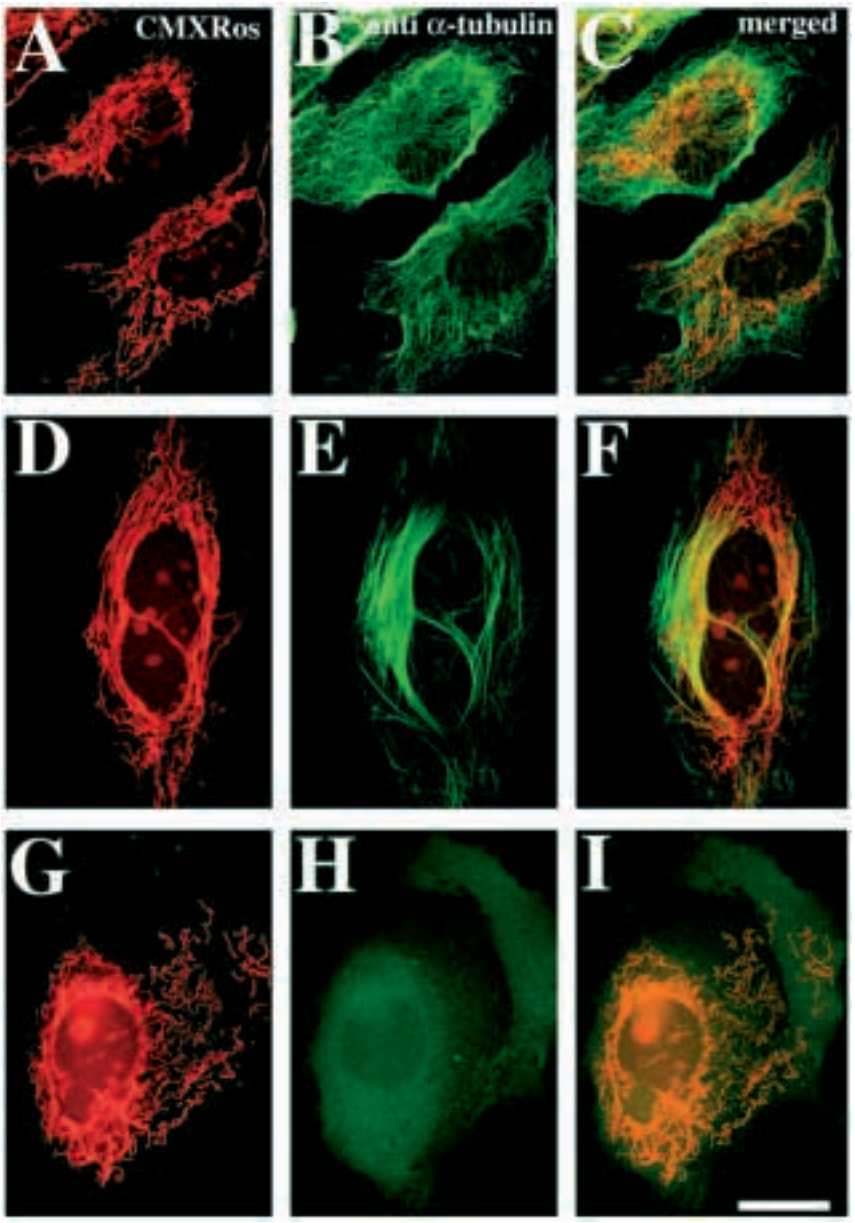

Fig. 5. Distribution of active mitochondria detected by CMXRos and the organization of MTs in taxol- or nocodazole-treated cells. Cells were incubated for 24 hours with DMSO (A-C), 2.5 μM taxol (D-F) or 10 μM nocodazole (G-I), then stained with CMXRos (A,D,G) and fixed, and MTs were visualized using anti-α-tubulin monoclonal antibodies (B,E,H). (C,F,I) Merged images. Scale bar: 20 μm.

Cell cycle analysis

Cell cycle distribution of the cells was detected using FACS assay of the propidium iodide (PI)-stained nuclei, essentially according to the method of Deitch et al. (Deitch et al., 1982). Cells collected by trypsinization were incubated with a buffer containing 50 μg/ml PI, 0.1% Na-citrate, 0.2% nonident P-40 (NP-40) and 25 U/ml RNAase A (Sigma) for 30 minutes at 4ºC in the dark, and then incubated further for another 15 minutes at 37ºC and analyzed by flow cytometry.

Protein determination

Protein was determined using BioRad DC Protein Assay Kit (BioRad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Data analysis

Data presented are mean±standard error (s. e. m.) of at least three different experiments. Since DMSO (0.1-0.2%) was used as the solvent for MADs, comparisons were made between DMSO-treated cells and MAD-treated cells. Student's *t*-test was used to evaluate significant differences. Values of experimental groups are statistically different from those of the control group at: a ($0.001<P<0.01$), b ($0.01<P<0.02$), c ($0.02<P<0.05$).

RESULTS

Microtubule-active drugs (MADs) modify the $\Delta\Psi_m$, volume and mass of mitochondria in 143B cells

Changes in the dynamics of MTs potentially exert profound effects on the homeostasis of mitochondria since the distribution and morphology of mitochondria and other organelles in mammalian cells are largely dependent upon the integrity of MTs (Heggeness et al., 1977; Tanaka et al., 1998). Thus, in this study we have addressed the question of how alterations of MTs modify the dynamics of mitochondria. To achieve this, we employed taxol as MT-stabilizing agent, and nocodazol and colchicine as MT-disrupting agents.

For the evaluation of functional states of mitochondria cells were cultured for up to 24 hours in the presence of MADs, stained with CMTMRos to detect $\Delta\Psi_m$ for flow cytometry (Fig. 1). When they were stained with the dye without the pre-treatment with CCCP, two peaks were invariably detected, while only one peak was detectable in the control cells: a tall peak with higher $\Delta\Psi_m$ than that of control, and a small peak with very low $\Delta\Psi_m$ (Fig. 1A-C). When cells were pre-treated with CCCP and then stained with CMTMRos, the peak $\Delta\Psi_m$ of the control cells shifted to the left. Similarly, tall peaks in MAD-treated cells, specified above, invariably shifted to the left resulting in the overlapping of the tall peaks and small ones except for the case of taxol (Fig. 1D-F). Shift of the tall peak of taxol-treated cells to the left was less distinct compared with that of nocodazole- or colchicine-treated cells, so that the peak intensity of the dye in taxol-treated cells remained higher than that of the control cells, and the small peak remained separated from the tall one. CCCP dissipates H^+ ion gradient generated by mitochondrial electron transfer chain necessary for the active accumulation of CMTMRos in mitochondria. Thus, the remaining uptake of the dye by mitochondria largely reflects the volume of mitochondria with a possible contribution of H^+-independent components of mitochondrial membrane potential (Leprat et al., 1990; Vander Heiden et al., 1997). The results with CCCP, described above, were reproducible when CCCP was replaced by valinomycin, which disrupted not only the

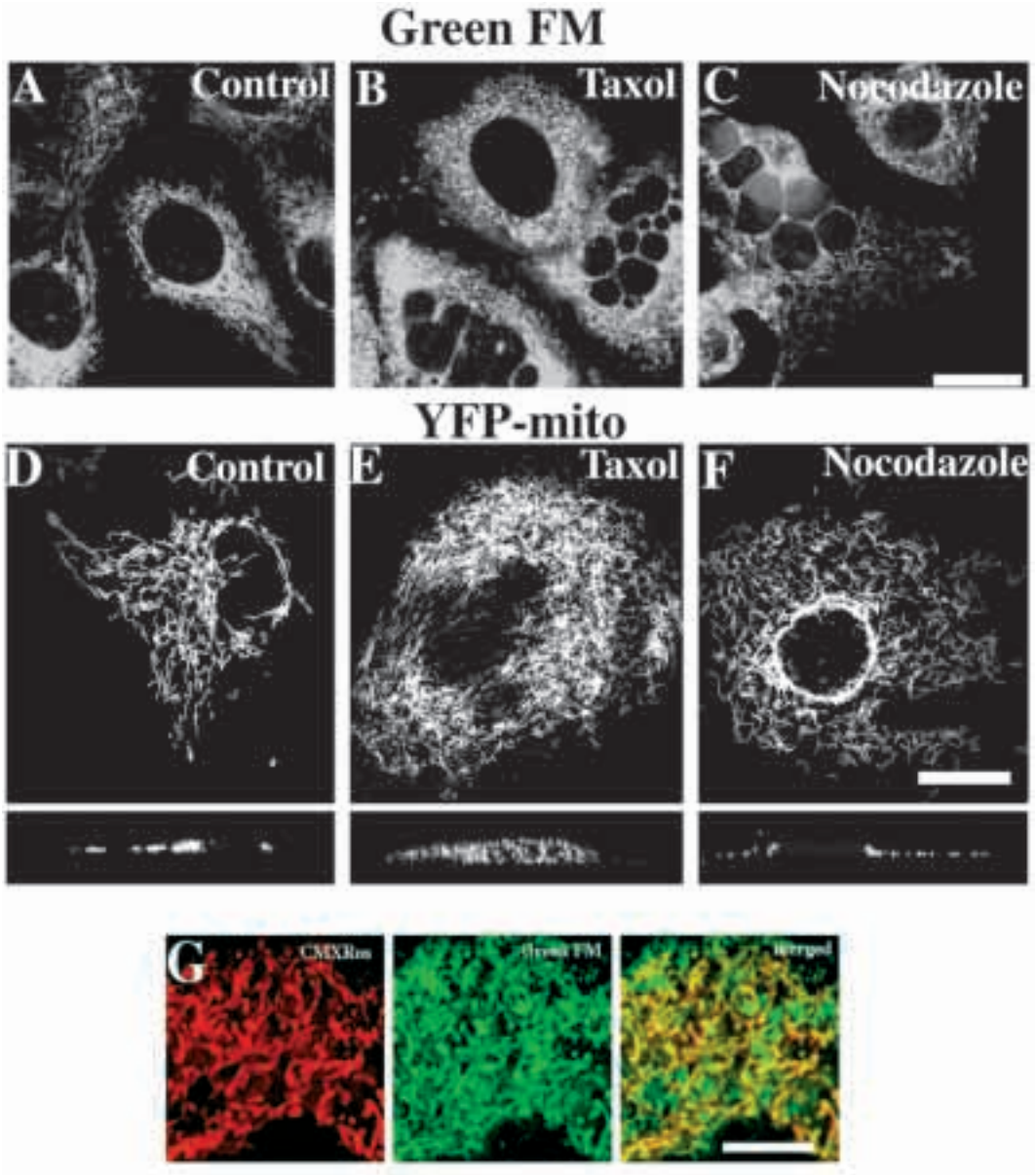

Fig. 6. Detection of active and non-active mitochondria by Green FM (A-C) or pEYFP Mito (D-F) in taxol- or nocodazole-treated cells. Cells were incubated for 24 hours with DMSO (A), 2.5 μM taxol (B) or10 μM nocodazole (C) and stained with Green FM. In D-F, cells were transfected with YFP targeted into mitochondria 24 hours before the treatment with taxol or nocodazole. 24 hours after the treatment, pEYFP-Mito was detected. Side view of the cells obtained by optical sectioning of the cells at every 0.2 μm are shown in lower part of the figures. In G,cells treated for 24 hours with 2.5 μM taxol were double-stained to detect mitochondria with CMXRos (red) and Green FM (green). Mitochondria stained with green and those stained orange to yellow are seen on the merged picture.

proton gradient but also gradients of other ions formed across the inner mitochondrial membrane (data are not shown). Thus, it might be reasonable to assume that $\Delta\Psi_m$ per cell measured in the presence of CCCP practically represents the total volume of mitochondria per cell, at least in the present experimental conditions. These results also indicated that the peak intensity of the dye obtained from cells without the pre-treatment with CCCP or valinomycin reflected not only $\Delta\Psi_m$ but also volume of mitochondria. Thus, data obtained with MAD-treated cells suggest that the total volume of mitochondria per cell is unchanged in major population of nocodazole- or colchicine-treated cells, whereas it is definitely increased in taxol-treated cells. The fluorescence intensity of small peak observed in taxol-treated cells remained essentially in the same position in the absence and presence of the CCCP- or valinomycin-pre-treatment indicating a possibility that the $\Delta\Psi_m$ of about 20% of taxol-treated cells became collapsed by apoptotic changes of the cell (this was confirmed by PI-staining). Population of cells forming the small peak in the case of nocodazole or colchicine also reached 20%. Apoptotic cells were excluded from further analyses.

In Fig. 2, a ratio of the mean fluorescence intensity of CMTMRos per cell measured in the absence of CCCP to that obtained in the presence of CCCP was plotted against the duration of time of the treatment with taxol or nocodazole. The ratio thus obtained indicates a relative $\Delta\Psi_m$ per unit volume of mitochondria (Mancini et al., 1997). Both taxol and nocodazole, especially the former, caused distinct decreases in the ratio compared with the control. Colchicine-treated cells showed similar results to those of nocodazole-treated cells (data are not shown). These data suggest that taxol causes an accumulation of mitochondria with distinctly low $\Delta\Psi_m$. However, taxol-induced increases in the total volume of mitochondria per cell could be due to two possibilities: simple swelling of each mitochondrion or actual proliferation of mitochondria. Taxol-treated cells were therefore stained with Green FM or NAO to detect the total mass of mitochondria per cell (Fig. 3). Green FM is essentially non-fluorescent

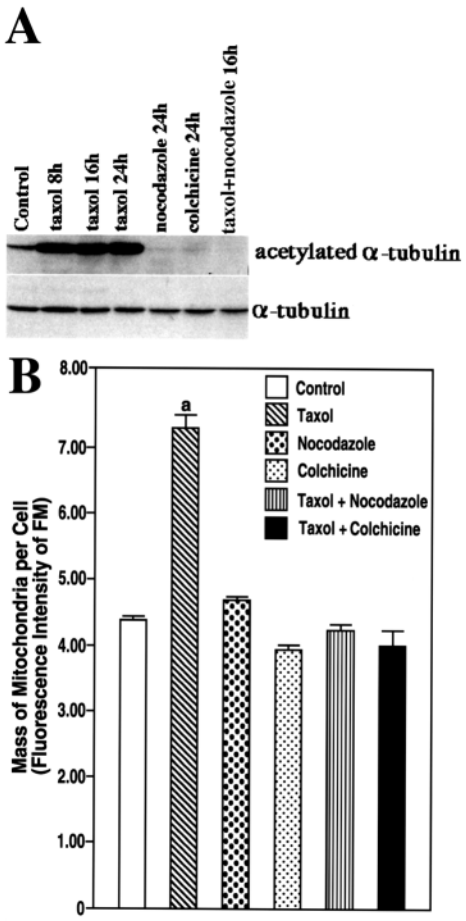

Fig. 7. Correlation between the level of acetylated α-tubulin and mass of mitochondria per cell in MAD-treated cells. (A) Cells were treated with taxol for up to 24 hours, nocodazole, colchicine, or taxol plus nocodazole for 16 hours, and the amounts of α-tubulin and acetylated α-tubulin were detected by SDS-PAGE and immunoblotting with antibodies against α-tubulin and acetylated α-tubulin. (B) Cells treated for 16 hours with MADs, specified in the figure, were stained with Green FM and the mitochondrial mass measured.

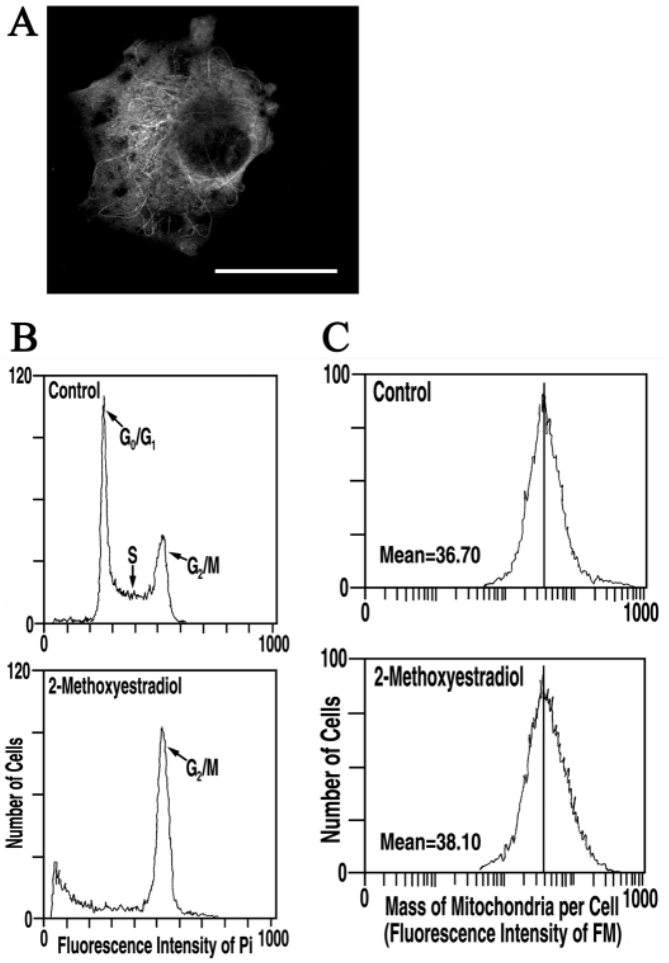

Fig. 8. Effects of 2-methoxyestradiol on the level of acetylated α-tubulin, cell cycle distribution and mass of mitochondria per cell. Cells treated for 24 hours with 2-methoxyestradiol were stained with anti-α-tubulin-antibodies for confocal microscopic analysis of MTs (A), stained with PI flow cytometric analysis of cell cycle distribution (B) or stained with Green FM to detect the mass of mitochondria per cell (C).

in aqueous solutions, and becomes fluorescent once it accumulates in lipid environment of mitochondrial membranes (Haugland, 1996). In Fig. 3A, relative fluorescence intensities of Green FM in taxol-treated cells are plotted against the duration of time of the experiment taking that of the control cells as 1.0. The mass of mitochondria per cell distinctly increased in the cells treated with taxol for 8 hours, reaching the maximum after 24 hours. Nocodazole, a MT-destabilizing drug, also caused increases in the mass of mitochondria when the treatment of the cells with drug was prolonged for up to 14-15 hours or longer, but the degree of the increment was far less distinct compared to the case of taxol. Taxol-induced increases in the mass of mitochondria per cell have been confirmed further by staining mitochondria with NAO, which interacts stoichiometrically with cardiolipin of the mitochondrial membrane (Petit et al., 1995) and independently of mitochondrial energy states (Maftah et al., 1989; Fig. 3B). There was a distinct shift of the peak intensity of NAO to the right in taxol-treated cells, whereas that in nocodazole-treated cells showed essentially the same fluorescence intensity as controls, although the former became somewhat broadened. Increases in the mass of mitochondria in taxol-treated cells detected by FM or NAO were further supported by electron microscopic observation of mitochondria. Mitochondria in taxol-treated cells showed no apparent sign of the swelling and remained intact (electron micrographs are not shown).

Replication of mtDNA in MAD-treated cells

In the present study we have examined whether or not BrdU is incorporated into mtDNA of MAD-treated cells to obtain a further evidence that increases in the mass of mitochondria detected by Green FM and NAO in taxol-treated cells are actually due to the accumulation of mitochondria. Using 143B(TK[-]) cells it is possible to detect in situ mtDNA replication by detecting the incorporation of the thymidine analog 5-bromo-2-deoxy-uridine (BrdU) into mtDNA (Davis and Clayton, 1996). We have applied this method to determine the rate of mtDNA replication in MAD-treated cells. Namely, cells were incubated for 24 hours with taxol or nocodazole in

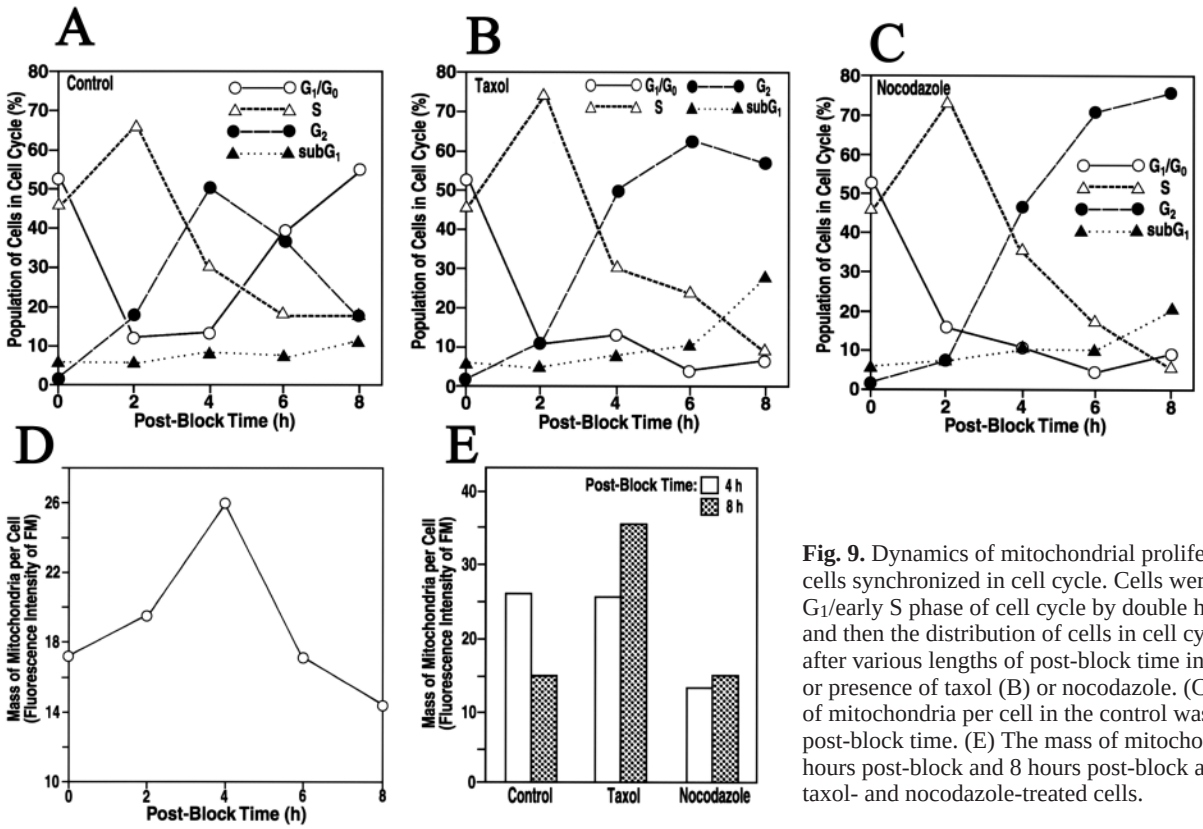

Fig. 9. Dynamics of mitochondrial proliferation in 143B cells synchronized in cell cycle. Cells were synchronized in G_1/early S phase of cell cycle by double hydroxyurea block, and then the distribution of cells in cell cycle was measured after various lengths of post-block time in the absence (A) or presence of taxol (B) or nocodazole. (C). (D) The mass of mitochondria per cell in the control was plotted against post-block time. (E) The mass of mitochondria per cell 4 hours post-block and 8 hours post-block are compared in taxol- and nocodazole-treated cells.

the presence of 15 μM BrdU, stained with CMXRos and anti-Brd monoclonal antibodies, and analyzed by confocal microscope (Fig. 4). In control cells, association of BrdU (green dots) (Fig. 4B) with mitochondria (Fig. 4A) was discerned clearly when both were visualized at the same time (Fig. 4C). In nocodazole-treated cells (Fig. 4D-F), the frequency of incorporation of BrdU into mtDNA became decreased distinctly (Fig. 4E) compared with that of the control, while that of taxol-treated cells (Fig. 4G-I) became remarkably increased (Fig. 4H). It should be noted that some of newly synthesized mtDNA in taxol-treated cells did not correspond to CMXRos-stained mitochondria (Fig. 4I, on the left upper corner of the cell). This may indicate an existence of a certain population of mitochondria with low $\Delta\Psi_m$, which could not accumulate CMXRos in an energy-dependent manner.

Stabilization of MTs by taxol results in the formation of two subpopulations of mitochondria

It has been shown that the treatment with taxol results in the random nucleation and disorganized distribution of stabilized MTs in different mammalian cells (Piperno et al., 1987; Ebneth et al., 1998). In addition, taxol causes bundling of MTs by unknown mechanisms (Turner and Margolis, 1984). However, the depolymerization of tubulin results in random cytoplasmic, or in some cell lines, perinuclear distribution of mitochondria (Heggeness, 1978; Tanaka et al., 1998). Thus, we have examined the correlation between changes in the distribution of MTs and those of mitochondria in the cell. Control 143B cells (Fig. 5A-C) and those treated for 24 hours with taxol (Fig. 5D-F) or nocodazole (Fig. 5G-I) were stained with CMXRos to detect mitochondria (Fig. 5A,D,G), and then fixed with aldehyde and processed for immunocytochemical detection of MTs (Fig. 5B,E,H). Double-fluorescence confocal images of mitochondria (Fig. 5A) and MTs (Fig. 5B) in the control cells showed that elongated, tubular mitochondria were distributed along fine networks of MTs (Fig. 5C). In taxol-treated cells, MTs formed thick bundles (Fig. 5E), and elongated, cable-like mitochondria (Fig. 5D) were detected running parallel with MTs (Fig. 5F). In nocodazole-treated cell, MTs were no longer detected (Fig. 5H), and randomly oriented, branching mitochondria were seen with higher density around the nucleus than the cell periphery (Fig. 5G).

In the present study we have detected increases in the mass of mitochondria in taxol-treated cells by flow cytometric analysis on Green FM- and NAO-stained mitochondria as well as by mtDNA replication. However, confocal micrographs of CMXRos-stained, taxol-treated cells have revealed no apparent sign of increases in the number of mitochondria per cell, as shown above, although their shapes and distribution in the cell were altered. One possibility is that two subpopulations of mitochondria may exist in taxol-treated cells: those with high $\Delta\Psi_m$, detectable with CMXRos, and those with low $\Delta\Psi_m$, undetectable with the dye. To determine this, cells were labeled with Green FM, or transfected with mitochondria-targeted-YFP for 24 hours, and then treated with taxol or nocodazole and analyzed by confocal microscopy. In both control (Fig. 6A) and nocodazole-treated (Fig. 6C) cells, mitochondria stained with Green FM were mainly filamentous. Furthermore, mitochondria visualized by YFP in control (Fig. 6D) and nocodazole-treated cells (Fig. 6F) were also mainly filamentous similar to those detected by CMXRos staining (compare Fig. 5A for the control with Fig. 5G for the nocodazole). In taxol-treated cells, increases in the number of

mitochondria per cell detected by Green FM (Fig. 6B) and YFP (Fig. 6E) were distinct. Vertical views of YFP-stained cells demonstrated at the bottom of Fig. 6D-F also demonstrated a distinct increase in the number of mitochondria in the case of taxol. Furthermore, two structurally different populations of mitochondria were detected in taxol-treated cells by Green FM or YFP: those with filamentous structures and those with granular ones. In contrast, CMXRos staining showed mitochondria that were exclusively filamentous (compare with Fig. 5D). When confocal images obtained from taxol-treated cells stained with CMXRos (Fig. 6G) and with Green FM were merged, granular mitochondria (stainable only with Green FM), were seen among filamentous ones (orange to yellow in color).

Co-treatment of cells with taxol and nocodazole or taxol and colchicine causes destabilization of MTs and at the same time suppresses taxol-induced proliferation of mitochondria

Treatment with MADs used in the present study invariably caused arrest of 143B cells in the G_2/M phase of cell cycle (data are not shown), but distinct increases in the mitochondrial mass were detected only in taxol-treated cells, as described above. This strongly suggests that the stabilization or destabilization of MTs may play a specific role in the regulation of the mitochondrial proliferation. It has been shown that a prolonged treatment of cells with taxol causes complete stabilization of MTs, concomitant with acetylation of α-tubulin (Piperno et al., 1987). We have also analyzed changes in the amount of stabilized and the total amount of tubulin polymer using anti-acetylated α-tubulin in MAD-treated cells (Fig. 7A). Data on immunoblotting shown in the Fig. 7 have clearly demonstrated that treatment of cells with taxol for 8 hours or longer causes distinct increases in the level of acetylated α-tubulin, while the total amount of α-tubulin remains essentially the same as that of the control cells. However, acetylated α-tubulin was not detected in nocodazole- or colchicine-treated cells. Furthermore, acetylated α-tubulin was undetectable in the cells co-treated with taxol and nocodazole. Thus, effects of co-treatment of cells with taxol and nocodazole or taxol and colchicine on the mitochondrial mass per cell were examined. In Fig. 7B, co-treatment with taxol and nocodazole or taxol and colchicine for 16 hours were stained with Green FM for flow cytometry. Taxol-induced distinct increases in the mitochondrial mass were no longer seen in these cells.

2-Methoxyestradiol as a possible natural regulator of MT-dependent mitochondrial proliferation

Next, we looked for natural products or metabolites that may control the stability of MTs in vivo, in line with the hypothesis that MTs may control the biogenesis of mitochondria. 2-Methoxyestradiol, a natural metabolic by-product in humans, has been shown to bind to the colchicine-binding site of tubulin, resulting in the alteration in the stability of MTs in MCF7 breast cancer cell line (Cushman et al., 1995). Thus, this estrogen metabolite can be described as an 'endogenous MAD'. In Fig. 8A, cells treated for 24 hours with 10 μM 2-methoxyestradiol were stained with anti-α-tubulin antibodies and analyzed by confocal miscroscopy. Increases in monomeric tubulin background and a distinct decrease in the number of MTs have confirmed its colchicine-like destabilizing effects on MTs. 2-Methoxyestradiol arrested about 80% of asynchronously growing 143B cells in G_2/M phase of cell cycle, while 20% of the control cells were in G_2/M phase (Fig. 8B). However, there was practically no difference in the total mass of mitochondria per cell between the control and 2-methoxyestradiol-treated cells (Fig. 8C). These data indicate that 2-methoxyestradiol, as in the case of nocodazole or colchicine, inhibits the mitochondrial mass increase, which should be distinct in G_2/M phase in normal conditions. Replication of mtDNA was also suppressed in 2-methoxyestradiol-treated cells (data are not shown).

Depolymerization of MTs inhibits the proliferation of mitochondria in cells synchronized in cell cycle

Finally, we examined effects of MADs on cell cycle progression using 143B cells synchronized in the cell cycle to obtain more information about the biogenesis of mitochondria. Cells were arrested in G_1/early S phase by double hydroxyurea block. Cell cycle distribution of MAD-treated cells were then analyzed by flow cytometry of PI-stained nuclei at different post-block times. At the same time, cells were stained with Green FM to detect changes in the mass of mitochondria per cell. As shown in Fig. 9A, more than 90% of control cells were synchronized in G_1/early S phase after the second period of incubation with hydroxyurea (details are described in Materials and Methods). The doubling of the amount of DNA per cell was detected 4 hours after the wash out of hydroxyurea, and most of the cells shifted to the second post-block G_1 phase 8 hours post release. The largest value in the mitochondrial mass per cell reached 165% of the initial value at 4 hours when about 50% of the cells were in G_2 phase, and it decreased to 82% of the initial value at 8 hours (Fig. 9D). These results indicate that during cell cycle progression in control 143B cells, increases in the mitochondrial mass per cell between G_1 and G_2 phase was linear without phase specificity. Neither taxol (Fig. 9B) nor nocodazole (Fig. 9C) affected the duration of time required for G_1 to G_2/M phase transition in cell cycle. However, the major population of taxol- and nocodazole-treated cells remained in G_2/M phase 8 hours post block indicating that the cell division was suppressed. In Fig. 9E, the cells synchronized in G_1/early S phase of cell cycle were treated with taxol or nocodazole immediately after hydroxyurea was removed from the culture medium. The mass of mitochondria per cell in these cells was estimated at 4 and 8 hours post block (when masses in control cells reach maximum and minimum, respectively). In taxol-treated cells, increases in the mass of mitochondria per cell proceeded during G_1 to G_2/M phase transition to the same degree as in the case of the control cells. However, mitochondria in these cells continued to proliferate after they were arrested in G_2/M phase. Conversely, increases in the mass of mitochondria per cell in nocodazole-treated cells during G_1 to G_2/M phase were suppressed almost completely, and remained unchanged for up to 8 hours post block.

DISCUSSION

Flow cytometric and confocal laser microscopic analyses on the mitochondrial mass per cell during cell cycle progression in taxol-treated cells using various mitochondria-specific fluorescent dyes have revealed that a prolonged treatment with

the drug causes abnormal accumulation of mitochondria in the cells arrested in G_2/M phase of cell cycle. At the same time, distinct increases in the intracellular level of acetylated α-tubulin were caused by taxol. Taxol-induced proliferation of mitochondria was evidenced also by the enhanced mtDNA replication and by the detection of pEYFP. On the contrary, nocodazole and colchicine inhibited mitochondrial proliferation during G_1 to G_2 phase progression and arrested cells in G_2/M phase and at the same time depolymerized MTs lowering the intracellular level of acetylated α-tubulin. A combined treatment of cells with taxol and nocodazole or taxol and colchicine suppressed taxol-induced proliferation of mitochondria.

Biogenesis of mitochondria has been extensively studied both on prokaryotic and eukaryotic cells (Luck, 1963; Luck, 1965; Bergeron and Droz, 1969; Pica-Mattoccia and Attardi, 1971; Pica-Mattoccia and Attardi, 1972; Posakony et al., 1977). Devey and Fuhr have shown using synchronized Chinese hamster cells that the number of mitochondria in M phase in cell cycle increases to twice that in G_1 phase (Devey and Fuhr, 1976). In early G_1 phase, fusion decreases the number of mitochondria to less than half before mitosis. While cells proceed through G_1 to S phase, mitochondria divide and their number increases. Similar phenomena have been observed in human leukemia cell line HL-60 (James and Bohman, 1981). In physiological conditions, mitochondria move towards the periphery of the cell using MTs as a railway, suggesting that stable MTs regulate the intracellular distribution of the organelles. The present study has shown that number of stable MTs is distinctly increased by taxol and yet MT-dependent intracellular distribution of mitochondria does not seem to be affected, indicating that MTs polymerized by taxol exert essentially the same cellular functions as 'normal MTs' as documented by others (Rogalski and Singer, 1984; Wiemer et al., 1997; Ebneth et al., 1998). Depolymerization of MTs, on the contrary, may affect intracellular transport of mitochondria as well as their contacts with other organelles, which may result in inability to synthesize new mitochondria: randomization of mitochondrial distribution in a variety of mammalian cells (Heggeness et al., 1977; Heggeness et al., 1978; Nangaku et al., 1998); clustering of peroxisomes in CV1 cells (Wiemer et al., 1997); and dispersion of Golgi apparatus from perinuclear compact configuration into cell periphery (Rogalski and Singer, 1984). Spatial distributions of both mitochondria and endoplasmic reticulum (ER) are also regulated by MTs so that they are extensively associated with each other (for a review see Bereiter-Hahn, 1990). Rizutto et al. have shown that the mitochondrial surface is specifically exposed to a higher concentration of Ca^{2+} than bulk cytosol after opening of inositol 1,4,5-triphosphate-gated channels of ER (Rizutto et al., 1998). Changes in the number of contact sites between two organelles may affect phospholipid exchanges between them resulting in changes in the dynamics of mitochondrial compartment formation. Frequency of the contact between mitochondria and ER was distinctly increased during the course of herbimycin A-induced abnormal proliferation of mitochondria in colon carcinoma cells (Mancini et al., 1997). Thus, we may deduce from the data shown in the present study, and from those by others, that changes in the physicochemical state of MTs can modify cellular environment surrounding mitochondria leading to changes in the rate of mitochondrial biogenesis.

It is interesting to note that the presence of stable MTs is most pronounced in various cellular processes which require a large amount of energy: neurite outgrowth (Baas and Black, 1990); myotube differentiation (Gunderson et al., 1989); formation of monolayer of epithelial cells (Pepperkok et al., 1990); pressure overloaded cardiac hypertrophy (Sato et al., 1997). Other evidence supporting a possible role of MTs in the regulation of the mitochondrial biogenesis is that the treatment with herbimycin A or H_2O_2, known to induce the accumulation of mitochondria in mammalian cells (Mancini et al., 1997; Lee et al., 2000), causes stabilization of MTs in 143B cells (J. H. S. and M. K., unpublished observations). Furthermore, the elevation of mitochondrial mass has been reported in HEP-2 cells cultured in the presence of cytotoxic necrotizing factor 1 (CNNF1), an activator of Rho GTPase (Fiorentini et al., 1998). Activation of Rho GTPase is an sufficient factor required for the stabilization of MTs (Cook et al., 1998). The present study has shown that 2-methoxyestradiol, which is structurally unrelated to nocodazole or colchicine, depolymerizes MTs and at the same time inhibits the proliferation of mitochondria. This may also suggest that MTs control the biogenesis of mitochondria and that 2-methoxyestradiol is a candidate for a controller of physicochemical properties of MTs in vivo. However, further studies are essential to clarify the correlation between biogenesis of mitochondria and depolymerization of MTs by 2-methoxyestradiol.

The present study has demonstrated the possibility that stability of MTs may control the biogenesis of mitochondria, using MADs and 2-methoxyestradiol as experimental tools. The possibility that taxol has induced the accumulation of mitochondria by toxic side effects and not by the modification of MTs could be eliminated by the fact that the duration of time required for the cell cycle progression is not affected by the drug. However, further studies are necessary to clarify exact correlation between the modification of MTs by MADs, and the biosynthesis of mitochondria.

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