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Structural changes of mitochondria during free radical-induced apoptosis

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The initial proposal for apoptosis stressed nuclear change (condensation of chromatin) and the intactness of intracellular organelles, including mitochondria, based on light and electron microscopic observations. However, data have accumulated to demonstrate that the opening of megachannels of mitochondrial membranes, resulting in the swelling of the organelles, notably by Ca^{2+} and free radicals, is the crucial step in the apoptotic processes of the cell. Application of fluorescent dyes to mitochondria, combined with flow cytometry, has made it possible to detect subtle changes in the structure and function of the organelles related to apoptosis. The present article overviews structural aspects of mitochondria related to apoptosis, including the free radical-induced formation of megamitochondria.

key words: apoptosis, megachannels, free radicals, megamitochondria, fluorescent dyes, flow cytometry

INTRODUCTION

Various cellular functions in mammalian cells are largely dependent upon ATP generated from mitochondria. Thus, changes in the structure and function of mitochondria in physiological and pathological conditions reflect those in the energy requirement of the cell: the mitochondria of various tissues in hibernating animals become enlarged (the formation of megamitochondria (MG)), resulting in decreases in the oxygen consumption by mitochondria, which may lead to decreases in the intracellular levels of ATP. In non-hibernating animals in physiological conditions, mitochondria consume a large amount of oxygen to generate ATP to maintain cellular activities resulting in the generation of a much larger amount of reactive oxygen species (ROS) than in hibernating animals. Since cells of various tissues in hibernating animals require the amount of ATP which is sufficient to maintain the basal metabolism, generation of a large amount of ROS due to

excessive ATP synthesis might be disadvantageous or harmful to the cell; cardiac mitochondria, which are required to produce a massive amount of ATP to maintain the cardiac function, are rich in cristae and contain a much larger number of cytochromes than those of other tissues such as the liver; diaphragm mitochondria form a reticular network ("mitochondria reticulare") serving as a cable to transport molecular oxygen and other substances across a large distance in the myocyte; adrenal cortex mitochondria are characterised by the presence of "vesicular cristae" which are common to those of steroid-producing organs. When steroidogenesis is suppressed (for example, by hypophysectomy), the transition of the cristal membranes of mitochondria from the vesicular configuration to the tubular one takes place.

Besides the central role described above of mitochondria as a high energy-producing machinery, data have accumulated to demonstrate that mitochondria play a key role in apoptotic processes of the cell

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via the following various components localised within the organelles: the megachannels (MGCs) [23,35,69,71,72,86], cytochrome c, apoptosis-inducing factor (APF) [70], caspase 9 [37], caspase 3 [45] and Bcl-2 family [1,6,14,21,51,52,76,80]. Pro-caspase-9 and pro-caspase-3 can reside either in the cytosol or within mitochondria, depending on cell types. A small portion of the total cellular pool of pro-caspase-3 enters the mitochondria [45]. When cytochrome c is released from the mitochondria into the cytoplasm, it binds the caspase-activating protein Apaf-1, stimulating binding of Apaf-1 to pro-caspase-9 resulting in the activation of the caspase [40].

According to the currently accepted hypothesis for the mechanism of the apoptotic processes of the cell [57,66], the following series of events take place: 1. Opening of MGCs of the inner mitochondrial membranes where the inner and outer membranes contact notably by Ca^{2+} and free radicals. 2. Swelling of mitochondria. 3. Release of cytochrome c, caspase 9 and APF from mitochondria into the cytoplasm. 4. Decreases in the membrane potential of mitochondria ($\Delta \Psi m$) 5. Activation of caspases by cytochrome c resulting in proteolysis in the cytoplasmic components and degradation of nuclear DNA by APF. The hypothesis described here emphasises the opening of MGCs by free radicals resulting in the swelling of mitochondria. However, several controversial data have been presented against the hypothesis in the past few years, which will be discussed later.

Recently, we have found that free radical-induced formation of megamitochondria (MG) is succeeded by apoptotic changes of the cell and that apoptosis is suppressed by scavengers of free radicals via the suppression of the MG formation [32,33]. This may strongly suggest that MG should be regarded as another important structural change of mitochondria besides "swelling" which is intimately related to apoptosis.

The present article overviews the structural changes of mitochondria during apoptotic changes of the cell based on the survey of the literature and on data obtained from our laboratory.

### Swelling of mitochondria as a result of the opening of megachannels (MGCs)

The opening of MGCs of mitochondria can be detected either by direct measurements with patch-clamp techniques [35], or by indirect measurements using a spectrophotometer [57] or electron microscopy. The latter indirect methods detect the swelling of mitochondria as a result of the opening of MGCs. The spectrophotometric technique is superior to electron microscopy when isolated mitochondria are used in that changes in absorbance at 520 nm (or 540 nm) reflect those of mitochondria in a large population (Fig. 1) [81]. Mitochondria isolated in a medium containing sucrose remain in the condensed configuration as long as the MGCs are closed. When the channels are opened mitochondria lose impermeability to sucrose and the transition from the condensed to orthodox configuration takes place. Thus, the swelling of isolated mitochondria can be easily detected also by electron microscopy (Fig. 2). However, quantitative evaluation of electron microscopic data is inevitably associated with some difficulties. When cells are used, spectrophotometric techniques for the analysis of mitochondrial swelling cannot be adopted since changes in absorbance at 520 nm (or 540 nm) reflect not only those obtained from mitochondria but also those from other organelles.

Mitochondria in culture cells or in tissues remain in the orthodox configuration since the aldehyde fixatives used for electron microscopy do not contain sucrose. It is easy to detect mitochondria which are swollen distinctly by electron microscopy since the mitochondrial matrix become distinctly pale and the cristae are pushed to the periphery in addition to increases in their sizes. However, small changes in the size of mitochondria are often overlooked and mitochondria which are only swollen slightly are misinterpreted as "intact". Since the routine electron microscopy using thin-sectioned specimens gives us information about the structure of mitochondria on one plane of section, even a subtle change in the size of mitochondria which are undetectable or difficult to be detected on one plane of section may indicate significant changes on the basis of their three dimensions. Thus, one of the reasons why mitochondrial intactness during apoptosis had been emphasised for more than 20 years [5,10,15,19,34,43,48,82] before the proposal of Petit et al. [57] and Skulachev [66] stressing the importance of the swelling of mitochondria may be partly due to a misinterpretation of mitochondrial structures observed on electron micrographs. This was inevitable since only routine light and electron microscopic techniques were available during that period to detect structural changes of mitochondria in situ. Recently, Jurgensmeier et al. [31] have shown that Bax, a proapoptotic member of the Bcl-2 family, induces cytochrome c release without causing the swelling of mitochondria. They judged the changes in the volume of mitochondria using a spectrophotometer.
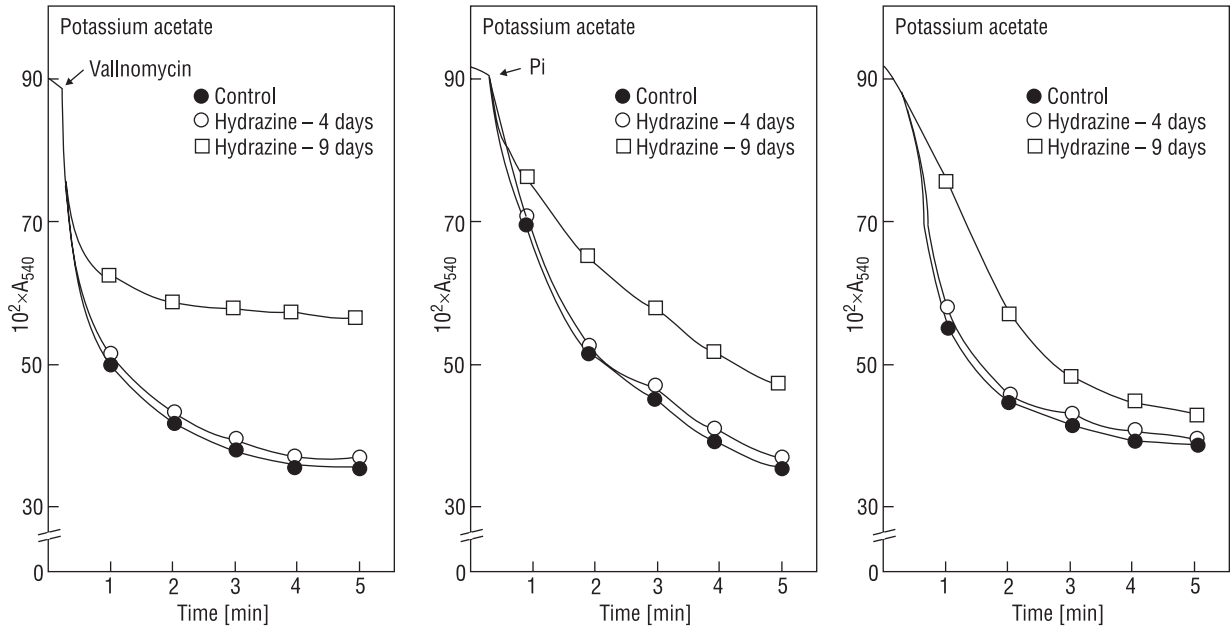
Figure 1. Detection of the swelling of mitochondria by a spectrophotometer. As a result of the opening of the megachannels. Mitochondria isolated from the livers of control rats, those of animals fed with a hydrazine-diet for 4 d or 9 d were exposed to various swelling media: potassium acetate (100 mM K-acetate, 0.1 mM EDTA, 15 mM Tris-HCl, pH 7.4, valinomycin, 0.5 mg/ml); ammonium succinate (100 mM ammonium succinate, 0.1 mM EDTA, 15 mM Tris-HCl, pH 7.4, 5 mM Pi); sodium perchlorate (500 mM sodium perchlorate), 0.1 mM EDTA, 15 mM Tris-HCl, pH 7.4, antimycin A, 0.5 mg/ml). Mitochondria obtained from control animals and those fed with a hydrazine-diet for 4 d swell distinctly whereas those with the toxic diet for 9 d swell less distinctly indicating that the latter mitochondria are swollen already before they are exposed to swelling media.
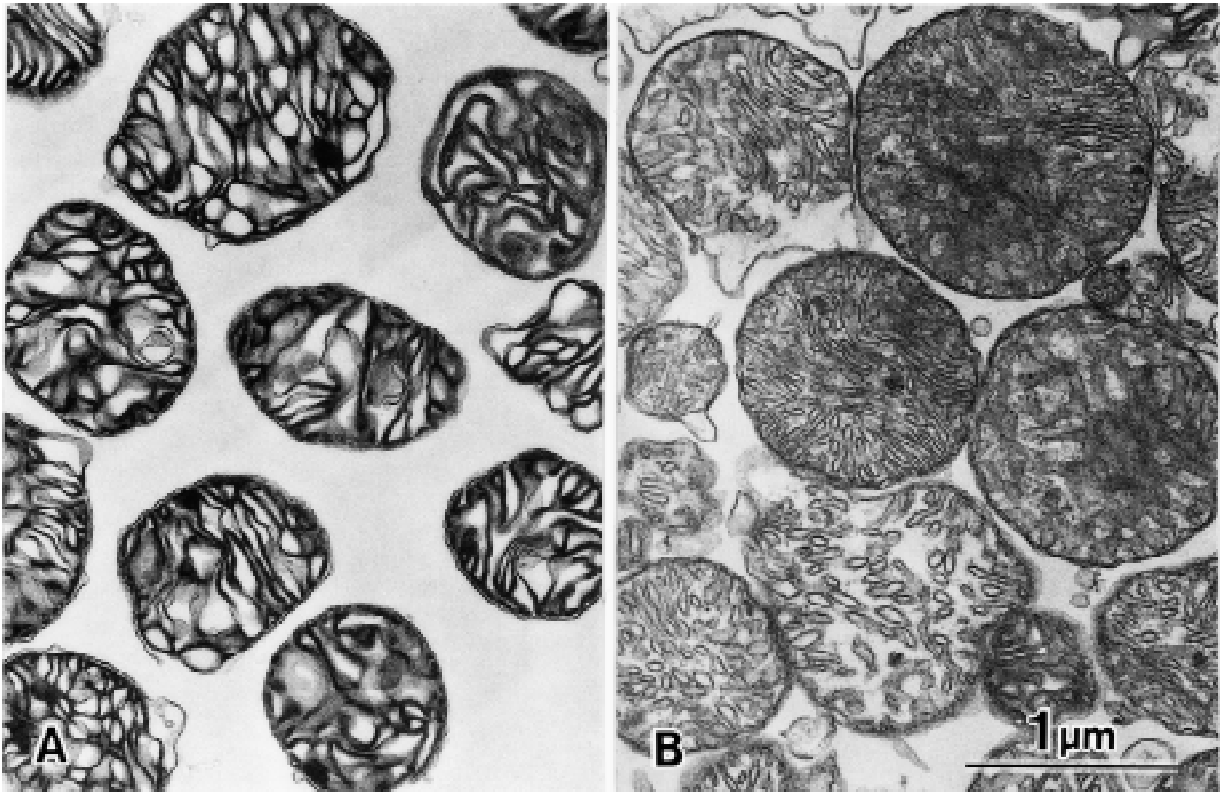
Figure 2. Transition from the condensed to orthodox mitochondria induced by Ca.
Beef heart mitochondria isolated in the condensed configuration (A) were treated with 10 mM Ca for 30 min at 30ºC (B).

63

## Structural changes of mitochondria during the phase of "secondary necrosis"

In vivo, apoptotic cells and apoptotic bodies are phagocytosed by mononuclear phagocytes or surrounding epithelial cells while culture cells undergoing apoptosis detach early on from the monolayer and float in the culture medium. These cells escape phagocytosis by neighbouring adherent cells and proceed to undergo spontaneous degenerative changes that result in the loss of cell membrane integrity and swelling of intracellular organelles including mitochondria. This process is called "secondary necrosis" [16,34,41,60,61,82]. Cells undergoing "secondary necrosis" as demonstrated in the literature have condensed nuclei as evidence of apoptosis whereas they are swollen extremely with the rupture of the cell membrane and with remarkable swelling of organelles including mitochondria. The cell demonstrated by Camilleri-Broet et al. [13] as an example of secondary necrosis exhibits a prominent mitochondrial swelling as the authors pointed out whereas it shows a typical feature of apoptosis with fragmented nuclei and the aggregation of chromatin. Swelling of mitochondria in this case is interpreted as a result of secondary necrosis [13]. However, we should not misinterpret these swollen mitochondria as a sign of secondary necrosis and the swelling of mitochondria in this case should be regarded as a morphological change of mitochondria intrinsic to apoptosis in advanced stage as long as the cell shows a characteristic feature of apoptosis. The cell may or may not undergo secondary necrosis with time.

Application of fluorescent dyes to the analysis of structural and functional changes of mitochondria in apoptosis

Disadvantages associated with spectrophotometric and electron microscopic techniques have been largely overcame by the application of various fluorescent dyes combined with flow cytometry to the research field of apoptosis. For example, using culture cells structural changes of mitochondria in the cell can be detected quantitatively and to some extent qualitatively using Mito Tracker CMXRos [58] or Mito Tracker CMTMRos [58] for confocal microscopy (Fig. 3A) combined with flow cytometry (Fig. 3B). Using either dye in the presence and absence of uncouplers of oxidative phosphorylation of mitochondria or ionophores, the $\Delta\Psi$m and volume changes of mitochondria per cell can be detected. In Fig. 3B, RL-34 cells were cultured for 48 h in the presence and absence of chloramphenicol (CP) and stained with CMTMRos with or without pretreatment with an ionophore, gramicidin. In control cells, the peak shifts to the left distinctly when they are pretreated with gramicidin (dotted peak), indicating that $\Delta\Psi$m measured in the absence of gramicidin is influenced by volume changes of mitochondria. Thus, actual $\Delta\Psi$m is obtained by calculating the difference between the mean fluorescence intensity of the dye measured in the absence and presence of gramicidin. However, the peak is much broader than that of the control overlapping partly the peak obtained in the absence of gramicidin. This indicates that $\Delta\Psi$m is decreased in a large population of CP-treated cells and that the total volume of mitochondria per cell is distinctly increased. When the total volume of mitochondria per cell is increased, there are two possibilities: this is either due to the swelling of mitochondria or due to the proliferation of the organelles (increases in the mass of mitochondria). Increases in the mass of mitochondria per cell are detected by the fluorescent dye Mito Tracker Green FM (FM) [75] or nonyl acridine orange (NAO) [42,75]. For example, the peak intensity of FM in the cells treated with CP for 22 h shifts to the right compared to that of those treated with CP for 12 h and it shifts further to the right when the CP treatment is prolonged to 36 h (Fig. 4A). In Fig. 4B, mitochondria isolated from control rats (A3) and those placed on a hydrazine-diet for 3 d (B3) and 7 d (C3), respectively, were applied to flow cytometry. Mitochondria in the liver of animals treated with hydrazine for 3 d (B1) were slightly enlarged compared to those of the control (A1). However, the peak intensity of FM in mitochondria isolated from the former animals remained essentially in the same position as that of control animals. Mitochondria became extremely enlarged in the liver of animals treated with hydrazine for 7 d (C1), and the peak intensity of FM of mitochondria isolated from these animals was distinctly broadened. When both the mass and volume of mitochondria per cell are increased we may conclude that the number of mitochondria per cell has increased with or without the swelling of the organelles.

The rate of the generation of reactive oxygen species (ROS) from mitochondria can be measured with H_2DCFDA [3,20,38] or carboxy-H_2DCFDA [33] using culture cells or isolated mitochondria. Superoxide can be specifically detected by dihydroethidium [44]. Overall intracellular level of ROS (mainly generated from mitochondria) and extra-mitochondrial generations of ROS in the cell can be detected
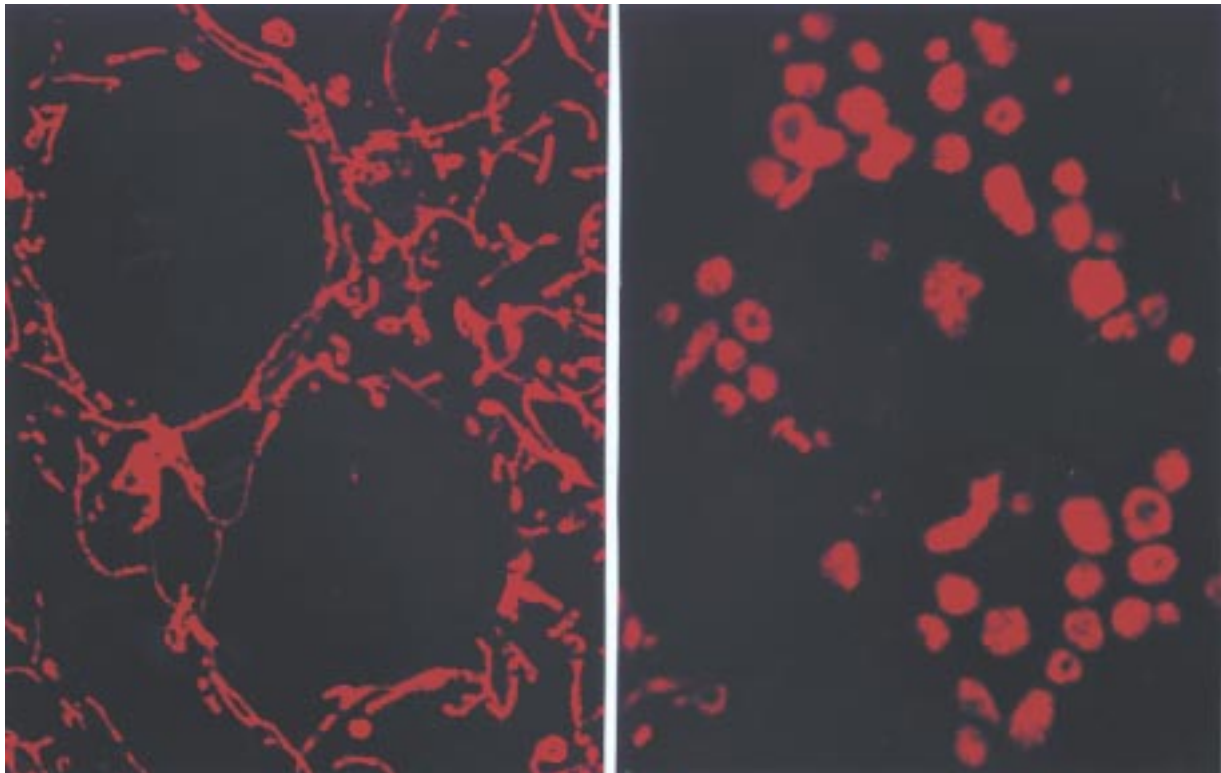
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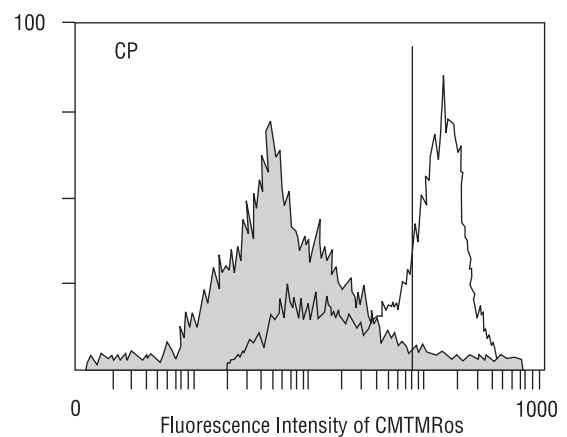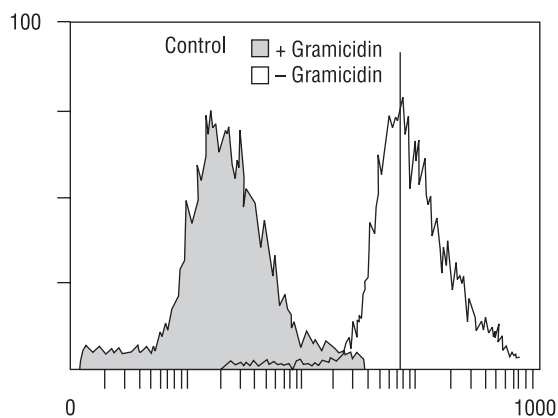
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Figure 3. Visualisation of mitochondria and detection of their ∆Ψm and volume changes by fluorescent dyes.
A) Visualisation of mitochondria by CMXRos. Cells cultured for 22 h in the absence (A) and presence of chloramphenicol (CP) (200 mg/ml) (B) were stained with CMXRos for confocal laser microscopy. B) Detection of the ∆Ψm and volume of mitochondria per cell by CMTMRos. RL-34 cells were cultured for 48 h in the presence and absence of CP and stained with CMTMRos in the presence and absence of gramicidin. (B was modified from ref. 33).

using carboxy-H$_2$DCFDA and CM-H$_2$TMRos, respectively (Fig. 5) [33]. In control cells cultured for 48 h, the intracellular level of ROS is slightly increased compared to that cultured for 22 h whereas that of the cells cultured for 48 h in the presence of CP is decreased compared to that cultured for 22 h in the presence of CP (A). Similar tendencies are obtained using CM-H$_2$TMRos both in control and CP-treated cells (B). Since CM-H$_2$TMRos does not react with mitochondria unless it is oxidized (CMTMRos), these results indicate extra-mitochondrial generation of ROS. The rate of the generation of ROS from mito-
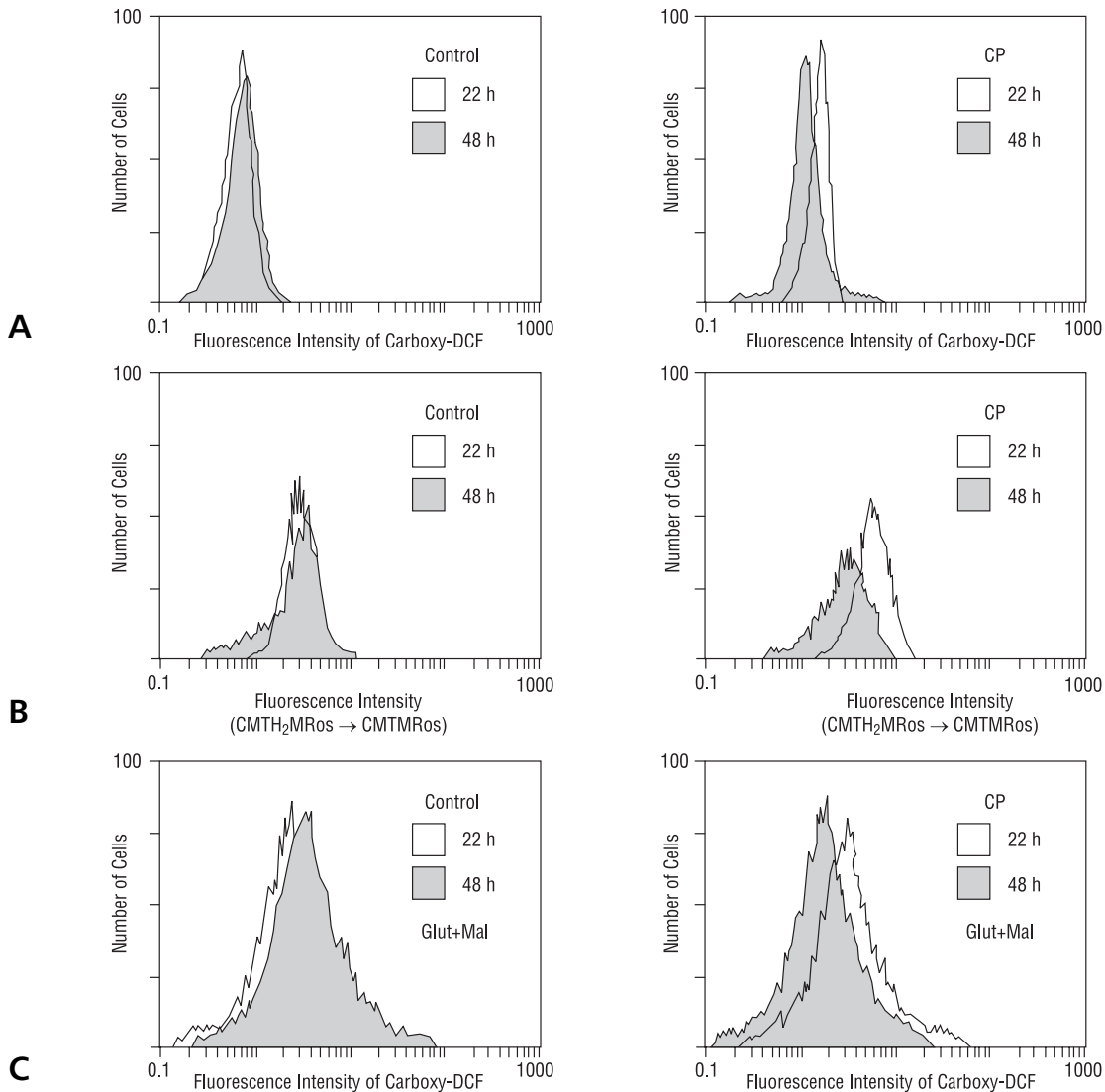
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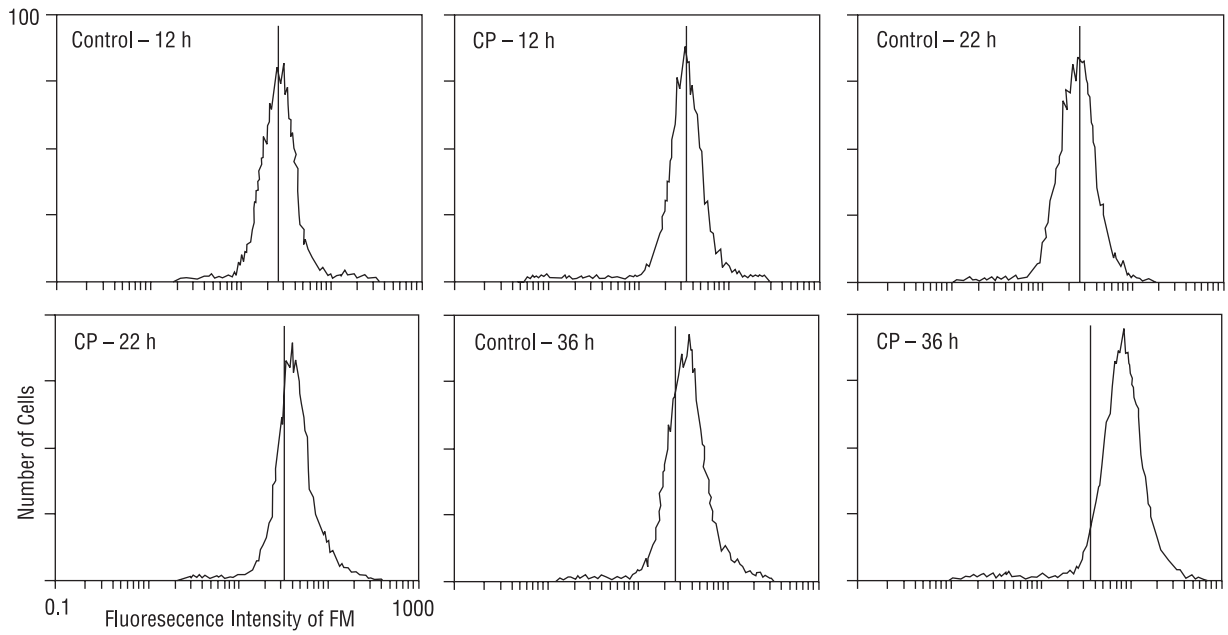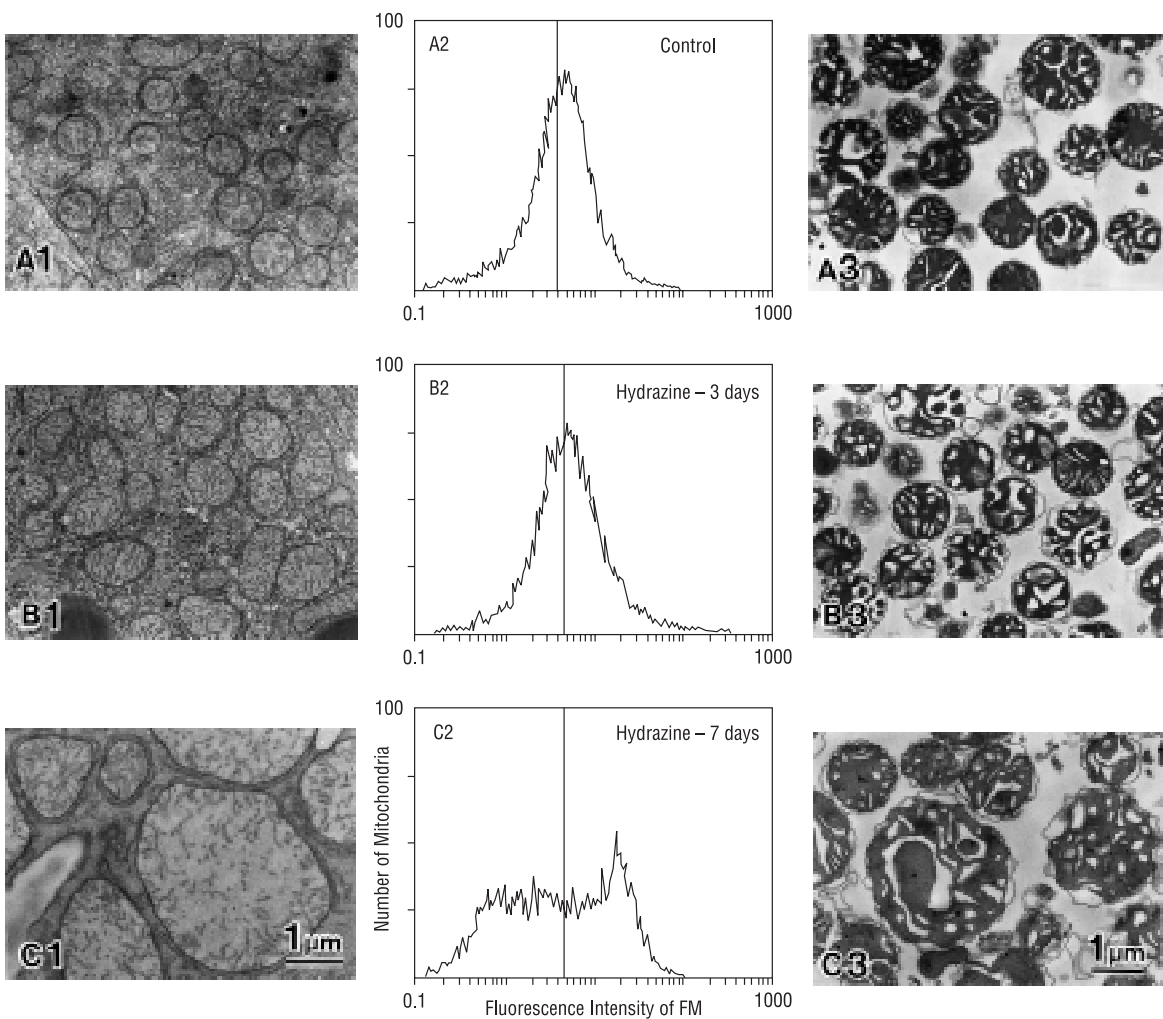
Figure 4. Detection of the total mass of mitochondria per cell.
A) RL-34 cells were cultured for 12 h, 22 h, and 36 h, respectively, in the presence of CP, and then stained with FM for flow cytometry (modified from ref. 32). B) Mitochondria isolated from the liver of control rats (A3), those treated with hydrazine for 3 d (B3) or 7 d (C3) were stained with FM.

Figure 5. Detection of overall intracellular level of ROS and the rate of mitochondrial and extra-mitochondrial generation of ROS. A) RL-34 cell cultured for 22 h and 48 h, respectively, in the presence and absence of CP were stained with carboxy-H$_2$DCFDA to detect intracellular level of ROS. B) Experimental conditions are the same as those shown in Fig. 5A except that cells were stained with CM-H$_2$TMRos to detect extra-mitochondrial generation of ROS; C) Mitochondria isolated from the cells used in Fig. 5A were stained with carboxy-H2-DCFDA to detect ROS (modified from ref. 32).

chondria isolated from the control and CP-treated cells (C) show similar results to those obtained using cells thus suggesting a possibility that increases in the overall intracellular level of ROS observed in the cells treated with CP for 48 h are partly due to decreases in the mitochondrial generation of ROS.

Free radical-induced formation of megamitochondria is succeeded by apoptosis

Recently, we have shown using culture cells of various sources of tissues that free radical-induced formation of megamitochondria (MG) is followed by apoptotic changes of the cell, and that the latter

has been successfully suppressed by scavengers of free radicals such as 4-OH-TEMPO (Figs. 6 and 7) [32,33]. For example, mitochondria in IAR-20 cells cultured for 22 h in the presence of CP become enlarged distinctly (Fig. 6B) compared to those of the control (Fig. 6A). When the cultivation time of these cells is prolonged to 48 h, the matrix of enlarged mitochondria becomes pale suggesting the swelling (Fig. 6C), and apoptotic cells characterised by the condensation of nuclear chromatin are detected (Fig. 6E). Appearances of apoptotic cells are not accidental since the population of apoptotic cells reaches about 45% in cells treated with CP for 72 h (Fig. 6F). The formation of MG and succeeding apoptotic chang-
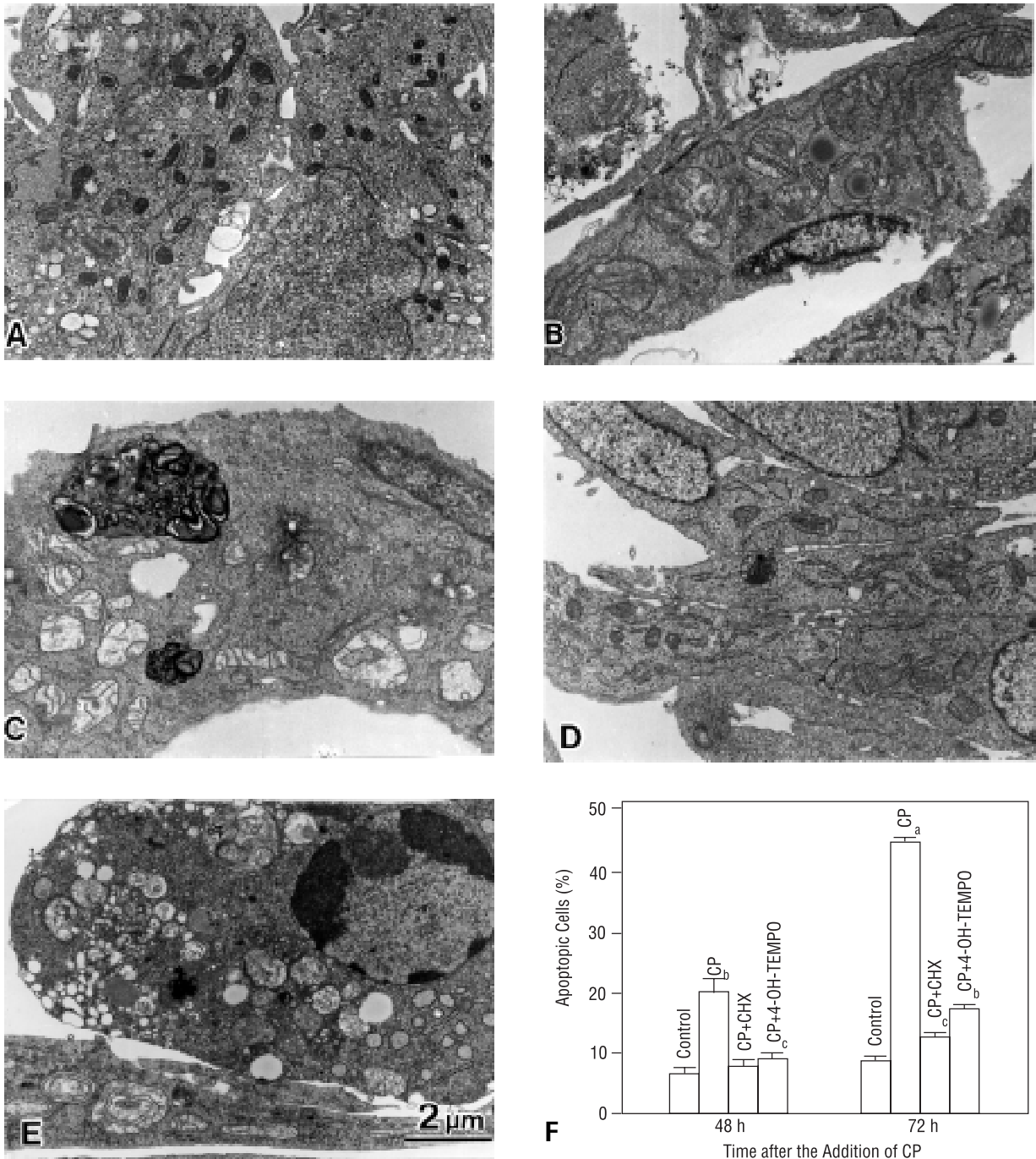
Figure 6. Induction of megamitochondria and succeeding apoptosis by CP.
A–E) IAR-20 cells were cultured in the absence (A) or presence of CP for 22 h (B), 48 h (C and D) or 72 h (E). Cells in Fig. 6D were pre-treated with 4-OH-TEMPO. F. RL-34 cells were cultured for 48 h and 72 h, respectively, in the presence of CP with or without the pretreatment with 4-OH-TEMPO or cycloheximide (CHX). Numbers of apoptotic cells were obtained by flow cytometric analysis of propidium iodide-stained cells. Presence of sub-G1cell population was used as an indicator of apoptosis. Data are the averages and standard error (mean±SE) of three different experiments. Values of experimental groups are statistically different from those of the control at: a ($0.001 < p < 0.01$); b ($0.01 < p < 0.02$), ($0.02 < p < 0.05$). (F, cited from ref. 33).

es of the cell are suppressed by 4-OH-TEMPO or cycloheximide. Similarly, mitochondria in the hepatocytes isolated from rat livers become distinctly enlarged when they are cultured for 24 h in the presence of hydrazine (Fig. 7B) compared to those of the control (Fig. 7A). Cultivation of these cells for 48 h causes a swelling of MG (Fig. 7C). Pretreatment of cells with 4-OH-TEMPO completely suppresses the formation of MG (Fig. 7D). We have also established the criteria for MG since there exists confusion in
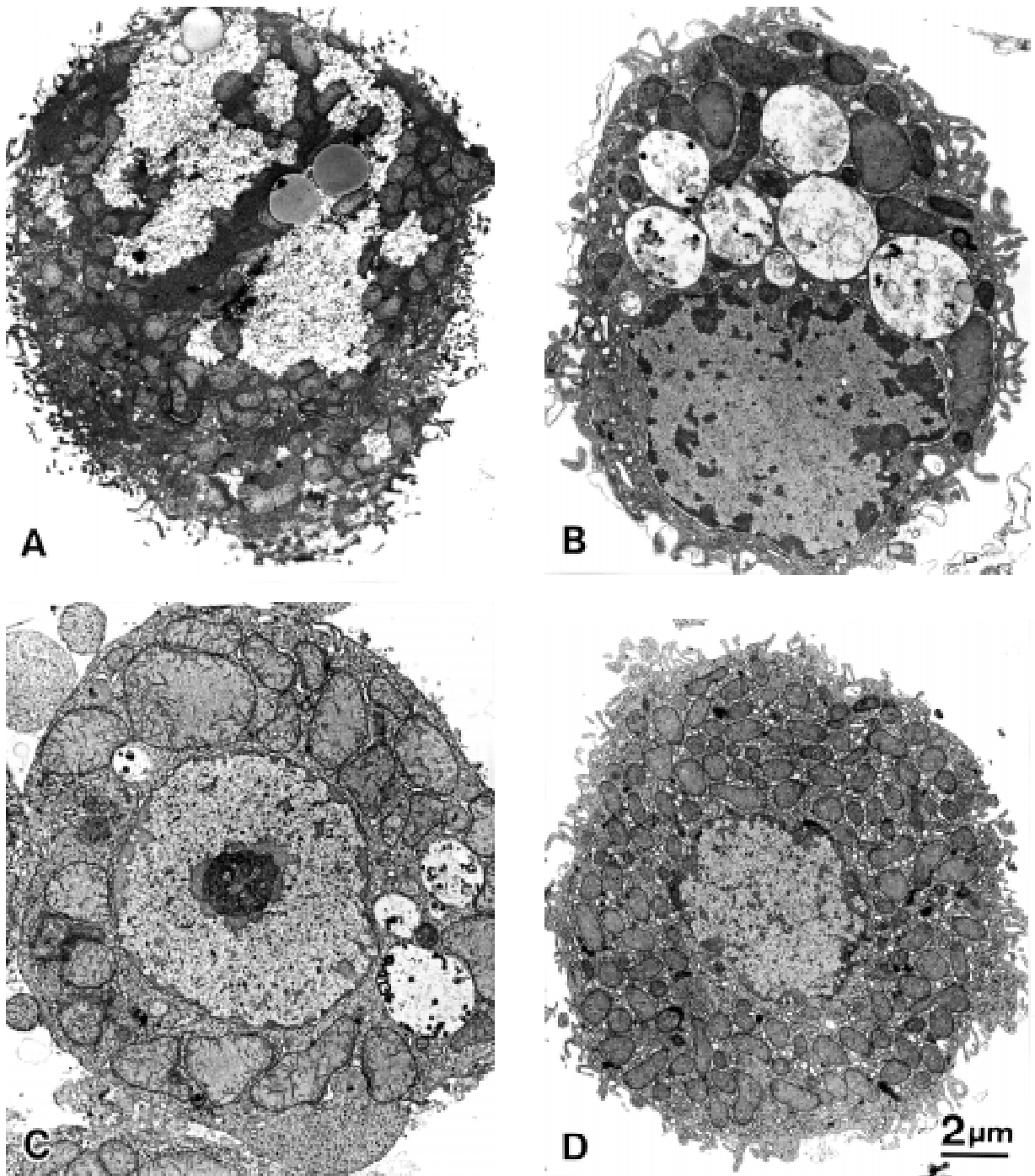
Figure 7. Induction of MG by hydrazine in rat hepatocytes.
Hepatocytes isolated from the liver of rats were cultured in the absence (A) or presence of hydrazine for 24 h (B) or 48 h (C, D) in the presence of 4-OH-TEMPO (D).

the literature concerning the description of structural changes of mitochondria during apoptosis [74]. When mitochondria are exposed to hypotonic media, they increase their sizes to at most 2–3 times larger than control mitochondria, and the outer mitochondrial membrane becomes ruptured beyond that point [74]. Thus, mitochondria enlarged more

than three times compared to the average sizes of control mitochondria should be designated as "MG". Survey of the literature describing structural changes of mitochondria during apoptosis discloses inappropriate descriptions in some cases. For example, Heiden et al. [78] have demonstrated an electron micrograph of mitochondria during apoptosis, which

they have described as "swollen mitochondria". Similarly, mitochondria enlarged extremely were described as "swollen mitochondria" in several cases [49,54]. However, these mitochondria should be designated as "MG" since the sizes of mitochondria exceed simple swelling.

Mitochondria consume more than 90% of oxygen taken up by the cell, and generate reactive oxygen species (ROS) via complex I, II and III linked to ATP synthesis [9,12,77]. Complex IV containing cytochrome $a+a_3$ (cytochrome oxidase), on the other hand, has superoxide dismutase activity [47]. When MG are newly formed by free radicals, the membrane potential of mitochondria ($\Delta\Psi$m) remain intact and the rate of the generation of ROS from MG is enhanced [33]. However, a prolonged treatment of cells with free radicals causes a swelling of MG resulting in decreases in ROS generation as well as further decreases in the content of cytochrome c besides that of cytochrome $a+a_3$ [74]. Typical example is obtained from hydrazine-induced MG (Fig. 8). The intracellular level of ROS in hepatocytes isolated from the liver of rats fed a hydrazine-diet for 4 d is slightly increased and becomes increased further in those isolated from the liver of animals treated with hydrazine for 9 d (A). The rate of the generation of ROS from mitochondria isolated from the liver of the former animals is increased whereas it becomes decreased in those of the latter animals (B). The $\Delta\Psi$m in the former animals is unchanged while it is distinctly decreased in the latter (C). We have interpreted these results as follows [74]: decreases in the content of cytochrome $a+a_3$ result in decreases in SOD activity of complex IV thus increase ROS generation from newly formed MG. Further exposure of MG to free radicals results in the opening of MGCs, namely the swelling of MG. Swelling MG in turn causes decreases in $\Delta\Psi$m and the content of cytochrome c. These changes may enhance the rate of the generation of free radicals from swollen MG. However, distinct decreases in the oxygen consumption by swollen MG result in decreases in ROS generation. Decreases in intracellular levels of ATP then lead to apoptotic changes of the cell.

Meaning of the MG formation induced under various pathological conditions has remained obscure for a long time. However, the results described above would strongly suggest the possibility that the
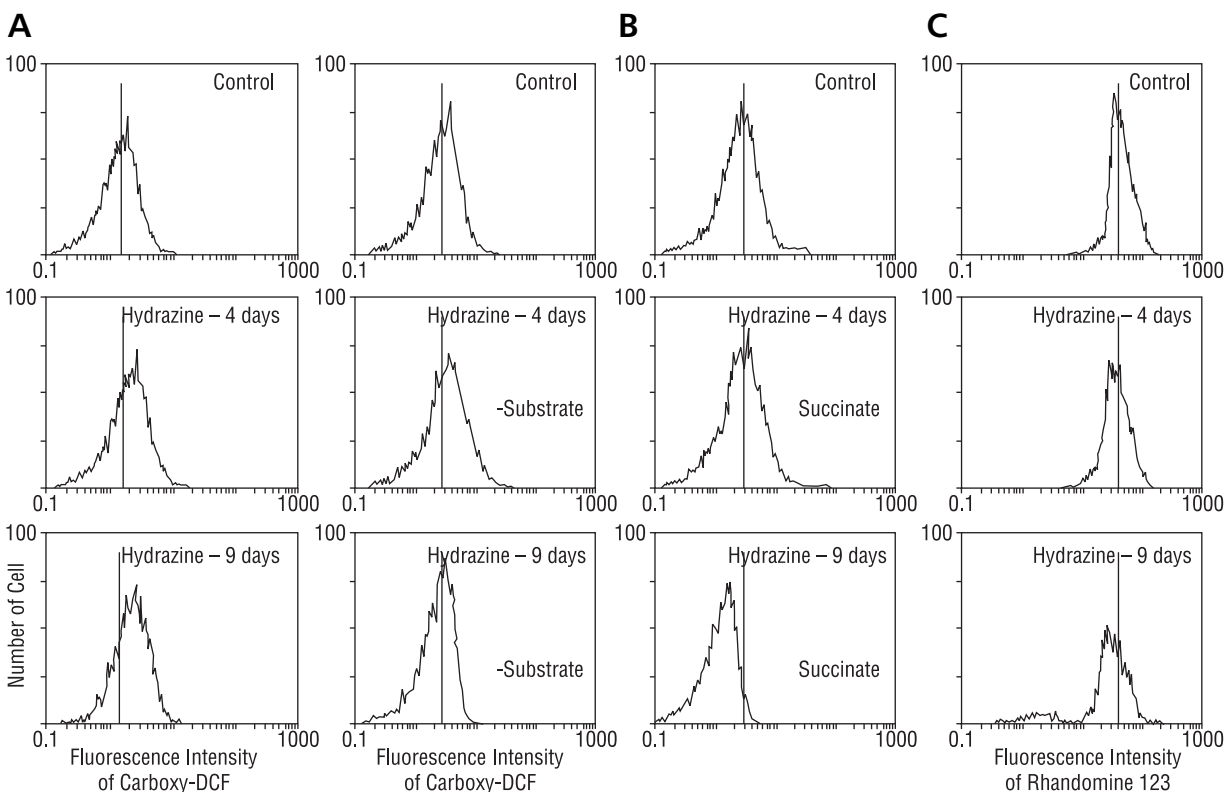
Figure 8. $\Delta\Psi$m and the rate of generation of ROS from "intact MG" and "swollen MG".
A) Hepatocytes were isolated from the liver of rats treated with hydrazine for 4 d when "intact MG" were induced similar to those shown in Fig. 6B, and 9 d when "swollen MG" were induced similar to those shown in Fig. 6D, respectively. They were stained with carboxy-H$_2$DCFDA for flow cytometry. B) Mitochondria were isolated from the livers of animals used in Fig. 8A, and stained with carboxy-H$_2$-DCFDA for flow cytometry. C) Hepatocytes used in Fig. 8A were stained with rhodamine 123 to detect $\Delta\Psi$m (modified from ref. 74).

MG formation is an adaptive process at an intracellular organelle level to unfavourable environments: if decreases in the rate of the generation of ROS from swollen MG are effective enough to lower intracellular level of ROS, swollen MG may return to normal both structurally and functionally and regain the ability to generate enough ATP to maintain various cellular activities. If not, the swelling of MG proceeds, intracellular levels of ATP decrease further, and cells become apoptotic.

### Problems to be solved concerning structural changes of mitochondria related to apoptosis

Data have accumulated that the size and distribution of mitochondria in the cell are controlled by a variety of genes detected mainly in yeast cells. Some of these genes exert their actions via the connection with certain components of cytoskeletons. *fzo* (*fuzzy onions*) in *Drosophila melanogaster* encodes a transmembrane GTPase that becomes detectable on spermatid mitochondria late in meiosis II and disappears soon after the fusion is complete ("Nebenkern" formation) [25]. Fzo1p is a mitochondrial integral membrane protein with GTPase domain localized to inner-outer membrane contact sites [25]. In yeast cells, Fzo 1p is required to maintain a tubular mitochondrial reticulum during mitotic growth [27]. This is the only gene at the moment which is capable of inducing MG under physiological conditions. On the other hand, dynamin-related GTPase, Dnm1p (yeast) is required to maintain the tubular network of mitochondria. Drp1 is a human dynamin-related protein which is essential for the tubular distribution of mitochondria in the cell [67]. *mdm 10* detected both in yeast cells and *Podospora arseria* [7,29,68] and *mmm 1* detected in yeast cells [4] are known to regulate the size of mitochondria. In both cases, MG are formed in mutants. Interestingly, it has been found that mutants lacking both *dnm 1* and *fzo 1* possess a normal mitochondrial network [62]. This result suggests as the authors have pointed out that there are balances between the fusion and fission of mitochondria controlled by these genes. Whether or not this is also the case with mammalian cells remains to be shown.

It is also well known that distribution of mitochondria in the cell is intimately related to cytoskeletons. For example, mitochondria in yeast cells are combined with actin microfilaments with actin-binding proteins (ABP) and move in the cell using these filaments as railroad tracks [7,11]. In mammalian cells, the association of mitochondria with microtubules has been emphasised [2,24,26,39,50,59]. For example, mouse kinesin superfamily KIF1B works as a motor for the transport of mitochondria along microtubules [50]. It should be stressed here that modifications of cytoskeletons result in perinuclear clustering of mitochondria [17,26]. In mutants defective of some genes, mitochondria aggregate around the nucleus: *mgm* [22,30,63]; *drp 1* [77]; *kif 1B* [50]; *kif 5B* [73]; *klp 67A* [55]; *clue A* [85]. Recently, we have found that microtubule-active drugs (MADs) such as taxol and nocodazole cause clustering of mitochondria around the nucleus (Fig. 9). Since these MADs arrest cells in the G_2/M phase of the cell cycle, one possibility is that mitochondria cluster around the nucleus just prior to the nuclear division. Mitochondrial clusterings around the nucleus induced by MADs are always highly condensed as shown in Fig. 9 whereas those in control cells are in the orthodox configuration (Wakabayashi, unpublished observation). This may suggest that these cells should not be related simply to cell cycle but that they are on the way to apoptosis.

CONCLUDING REMARKS

We have discussed structural changes of mitochondria during apoptosis focussing on "the swelling of mitochondria". We have also demonstrated data to indicate that the formation of MG is an early sign of apoptosis. We emphasise that structural changes of mitochondria during apoptosis should be analysed using various techniques to obtain correct information. Since the proposal of Petit et al. [57] for the apoptotic processes of the cell stressing the swelling of mitochondria, several arguments have been raised concerning the mechanism of the release of cytochrome *c* from mitochondria: Bcl-2 was thought to block apoptosis by preventing decreases in $\Delta\Psi$m thus preventing the release of cytochrome *c* from mitochondria [46,53,56,84]. However, it has been shown that cytochrome *c* release is unaccompanied by changes in $\Delta\Psi$m [8,18,36]. Decreases in $\Delta\Psi$m is preceded by the release of cytochrome *c* from mitochondria into the cytosol [83]. Changes in the configuration of cytochrome *c* instead of its release from mitochondria was stressed by others [79]. The role of free radicals as a triggering factor for apoptosis was questioned [28,64,64,65]: apoptosis was observed in the cells deprived of oxygen and Bcl-2 or Bcl-xL prevented apoptotic changes of the cells [65]. This may suggest that ROS is not always a triggering factor for apoptosis and that Bcl-2 and Bcl-XL exert

71
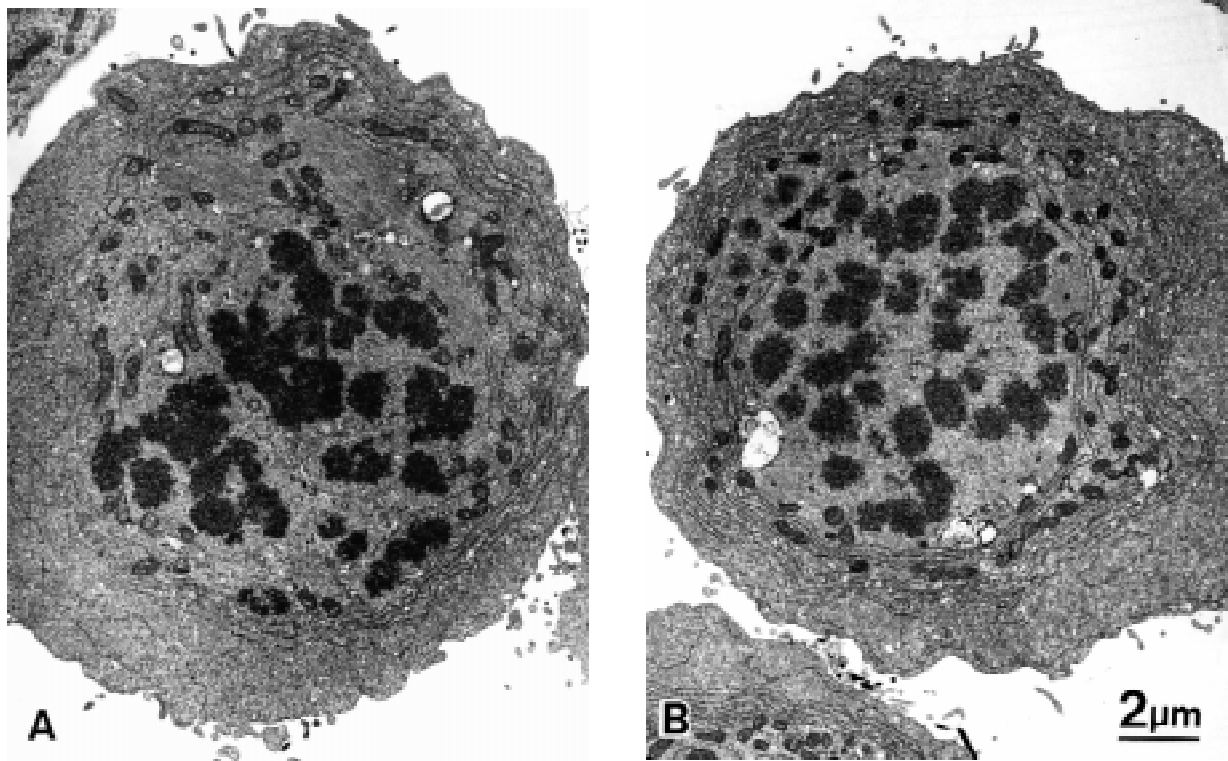
Figure 9. Perinuclear clustering of mitochondria induced by microtubule-active drugs. Human osteosarcoma-derived 143B cell were treated with taxol (A) or nocodazole (B).

an anti-apoptotic function by a mechanism other than regulation of ROS activity in some cases. Furthermore, the swelling of mitochondria was questioned as described above. We are still in the midst of *chaos* as far as the research field of apoptosis is concerned, and further studies are definitely required.

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73

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