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Review

Structural changes of mitochondria related to apoptosis: Swelling and megamitochondria formation[✪]

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Recently we have found that the formation of megamitochondria in culture cells of various sources, induced by chemicals capable of generating free radicals, is followed by apoptotic changes of the cell. Detailed analysis on functional and morphological aspects of megamitochondria has enabled us to speculate that the formation of megamitochondria may be a prerequisite for free radical-mediated apoptosis: free radicals modify the mitochondrial membranes resulting in the fusion of adjacent mitochondria (megamitochondria formation). If the intracellular level of free radicals is continuously kept high, the permeability transition pores of the megamitochondria membranes are opened and megamitochondria become swollen. Oxygen consumption and the ability to synthesise ATP by swollen megamitochondria decrease distinctly. At the same time, cytochrome *c* is released from swollen megamitochondria into the cytoplasm. If lowered rates of the generation of reactive oxygen species from swollen megamitochondria, possibly due to decrease in their oxygen consumption, are effective enough to lower the intracellular level of free radicals, megamitochondria may return to normal. If not, decrease in the membrane potential of megamitochondria membranes causes the release of apoptosis-inducing factor into the cytoplasm. Cytochrome *c* and apoptosis-inducing factor thus released into the cytoplasm may cause cytoplasmic and nuclear apoptotic changes. Experimental data to support this hypothesis are presented.

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Abbreviations: Drp, human dynamin-related protein; 4-OH-TEMPO, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; MG, megamitochondria; PT, permeability transition; ROS, reactive oxygen species.

Apoptosis (programmed cell death) plays a crucial role in normal development and differentiation of multicellular organisms and is essential for embryogenesis and metamorphosis. Apoptosis is also induced by various pathologic conditions and a variety of agents such as serum deprivation, ionizing radiation, oncogene products, reactive oxygen species (ROS) or ROS-producing toxic substances [1-7]. Data have accumulated to demonstrate that mitochondria are intimately related to the initiation and progression of apoptotic processes: apoptosis is regulated by a number of genes, in particular those of *Bcl-2* family, originally identified with follicular B-cell lymphoma [8]. Bcl-2 is an integral protein located mainly in the outer membrane of mitochondria [9-10]. The detailed mechanism by which Bcl-2 regulates apoptosis remains to be solved. However, the localization of Bcl-2 in the outer mitochondrial membranes raises a possibility that its function may be related to the functions of mitochondria which have been implicated in apoptosis [11-13].

Cytochrome *c*, electrostatically bound to the intermembrane space side of the inner membrane of mitochondria, is released from mitochondria into the cytoplasm and can trigger the activation of CED-3 family proteases and apoptotic effects in cell-free systems containing cytosol [14-15]. Bcl-2 was found to prevent the release of cytochrome *c* from mitochondria and thus caspase activation [15]. Recent works indicate that a crucial common step in the initiation of apoptosis is the opening of the mitochondrial "megachannels" or "permeability transition (PT) pores" [16-18]. Alterations in mitochondrial permeability transition (opening of PT pores) linked to disruption of the membrane potential is an early change of apoptosis preceding nuclear and plasma membrane changes [19]. Bcl-2 prevents the PT-mediated membrane potential collapse, both in cells and in isolated mitochondria [18]. Bcl-2 has antioxidant properties and inhibits apoptosis by suppressing the formation or effects of ROS [20, 21]. Thus, it seems now generally accepted that the opening of PT pores, notably by free radicals, is a key event for the apoptotic processes of the cell, resulting in the swelling of mitochondria followed by a series of events linked to this process.

STRUCTURAL CHANGES OF MITOCHONDRIA RELATED TO APOPTOSIS

Mitochondria undergo various structural changes concomitant with their functional changes in pathological conditions. These changes are classified into three categories: simple swelling, the formation of megamitochondria (MG), and the formation of paracrystalline or crystalline structures within either intracrystal space or matrix space. The former two categories are discussed here since they are related to apoptosis.

The swelling of mitochondria and the formation of MG should be precisely distinguished from each other, since the underlying mechanisms responsible for the two categories, described above, are completely different, and details in this point will be discussed later. When mitochondria reach or even exceed the size of nucleus, one may call them MG. On the other hand, when mitochondria become 5-6 times as large as the control, one may hesitate to call them MG and call them "swollen mitochondria" without knowing precisely to what extents mitochondria can be enlarged by the swelling since definite criteria for MG are not available in the literature. We can determine to what extent mitochondria are enlarged by a simple swelling by the following simple experiments: isolated rat hepatocytes or established rat liver cell line RL-34 at 30°C are incubated in a hypotonic medium for various lengths of time and fixed for electron microscopy (Fig. 1). After 10 min, mitochondria in the rat hepatocyte become round, losing the density of the matrix moderately (B) compared to those in the control cells (A). After

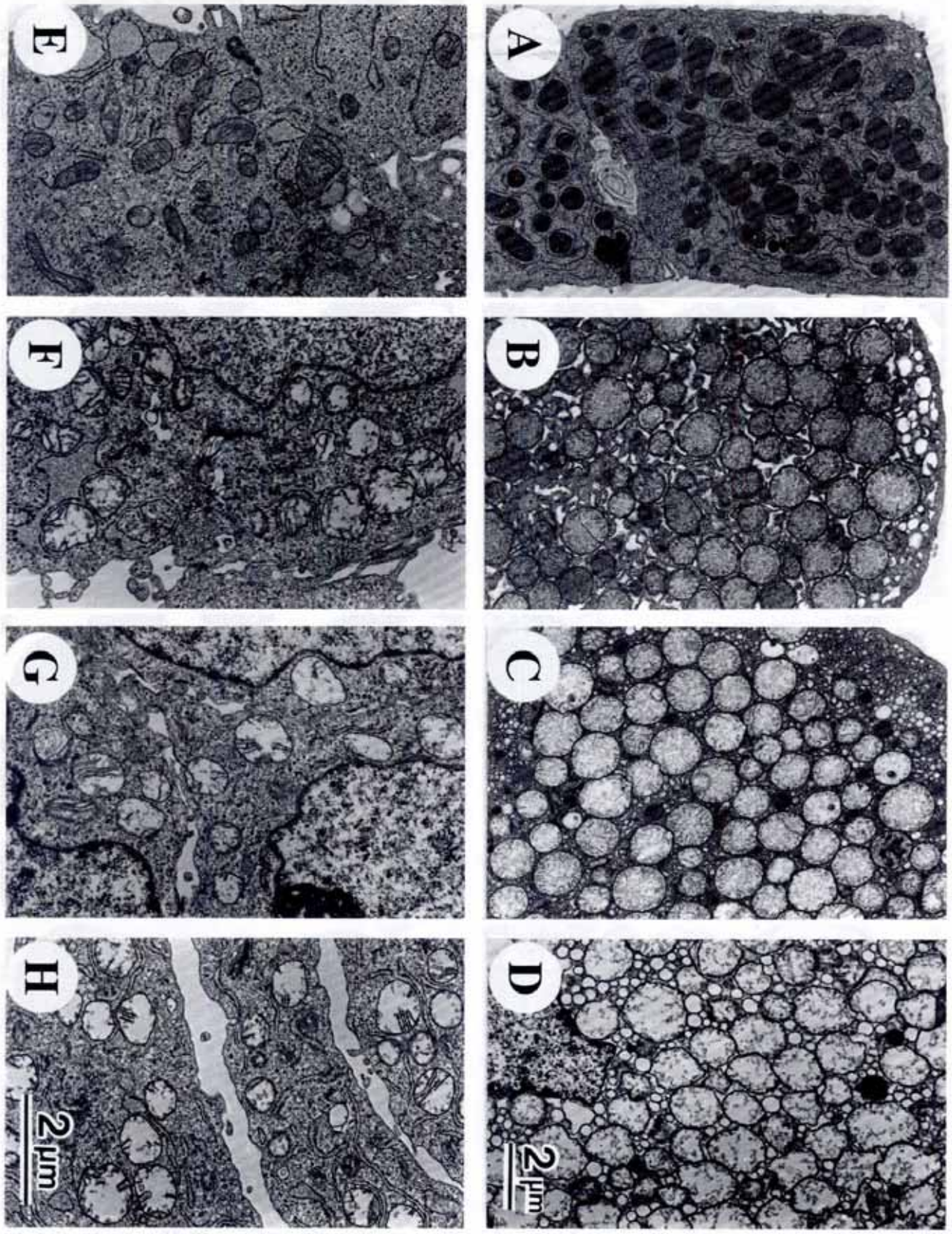

Figure 1. Ultrastructural appearances of isolated rat hepatocytes and RL-34 cells incubated with a hypotonic solution.

Rat hepatocytes (A–D) and RL-34 cells (E–H) were incubated in a medium containing 15 mM Na-acetate and 5 mM Tris/acetate, pH 7.4, at 30°C for 10 min (B, F), 20 min (C, G) and 30 min (D, H). Controls, (A) and (E).

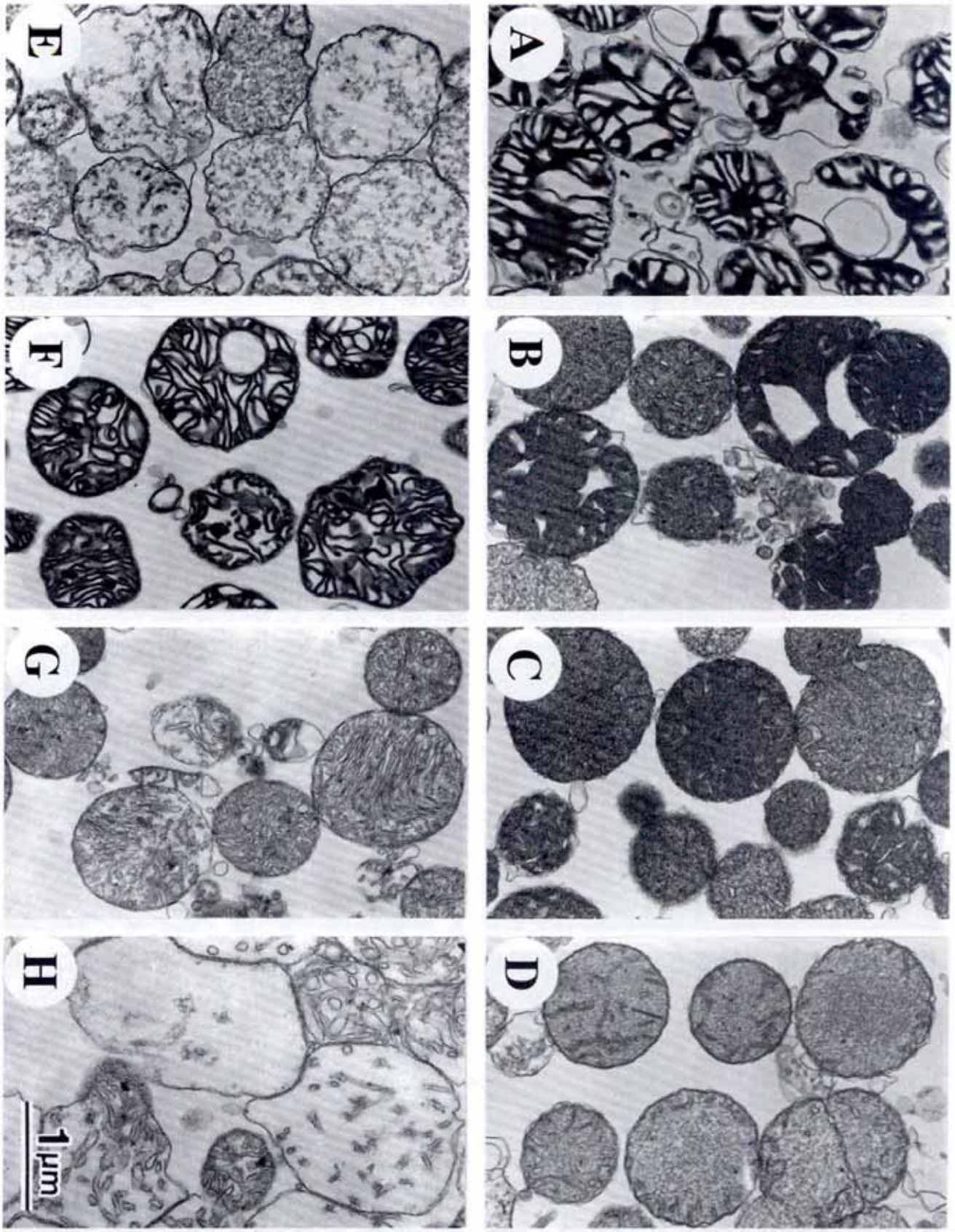

Figure 2. Ultrastructural appearances of isolated rat liver (A–E) and beef heart (F–H) mitochondria incubated with a hypotonic solution.

Mitochondria were incubated in the solution specified in the legend to Fig. 1 at 25°C for 15 s (B), 30 s (C), 2 min (D, G) and 5 min (E, H). Controls, (A) and (E).

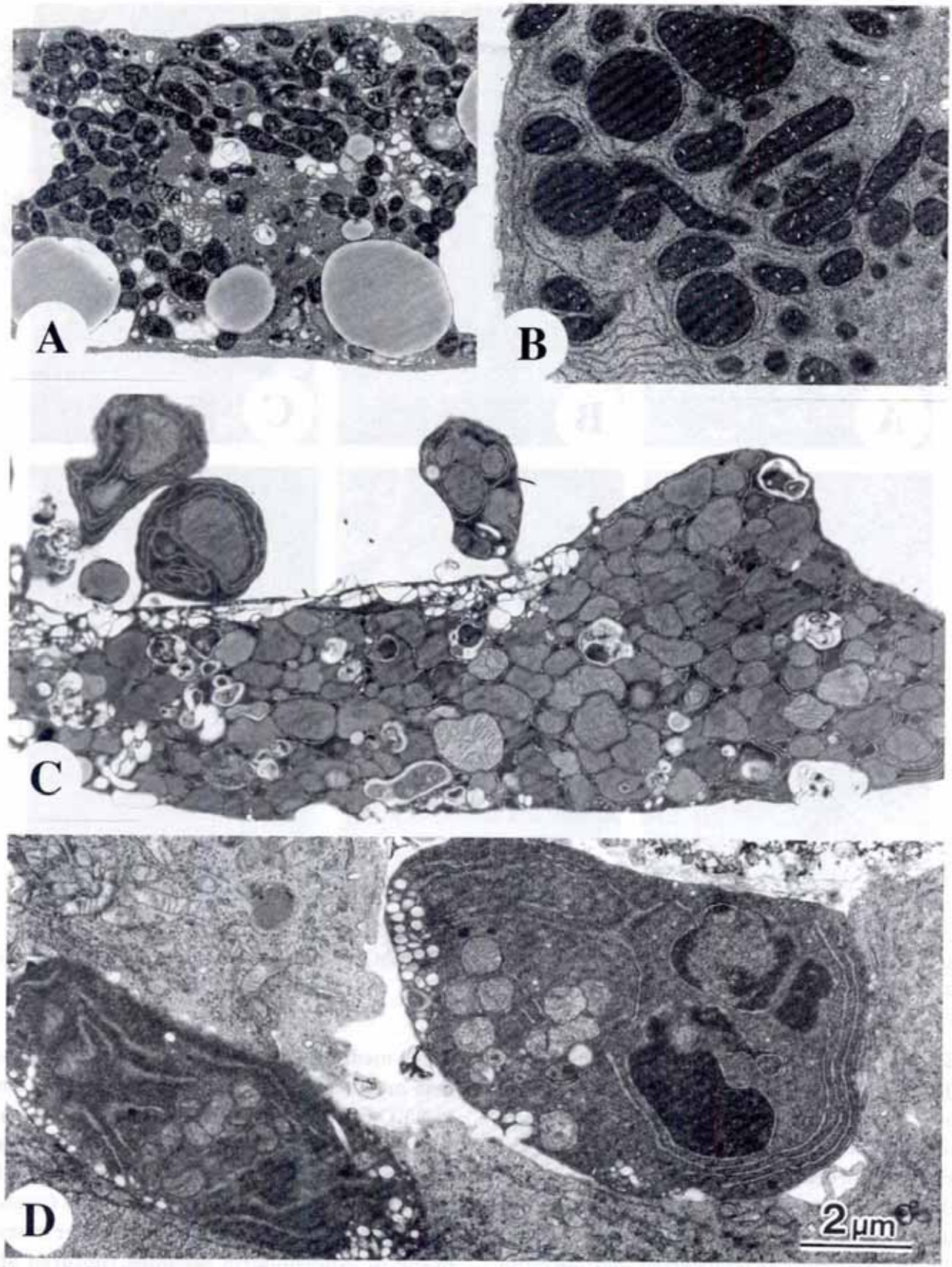

Figure 3. Hydrazine- and chloramphenicol-induced apoptotic changes of the cell.

Rat hepatocytes were cultured for 22 h (B) and 72 h (C) in the presence of 2 mM hydrazine. Control, (A). RL-34 cells (D) were culture for 72 h in the presence of chloramphenicol (300 μg/ml).

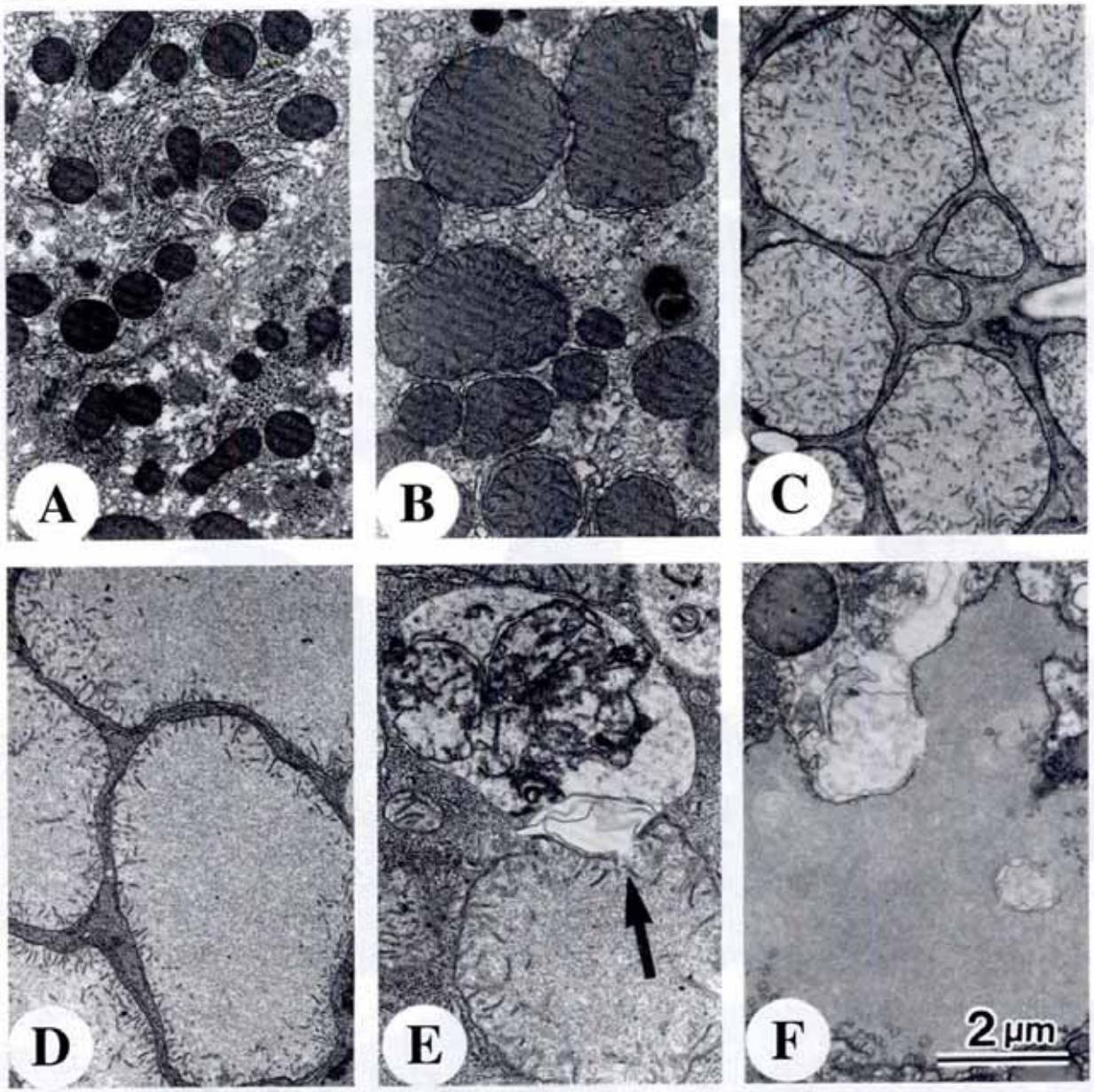

Figure 4. Hydrazine-induced formation of megamitochondria in rat liver.

Rats were placed on a diet containing 1% hydrazine for 4 days (B) and 8 days (C–F). Arrow in (E) indicates the rupture of the outer membrane of mitochondria. Control liver, (A).

20 min, the matrix of these swollen mitochondria becomes more pale, and the outer membrane of mitochondria becomes ruptured after 30 min (D). In the case of RL-34 cells the swelling process of mitochondria is faster than that of rat hepatocytes: after 10 min, mitochondria become swollen losing the matrix density distinctly (F) indicating that the outer membrane of mitochondria has been already ruptured. Similarly, isolated rat liver mitochondria (Fig. 2 A–E) or beef heart mitochondria (Fig. 2 F–H) become swollen in a hypotonic medium. When the former mitochondria are incubated with a hypotonic medium at 25°C for 60 s, the transition from the condensed to the orthodox configuration takes place indicating that the impermeability of the inner membranes to sucrose is lost (opening of the megachannels) (D). The outer membrane of mitochondria becomes ruptured after 2 min (E). Similarly, the outer membrane of beef heart mitochondria incubated for 5 min with a hypotonic medium is broken and the content of the matrix is lost almost com-

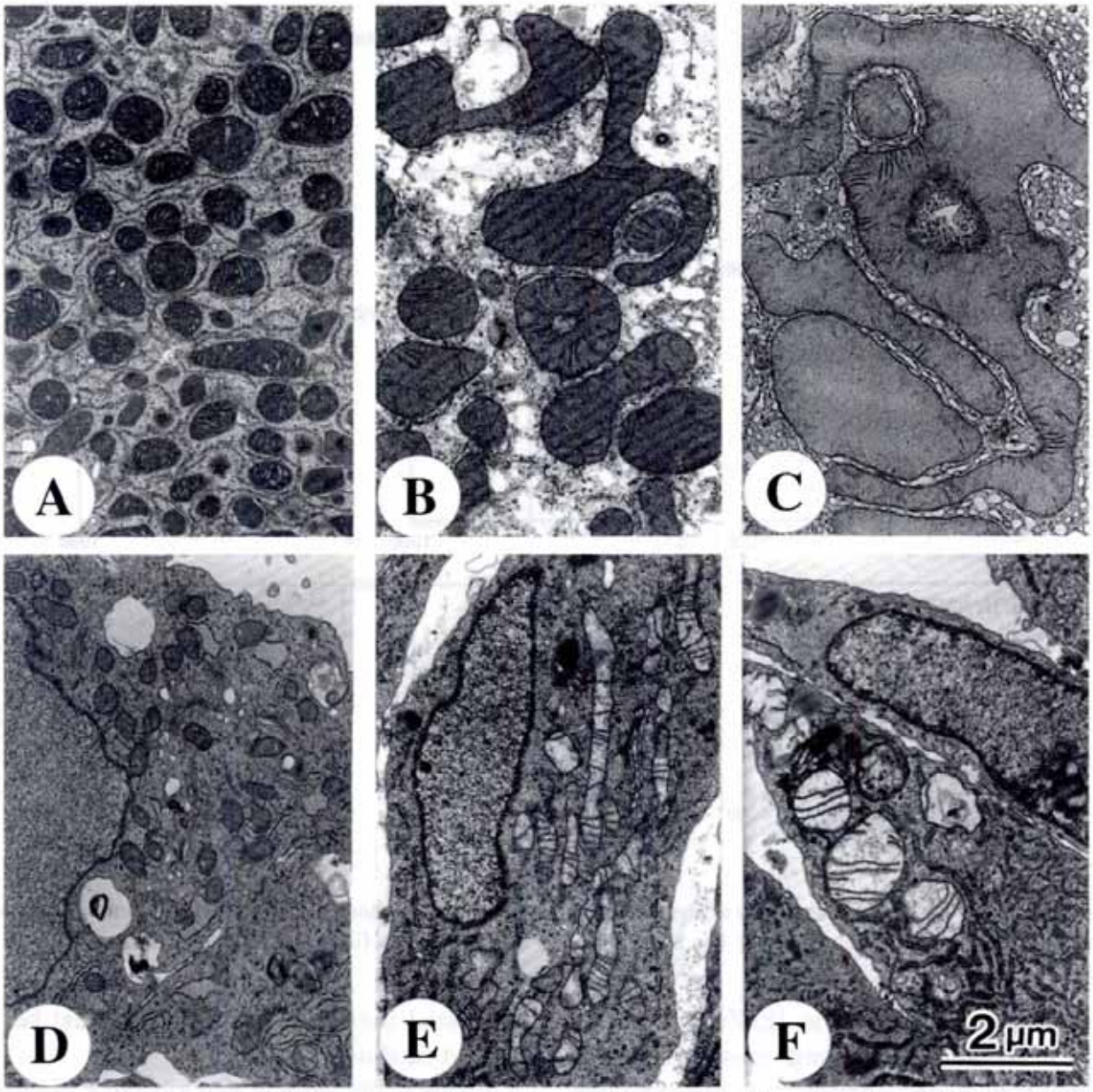

Figure 5. Chloramphenicol-induced formation of megamitochondria in mouse liver (A–C) and RL-34 cells (D–F).

Mice were fed a 2% chloramphenicol-containing diet for 10 days (B) and 15 days (C). RL-34 cells were cultured for 22 h (E) and 48 h (F) in the presence of chloramphenicol (300 μg/ml). Controls, (A) and (D).

pletely (H). It is obvious from these results that, due to the swelling, mitochondria become at most three times as large as their original size in a hypotonic medium.

MECHANISM OF THE MEGAMITOCHONDRIA FORMATION

Mitochondria become extremely enlarged in various human diseases including alcoholic intoxication and neuro-muscular diseases [22–26]. Megamitochondria are also induced experimentally using hydrazine, chloramphenicol and ethanol [27–31]. Proposed mechanisms for the formation of MG are [29]: (1) Suppression of the mitochondrial dividing process due to the inhibition of mitochondrial protein synthesis. Chloramphenicol-induced formation of MG has been assumed as a typical example of this case since chloramphenicol is known to inhibit specifically protein

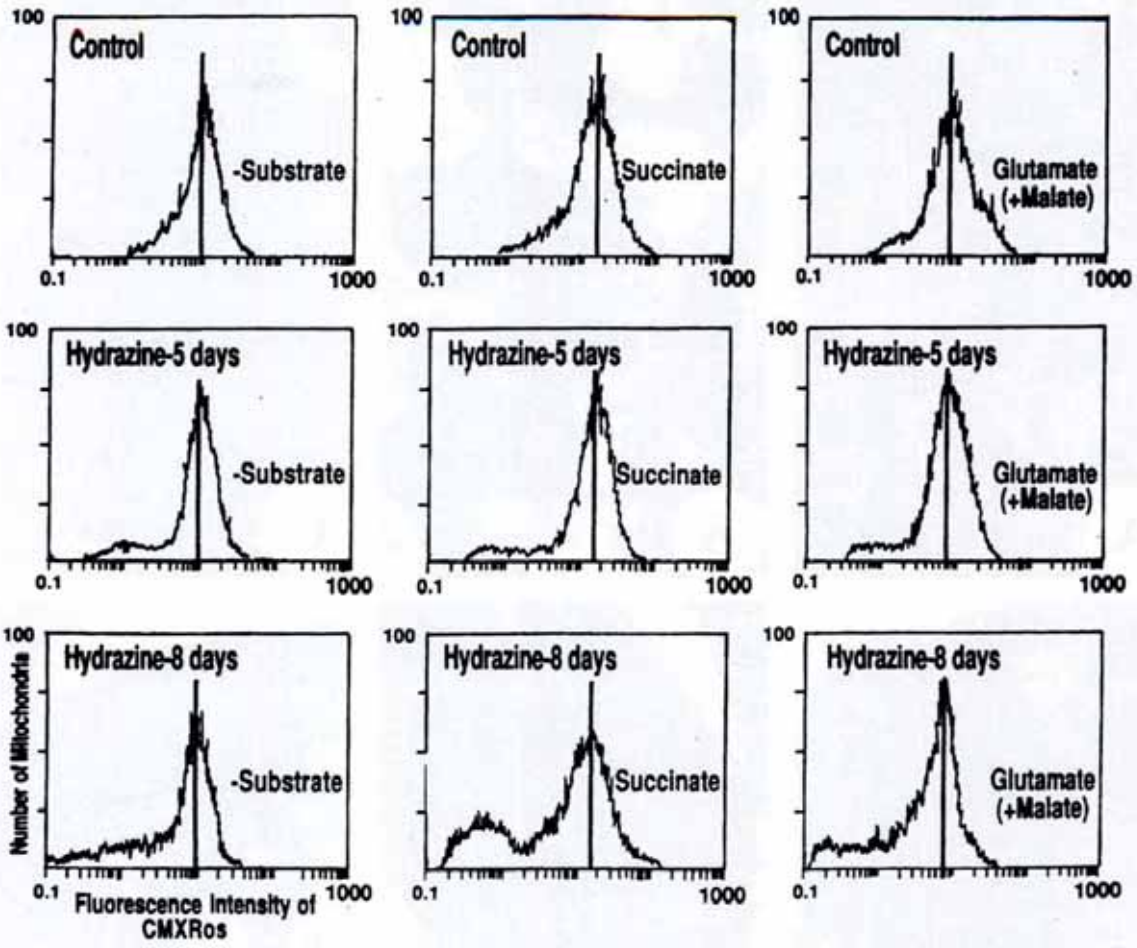

Figure 6. Flow cytometric analysis of the membrane potential of mitochondria isolated from the liver of rats fed with a 1% hydrazine-diet for 5 days and 8 days, using a fluorescent dye CMXRos.

synthesis in mitochondria. (2) Fusion of adjacent mitochondria. We have presented a body of evidence to demonstrate that distinct physicochemical and biochemical changes in the mitochondrial membranes take place during the formation of MG induced by ethanol, chloramphenicol and hydrazine which are favorable for the membrane fusion [29, 31–37]. These changes include: increase in the relative amounts of phosphatidylethanolamine and acidic phospholipids in phospholipid subclasses; increase in the ratio of unsaturated to saturated fatty acids in phospholipid domains of mitochondrial membranes; increase in the content of Ca^{2+} in mitochondria; increase in the mitochondrial membrane fluidity. Furthermore, freeze-fracture studies on the mitochondrial membranes during the formation of hydrazine-induced MG have also strongly indicated that membrane fusion must be involved in this process [29].

A body of evidence is also available in the literature to suggest that free radicals are the underlying mechanism common to ethanol-, chloramphenicol- and hydrazine-induced formations of MG [37–40]. Increase in the level of lipid peroxides, decrease in the level of reduced form of glutathione and increase in the oxidized form of glutathione are common findings in the experimental conditions for the MG formation. Actually, ethanol-, hydrazine- and chloramphenicol-induced MG formations have been successfully suppressed by scavengers of free radicals including α-tocopherol, coenzyme Q_{10} (CoQ_{10}) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (4-OH-TEMPO) [38–41]. Thus, it is very probable that "oxidative stress state" is closely re-

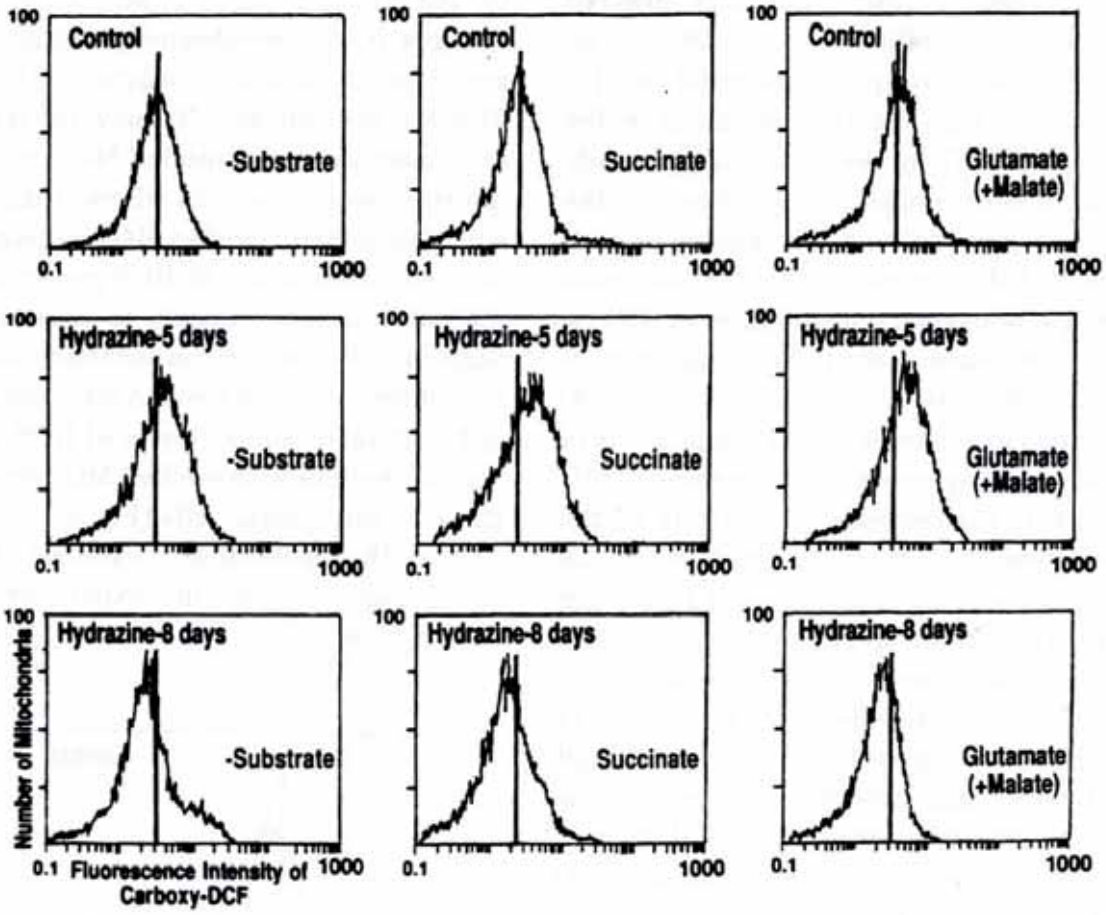

Figure 7. Flow cytometric analysis of the rate of the generation of reactive oxygen species in hydrazine-induced megamitochondria.

Experimental conditions were the same as those described in the legend to Fig. 6 except that mitochondria were stained with a fluorescent dye carboxy-H_2-DCFDA.

lated to the mechanism of the formation of MG, although the molecular mechanism by which free radicals modify physicochemical and biochemical properties of the mitochondrial membranes favorable for the membrane fusion, as described above, has not been solved yet.

CORRELATION BETWEEN THE FORMATION OF MEGAMITOCHONDRIA AND APOPTOSIS

Recently, we have established an *in vitro* experimental model to study the mechanism of the formation of MG using cultured cells from various sources and inducers of free radicals [42]. For example, mitochondria in rat hepatocytes cultured for 22 h in the presence of hydrazine or chloramphenicol become distinctly enlarged. The prolongation of the cultivation time to 48 h or longer induced apoptotic changes of these cells in the presence of MG. Similarly, chloramphenicol induced apoptotic changes in RL-34 cells (Fig. 3). In the case of chloramphenicol, the population of apoptotic cells has reached 49% [43]. The formation of MG and succeeding apoptosis have been suppressed by 4-OH-TEMPO or cycloheximide, an antiapoptotic agent [43]. Then a question arises: What is the correlation between MG formation and apoptosis?

One of the functional changes common to MG induced by various experimental condi-

tions and human diseases is a decrease in the rate of oxygen consumption with lowered phosphorylating abilities [22, 24-26, 29, 33, 37, 44-49]. We have speculated as follows: (1) Mitochondria fuse together resulting in the formation of MG by the action of free radicals. (2) In early stages of MG formation, the membrane potential and the content of cytochrome *c* of MG are maintained. (3) Decrease in the rate of oxygen consumption by MG results in a decrease in the intracellular level of ATP, and cells are arrested in G1 phase. (4) Continuous exposure of cells to high levels of free radicals may result in the swelling of MG secondarily. (5) Decrease in the rate of the oxygen consumption by MG may lead to decreased rates of the generation of ROS from MG. (6) If decrease in ROS generation from MG is effective enough to lower the intracellular level of free radicals, MG may return to normal mitochondria both structurally and functionally, and mitochondria regain the ability to actively synthesize ATP. If such effects are incomplete and intracellular levels of free radicals are kept high, MG become further swollen, and the release of cytochrome *c* from MG into the cytoplasm, decrease in the membrane potential of mitochondrial membrane and decrease in the intracellular level of ATP proceed, and apoptotic changes of the cell become distinct.

When rats are fed with a diet containing 1% hydrazine for 4-5 days, MG are formed in the liver (Fig. 4). At this stage, the density of the matrix of MG is well preserved (B) compared to that of the control mitochondria (A). Prolongation of the treatment of animals with this toxic diet for up to 8-9 days induces MG with extremely pale matrix suggesting that MG become extremely swollen (C, D). In some cases the outer membrane of MG becomes ruptured (E) and MG become collapsed (F). Similarly, the prolongation of the treatment of mice with a 2% chloramphenicol-diet for up to 14-15 days causes the swelling of MG (Fig. 5 C-D). Similar results are also obtained using RL-34 cells cultured in the presence of chloramphenicol (Fig. 5 D-F). Corresponding to the changes in the structure of MG, changes in the membrane potential (Fig. 6) and those in the ability to generate ROS (Fig. 7) of MG are detected. Namely, the mitochondrial membrane potential of MG with a dense matrix is well preserved whereas that of MG with pale matrix (swollen MG) is distinctly decreased. The amount of ROS generated from MG with a dense matrix is essentially the same as that of control mitochondria whereas that generated from swollen MG is decreased, although intracellular levels of ROS are kept high in the cells with swollen MG compared to those of the control cells (Fig. 8).

Thus, the speculation described above is partly supported by the experimental data shown here.

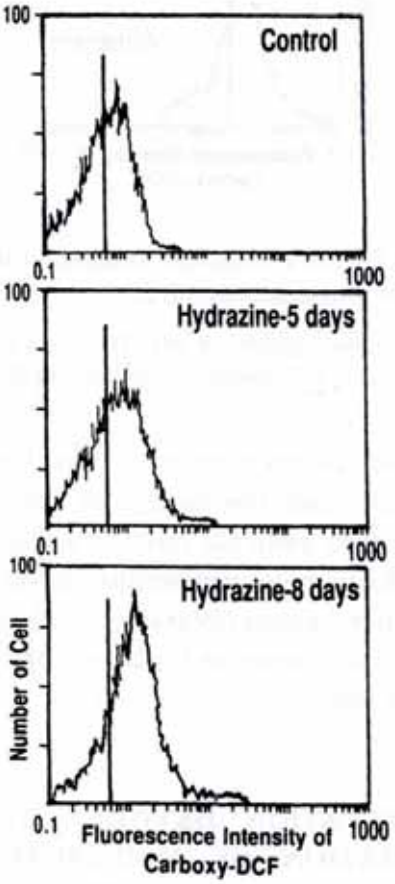

Figure 8. Flow cytometric analysis of intracellular levels of free radicals in hepatocytes isolated from the liver of rats fed with a 1% hydrazine-diet for 5 days and 8 days, using a fluorescent dye carboxy-H_2-DCFDA.

GENES CONTROLLING THE SIZE AND DISTRIBUTION OF MITOCHONDRIA IN THE CELL

A variety of genes controlling the size and distribution of mitochondria in the cell have been identified in yeast mutant cells [50–62]. For example, mutant cells lacking MMM1 or MDM10 are endowed with MG [54, 55, 59]. In mammalian cells, Drp1 (human dynamin-related protein) was identified in human stromal cells and a mutation in the GTPase domain caused alterations in mitochondrial morphology: mitochondria were dispersed throughout the cytoplasm in normal cells whereas cells with mutant Drp1 were endowed with a cluster of mitochondria near the nucleus [61]. In contrast to genes described above, *fuzzy onions* (*fzo*) gene encodes a protein which mediates the mitochondrial fusion first identified during *Drosophila* spermatogenes [57] and also identified during yeast mating [56]. *Drosophila* FZO1 is detected only in sperm mitochondria during a short period of time when mitochondria fusion is occurring to form "Nebenkern" [57].

Data have been accumulated to demonstrate an intimate correlation between mitochondria and the cytoskeleton. In physiological conditions, mitochondria in the hepatocyte are scattered evenly throughout the cytoplasm and never aggregated in certain portions of the cytoplasm. This is also the case with adrenal cortex mitochondria. These facts strongly suggest that there must be a control mechanism (genes?) to regulate the distribution of mitochondria in the cell possibly *via* the cytoskeleton.

PROBLEMS TO BE SOLVED

Based on the experimental data, we may conclude that free radicals are deeply involved in the mechanism of the formation of megamitochondria which are induced under apparently different pathological conditions and that megamitochondria may cause apoptotic changes of the cell. However, further investigations are absolutely necessary to answer the following questions: (1) How do free radicals modify the physicochemical and biochemical properties of the mitochondrial membranes resulting in the fusion of adjacent mitochondria? (2) Is/are there gene(s) which control(s) the size and the distribution of mitochondria in mammalian cells? (3) If so, how are the gene(s), the components of the cytoskeleton and the mitochondrial membranes related to each other in the formation of megamitochondria?

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REFERENCES

1. Collins, M., Marbel, J., Malde, P. & Rivas, A. (1992) Interleukin 3 protects murine bone marrow cells from apoptosis induced by DNA damaging agents. *J. Exp. Med.* **176**, 1043–1051.

2. Bisonette, R.P., Echeverri, R.H., Mahboubi, A. & Green, D.R. (1992) Apoptotic cell death induced by *c-mic* is inhibited by *bcl-2*. *Nature* **359**, 552–554.

3. Fanidi, A., Harrington, E.A. & Evans, G.I. (1992) Cooperative interaction between *c-myc* and *bcl-2* proto-oncogenes. *Nature* **359**, 554–556.

4. Oliver, F.C., Marvel, J., Collins, M.K. & Lopez-Rivas, A. (1993) *Bcl-2* oncogene protects a bone marrow-derived pre-B-cell line from 5'-fluor-2-deoxyuridine-induced apoptosis. *Biochem. Biophys. Res. Commun.* **194**, 126–132.

5. Zhong, L.T., Sarafian, T., Kane, D.J., Charles, A.C., Mah, S.P., Edward, R.H. & Bredesen, D.E. (1993) *Bcl-2* inhibits death of central neural cells induced by multiple agents. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4533-4537.

6. Lotem, J. & Sachs, L. (1993) Regulation by *bcl-2*, *c-myc*, and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation-competent and -defective myeloid leukemic cells. *Cell Growth & Differ.* **4**, 41-47.

7. Slater, A.F.G., Nobel, C.S.I., Maellaro, E., Bustamante, J., Kimland, M. & Orrenius, S. (1995) Nitrone spin traps and a nitroxide antioxidant inhibit a common pathway of thymocyte apoptosis. *Biochem. J.* **306**, 771-778.

8. Korsmeyer, S.J., Yin, X.M., Oltvai, Z.N., Veis-Novak, D.J. & Linette, G.P. (1995) Reactive oxygen species and the regulation of cell death by the Bcl-2 gene family. *Biochim. Biophys. Acta* **1271**, 63-66.

9. Clearly, M.L. & Sklar, J. (1985) Nucleotide sequence of a t(14:18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7439-7443.

10. Miyashita, T. & Reed, J.C. (1992) *Bcl-2* transfer increases relative resistance of S49.1 and WEHI7.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res.* **52**, 5401-5411.

11. Hockenbery, D.M., Oltvai, Z.N., Yin, X.-M., Milliman, C.L. & Korsmeyer, S.J. (1993) *Bcl-2* functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**, 241-251.

12. Newmeyer, D.D., Farschon, D.M. & Reed, J.C. (1994) Cell-free apoptosis in Xenopus egg extracts: Inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell* **79**, 353-364.

13. Srinivasula, S.M., Fernandes-Alnemri, T., Zangrilli, J., Robertson, N., Armstrong, R.C., Wang, L., Trapani, J.A., Tomaselli, K.J., Litwack, G. & Alnemri, E.S. (1996) The Ced-3/interleukin 1β converting enzyme-like homolog Mch6 and the lamin-cleaving enzyme Mch2α are substrates for the apoptotic mediator CPP32. *J. Biol. Chem.* **271**, 27099-27106.

14. Shimizu, S., Eguchei, Y., Kamiike, W., Matsuda, H. & Tsujimoto, Y. (1996) Bcl-2 expression prevents activation of the ICE protease cascade. *Oncogene* **12**, 2251-2257.

15. Yang, J., Liu, X., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.-I., Jones, D.P. & Wang, X. (1997) Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. *Science* **275**, 1129-1132.

16. Kluck, R.M., Bossy-Wetzel, E., Green, D.R. & Newmeyer, D.D. (1997) The release of cytochrome c from mitochondria: A primary site for Bcl-2 regulation of apoptosis. *Science* **275**, 1132-1136.

17. Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J.L., Petit, P.X. & Kroemer, G. (1995) Reduction in mitochondria potential constitutes an early irreversible step of programmed lymphocyte. *J. Exp. Med.* **181**, 1661-1672.

18. Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S.-A., Petit, P.X., Mignotte, B. & Kroemer, G. (1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J. Exp. Med.* **182**, 367-377.

19. Zamzami, N., Susin, S.A., Marechetti, T., Hirsch, T., Castedo, M. & Kroemer, G. (1996) Mitochondrial control of nuclear apoptosis. *J. Exp. Med.* **183**, 1533-1544.

20. Petit, P.X., Susin, S.A., Zamzami, N., Mignotte, B. & Kroemer, G. (1996) Mitochondria and programmed cell death: Back to the future. *FEBS Lett.* **396**, 7-13.

21. Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E.B., Valentine, J.S., Ord, T. & Bredesen, D.E. (1993) Bcl-2 inhibition of neural death: Decreased generation of reactive oxygen species. *Science* **262**, 1274-1277.

22. Gimeno, A., Trueba, J.L., Blanco, M. & Gosalvez, M. (1973) Mitochondrial functions in five cases of human neuromuscular disorders. *J. Neurol. Neurosurg. Psychiat.* **36**, 806-812.

23. Worsfold, M., Park, D.C. & Pennington, R.J. (1973) Familial "mitochondrial" myopathy. A myopathy associated with disordered oxidative metabolism in muscle fibers. 2. Biochemical findings. *J. Neurol. Sci.* **19**, 261-274.

24. Cederbaum, A.I., Lieber, C.S. & Rubin, E. (1974) Unexpected oxidation of a nitroxide alcohol with *m*-chlorobenzoic acid. *Arch. Biochem. Biophys.* **165**, 560-569.

25. Schotland, D.L., DiMauro, S., Bonilla, E., Scarpa, A. & Lee, C.-P. (1976) Neuromuscular disorder associated with a defect in mitochondrial energy supply. *Arch. Neurol.* **33**, 475-479.

26. DiMauro, S., Bonilla, E., Lee, C.-P., Schatland, D.L., Scarpa, A., Conn, H., Jr. & Chance, B. (1976) Luft's disease. Further biochemical and ultrastructural studies of skeletal muscle in the second case. *J. Neurol. Sci.* **27**, 217-232.

27. Wakabayashi, T., Asano, M. & Kawamoto, S. (1979) Induction of megamitochondria in the mouse liver by isonicotinic acid derivatives. *Exp. Mol. Pathol.* **31**, 387-399.

28. Wakabayashi, T., Horiuchi, M., Sakaguchi, M., Onda, H. & Misawa, K. (1983) Induction of megamitochondria in the mouse and rat livers by hydrazine. *Exp. Mol. Pathol.* **39**, 139-153.

29. Wakabayashi, T., Horiuchi, M., Sakaguchi, M., Misawa, K., Onda, H., Iijima, M. & Allmann, D.W. (1984) Mechanism of hepatic megamitochondria formation by ammonia derivatives. Correlation between structure of chemicals and their ability to induce the formation of megamitochondria. *Eur. J. Biochem.* **143**, 455-465.

30. Wakabayashi, T., Adachi, K. & Popinigis, J. (1991) Effects of alkyl alcohols and related chemicals on rat liver structure and function. I. Induction of two distinct types of megamitochondria. *Acta Pathol. Jpn.* **41**, 405-413.

31. Matsuhashi, T., Liu, X.-R., Usukura, J., Woźniak, M. & Wakabayashi, T. (1996) Mechanism of the formation of megamitochondria in the mouse liver induced by chloramphenicol. *Toxicol. Lett.* **86**, 47-54.

32. Wakabayashi, T., Yamashita, K., Adachi, K., Kawai, K., Iijima, M., Gekko, K., Tsudzuki, T., Popinigis, J. & Momota, M. (1987) Changes in physicochemical properties of mitochondria membranes during the formation process of megamitochondria induced by hydrazine. *Toxicol. Appl. Pharmacol.* **87**, 235-248.

33. Adachi, K., Wakabayashi, T. & Popinigis, J. (1991) Effects of alkyl alcohols and related chemicals on rat liver structure and function. II. Some biochemical properties of ethanol-, propanol- and butanol-treated rat liver mitochondria. *Acta Pathol. Jpn.* **41**, 414-427.

34. Adachi, K., Momota, Y., Teranishi, Y., Ueki, R., Hagiwara, T. & Wakabayashi, T. (1992) Effects of alkyl alcohols and related chemicals on rat liver structure and function. III. Physicochemical properties of ethanol-, propanol- and butanol-treated rat liver mitochondria membrane. *Acta Pathol. Jpn.* **42**, 544-557.

35. Adachi, K., Matsuhashi, T., Nishizawa, Y., Usukura, J., Momota, M., Popinigis, J. & Wakabayashi, T. (1994) Further studies on physicochemical properties of mitochondrial membranes during the formation process of megamitochondria in the rat livers by hydrazine. *Exp. Mol. Pathol.* **61**, 134-151.

36. Matsuhashi, T., Karbowski, M., Liu, X.-R., Usukura, J., Nishizawa, Y., Woźniak, M. & Wakabayashi, T. (1998) Complete suppression of ethanol-induced formation of megamitochondria by 4-hydroxy-2,2,6,6-tetramethyl-

piperidine-1-oxyl (4-OH-TEMPO). *Free Radical Biol. Med.* **24**, 139–147.

37. Matsuhashi, T., Liu, X.-R., Nishizawa, Y., Karbowski, M., Antosiewicz, J. & Wakabayashi, T. (1997) Role of free radicals in the mechanism of the hydrazine-induced formation of megamitochondria. *Free Radical Biol. Med.* **23**, 285–293.

38. Antosiewicz, J., Nishizawa, Y., Liu, X.-R., Usukura, J. & Wakabayashi, T. (1994) Suppression of the hydrazine-induced formation of megamitochondria in the rat liver by α-tocopherol. *Exp. Mol. Pathol.* **60**, 173–187.

39. Adachi, K., Matsuhashi, T., Nishizawa, Y., Usukura, J., Popinigis, J. & Wakabayashi, T. (1995) Suppression of the hydrazine-induced formation of megamitochondria in the rat liver by CoQ_{10}. *Toxicol. Pathol.* **23**, 667–676.

40. Matsuhashi, T., Karbowski, M., Liu, X.-R, Usukura, J., Woźniak, M. & Wakabayashi, T. (1998) Complete suppression of ethanol-induced formation of megamitochondria by 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (4-OH-TEMPO). *Free Radical Biol. Med.* **24**, 139–147.

41. Wakabayashi, T., Adachi, K., Matsuhashi, T., Woźniak, M., Antosiewicz, J. & Karbowski, M. (1997) Suppression of the formation of megamitochondria by scavengers for free radicals. *Molec. Aspects Med.* **18** (Suppl.) s51–s61.

42. Karbowski, M., Kurono, C., Nishizawa, Y. & Wakabayashi, T. (1997) Induction of megamitochondria by some chemicals inducing oxidative stress in primary cultured rat hepatocytes. *Biochim. Biophys. Acta* **1349**, 242–250.

43. Karbowski, M., Kurono, C., Woźniak, M., Ostrowski, M., Teranishi, M., Soji, T. & Wakabayashi, T. (1999) Cycloheximide and 4-OH-TEMPO suppress chloramphenicol-induced apoptosis in RL-34 cells *via* the suppression of the formation of megamitochondria. *Biochim. Biophys. Acta* **1449**, 25–40.

44. Bernstein, J.D. & Pennial, R. (1978) Effects of chronic ethanol treatment upon rat liver mitochondria. *Biochem. Pharmacol.* **27**, 2337–2342.

45. Wakabayashi, T. & Green, D.E. (1974) On the mechanism of cuprizone-induced formation of megamitochondria. *Bioenergetics* **6**, 179–192.

46. Wakabayashi, T., Horiuchi, M., Sakaguchi, M., Onda, H. & Misawa, K. (1983) Induction of megamitochondria in the mouse and rat livers by hydrazine. *Exp. Mol. Pathol.* **39**, 139–153.

47. Thayer, W.S. (1987) Effects of ethanol on proteins of mitochondrial membranes. *Ann. N.Y. Acad. Sci.* **492**, 193–206.

48. Quintaniella, M.E. & Tampier, L. (1971) Ethanol intake effect on liver and brain mitochondrial function and acetaldehyde oxidation. *Alcoholism* **9**, 375–380.

49. Adachi, K., Matsuhashi, T., Nishizawa, Y., Usukura, J., Popinigis, S. & Wakabayashi, T. (1995) Studies on urea synthesis in the liver of rats treated chronically with ethanol using perfused livers, isolated hepatocytes and mitochondria. *Biochem. Pharmacol.* **50**, 1391–1399.

50. McConnell, S.J., Stewart, L.C., Talin, A. & Yaffe, M.P. (1990) Temperature-sensitive yeast mutants defective in mitochondrial inheritance. *J. Cell Biol.* **111**, 967–976.

51. McConnell, S.J. & Yaffe, M.P. (1992) Nuclear and mitochondrial inheritance in yeast depends on novel cytoplasmic structures defined by MDM1 protein. *J. Cell Biol.* **118**, 385–395.

52. Jones, B.A. & Fangman, W.L. (1992) Mitochondrial DNA maintenance in yeast requires a protein containing a region related to the GTP-binding domain of dynamin. *Genes Dev.* **6**, 380–389.

53. Guan, K., Fahr, L., Marshall, T.K. & Descenes, R.J. (1993) Normal mitochondrial structure and genome maintenance in yeast requires the dynamin-like product of the MGM1 gene. *Curr. Genet.* **24**, 141–148.

54. Sogo, L.F. & Yaffe, M.P. (1994) Regulation of mitochondrial morphology and inheritance by Mdm10p, a protein of the mitochondrial outer membrane. *J. Cell Biol.* **126**, 385–395.

55. Burgess, S.M., Delannoy, M. & Jensen, R.E. (1994) MMM1 encodes a mitochondrial outer membrane protein essential for establishing and maintaining the structure of yeast mitochondria. *J. Cell Biol.* **126**, 1375–1391.

56. Hermann, G.T., King, E.J. & Shaw, J.M. (1997) The yeast gene, MDM20, is necessary for mitochondrial inheritance and organization of the actin cytoskeleton. *J. Cell Biol.* **137**, 141–153.

57. Hales, K.G. & Fuller, M.T. (1997) Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. *Cell* **90**, 121–129.

58. Berger, K.H., Sogo, L.F. & Yaffe, M.P. (1997) Mdm12p, a component required for mitochondrial inheritance that is conserved between budding and fission yeast. *J. Cell Biol.* **136**, 545–553.

59. Boldogh, I., Vojtov, N., Karmon, S. & Pon, L.A. (1998) Interaction between mitochondria and the actin cytoskeleton in budding yeast requires two integral mitochondrial outer membrane proteins Mmm1p and Mdm10p. *J. Cell Biol.* **141**, 1371–1381.

60. Otsuga, D., Keegan, B.R., Brisch, E., Thatcher, J.W., Hermann, G.J., Bleazard, W. & Shaw, J.M. (1998) The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast. *J. Cell Biol.* **143**, 333–349.

61. Smirnova, E., Shurland, D.-L., Ryazantsev, S.N. & van den Bliek, A.M. (1998) A human dynamin-related protein controls the distribution of mitochondria. *J. Cell Biol.* **143**, 351–358.

62. Hermann, G.T., Thatcher, J.W., Mills, J.P., Hales, K.G., Fuller, M.T., Nunnari, J. & Shaw, J.M. (1998) Mitochondrial function in yeast requires the transmembrane GTPase Fzo1p. *J. Cell Biol.* **143**, 359–373.