Segregation of nucleolar components coincides with caspase-3 activation in cisplatin-treated HeLa cells

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SUMMARY

We studied morphological changes of the nucleoli in HeLa cells treated with cisplatin and compared them with induction of markers of programmed cell death and TUNEL staining. We used different light microscopic nucleolar staining methods allowing us to visualize not only nucleolar proteins but also nucleolar RNA. Our results show predominantly compact, centrally localized nucleoli in intact control HeLa cells. In cisplatin-treated HeLa cells, we found an early onset of nucleolar segregation of proteins detected by argyrophilic nucleolar organizer regions and anti-nucleolar monoclonal antibody as well as an increased immunoreactivity for activated caspase-3 after 6 hours. Staining with Toluidine Blue and Methyl-green Pyronine revealed segregated nucleoli 12 hours after the treatment with cisplatin. TUNEL positivity in cisplatin-treated HeLa cells was accompanied by the aggregation of the argyrophilic proteins in the central portion of nucleus, disappearance of nucleolar RNA and shrinkage of the nucleus after 24 hours. Monitoring of the biochemical changes by immunoblotting revealed that activation of distinct caspases and degradation of their downstream protein substrates is executed in two phases. During an early apoptotic stage beginning 4.5 hours post treatment an activation of caspase-9 and caspase-3 was observed. This was accompanied by proteolytic cleavage of poly(ADP-ribose) polymerase-1 (PARP-1). The caspase-9 activation seems to be mediated by recruitment by the activating factor Apaf-1 because the increased accumulation of Apaf-1 and cytochrome C in cytosol preceded the generation of mature caspase-9 form. A second phase of apoptosis occurring between 10 and 15 hours post treatment was characterized by degradation of other nucleolar and nuclear proteins such as nuclear lamins, topoisomerase I and B23.

In conclusion, remarkable segregation of nucleolar argyrophilic proteins, nucleolar RNA and a simultaneous activation of the cascade of caspases markedly preceded the TUNEL positivity in cisplatin-treated HeLa cells thereby substantiating the hypothesis that the nucleolus is a preferred target for caspase-3-dependent proteolysis in cisplatin-treated HeLa cells.

Key words: Apoptosis, Caspase-3, Caspase-9, Nucleolus, Nucleolar segregation, Nuclear lamins, B23, PARP-1, Topoisomerase I, TUNEL

INTRODUCTION

Apoptosis is a multistep process of active cell death that involves the action of specific and highly conserved enzymes responsible for proteolysis and DNA cleavage. This type of cell death is essential for the maintenance of homeostasis and cell number within organs under physiological conditions and also serves to eliminate damaged or initiated neoplastic cells under pathological conditions. Induction of programmed cell death (PCD) is also a critical and desired effect in the treatment of tumours by cytostatics, e.g. cisplatin. Cisplatin (CP) is one of the most effective and broadly used anticancer drugs. Apoptosis induced by CP is generally considered to be generated by formation of covalent DNA adducts, which block replication and transcription, as well as reactive oxygen intermediates (Maldonado et al., 1997).

Recent studies have provided information about the critical role of caspases in the execution of PCD as well as their involvement in the CP-induced apoptosis. Caspases, a growing family of cysteine proteases, specifically target proteins possessing a characteristic tetrapeptide motif cleaving them precisely after aspartate residues. All caspases exist as inactive proenzymes, which after proteolysis are processed to the active heterodimeric form. Procaspases can undergo autocatalysis or cleave other caspase zymogens, thus initiating an orchestrated cascade of events triggering several unique morphological changes such as chromatin condensation and nuclear fragmentation (Sahara et al., 1999). A reduced expression of ICE-like proteases has been associated with drug resistance (Eichholtz-Wirth et al., 1997).

The nucleolus represents a highly dynamic nuclear compartment, easily visible by light microscopy. Nucleolar morphology can be evaluated histologically using the silver staining method for nucleolar organizer regions (NORs). NORs are loops of ribosomal DNA within the nucleus that transcribe ribosomal RNA and are usually tightly aggregated.
within the nucleoli in interphase cells. Associated with NORs are argyrophilic proteins (RNA polymerase I, nucleolin, B23) that have the ability to bind silver ions selectively (Goezens, 1984; Hernandez-Verdun, 1991). Alterations in the number and configuration of NORs have been demonstrated in numerous types of human neoplasms and related to progression similarly as proliferative markers (Smith et al., 1993). Currently, the morphological description of apoptosis is characterized by the segregation of the nucleolus. By studying apoptosis in FDCP-Mix, a pluripotent murine haemopoietic stem cell line, it was found that electron dense material became apparent at the nuclear envelope as a result of the segregation of nucleoli in association with the nuclear membrane. The remaining electron dense and homogenous bulk of the nucleolus labels for Rnase-gold, but even more intensely for Dnase-gold. The labelling of the electron dense material for DNase-gold could be explained by a migration of DNA into the bulk of the nucleoli at an early stage of active cell death (Reipert et al., 1999).

PARP-1 (poly(ADP-ribose) polymerase-1), an ubiquitous nuclear enzyme is a unique sensor of DNA breaks (for review, see D’Amours et al., 1999). This property to detect even negligible DNA lesions is important for protection of cells from DNA damage and for the maintenance of genome integrity. PARP-1 is localized in the nucleolus (Leitinger et al., 1993; Mosgoeller et al., 1996) and accumulates in foci within the dense fibrillar component. Such foci are seen in close spatial association with sites of nucleolar transcription as revealed by high resolution immunodetection of bromodeoxyuridine uptake (Mosgoeller et al., 1996).

We hypothesized that down-regulation of cellular biosynthetic activities, induced by caspase-dependent degradation of vitally important nuclear proteins in the onset of apoptosis, would not exclude the nucleolus. Thus, active caspases should be associated with the alteration of nucleolar phenotype. We detected early morphological changes of the nucleoli in CP-induced apoptosis of HeLa cells. For visualization of nucleolar proteins, we used histochemical staining for argyrophilic nucleolar organizer regions (AgNOR) and immunohistochemical staining of nucleolar antigens. We also detected nucleolar RNA using basic dyes, Toluidine Blue (TB) and Methyl-green Pyronine (MGP). For in situ detection of apoptosis, we used polyclonal sera against activated caspase-3 and TUNEL (TdT-mediated X-DUTP nick end labeling) assay. Shrinkage of cells determined by CASY (Cell Analysis System) was clearly visible at 10 and 15 hours after cisplatin administration. The activation of caspases-9 and caspase-3 followed by degradation of PARP-1, a sensor of DNA damage, was detected at an early stage of apoptosis, whereas cleavage of nuclear lamins and topoisomerase I induced by other specific caspases occurred in the second stage of apoptosis. The kinetics of nucleolar segregation coincided with that of activation of caspase-9 and caspase-3 and with dramatic degradation of PARP-1. Our results indicate that nucleolus is involved in initiation of the early events during CP-induced apoptosis.

**MATERIALS AND METHODS**

**Antibodies**

We used the following antibodies: monoclonal anti-PARP-1 antibodies (C-2-10) from Dr G. G. Poirier (Laval University, Quebec); polyclonal anti-caspase-3 and monoclonal anti-bcl-2 (clone 124) antibodies from DAKO (Glostrup, Denmark), monoclonal anti-caspase-9 (clone 1-2) and anti-lamin B (Clone 101 B7) antibodies were from Oncogene Research Products (Cambridge, MA), monoclonal anti-cytochrome c antibodies (clone 7H8.2C12) were from Pharmingen (San Diego, CA), polyclonal anti-B23 antibodies were from Santa Cruz Biotechnology, (Santa Cruz, CA), monoclonal anti-actin (Clone C4) antibodies were from ICN Biochemicals (Aurora, OH) and monoclonal anti-lamin A/C antibody 41CC was a kind gift from Dr B. Burke. Polyclonal anti-APAF-1 antibodies were kindly gift from Dr Wang, Texas University. Anti-topoisomerase I antibodies were purified from human serum from scleroderma patients by affinity chromatography (Mosgoeller et al., 1998) using immobilized full length human topoisomerase I derived from insect SF9 cells infected with recombinant baculovirus expressing human topoisomerase I (Whyte et al., 1995).

**Cell culture**

Human cervical carcinoma cell line HeLa were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum. Cells were grown to 70% confluence and then treated with CP (Lachema, Czech Republic) at a final concentration of 40 μM for 1, 3, 4.5, 6, 9, 10, 12, 15 and 24 hours.

**TUNEL assay**

The extent of apoptosis in HeLa cells was assessed by TUNEL (Boehringer Mannheim, Germany).

**Immunohistochemistry**

For microscopic investigations, cells were plated on slides in chambers and appropriately cultivated. After treatment for the indicated time, cells were washed three times in PBS and immediately fixed in cold methanol-acetone (3:2) mixture. The immunohistochemical procedure was performed using a highly sensitive peroxidase-labeled avidin-biotin system. The antibodies used were rabbit polyclonal antibody against the peptide (residues 178-192) of human caspase-3 (Upstate Biotech, USA), which recognizes the p12 cleavage product and anti-nucleolar antibody (Biogenex, USA) against nucleolar antigens. To avoid crossreactivity with the inactive precursor of caspase-3, the cells were also stained with polyclonal serum against residues 1-14 of the inactive precursor of human caspase-3. The cells were incubated with the primary antibodies overnight at 4°C in humidity chambers at the dilutions 1: 2000 and 1:5000. As second antibody, we used anti-rabbit IgG biotin conjugate (Dako, Denmark) at 1:50 dilution (45 minutes). The morphology of cells was examined by light microscopy. We evaluated nucleolar morphology in 200 randomly selected nuclei.

**Nucleolar staining**

For detection of AgNORs, we used the silver staining technique described by Smith (Smith et al., 1993). The slides were incubated in 33% silver nitrate colloid solution at 37°C for 15 minutes. The silver colloid was then washed out with distilled deionized water. Nucleolar RNA was stained with TB at pH 5 without previous fixation to visualise the RNA-containing structures. MGP was used according to the manufacturer’s instructions. All chemicals were purchased from Sigma (USA).

**Cell fractionation**

Isolation of nucleoli was performed as previously described in detail (Wesierska-Gadek et al., 1992). During all isolation steps phenylmethylsulfonylfluoride (PMSF) and Pefabloc were included at a final concentration of 1 mM and 50 μM, respectively. Briefly, PBS-washed cells were suspended in ice-cold low salt buffer, allowed to swell, and after addition of detergents (NP-40 and sodium deoxycholate), were homogenized. After centrifugation through a...
Segregation of nucleolar components

Sucrose cushion, pelleted nuclei were resuspended in 0.34 M sucrose solution and sonicated until no nuclei remained intact. The sonicate was underlaid with 0.88 M sucrose and centrifuged to sediment the purified nucleoli. Supernatant collected after sedimentation of nucleoli was designated as chromatin.

In experiments designed to examine the integrity of mitochondria, cells swollen in cold hypotonic medium for 20 minutes were fractioned by successive centrifugation steps yielding three subcellular fractions: crude nuclei pelleted after centrifugation at 800 \( g \) for 15 minutes, mitochondria after centrifugation at 8500 \( g \) for 15 minutes and cytosol (S-100) representing supernatant after centrifugation at 100,000 \( g \) for 60 minutes (Wesierska-Gadek et al., 1998).

**Electrophoretic separation of proteins and immunoblotting**

Total cellular proteins or proteins of the distinct subcellular fractions dissolved in SDS sample buffer were separated on 10% or 15% SDS slab gels and transferred electrophoretically onto polyvinylidene difluoride membrane (PVDF) (Amersham International). Equal protein loading was confirmed by Ponceau S staining. Blots were incubated with specific primary antibodies and the immune complexes were detected autoradiographically using appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescent detection reagent ECL+ (Amersham International) (Wesierska-Gadek et al., 1995). In some cases, blots were stripped and used for several sequential incubations.

**RESULTS**

**Nucleolar structure**

Normal HeLa cells contain intact nuclei with no condensation of cytoplasmic and nuclear content. Cells without nuclei and multinucleate cells were not included. Intact HeLa cells had generally one or two compact nucleoli (AgNORs as well as nucleolar RNA) with an average of three satellites localized in the central portion of the nucleus (Figs 1A,B, 2A,B).

In CP-treated cells (6, 9, 12 hours), we found small nucleolar fragments (6-20 dots) scattered across the whole nucleus as well as partially preserved larger centrally localized nucleoli (Fig. 1C). In the later stages (24 hours), large single aggregates (AgNORs) were also present in the central portion of the nucleus. Using the anti-nucleolar monoclonal antibody, we also found loss of stainability of the centrally localized nucleoli and positive dots at the periphery of the nuclei in CP-treated cells Fig. 1D).

Nucleolar RNA of CP-treated HeLa cells, detected by TB and MGP, was segregated after 12 hours (Fig. 2C,D). This type of quiescent nucleolar phenotype was previously described as micronucleoli (Smetana and Likovský, 1971; Smetana et al., 1975).

**Caspase-3 and TUNEL staining**

Untreated HeLa cells were negative for both activated caspase-3 and TUNEL staining. However, we found cytoplasmic and perinuclear positivity in CP treated cells after 6 hours (Table 1).

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Caspase-3 (%)</th>
<th>TUNEL (%)</th>
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<tr>
<td>3</td>
<td>5</td>
<td>72</td>
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<tr>
<td>6</td>
<td>8</td>
<td>69</td>
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<td>72</td>
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<td>24</td>
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**Fig. 1.** Visualization of nucleolar proteins in HeLa cells using histochemical staining for AgNOR (A,C) and anti-nucleolar antibody (B,D). (A,B) Intact HeLa cells with two prominent, centrally located nucleoli. (C,D) Nucleolar segregation in CP-treated HeLa cells after 6 hours; note a large number of fragments (black arrowhead) and a shift of nucleolar content to the periphery of nucleus (white arrowhead).

**Fig. 2.** Visualization of nucleolar RNA in HeLa cells using TB and MGP. (A,B) Intact HeLa cells with two prominent, centrally located nucleoli. (C,D) Nucleolar segregation in CP-treated HeLa cells after 12 hours (black arrowhead).
1), and they were TUNEL positive from 12 hours onwards (Table 1).

**Reduction of cell size after CP treatment**

The well defined sequence of apoptotic changes is characterized not only by morphological nuclear changes but also by alteration of plasma membrane resulting in surface blebbing and by cytoplasmic contraction. To investigate the effect of CP on the cell size, we determined the volume of cells in suspension by CASY. As shown in Fig. 3, a short CP treatment did not affect the size of HeLa cells. A slight reduction of cellular diameter was observed after 6 hours.

![Cell size measurements](image)

**Fig. 3.** Shrinkage of HeLa cells after prolonged treatment with CP. Untreated controls and cells treated with 40 μM CP for indicated periods of time were harvested and the cell size was determined by CASY. Measurements were performed in triplicate. The Y axis represents cell number and x-axis represents cell diameter (μm). The mean cell diameter is also listed for easy comparison of cell size after CP treatment.
Segregation of nucleolar components

However, after prolonged CP action (10 and 15 hours) HeLa cells were markedly shrunken, resulting in an approx. 15% reduction in cell size. Remarkably, the morphological changes of nucleoli in response to CP treatment preceded the reduction in cell size.

**Early degradation of PARP-1**

To assess the biochemical changes induced by CP treatment and their kinetics, total cell lysates or distinct subcellular fractions isolated from HeLa cells treated with CP for increasing periods of time were analyzed. As shown in Fig. 4, a very strong degradation of PARP-1 occurred by 4.5 hours post treatment and could be detected in total cell lysates. At 6 hours the intensity of the carboxy-terminal 89 kDa proteolytic fragment was greater than that of the intact enzyme. Sequential incubation of the blot with anti-actin antibodies confirmed the equal protein loading. The next immunoblotting experiments performed with proteins of isolated nuclei and nucleoli revealed that PARP-1 degradation proceeded very rapidly and after 10 hours CP treatment no intact PARP could be detected. Notably, the chromatin-bound and nucleolar PARP-1 were proteolytically processed with comparable kinetics and intensity. The sequential incubation of the blot with anti-B23 antibodies revealed that the nucleolar level of B23 phosphoprotein was diminished after prolonged CP action (at 10 and 15 hours). The reduction of nucleolar B23 level was due to its translocation into the nucleoplasm as described previously for another drug (Yung et al., 1990).

**Late cleavage of other nuclear proteins**

In the next step we examined whether other nuclear proteins previously reported to be apoptotic substrates are also affected in HeLa cells after CP administration. Indeed, topoisomerase I, nuclear lamins A and C and lamin B were also proteolytically processed (Fig. 5). However, the onset and even the extent of their degradation markedly differed from those of PARP-1. The cleavage of these proteins was retarded by about 6 hours as compared to PARP-1 and began 10 hours after drug administration. Furthermore, only a portion of the corresponding protein was fragmented whereas substantial amounts of the nuclear lamins and topoisomerase I remained unaffected and appeared as full-length protein bands. The proteolytic processing proceeded relatively slowly since after a further 5 hours the intensity of apoptotic cleavage fragments did not essentially increase. Moreover, a comparison of the lamin A and C processing in subnuclear compartments showed more rapid terminal degradation in chromatin than in the nucleolus (Fig. 5).

**Early activation of the caspases 3 and 9**

The analysis of the action of apoptotic proteases on specific substrates revealed that they are not chaotically and randomly degraded but are processed in a systematic and orchestrated manner. Therefore, we determined the temporal and spatial pattern of the activation of caspase-3 specifically targeting PARP-1, the major substrate of the early apoptosis after CP administration. Conversion of the proenzyme into the activated form was detected in total cell lysates 4.5 hours post treatment (Fig. 6; right panel). During prolonged CP treatment 32 kDa
band disappeared even in the cytosol fraction (Fig. 6, left panel) indicating that the proenzyme was almost completely processed to the activated form. The cleavage of procaspase-3 and simultaneous generation of immunoreactive low molecular mass fragments p20/p17, detected by immunoblotting, preceded the appearance of the positive signals specific for activated caspase-3 in the cytoplasm as shown by immunohistochemical staining (Table 2). This result reflects the higher sensitivity of the immunoblotting test. Moreover, procaspase-3, found exclusively in the cytoplasm of control untreated HeLa cells, moved into the nucleus in response to CP action. As shown in Fig. 7 a substantial portion of procaspase-3 and its activated form was detected in the isolated nuclei 6 hours post-treatment thereby explaining how the nuclear and nucleolar PARP-1 comes into contact with apoptotic protease normally localized in the cytoplasm.

**Activation of caspase-9 precedes the maturation of caspase-3**

Caspase-3 is known to be predominantly activated by caspase-9, although some alternative pathways have also been described. We examined the activation of the caspase-9 and found that the earliest generation of the activated heterodimers, 3 hours after drug administration, slightly preceded the activation of caspase-3 (Fig. 8). Furthermore, we additionally monitored the cytoplasmic levels of Apaf-1 and cytochrome c in response to CP treatment. To assess the release of cytochrome c from the mitochondria, we analysed the S-100 cytosolic fraction. As shown in Fig. 9A CP treatment resulted in stepwise release of cytochrome c from the mitochondria into the cytosol. A marked cytochrome c accumulation in cytosol was observed 6 hours after CP treatment and coincided with the increase in the level of Apaf-1 (Fig. 9B). The simultaneous accumulation of cytochrome c and Apaf-1 in cytosol provides evidence that mature caspase-9 is derived from its procaspase precursor as a result of recruitment by the activating factor Apaf-1 in cooperation with the released cytochrome c.

**DISCUSSION**

The present study shows an association of caspase-3-induced degradation of PARP-1 with an alteration of nucleolar structure (segregation) in the onset of PCD.

The activation of distinct caspases (Wesierska-Gadek et al., 1991; Wesierska-Gadek et al., 1999) and degradation of their downstream protein substrates was executed in two phases (D’Amours et al., 1999). During an early apoptotic stage beginning 4.5 hours post-treatment, activation of caspase-9 and caspase-3 was observed. This was accompanied by proteolytic cleavage of PARP-1. The procaspase-9 activation seemed to be mediated by recruitment by the apoptotic protease-activating factor, Apaf-1, because the increased accumulation of Apaf-1 and cytochrome c in cytosol preceded the generation of the mature caspase-9 form (Wesierska-Gadek et al., 1996). A second phase of apoptosis occurring between 10 and 15 hours

**Table 2. Detection of nucleolar segregation in CP-treated HeLa cells (%)**

<table>
<thead>
<tr>
<th>Staining methods for nucleoli</th>
<th>CP treatment of HeLa cells (hrs)</th>
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<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>NOR</td>
<td>–</td>
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<tr>
<td>ANA*</td>
<td>–</td>
</tr>
<tr>
<td>TB</td>
<td>–</td>
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<tr>
<td>MGP</td>
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*ANA, anti-nucleolar antibody.

**Fig. 6** Maturation of caspase-3 in response to CP treatment. Proteins of the crude cytosol fraction obtained as postnuclear supernatant or total cell lysate (20 μg/lane) were separated on 15% SDS gel. Immunoblotting was performed with polyclonal anti-caspase-3 antibodies and subsequently with monoclonal anti-bcl antibodies. Sequential incubation with anti-bcl-2 confirmed the equal protein loading. M-carbonic anhydrase (30 kDa) was loaded as a molecular mass marker.

**Fig. 7** A partial translocation of caspase-3 into the nucleus after CP treatment. Proteins of crude cytosol (C) fraction and of isolated nuclei (N; 20 μg/lane) were resolved on 15% SDS slab gel. The blot was incubated with polyclonal anti-caspase-3 antibodies.
post-treatment was characterized by degradation of other nucleolar and nuclear proteins such as nuclear lamins, topoisomerase I and B23.

Our biochemical analysis of CP-induced apoptosis in HeLa cells conforms with the view that PCD is executed in three chronologically distinct phases. The first phase is a reversible sequence of metabolic and cell cycle adaptation steps (e.g. mitochondrial cytochrome c production and bcl-2 gene activation). The second phase is irreversible and derives from a cascade of proteolytic signals originating from mitochondria and translocated into the nucleus. The third phase of apoptosis is manifested by the macromolecular degradation of chromosomal DNA and catalyzed by caspase-activated death factor. The key nuclear proteins (e.g. PARP) are degraded by caspase-3 in an early stage of apoptosis. This leads to the disintegration of the nucleus.

It suggests that nucleolar PARP-1 plays a role in preribosome processing via modification of specific nucleolar proteins that bind to nascent transcripts. This theory is additionally supported by the fact that two major nucleolar proteins, B23 and C23, are ADP-ribosylated in HeLa cells (Leitinger and Wesierska-Gadek, 1993). Modification of both proteins has been found to occur if nuclei or isolated nucleoli are incubated with radioactively labeled substrate (Leitinger and Wesierska-Gadek, 1993) thereby additionally substantiating the nucleolar localization of PARP-1. Nucleoli associated enzyme seems to represent a distinct subdivision of the nuclear PARP-1. There are at least three independent lines of evidence for the distinctiveness of the nucleolar PARP-1. First, a monoclonal anti-PARP-1 antibody, F-2, has been characterized to recognize only nucleoli-associated enzyme in cell preparations. A second line of support for the distinctiveness of nucleolar PARP-1 comes from studies with human autoimmune sera. Some autoimmune sera monospecific for PARP-1 have been shown to stain only the nucleoli (Wesierska-Gadek et al., 1991; Yamanaka et al., 1987). Finally, an overexpression of mutant temperature-sensitive p53 in primary rat cells led to cytoplasmic sequestration of PARP-1 by mutant p53 (Wesierska-Gadek et al., 1996). However, immunostaining of the cells revealed recruitment of only chromatin-bound PARP-1 whereas nucleoli-associated enzyme remained unaffected (Wesierska-Gadek et al., 1996). Activated caspase-3, recognizing a DEVD tetrapeptide motif within the amino-terminal domain of PARP-1, generates two proteolytic products, a 29 kDa amino-terminal fragment encompassing the DNA-binding domain and an 89 kDa carboxyl terminus harboring the substrate binding pocket. Caspase-3 induced PARP-1 cleavage separates the DNA-binding domain, essential for the stimulation of the enzyme, and the catalytic domain.

For evaluation of nucleolar morphology, we used histochemical staining of AgNORs, which contain critical

Fig. 8. Activation of caspase-9. Total cell lysates were separated on 15% SDS gel. Blots were incubated with anti-caspase-3 antibodies (left panel) and with anti-caspase-9 antibodies. Equal protein loading was confirmed by sequential incubation with anti-actin antibodies.

Fig. 9. (A) Release of cytochrome c coincides with accumulation of APAF-1 in cytosol. Proteins of isolated cytosol (S-100) and mitochondria were separated on 15% SDS gels. Immunoblotting was performed with monoclonal anti-cytochrome c antibodies. (B) Proteins of isolated cytosol (S-100) were resolved on 10% SDS gel. Membrane was incubated with anti-APAF polyclonal antibody (upper panel). Ponceas S stain of the blot (lower panel) confirms nearly equal protein loading. Rainbow marker was loaded in lane M. Distinct bands of defined molecular mass are shown.
transcriptional factors (UBF, RNA polymerase) showing avidity to bind silver ions (Scheer and Benavente, 1990; Wachtler et al., 1986). MGP and TB were used to visualize nucleolar RNA content and discriminate transcriptional activation (nucleoli with nucleolonemata) and inactivation (micronucleoli) (Smetana and Likovský, 1972). We observed a rapid onset of nucleolar segregation of argyrophilic proteins as well as nucleolar RNA (micronucleoli), increased immunoreactivity of caspase-3 (at 6, 9 and 12 hours) and TUNEL positivity at 12 and 24 hours. Similarly, we detected a remarkable shrinkage of nuclei by 15% after 10 hours of CP treatment.

Recent immunohistochemical studies have revealed a nucleolar localization of a variety of, so called, death effector domain molecules resopible for execution of an early proteolysis in the onset of active cell death (e.g. caspase-8, MADD). For example, there are studies showing alteration of nucleolar structure induced by anticancer drugs, e.g. 5-fluorouracil-induced title aggregation of NORs in the nuclei of MCF-7 human breast carcinoma cell line (Nishihara et al., 1996). Similarly, NORs have been shown to be altered cardiac myocytes treated with doxorubicin (Leblanc et al., 1991).

Our biochemical and morphological findings agree with the above noted experiments showing a rapid onset of apoptosis and proving involvement of nucleoli, by the rapid disintegration of nucleolar structure (6 hours) that coincides not only with in situ nuclear positivity for active caspase-3 but also with our biochemical data showing a rapid degradation of PARP-1 driven by caspases and subsequent phase of proteolysis of nuclear/nucleolar proteins (B23, topoisomerase I and lamins).

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