



Ultrastructural localization of cytochrome c in apoptosis demonstrates mitochondrial heterogeneity

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Abstract

Release of apoptogenic factors into the cytosol including cytochrome c is triggering the execution phase of apoptosis through activation of cytoplasmic effector caspases. How loss of function of the electron transport chain can be reconciled with an adequate energy supply necessary for executing the apoptotic program was studied in granulosa cell (GC) sheets cultured up to 72 h without gonadotrophic support. Cytochrome c was localized ultrastructurally by oxidation of diaminobenzidine tetrahydrochloride both in living and fixed cells. In uncultured GC sheets all cells show staining over their entire mitochondrial population. In 72 h cultured sheets in the absence of FSH pre-apoptotic GC's display two subsets of mitochondria: normal sized stained mitochondria and small orthodox mitochondria without demonstrable cytochrome function. Apoptotic cells contain several mitochondria with preservation of respiratory function besides unstained orthodox mitochondria. The cytochrome c containing mitochondria typically display dilated intracristal spaces, a mitochondrial conformation related to increased ATP production. Cytochrome c release was confirmed by Western blotting. In 72 h cultures supplemented with FSH, GC's displayed staining over their entire mitochondrial population. In cultures lacking FSH, but partially protected from apoptosis through caspase inhibition, the cytochrome c release was not inhibited. Thus in the present studied model dysfunction of only a subset of mitochondria is instrumental to initiate the apoptotic program while a functional electron transport chain is maintained until the degradation phase in a subset of respiring mitochondria. *Cell Death and Differentiation* (2000) 7, 331–337.

Keywords: cytochrome c; apoptosis; programmed cell death; granulosa; follicular atresia; caspases

Abbreviations: AI, apoptotic index; DAB, diaminobenzidine tetrahydrochloride dihydrate; FSH, follicle stimulating hormone; GC, granulosa cell; zVAD-fmk, Z-Val-Ala-Asp-fluoromethylketone

Introduction

Preservation of morphologically intact mitochondria has been considered a hallmark of apoptotic cell death.¹ However, mitochondria are known to be implicated in apoptosis since the localisation of the anti-apoptotic Bcl-2 gene product at the outer mitochondrial membrane.² Newmeyer and colleagues showed that it was the mitochondrial fraction of the *Xenopus* oocyte extract which induced apoptosis.³ At the other it was reported that in cell-free systems nuclear apoptosis was dependent on the presence of ATP.⁴ Intracellular ATP levels have been implicated both *in vitro* and *in vivo* as a determinant of the cell's decision to die by apoptosis or necrosis.^{5–7} In discordance with the need for ATP are the studies showing that an early common event is the dissipation of the transmembrane potential with uncoupling of the electron transport from ATP production.⁸ A link between mitochondrial events and the activation of the proteolytic caspase cascade is cytochrome c efflux from the mitochondria,⁹ although the mechanism by which cytochrome c is able to escape from the mitochondria is still a matter of debate.^{10,11} In any case, the efflux of cytochrome c is under Bcl-2 control.^{12–14} The discrepancy between mitochondrial dysfunction and the need for ATP to execute the apoptotic program can only be resolved if either the mitochondrial changes are only involving a subset of mitochondria; or the escaping cyt c is only a small fraction of mitochondrial cyt c, not impairing the electron transport chain; or the energy supply is maintained by glycolysis. The present study addresses the following questions: (1) are mitochondrial changes (as revealed by cytochrome function) an early instrumental event during gonadotropin-withdrawal elicited apoptosis in the granulosa cell sheet model?; (2) are all mitochondria equally involved in the cytochrome c efflux?; and (3) is cytochrome c release an event upstream of caspase activation?

Previous studies showed that apoptosis is initiated in the granulosa cell sheet model after 24 h of serum-free culture in the absence of gonadotrophic support, increasing thereafter with a maximum at 72 h.^{15,16}

Results

Cytochrome oxidase activity

The diaminobenzidine technique used in the present study localizes cytochrome c in individual mitochondria, but requires cytochrome oxidase activity to oxidize the reduced cytochrome c by means of O₂ with the generation of H₂O as

shown in Figure 1. Because of the implication of both cytochrome c and cytochrome oxidase, loss of either enzyme can cause a loss of staining. Therefore it was crucial to determine cytochrome oxidase activities in 72 h cultures in the absence/presence of FSH, i.e. in apoptotic cultures and cultures in which apoptosis was inhibited. We found no significant difference between cytochrome oxidase activities expressed in ng/min/mg protein in 72 h cultures in the absence/presence of FSH (five independent experiments; see Table 1).

Light microscopical observations

In uncultured GC sheets mitochondrial staining is intense and the pattern is uniform throughout the sheet. In 72 h cultured GC sheets in the absence of FSH the overall staining decreases and is not uniform throughout the cell layer: i.e. some cells with normal nuclei are surrounded by very few stained mitochondria, while other adjacent cells display a sizable number of reactive mitochondria. At this culture stage GC-sheets display an A.I. of $40\% \pm 9$ S.E.M. as calculated on DAPI stained sheets in four independent experiments. A number of apoptotic cells identified by their condensed chromatin contain reactive organelles. In 72 h FSH-supplemented cultures the apoptotic process is inhibited significantly (A.I. $< 5\%$) and the heterogeneous pattern found in the serum-free cultures was absent: i.e. all normal cells showed a similar number of stained organelles (Figure 2A,B).

Granulosa cell sheets cultured for 48 h in the absence of FSH (AI of circa 20%) were partially protected from apoptosis through caspase inhibition ($50 \mu\text{M}$ zVAD-fmk). Comparison between zVAD-fmk treated and untreated cultures both in the absence of FSH yielded a significant inhibition of the AI. In 24 h cultures zVAD-fmk treatment reduced the apoptotic index by $71\% \pm 11$ S.E.M. (see Figure 3) while at 48 h culture a reduction of 60% was calculated (data not shown). In the 48 h cultures under zVAD treatment the cytochrome c staining was similar to

untreated cultures. Again a heterogeneous pattern of staining in normal non apoptotic cells was apparent, with some cells containing very few reactive organelles adjacent to cells containing a sizable number of reactive organelles (data not shown).

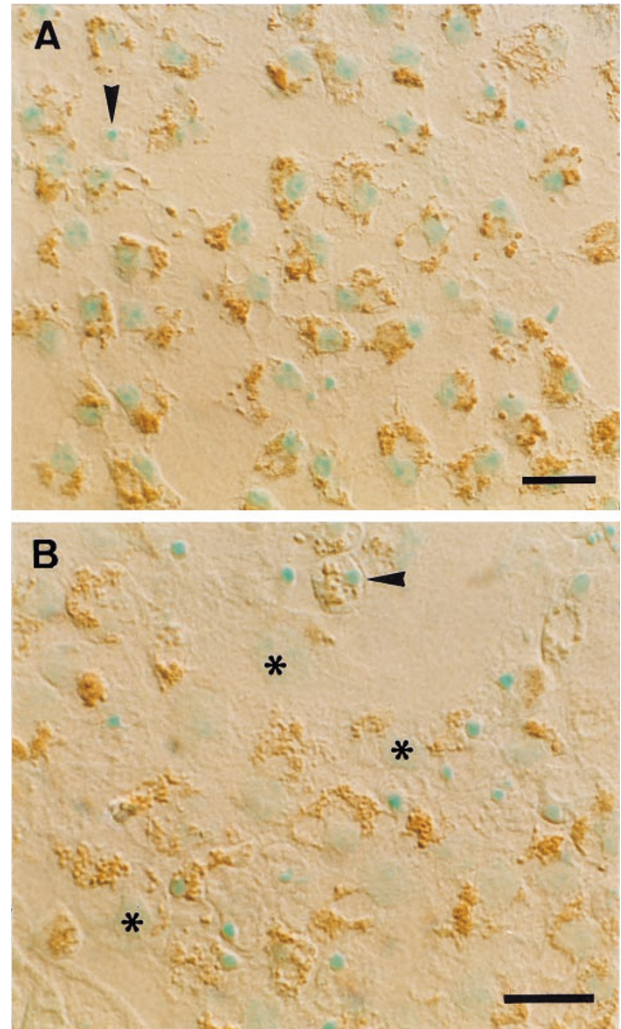


Figure 2 Light microscopy (interference contrast); cytochrome c localization (brown) in 72h living GC sheet, methylgreen counterstaining. (A) FSH supplemented GC sheet. All GC's contain a sizable number of reactive mitochondria. Rare apoptotic nuclei can be found under FSH supplementation (arrowhead). (B) GC sheet cultured in the absence of FSH; normal nuclei faintly stained are indicated by asterisks. In one apoptotic cell with condensed chromatin (green mass) several reactive mitochondria are seen (arrowhead), while other apoptotic bodies are devoid of stained mitochondria. Remark the presence of some normal nuclei surrounded by few or no reactive organelles. Bars: $10 \mu\text{m}$

Table 1 Cyt oxidase activity

Variable	Number of experiments	Mean	S.D.	S.E.M.
		(ng/min/mg protein)		
72 h	5	66.08	18.49	8.27
72 h+FSH	5	84.30	14.11	6.31

Wilcoxon Rank Sum Test 2-Tailed $P=0.1745 > 0.05$. Cytochrome oxidase activity in the 72 h serum-free cultured sheets is not significantly different from the FSH inhibited cultures

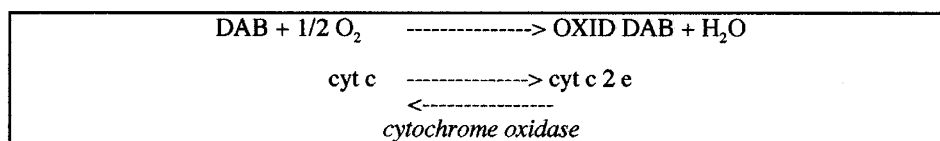


Figure 1 Cytochrome c localization based on diaminobenzidine (DAB) oxidation. This reaction localizes cytochrome c and is dependent on cytochrome oxidase activity^{41,43}

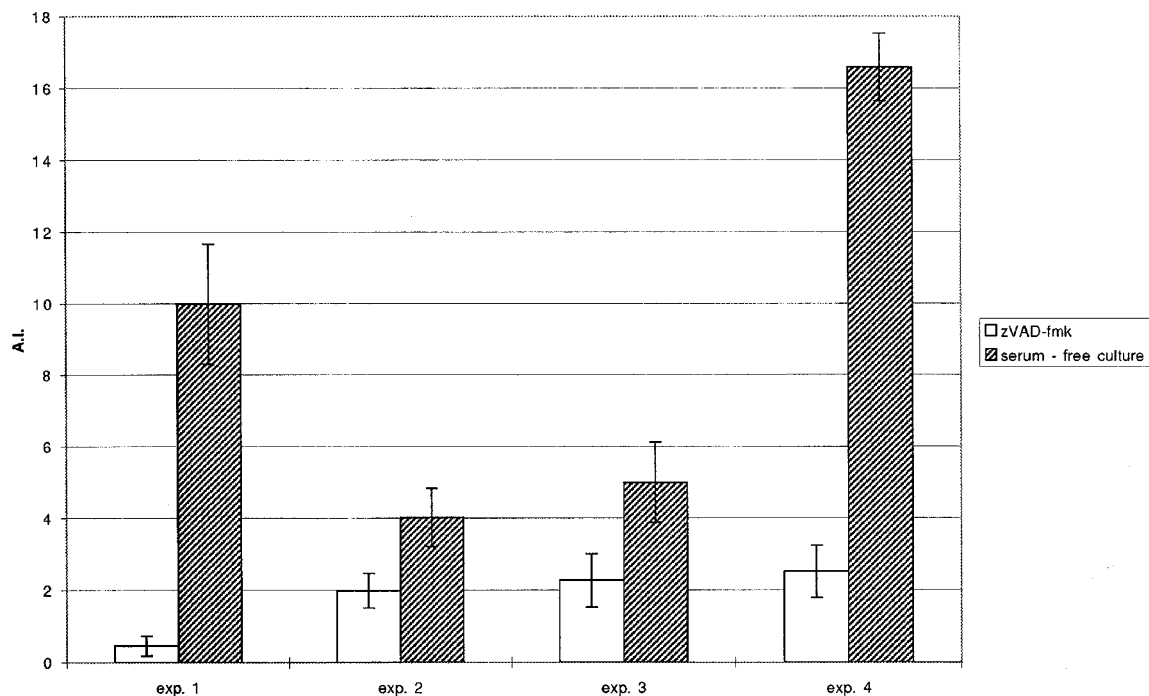


Figure 3 Effect of 50 μ m zVAD-fmk on the apoptotic index of 24 h cultured GC-sheets in the absence of FSH. Apoptotic indexes (AI) were scored after DAPI staining in four independent experiments (values represent mean \pm S.E.M.). A similar decrease of the AI was obtained at 48 h culture with zVAD-fmk

Ultrastructural observations

In uncultured GC sheets the mitochondria appeared in the orthodox conformation; the reaction product was localized in the intermembrane space and at the intracristal spaces of all mitochondria (Figure 4).

In 72 h cultures in the absence of FSH, a fraction of cells with normal nuclear morphology showed a number of unreactive mitochondria (Figure 5A). Importantly these unreactive mitochondria displayed an orthodox configuration of their membranes and matrix, often had elongated forms and seemed smaller than the reactive organelles. Discontinuities of outer mitochondrial membranes were not identified.

Sections through apoptotic cells showed several stained organelles besides a number of unstained but ultrastructurally normal small mitochondria (Figure 5B). Typically the reaction product was most obvious in the intracristal space which was enlarged (intracristal swelling or ballooning) as compared to unreactive organelles. In some instances the reaction is confined to the intracristal spaces with no reactivity at all in the intermembrane space.

In the 72 h FSH-supplemented cultures, where the apoptotic process was inhibited, all mitochondria in normal cells behaved similarly, i.e. they were reactive.

In 48 h cultured GC sheets in the absence of FSH but treated during the last 24 h with the pancaspase inhibitor zVAD-fmk the cytochrome c release seemed unaffected. Again the two subsets of mitochondria were recognized: small unreactive mitochondria besides larger staining mitochondria with dilatated intracristal spaces (Figure 6).

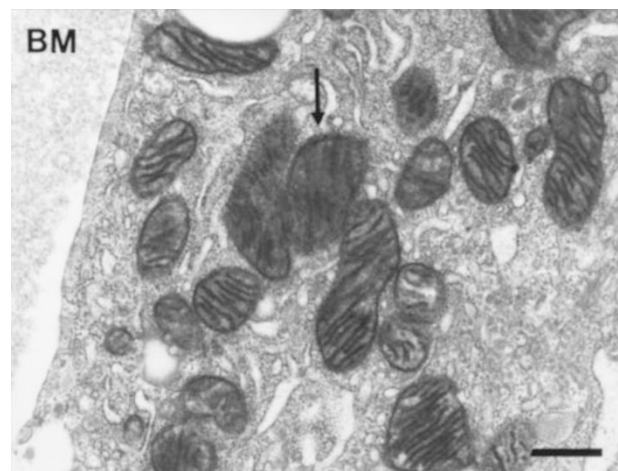


Figure 4 Electron microscopy; cytochrome c localization (osmiophilic reaction product) in uncultured GC sheets. Mitochondria display the orthodox configuration; all are stained, reaction product confined to intracristal spaces and intermembrane space. Some mitochondria are cut tangentially and therefore do not show a sharply delineated reaction product (arrow); BM: basement membrane. Bar: 0.5 μ m

Importantly, now the mitochondria with enlarged intracristal spaces were not only present in clear-cut apoptotic cells, but also in the population of ultrastructurally normal cells. Typically apoptotic nuclear morphology in zVAD-fmk treated cultures was aberrant: many nuclear fragments remained enclosed within one cytoplasm; i.e. fragmentation of apoptotic cells in apoptotic bodies seemed inhibited.

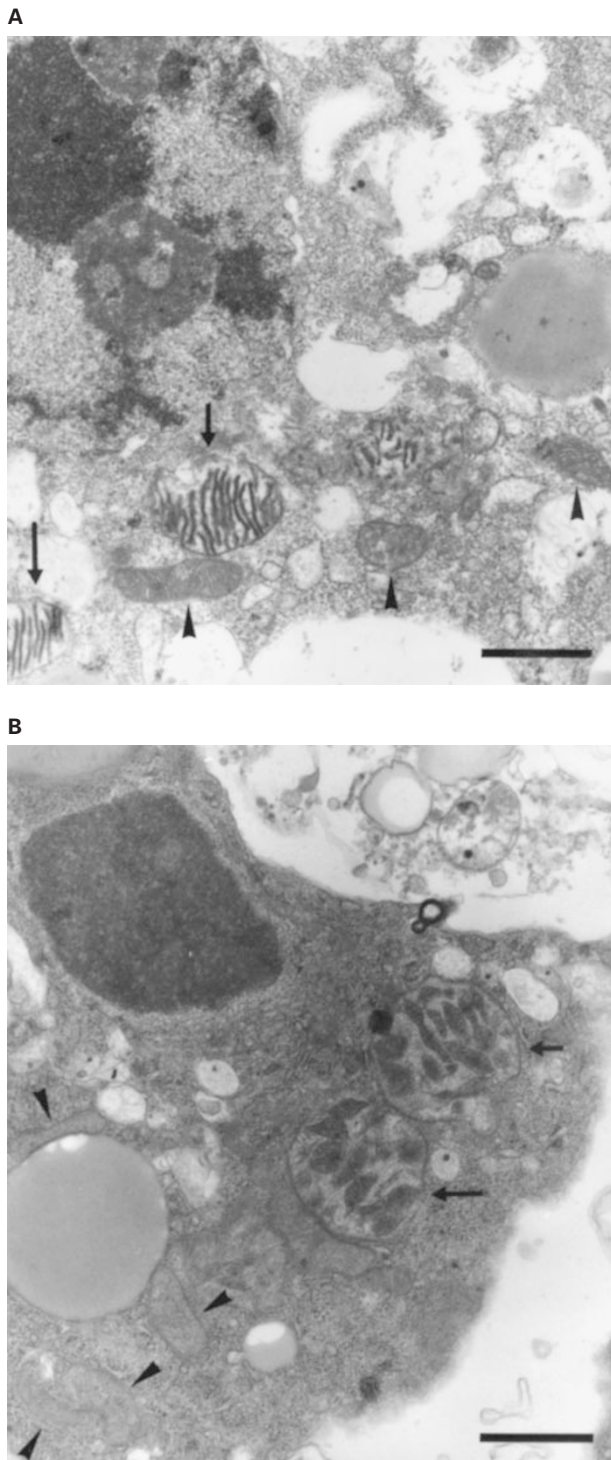


Figure 5 Electron microscopy; cytochrome c localization (osmiophilic reaction product) in 72h living GC sheet cultured in the absence of FSH. (A) Pre-apoptotic cells with normal nuclear morphology contain two subsets of mitochondria: small unreactive mitochondria with orthodox configuration (arrowheads) and normal sized reactive mitochondria (arrows). (B) Apoptotic cell with dense cytoplasm and condensed chromatin mass. Reaction product is prominent in dilated intracristal spaces of two reactive mitochondria, while four mitochondria with orthodox configuration are unreactive (arrowhead). Bars: 1 μ m

Incubations performed on prefixed tissue (data not shown) yielded similar results as those performed on living tissue (Figures 2A,B, 4, 5A,B and 6).

Biochemical confirmation of cytochrome c release

In order to confirm that the unreactive mitochondria in pre-apoptotic and apoptotic cells (Figure 5A,B) have released cytochrome c, cytosolic extracts of 72 h cultured granulosa cell sheets were prepared under conditions that keep mitochondria intact and compared to extracts of the pellet fraction in which mitochondrial cyt c is detected. This procedure was used for apoptotic cultures (72 h cultures in the absence of FSH) and for controls (uncultured granulosa cell sheets). Cytosol from the control cultures did not contain any detectable cytosolic cytochrome c protein (Figure 7, lane 3) indicating that the procedure did not induce mitochondrial contamination. In contrast, cytosolic cytochrome c accumu-

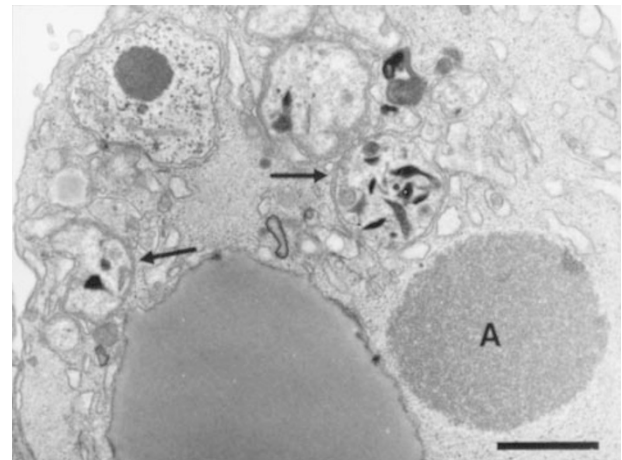


Figure 6 Electron microscopy; cytochrome c localization (osmiophilic reaction product) in 48h GC sheet cultured in the absence of FSH under zVAD-fmk treatment. In the remaining apoptotic cells identical activation of mitochondria with preserved cytochrome function (reaction product in dilated cristal spaces (arrows)) could be observed. These activated mitochondria were also found in non-apoptotic cells (not shown). Note apoptotic nuclear fragments (A). Bar: 1 μ m

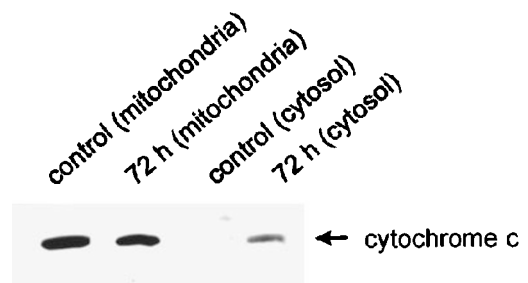


Figure 7 Cytochrome c is released in the 72h CC sheets cultured in the absence of FSH and not in control (uncultured) GC sheets; compare lanes 3 and 4. Cytochrome c present in the cytosol was determined after cell lysis in the presence of 0.075% digitonin. The amount of cytochrome c that is left in the pellet fraction (released by 1% NP 40) represent the mitochondrial cytochrome c present in the cells (lanes 1,2)

lated significantly in the 72 h GC sheets cultures in the absence of FSH (Figure 7, lane 4).

Morphometrical analysis

In order to validate the impression that the negative subset of mitochondria were smaller as compared to the reactive ones, the mean square of the two subsets of mitochondria was calculated.

From this analysis it appears that the negative mitochondria are significantly smaller as compared to the reactive subset (see Table 2).

Discussion

The implication of cytochrome c in apoptosis is studied by various techniques, but most approaches fail to provide data on individual mitochondria in intact cells. Indeed, it was reported that 'it is not known until now whether all mitochondria within each cell are affected to a similar extent'.¹⁷ Several investigators have already suggested the possibility of mitochondrial heterogeneity with respect to cytochrome c efflux or mitochondrial dysfunction as revealed by dissipation of transmembrane potential or permeability transition.^{18–24} In two landmark papers cytochrome c release was shown by subcellular fractionation.^{12,13} Other groups demonstrated the cell-specific induction of apoptosis by microinjection of cytochrome c in cells expressing caspase 3^{24,25} or localized cytochrome c in the cytoplasm by immunofluorescence.^{14,26} By the latter technique, cells with a mixed punctuate/diffuse pattern could be observed very rarely.²⁷ As far as we know cytochrome c was not yet localized in individual mitochondria of apoptotic cells by immunogold technique. Data concerning the extent of cytochrome c release or the compartment within the mitochondria from which cytochrome c is released are scarce. It was reported that 40% of total cytochrome c is released in a cyclosporin A-insensitive manner from brain mitochondria induced by calcium overload, however this study did not resolve the issue whether all mitochondria of a given cell are equally involved in this efflux.²⁸

In Bax expressing yeast cells cytochrome c release was accompanied by a decrease in the amount of cytochrome oxidase which could be prevented by co-expression of Bcl-XL.²⁹ Several studies assaying cytochrome c in the cytosol have also assayed cytochrome oxidase to exclude mitochondrial contamination; efflux of cytochrome oxidase was never shown.^{17,30} Surprisingly high levels of cytochrome oxidase activity in mitochondria were observed

cytochemically in pre-apoptotic and apoptotic hepatocytes.³¹ We assayed cytochrome oxidase in 72 h cultured sheets in the absence/presence of FSH. Absence of a significant difference suggests that the appearance of an unstained subset of mitochondria during apoptosis is not due to a decrease in cytochrome oxidase activity but to either inactivation of cytochrome c *in situ* or to the efflux of cytochrome c. The latter proposal being confirmed by the Western blots showing indeed cytosolic cytochrome c in the 72 h serum-free cultures. To be rigorous we should consider the possibility that the small non respiring mitochondria are defective at the biosynthetic level. Indeed, accumulation of newly synthesised mitochondria with impaired function was reported in Colo-205 cells treated with herbimycin A. These mitochondria have a dissipated mitochondrial transmembrane potential, they are electron dense and have a reduced volume.³² In this case the cytosolic cytochrome c detected in the 72 h cultured GC sheets would be derived from the positively staining subset. In any case the appearance of a subset of non-respiring but intact mitochondria is tightly linked to the apoptotic process as evidenced by the absence of negative mitochondria in 72 h FSH-supplemented cultures.

In two complementary studies it was shown that a reversible transition of mitochondrial morphology from an orthodox conformation to one with dilated intracristal spaces is associated with transition from a resting energy state to a state of increased oxidative phosphorylation.^{33,34} In the apoptotic cells we found two subsets of mitochondria, and in addition the respiring mitochondria display now the conformation related to increased ATP generation. Hackenbrock called this the 'condensed conformation', but also stresses that a more specific sign of stimulated mitochondria is the intracristal dilatation.³⁴ In fact a previous study on etoposide treated THP.1 cells showed intracristal dilatation in the 'ultracondensed' mitochondria.¹⁷ Moreover, besides the altered mitochondria, these authors reported casually the persistence of mitochondria with normal ultrastructural configuration. The presence of a subset of respiring mitochondria until late in the apoptotic process is in agreement with the late drop in ATP measured during apoptosis.²⁶

By morphometrical analysis we have shown that the cytochrome c depleted mitochondria are smaller than the cytochrome c containing subset. Others have reported the shrinkage of mitochondria after cytochrome c depletion in NGF-deprived sympathetic neurons.³⁵ Our subset of non-respiring mitochondria do not show ultrastructural features of dysfunction: they do not show discontinuities of the outer mitochondrial membrane,³⁶ they are not swollen and show the orthodox conformation.

The maintained cytochrome c release and the ultrastructural changes in mitochondria of the caspase-inhibited cultures suggest that the cyt c release is upstream of caspase activation in our model system, as is the case in most apoptotic systems.^{12,26,37} Moreover, it suggests that the ultrastructural changes (intracristal dilatation) are not a late byproduct of caspase activation. This finding contrasts with the 'ultracondensed' mitochondria reported by Zhuang and colleagues; here the ultrastructural change (zVAD-fmk

Table 2 Mitochondrial surface area

Variable	Number of cases	Mean	S.D.	S.E.M.
Positive mit.	62	0.4537 mm ²	0.372	0.047
Negative mit.	42	0.1757 mm ²	0.090	0.014

Morphometrical analysis of the two subsets of mitochondria: cytochrome c containing mitochondria are significantly larger than the mitochondria that lost their cyt c

inhibitable) was clearly an event occurring downstream of the activation of caspases.¹⁷

The aberrant morphology of the apoptotic cells under zVAD-fmk protection needs to be further investigated. Although the latter suggests a role for caspases in fragmentation of apoptotic cells, zVAD-fmk mediated inhibition of non-caspases (as for example cathepsin B)³⁸ might also be responsible for this observation.

In conclusion we have shown that during apoptosis in the granulosa cell sheet model a subset of mitochondria retains cytochrome c function and hence the possibility for ATP production up to the stage of chromatin condensation and fragmentation. A second subset of mitochondria is non respiring; their normal ultrastructural appearance points to a selective protein channel as the mechanism for cytochrome c efflux.

Materials and Methods

Isolation and culture of GC sheets

GC sheets were prepared from ovarian follicles of adult regularly laying Japanese quail (*Coturnix coturnix japonica*). The animals were reared under continuous artificial illumination, with food and water *ad libitum*. The monolayered granulosa layer of the largest preovulatory follicle (F1) was isolated from the surrounding thecal covering as previously described.³⁹ This method provides large sheets of vital GC's, sandwiched between their basement membrane and vitelline membrane. Granulosa cell sheets of circa 4 mm² were maintained in 35 mm culture dishes under serum-free conditions for up to 72 h in humidified room air at 37°C. The culture medium was M199 (Sigma, Bornem, Belgium) supplemented with 0.1% bovine serum albumin (fraction V, Sigma), 6.0 g/l HEPES, 50 U/ml penicillin and 50 mg/ml streptomycin at pH 7.4. The medium was changed every 24 h. In order to inhibit the apoptotic process culture medium was supplemented with 100 ng/ml sheep pituitary FSH (NIH-FSH-S17 U/mg Sigma) in control cultures.⁴⁰

Enzyme histochemistry

Living GC sheets were stained at 37°C, pH 7.4 during 40 to 90 min with 2 mg/ml DAB (3-3' diaminobenzidine-tetrahydrochloride dihydrate) that was freshly prepared in M199 culture medium according to a modification of the technique previously described.⁴¹ Oxidation of DAB is effected by cytochrome c which is itself reoxidized by cytochrome oxidase. The reaction thus requires both the presence of cytochrome c and cytochrome oxidase and will localize only the co-presence of both enzymes. An excess of catalase is added to the medium, in order to exclude reaction products caused by peroxidatic reactions. Controls were incubated in DAB-medium containing 10⁻³ M KCN as cytochrome oxidase inhibitor. After the incubation the unfixed sheets were fixed in a 4% formaldehyde solution buffered with Na-cacodylate containing 1% CaCl₂; all the sheets were postosmicated for 1 h in 1% osmiumtetroxide in water and after dehydration embedded in epon. The same cytochrome c incubation procedure was performed on GC-sheets prefixed during 5 min in the above mentioned formaldehyde fixative. Ultrathin sections were contrasted with uranylacetate and lead and analysed on a Jeol electron microscope.

For light microscopical evaluation whole mounts of the sheets were counterstained with methylgreen.

Cytochrome oxidase assay

Cytochrome oxidase activity was measured spectrophotometrically according to a previously validated technique.⁴²

Detection of cytochrome c release by Western blotting

GC sheets cultured for 72 h under serum-free conditions and uncultured (control) GC sheets were incubated in medium containing 0.2% collagenase and rotated for 10 min at 37°C. Cells were harvested by centrifugation and washed four times with cold PBS. For the detection of cytosolic cytochrome c, cells were lysed in lysis buffer containing 0.075% digitonin, 10 mM HEPES-NaOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH₂PO₄, 0.5 mM EGTA, 2 mM MgCl₂, 5 mM pyruvate, 0.1 mM PMSF and 1 mM dithiothreitol. Insoluble material was pelleted by centrifugation and the supernatant was retained as a source for cytosolic cytochrome c. The cell pellet was further extracted with 10 mM Tris/HCl pH 7, 1% NP40, 200 mM NaCl, 5 mM EDTA and 1 mM PMSF to determine the mitochondrial cytochrome c. Proteins were subjected to SDS-PAGE and transferred to Hybond nitrocellulose filters (Amersham Pharmacia Biotech, Upssala, Sweden), blocked with 5% dry milk in PBS containing 0.3% Tween 20 and probed with anti-cytochrome c (clone 7H8.2C12, Pharmingen, San Diego, CA, USA) as described by the manufacturer's protocol. Blots were developed with the chemiluminescence method (NENTM Renaissance, NEN Life Science Products, Inc.).

Quantitation of apoptosis by DAPI staining

At the end of each culture period (24, 48, 72 h), GC-sheets were rinsed in PBS and fixed in 4% buffered formaldehyde. Then GC-sheets were washed again in PBS and stained with DAPI (4',6'-diamidino-2-phenylindole; 1 mg/ml) in distilled water at room temperature for 10 min. Subsequently GC sheets were mounted on slides and samples were observed under an epifluorescence microscope (Leitz) using the DAPI bandpass. Apoptotic cells were identified as cells showing condensed chromatin or fragmented nuclei. A minimum of 1000 cells were scored for each condition and apoptosis was expressed as the per cent apoptotic nuclei. Nuclear fragments lying in close proximity were counted as one apoptotic nucleus.

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