Abstract. In the light of both the major role played by released cytochrome c in apoptosis of a variety of cells and the availability of cerebellar granule cells as a model system to investigate apoptosis as a function of time from induction to cell death, we review data aimed at elucidating the events dealing with cytochrome c release from mitochondria as well as its role outside mitochondria. We report cytochrome c release in the apoptosis time course as dependent on the function of both the antioxidant and proteolytic systems. We show that, beside the role played by cytochrome c in participating in apoptosome formation and in triggering the caspase cascade, at least in cerebellar granule cells, released cytochrome c can maintain its ability to work as an electron carrier, being a scavenger of reactive oxygen species and an electron donor to cytochrome oxidase, thus driving the ATP synthesis.

Mitochondria are sites of cellular energy production which may also influence life and death decisions by initiating or inhibiting cell death. Mitochondrial depolarization and the subsequent release of pro-apoptotic factors, including cytochrome c (cyt c), have been suggested to be required for the activation of a cell death program in some forms of neuronal apoptosis. Despite the large body of literature dealing with cyt c release from mitochondria in cells undergoing apoptosis (1-9) and its role in caspase activation (10, 11), knowledge of the relationship between apoptosis and cyt c remains incomplete. For instance, it has not been well established: a) whether cyt c release occurs from uncoupled/damaged mitochondria or cyt c release takes place before any mitochondrial dysfunction, b) how cyt c release is regulated, c) the release time course, d) whether and how the cytosolic level of released cyt c changes during apoptosis, and 5) the role played by the released cyt c in apoptosis.

Since the apoptosis pathways can differ from each other, depending on the cells investigated as well as on the manner by which apoptosis is induced, a variety of response is expected. The above points can be described by using as a model system rat cerebellar granule cells (CGCs).

Dissociated CGCs from early postnatal rats can be maintained in serum-containing medium in the presence of elevated potassium levels (25mM) (12) or by adding a low concentration of N-methyl-D-aspartic acid (NMDA) to the culture medium (13). Both a low concentration of NMDA and depolarization are assumed to mimic endogenous excitatory activity (14) with survival promotion mediated by increases in intracellular calcium level. Dissociated cerebellar granule cells develop characteristics of mature cerebellar granule cells in vivo, including an extensive neurite network, expression of excitatory amino acids receptor and production and release of L-glutamate (14). CGCs undergo apoptosis after serum removal, in the presence of low K+ concentrations (5mM) (15), while over stimulation of glutamate receptors on granule cells leads to excitotoxic death (16-18). Therefore, these neurons are a versatile system to dissect the mechanisms which are common to apoptosis and necrosis and those which are unique. Apoptotic cell death, which is accompanied by DNA fragmentation and is dependent on cell biosynthesis (15, 19), probably mimics the naturally occurring death of 20-30% of granule cells (20), which is important for matching the number of granule cells with Purkinje cells, which occurs during the third to fifth postnatal weeks (21, 22).
We have shown that in cerebellar granule cells, apoptosis proceeds in two separate phases, here defined as early and late. The early-phase (0-3 h) of CGCs apoptosis is characterized by reactive oxygen species (ROS) production, increase of proteasome (23) and antioxidant enzymes activities (24). Notice that one of the earliest events taking place during CGCs apoptosis is the release of cyt c from the mitochondrial intermembrane space to the cytosol. In the late-phase (3-8 h) ROS production remains constant, with a progressive decrease in proteasome and antioxidant enzymes activities; moreover decrease of the cytosolic cyt c content takes place as a result of caspase activation. Interestingly, we have found that the released cyt c can still work as an electron carrier, in particular as a ROS scavenger and can still transfer electrons to cytochrome oxidase thus driving ATP synthesis, which is necessary for apoptosis to occur. These points will be described separately.

In cerebellar granule cells undergoing apoptosis cyt c is released from coupled mitochondria in a manner dependent on ROS production which relies on the function of both the antioxidant and the proteolytic system

When the cytosolic fractions of apoptotic CGCs isolated at different times after apoptosis induction were compared to the controls with respect to their cyt c content, progressive increase was detected as soon as 30 min after apoptosis induction with a maximum reached after 3 h (Figure 1). A progressive decline occurs in the later phase. Consistently, the amount of cyt c in the corresponding mitochondrial fractions is characterized by a progressive decline up to 3 h; until this time the increase in the extra-mitochondrial phase and the decrease in the mitochondria are in fairly good correlation. A control to see that this cyt c release is specifically associated with the apoptotic process was done by checking the capability of both actinomycin D (ActD) and cycloheximide (CHX), known inhibitors of apoptosis in CGCs, to prevent cyt c release. Since in this time range mitochondria maintained their capability to drive oxidative phosphorylation (see below), cyt c release proved to occur from coupled intact organelles. In agreement with such a conclusion, in other reports it was suggested, in the light of the lack of mitochondrial swelling, that the mitochondrial permeability transition pore is not involved in cyt c release from mitochondria during the apoptotic death of cerebellar granule neurons (25). Colombaioni et al. (26) analyzed the cellular events associated with apoptosis induced by serum deprivation and observed translocation of Bax from cytosol to mitochondria and cyt c release from mitochondria, these events occurring without mitochondrial membrane potential loss with maintenance of mitochondrial functionality. That cyt c release also occurred in the absence of mitochondrial depolarization was also shown by Krohn et al. (27).
On the other hand, cyt c release was proposed as occurring from uncoupled/damaged mitochondria by a number of authors in studies carried out using different cell types, where mitochondrial swelling with external membrane rupture (28, 29), involvement of the permeability transition pore (PTP) opening (30) with loss of ΔΨ (31), or formation of a specific channel by Bcl-2 family-proteins in the outer mitochondrial membrane (4) were shown. Bilirubin and amyloid-beta peptide were reported to induce cyt c release through mitochondrial membrane permeabilization. (32), while mitochondrial permeability transition and cyt c release prevention by Bcl-2 was reported to occur via maintenance of reduced pyridine nucleotides (33); similarly cyt c release from brain mitochondria was proposed to be associated with the permeability transition and rupture of the outer mitochondrial membrane (34-36).

The occurrence of two distinct mechanisms for mitochondrial cyt c release, a) active cyt c release associated with early mitochondrial hyperpolarization, leading to neuronal apoptosis, and b) passive cyt c release secondary to mitochondrial depolarization and matrix swelling (37), could account for the observed data. Given that most reports describe cyt c release as being measured many hours after apoptosis induction, the short-term release should be investigated in more detail.

The finding that the cyt c release is strongly inhibited by adding CGCs to the antioxidant system (AOS), including superoxide dismutase (SOD), catalase and glutathione peroxidase, shows that cyt c release depends on the increase of ROS, which is measured consistently with a fairly good time correspondence. Consistently, when apoptotic CGCs were pre-treated with captopril and NH2-triazole (NH2TZ), inhibitors of SOD and catalase respectively (24), a further increase in O2- production was observed with increase in the rate of cell apoptosis.

ROS involvement in cell death is not unique for apoptosis: in CGCs undergoing death via necrosis due to glutamate-dependent neurotoxicity, we have found that cyt c release from mitochondria occurs due to ROS generated outside mitochondria (38, 39). Thus, both in necrosis and in apoptosis, ROS formation must be considered to be the mediator of intracellular signalling cascades (40) as well as to play a crucial role in the progression of cell death (17, 19, 39, 41-47).

It has been reported that overexpression of superoxide dismutase can prevent apoptosis in primary neuronal

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**Figure 2.** The cytochrome c release is regulated by the antioxidant system and proteasomes.
cultures (41) as well as cyt c release from mitochondria (48, 49), while glutathione depletion proved to result in an increase of ROS levels (50). These results pose the question as to whether and how the cell AOS, ROS production and cyt c are regulated during apoptosis.

Interestingly, in apoptotic CGCs, the activity of SOD and catalase together with the GSSG/GSH ratio, which is a measure of thiol oxidation state, were found to increase with respect to control cells maintained in high potassium as soon as 30 min, with maximum enzyme activity increase measured 3 h after potassium shift: a time at which cells survive even if ROS production has already increased. At longer times, i.e. 15 h after K+ shift, a progressive reduction of all three AOS activities was accomplished. This occurs due to the existence of the proteasome, a proteolytic system which has been found to play a crucial role during the early commitment phase of apoptosis (51-53) and particularly in neuronal apoptosis (23, 54).

Proteasome plays a major role in protein degradation in all eukaryotic cells (55, 56), where it exhibits chymotrypsin-, trypsin- and caspase-like proteolytic activities. This applies to apoptosis in CGCs (7, 23) with a slight but consistent increase in the three peptidase activities of proteasome in the early phase of cell apoptosis, whereas the proteasome activities decline in the late phase.

Interestingly, both MG132 and Lactacystin (LC), reversible and irreversible proteasome inhibitors respectively (57), were found to prevent cyt c release in CGCs undergoing apoptosis. Consistently MG132 and LC were found to rescue cells from apoptosis (Figure 2). The above reported results show that the steady-state ROS level derives from the cell balance between ROS-producing and ROS-scavenging enzymes which are SOD, catalase and glutathione peroxidase, being all together the cell antioxidant system which, in turn, are substrate of the proteasome activity.

In opposition to the 0-3h apoptosis situation, in the 3-15h time-range the cyt c amount remains constant in the mitochondria, but it strongly decreases in the cytosol; since 3-4 h after apoptosis induction caspase activation occurs, the caspase-dependent proteolysis of cyt c can be assumed to occur. Indeed, there is general consensus that cyt c release and the activity of caspases are strictly correlated. Once the cyt c level in cytosol rises above a certain threshold, interaction with other cytoplasmic factors such as Apaf-1 and ATP initiates the activation of caspase-9 (11, 58, 59). It is well known that caspase-3 is the most prominently involved in neural tissue (60-62) and its activation is reported to be a key event in the execution of neuronal apoptosis.

When investigated in CGCs, the time course activation of caspase-3 shows a progressive increase which correlates with
Figure 4. The apoptosis time course. Taken with permission from (24).
1. EARLY PHASE (0-3 h)
1.1 ROS production
1.2 Cytochrome c release from mitochondria
1.3 Antioxidant system activity activation
1.4 Proteasome activity activation
2. LATER PHASE (3-8 h)
2.1 Steady-state ROS level
2.2 Cytochrome c-dependent caspase activation
2.3 Proteasome/caspase-dependent antioxidant system degradation
2.4 Caspase-dependent cytochrome c degradation
2.5 Caspase-dependent proteasome degradation
apoptosis progression (24, 63, 64). In detail, we show that the increase in caspase-3 activity strictly correlates with the timing of cyt c release, being caspase undetectable in the first 3 h of apoptosis, when cyt c reached its maximum cytosolic level, and already measurable after 4 h with a maximum reached at 8 h. This result shows that in CGCs, caspases can be involved in the degradation of the cytosolic cyt c rather than in its release from mitochondria as found in other apoptotic paradigm (65). Consistently, the addition to CGCs of zVAD-fmk, a broad-range specific caspase inhibitor, could not prevent the release of cyt c (1, 6, 66), but only the decrease of cyt c level into the cytosol in the late phase, with simultaneous blocking of the apoptotic process (6, 67). This finding shows, for the first time, that in apoptotic CGCs, cyt c is degraded in a caspase-dependent way (Figure 3). Different results have been reported in studies carried out with a different neuronal cell type (66).

The apoptosis time course in CGCs, as derived from the above findings, is shown in Figure 4. In an EARLY-phase ranging from 0 to 3 h after the apoptotic stimulus, ROS production increases and the cell components, including AOS and proteasomes, are evoked to maintain sufficient ROS production for the correct application of the programmed death. The caspases have no role in this phase. In this situation cyt c release occurs from still coupled (7) mitochondria, in order to begin the activation of the caspase cascade, (2, 45, 58, 59, 68) as well as to drive ATP synthesis (25, 69, 70). In the LATE-phase, ranging between 3 and 15 h, proteasome activities decrease slowly (7, 23), as well as AOS enzyme activities, but caspase-3 activity increases. The resulting caspase-cascade (71, 72) contributes to cyt c (6), AOS (24) and proteasome degradation (23). As a result of this, ROS levels still remain high and the cells are committed to death.

A different situation occurs when the AOS is totally blocked. In this condition cyt c release takes place as well as a further superoxide production. Interestingly, a strong reduction of the caspase-3 activity was found, nonetheless cells die. Since we can exclude that both inhibitors have any effect on the caspase activity in vitro, while zVAD strongly inhibited it, the only possible scenario is one in which an apoptosis/necrosis shift takes place when ROS production is high, i.e. not regulated by cell AOS (Figure 5).

The released cytochrome c works as ROS scavenger and electron donor to cytochrome oxidase, thus driving ATP synthesis

Once released in the cytosol, cyt c participates in the formation of apoptosome and in the consequent activation of the caspase cascade that will finally lead the cell to death (11, 59, 68). Interestingly, also in its new cytosolic location, cyt c proved to play a key role in mitochondrial energy production, still being able to act as a ROS scavenger, to supply electrons to cytochrome oxidase and to drive the synthesis of ATP (25, 69, 70).

In order to start caspase activation, cyt c must be released into the cytosol as a holoenzyme, apo-cytochrome c being shown ineffective in doing this (73). On the other hand, the redox state of cyt c appears not to be important for apoptosis, since oxidized and reduced cyt c induce caspase activity with similar efficiency (74).

Consistently, we found that in CGCs the cyt c is released as a fully functioning protein from mitochondria that were essentially coupled and intact. Indeed, under our conditions neither glutamate dehydrogenase nor adenylate kinase were significantly released, thus excluding that the early cyt c release can result from mitochondrial damage (6). The stimulation of
the oxygen consumption rate due to either ADP or FCCP addition to mitochondria releasing cyt c (7, 75, 76) further confirms that that cyt c movement across the outer mitochondrial membrane occurs in intact and coupled mitochondria.

The coupling and function of mitochondria is in good accordance with their role in the maintenance of the ATP level necessary to trigger apoptosis. This point has been investigated in detail in in vitro experiments carried out using cytosolic fractions obtained from CGCs undergoing apoptosis for times ranging from 10min to 8h, i.e. cytosolic fractions in which the released cyt c is present in different amounts together with other released proteins (77). The cyt c within the cytosolic fraction (CCCF, cyt c-containing fraction), was shown to work as a ROS scavenger, being able to oxidize superoxide anion produced in vitro by the externally added xanthine/xanthine oxidase system (70). Moreover, once reduced, CCCF was shown to work as a respiratory substrate for mitochondria isolated from CGCs, transferring reducing equivalents to cytochrome oxidase (70) and determining a cyanide-sensitive oxygen consumption (Figure 6).

The direct oxidation of cyt c by cytochrome oxidase at mitochondrial contact sites (78) is probably due to the existence of a structurally distinct type of contact site, named bridge contact sites and revealed in brain mitochondria (79), which might play a role in maintaining the structural integrity of the outer and inner membrane systems as well as in allowing cyt c oxidation. Consistently, CCCF reduced in vitro by the non-permeating reducing compound ascorbate, was also shown

Figure 6. The released cytochrome c acts as a ROS scavenger and electron donor to cytochrome oxidase thus driving ATP synthesis. MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; I, III and IV, respiratory chain complexes (NADH-CoQ reductase, CoQH2-cytochrome c reductase and cytochrome c oxidase, respectively); Q, coenzyme Q. ANT, adenine nucleotide translocator; Pi, phosphate carrier.
to determine proton efflux in the extra-mitochondrial phase and mitochondrial protonotive force (ΔΨ) generation, when added to CGCs mitochondria isolated after 3h from apoptosis induction, in the presence of complex I and complex III inhibitors. Moreover, reduced CCCP can also drive both ATP synthesis and export from mitochondria isolated from apoptotic CGCs, after ADP addition, in those conditions in which the electron flow along complex I and complex III is completely inhibited and ATP synthesis driven by endogenous substrates does not take place (70) (Figure 6).

This shows that cyt c released in the cytosol is still a functional protein and plays an important role during apoptosis, both as a ROS scavenger and as a respiratory substrate for energy production by oxidative phosphorylation, as previously shown in CGCs undergoing glutamate-dependent necrosis (38). However, the presence of a variety of proteins/factors released from mitochondria during apoptosis (80-83) poses the question as to whether the mitochondrial energization may be due to the combined action of several proteins. This was excluded on the basis of the evidence that the cyt c-depleted cytosolic fraction (CF), obtained after treating the cytosolic fraction of apoptotic neurons with the monoclonal anti-cyt c antibody, can neither energize mitochondria nor drive ATP synthesis and export, while cytochrome c bound to the antibody can, both in the absence or presence of CF. Thus, the released cyt c can itself provide the energy needed for ATP synthesis and ADP/ATP exchange to occur (70).

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