Nuclear Apaf-1 and cytochrome *c* redistribution following stress-induced apoptosis

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Abstract Apoptotic protease activating factor-1 (Apaf-1) and cytochrome *c* are cofactors critical for inducing caspase-9 activation following stress-induced apoptosis. One consequence of caspase-9 activation is nuclear–cytoplasmic barrier disassembly, which is required for nuclear caspase-3 translocation. In the nucleus, caspase-3 triggers proteolysis of the caspase-activated DNA nuclease (CAD) inhibitor, causing CAD induction and subsequent DNA degradation. Here we demonstrate that apoptotic cells show perinuclear cytochrome *c* aggregation, which may be critical for nuclear redistribution of cytochrome *c* and Apaf-1. We thus indicate that the nuclear redistribution of these cofactors concurs with the previously reported caspase-9-induced nuclear disassembly, and may represent an early apoptotic hallmark. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Apaf-1; Caspase-9; Cytochrome *c*; Stress-induced apoptosis; Subcellular localization

1. Introduction

The execution mechanism of stress-induced apoptosis is mediated by a group of proteases known as caspases (cysteinyl aspartate-specific proteinases) [1], which carry out the apoptotic program through a sequential activation cascade of initiator and executioner caspases [2]. Apaf-1 (apoptotic protease activating factor-1) is a cofactor that induces activation of initiator caspase-9 [3,4]. Apaf-1 binds caspase-9 via the caspase recruitment domains at their NH_2 termini, triggering the formation of a supramolecular complex called the apoptosome [3,4]. When activated, initiator caspase-9 triggers subsequent proteolytic activation of executioner caspase-3 [3].

During stress-induced apoptosis, caspase activation also requires a large number of post-translational mechanisms, including translocation to other organelles [5]; an example of these mechanisms is caspase-9 translocation to the nucleus [6,7]. In the nucleus, caspase-9 has been implicated in nuclear–cytoplasmic barrier disassembly, allowing an increase in the diffusion limit of nuclear pores [8]. This process induces caspase-3 to enter the nucleus by diffusion [8], where it triggers caspase-activated DNA nuclease by proteolysis of its inhibitor, causing DNA degradation [9,10].

Since caspase-9 redistributes from cytosolic compartments to the nucleus during stress-induced apoptosis, and caspase-9 activation requires Apaf-1 to form an active holoenzyme [11], we studied whether this cofactor also redistributes to the nucleus during apoptosis. Here we demonstrate that apoptotic cells show perinuclear cytochrome *c* aggregation as well as apparent Apaf-1 redistribution to the nucleus after stress-induced apoptosis. Taken together, these results corroborate previous reports showing caspase-9 translocation to the nucleus and indicate that, during stress-induced apoptosis, Apaf-1 appears to translocate from cytoplasmic organelles to the rest of the cell, including the nucleus. This redistribution may be required to allow the increase in the local Apaf-1 concentration necessary for caspase-9 triggering of nuclear–cytoplasmic barrier disassembly.

2. Materials and methods

2.1. Cell culture

NIH-3T3 fibroblasts and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES and 50 μM 2-mercaptoethanol (Sigma, St. Louis, MO, USA), and maintained at 37°C in a humidified 5% CO_2 atmosphere.

2.2. Antibodies and reagents

Anti-active caspase-3 antibody was purchased from Promega (Madison, WI, USA), human anti-mitochondrial antibody was used as described [12], and monoclonal anti-cytochrome *c* antibody was from Pharmingen (San Diego, CA, USA). Mouse anti-β-tubulin (clone tub 2.1), actinomycin D and cisplatin were from Sigma. Rabbit anti-Apaf-1 antibody was used as described [13].

2.3. Immunofluorescence and image acquisition

For immunofluorescence, cells were cultured in chamber slides, washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (15 min, room temperature), pre-incubated in 2% bovine serum albumin (BSA), and incubated for 1 h with primary antibody in PBS containing 0.5% BSA and 0.1% Triton X-100. Cells were washed three times in the same buffer and incubated for 1 h with Cy2-, Cy3- or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). After washing, samples were incubated with TOPRO-3 (Molecular Probes, Eugene, OR, USA) in PBS for DNA staining. Serial Z-sections were obtained with an Ar–Kr laser and a TCS-NT Leica confocal imaging system equipped with a 63×1.4 oil PLAPO objective. Cy2 was analyzed at 488 nm, Cy3 at 568 nm, and Cy5/TOPRO-3 at 647 nm. Images for each channel were captured separately and assembled into a single file with TCSMERGE software (Leica Microsystems, Heidelberg, Germany) prior to analysis. All confocal images were analyzed using the Leica Vista+ (Beta Release 2) program. Images were processed digitally using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

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Abbreviations: Apaf-1, apoptotic protease activating factor-1

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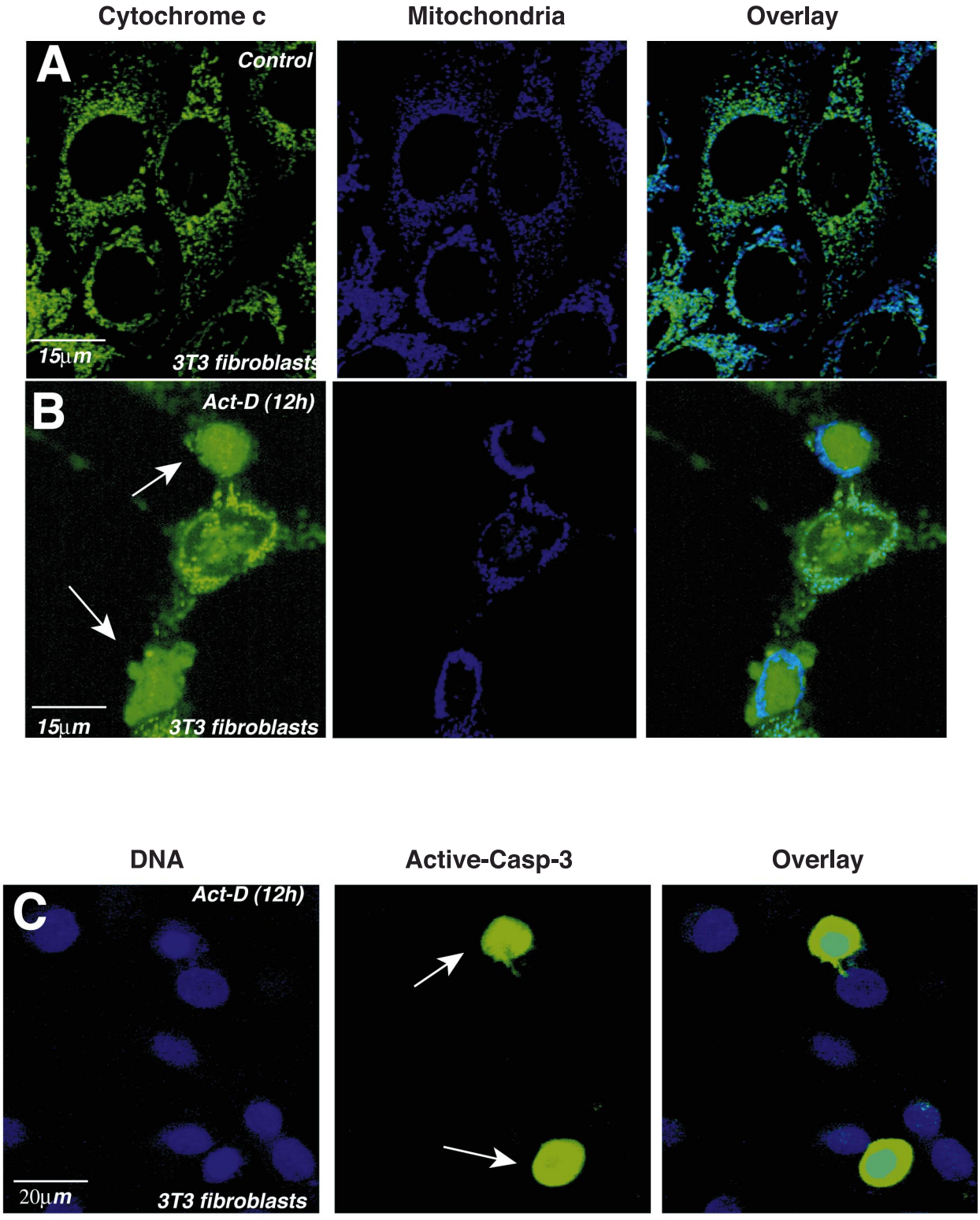

Fig. 1. Nuclear caspase-3 activation during stress-induced apoptosis. A: Untreated control NIH-3T3 fibroblasts. NIH-3T3 fibroblasts were cultured and fixed as described (Section 2), then incubated with mouse anti-cytochrome *c* and human anti-mitochondrial antibodies (Section 2); Cy2-conjugated anti-mouse (green) and Cy5 anti-human secondary antibodies (blue) were used. B: Actinomycin D-treated NIH-3T3 fibroblasts (5 μg/ml, 12 h) were fixed and stained as above. C: NIH-3T3 fibroblasts as in B were pretreated as before, then incubated with rabbit anti-active caspase-3 antibody; Cy2-conjugated anti-rabbit secondary antibody (green) was used. Samples were washed, incubated with TOPRO-3 (blue) in PBS, then analyzed by confocal microscopy (Section 2).

2.4. Measurement of caspase-9 activity

Cytosolic protein (20 μg) was diluted five-fold in caspase buffer (25 mM HEPES pH 7.5, 0.1% CHAPS, 10% sucrose, 10 mM dithiothreitol and 0.1 mg/ml ovalbumin) and incubated (1 h, 37°C), with the fluorescent substrate Ac-LEHD-AMC (acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin; 10 mM); reactions were terminated by adding HPLC buffer (water/acetonitrile 75/25, 0.1% trifluoroacetic acid). Cleaved substrate fluorescence was determined by C_{18} reverse phase HPLC using fluorescence detection (338 nm excitation, 455 nm emission). Control experiments confirmed linearity of substrate release with time and protein concentration.

3. Results and discussion

Caspase-9 triggers nuclear–cytoplasmic barrier disassembly, causing caspase-3 to enter the nucleus by diffusion [8]. According to this model, active caspase-3 is localized in the nucleus during apoptosis. To validate this hypothesis, we treated NIH-3T3 cells with actinomycin D, which causes stress-induced apoptosis, including cytochrome *c* release from mitochondria and caspase-9 activation [14]. We analyzed whether cytochrome *c* redistributes during actinomycin D-induced apoptosis in NIH-3T3 cells. Co-localization analysis in healthy NIH-3T3 cells showed cytochrome *c* in mitochondria (Fig. 1A); in apoptotic NIH-3T3 cells, however, there was striking perinuclear mitochondrial aggregation, as well as cytochrome *c* redistribution to the nucleus (Fig. 1B). These data indicate that cytochrome *c* is released from mitochondria during actinomycin D-induced apoptosis. Moreover, we studied the localization of active caspase-3 during actinomycin D-induced apoptosis using an anti-caspase-3 antibody that specifically stains active caspase-3 in apoptotic cells. Analysis of actinomycin D-treated NIH-3T3 cells showed active caspase-3 localization in nuclei (Fig. 1C), confirming nuclear caspase-3 localization in apoptotic cells and thus validating the model.

Caspase-9 activation requires the cofactor Apaf-1 to form an active holoenzyme [11]. We analyzed whether, during apoptosis, Apaf-1 undergoes subcellular redistribution, which may be required for caspase-9-induced nuclear–cytoplasmic barrier disassembly. Caspase activation induces cytoskeletal collapse during apoptosis; this process is due to the degradation of multiple structural proteins associated with the cytoskeletal network [15]. To study whether Apaf-1 redistributes to the nucleus during apoptosis, we used cytoskeletal collapse as an indicator of actinomycin D-induced apoptosis. Cytoskeletal collapse is detected during apoptosis using the β-tubulin microtubule marker. Confocal microscopy analysis of Apaf-1 and β-tubulin in untreated cells indicated that Apaf-1 did not co-localize with the microtubule network (Fig. 2A). Apaf-1 and β-tubulin localization was analyzed by immunostaining after 12 h of actinomycin D treatment. In actinomycin D-treated cells, both a marked collapse in the microtubule network and cell shrinkage were observed (Fig. 2B). We also detected Apaf-1 in the nucleus of apoptotic cells, indicating apparent Apaf-1 redistribution during actinomycin D-induced apoptosis (Fig. 2B). These data suggest that, during stress-induced apoptosis, Apaf-1 translocates from cytoplasmic organelles to the rest of the cell, including the nucleus.

To test whether Apaf-1 and cytochrome *c* accumulated within the nucleus or were adsorbed to the nuclear envelope early in apoptosis, we studied Apaf-1 and cytochrome *c* redistribution in HeLa cells at different times after cisplatin treatment. We found that cytochrome *c* redistributes 4 h after cisplatin treatment, forming a ring structure (Fig. 3A,B). In addition, we detected a diffuse cytochrome *c* pattern in the nucleus at 8 h (Fig. 3C), a time point at which more than 60% of cells are clearly apoptotic (not shown). These data indicate that the cytochrome *c* release process may have two stages; in

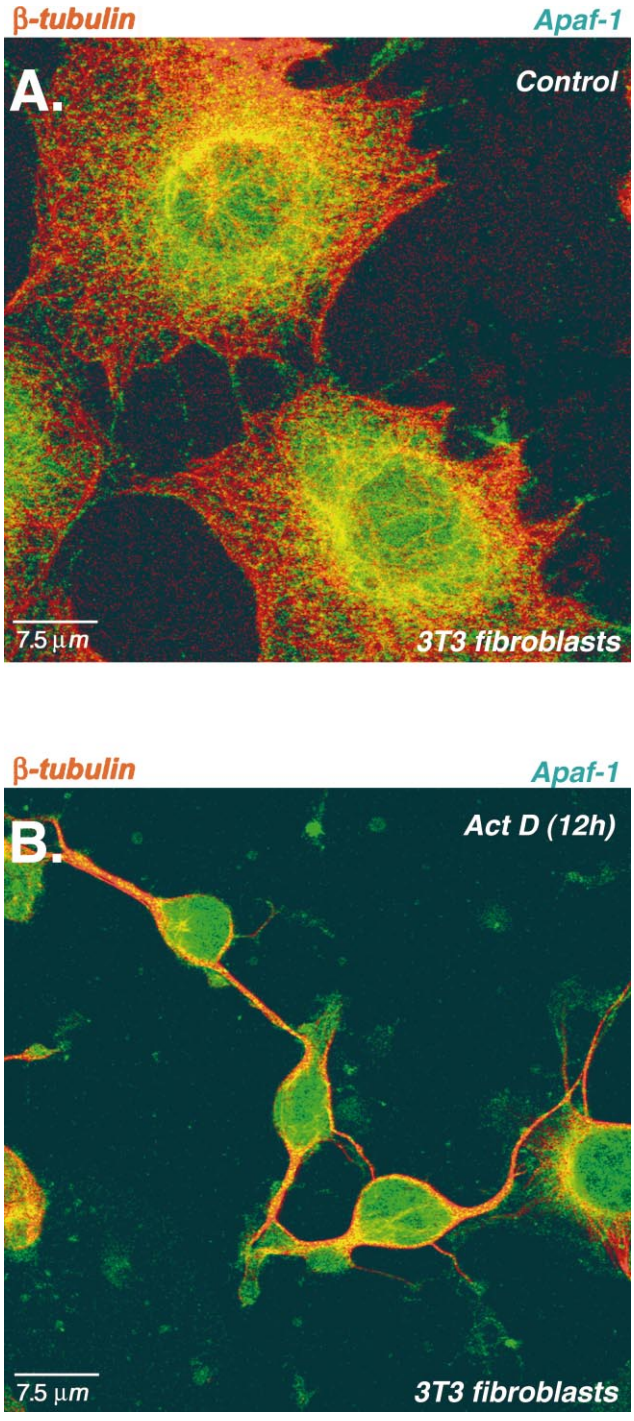

Fig. 2. Cytoskeletal collapse and nuclear redistribution of Apaf-1 during stress-induced apoptosis. A: Untreated NIH-3T3 fibroblasts were pretreated as in Fig. 1, then incubated (1 h) with rabbit anti-Apaf-1 (green) and mouse anti-β-tubulin (red). After incubation, samples were washed, then incubated (1 h) in Cy2 anti-rabbit and Cy3 anti-mouse antibodies. After washing, samples were analyzed and serial Z-sections obtained as above. B: Cells were actinomycin D-treated for 12 h, then processed as above. The data are representative of the total cell population.

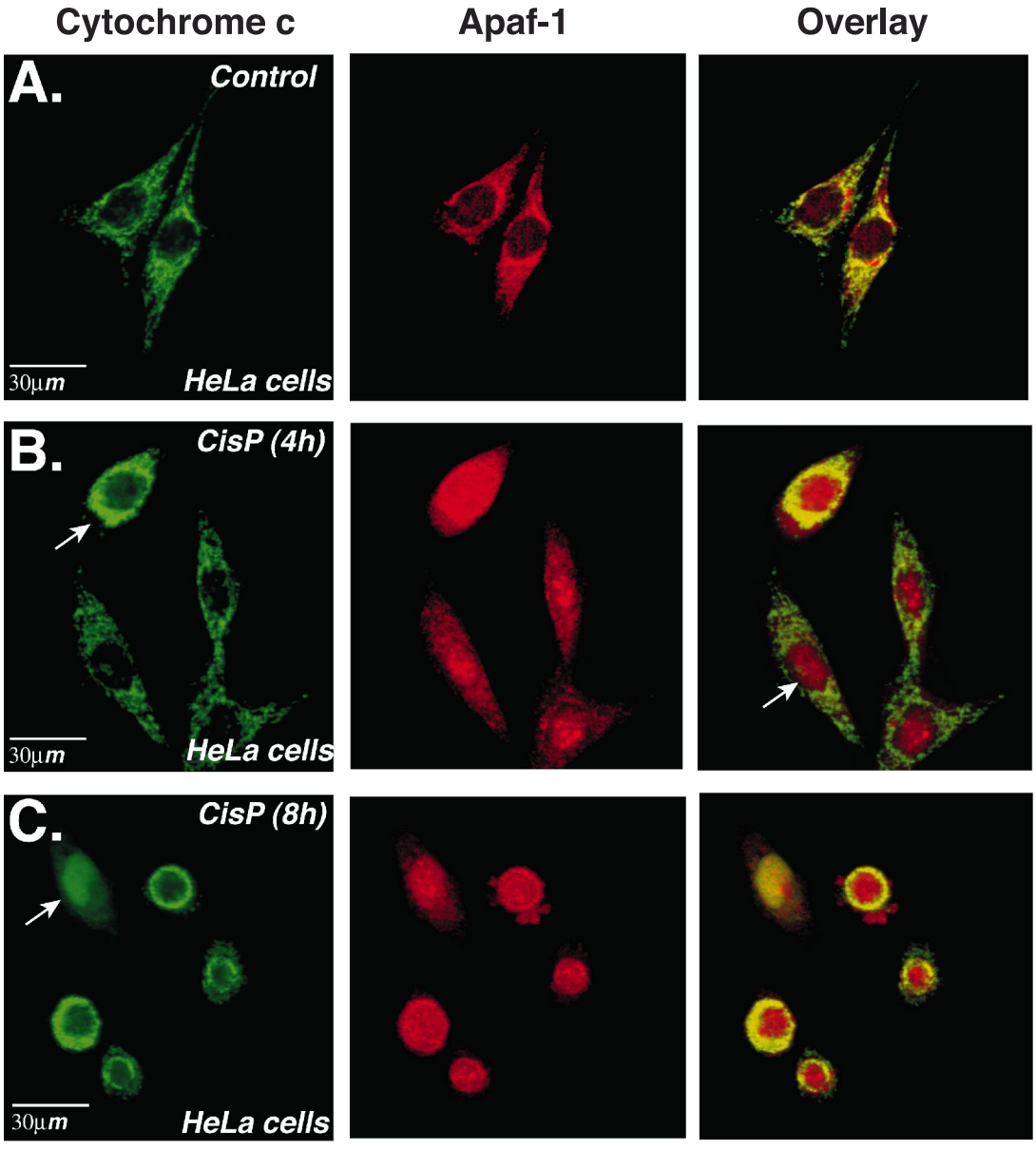

Fig. 3. Apaf-1 redistributes earlier than cytochrome *c*. A: Untreated HeLa cells. B: Cisplatin-treated HeLa cells (10 μg/ml, 4 h). C: Cisplatin-treated HeLa cells (10 μg/ml, 8 h). After treatment, cells were fixed and pretreated as in Fig. 1, then incubated (1 h) with rabbit anti-Apaf-1 (red) and mouse anti-cytochrome *c* antibodies (green). After washing, samples were incubated (1 h) in Cy3 anti-rabbit and Cy2 anti-mouse antibodies, and analyzed as before.

the first, cytochrome *c* would move to the nuclear envelope, in the second, it would be redistributed within the nucleus. Apaf-1 is nonetheless detected inside the nucleus as early as 4 h after cisplatin treatment, a time at which cytochrome *c* does not show nuclear localization.

We next analyzed whether Apaf-1 and cytochrome *c* redistribution is associated with caspase-9 activation. NIH-3T3 cells were cisplatin-treated at several time points, after which Apaf-1 and cytochrome *c* localization were analyzed by immunostaining; caspase-9 activity was measured in parallel. We found caspase-9 activity as well as Apaf-1 redistribution at a time at which cytochrome *c* did not show nuclear localization, suggesting that caspase-9 activation and Apaf-1 translocation occur earlier than this characteristic pattern of cytochrome *c* release (Fig. 4A,B).

A growing number of studies suggest that relocalization and aggregation of apoptotic signaling proteins are important steps in caspase activation. Apaf-1 triggers its apoptotic effect by oligomerization and subsequent activation of caspase-9 [16]. The fact that Apaf-1 is a transcriptional target of p53 also indicates that the cellular concentration of Apaf-1 is an important molecular determinant of the stress-induced apoptosis threshold [17–19]. In this study, we show apparent subcellular redistribution of Apaf-1, which may be required to allow the increase of the local Apaf-1 concentration necessary for caspase-9 triggering of nuclear–cytoplasmic barrier disassembly. Further studies are needed, however, to determine whether the relationship described here between Apaf-1 subcellular redistribution and stress-induced apoptosis has a role in in vivo caspase-9 activation.

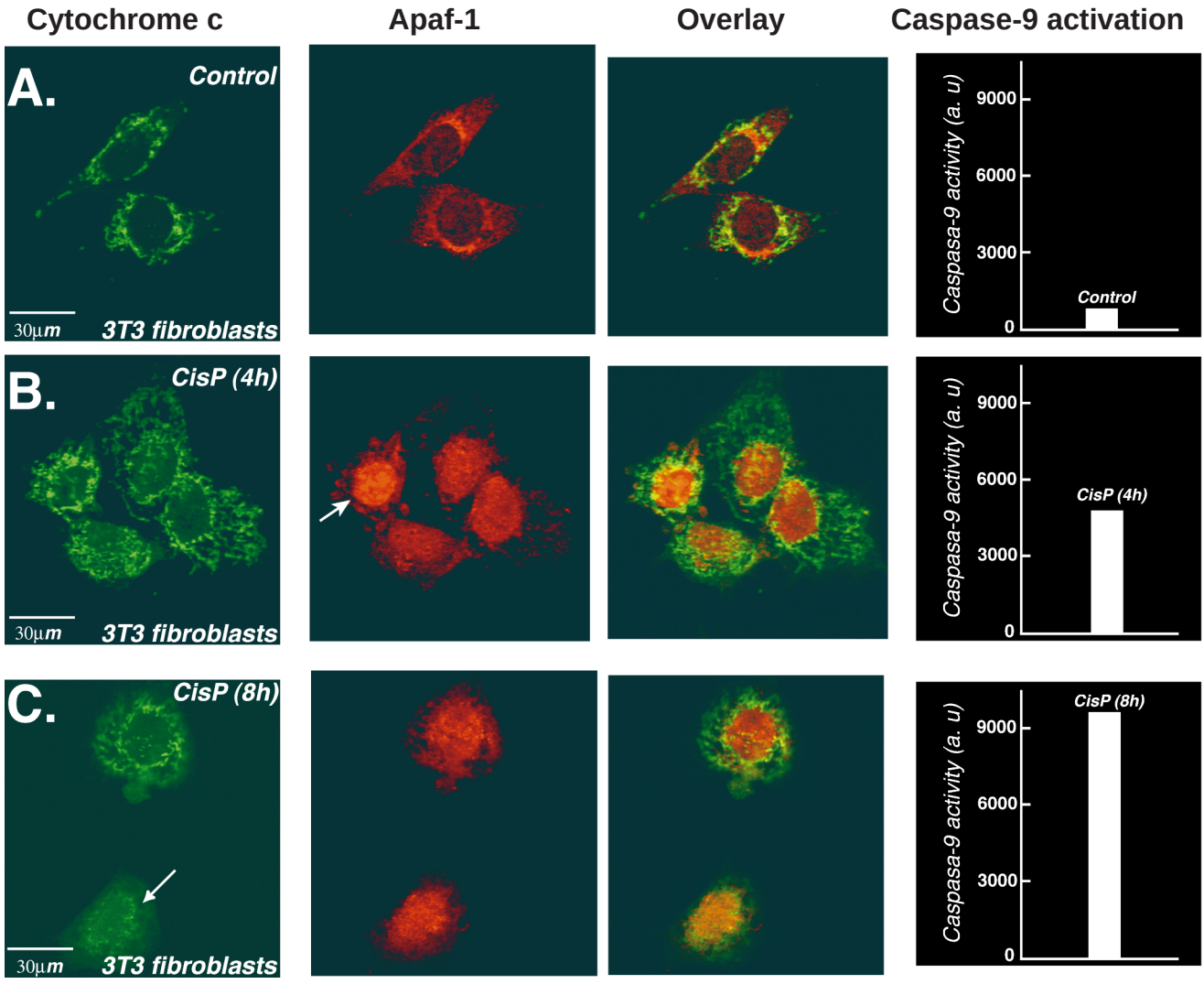

Fig. 4. Apaf-1 redistribution is associated with caspase-9 activation. A: Untreated NIH-3T3 fibroblasts. B: Cisplatin-treated NIH-3T3 fibroblasts (10 μg/ml, 4 h). C: Cisplatin-treated NIH-3T3 fibroblasts (10 μg/ml, 8 h). After treatment, cells were fixed, pretreated, stained and analyzed as in Fig. 3C. Untreated and cisplatin-treated cells were lysed in parallel to measure caspase-9 activity.

In the nematode *Caenorhabditis elegans*, the CED-4 protein (the Apaf-1 homologue) triggers activation of CED-3 (caspase homologue) following CED-4 nuclear redistribution [20]. This phenomenon is observed here in mammalian cells, suggesting the conservation of this Apaf-1 function throughout evolution.

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References

[1] Ruiz-Vela, A. and Martínez-A, C. (2001) Inmunología 20, 143–152.
[2] Salvesen, G.S. and Dixit, V.M. (1997) Cell 91, 443–446.
[3] Zou, H., Henzel, W.J., Liu, X., Lutschg, A. and Wang, X. (1997) Cell 90, 405–413.
[4] Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cell 91, 479–489.
[5] Porter, A.G. (1999) Trends Cell Biol. 9, 394–401.
[6] Krajewski, S., Krajewska, M., Ellerby, L.M., Welsh, K., Xie, Z., Deveraux, Q.L., Salvesen, G.S., Bredesen, D.E., Rosenthal, R.E., Fiskum, G. and Reed, J.C. (1999) Proc. Natl. Acad. Sci. USA 96, 5752–5757.
[7] Ritter, P.M., Marti, A., Blanc, C., Baltzer, A., Krajewski, S., Reed, J.C. and Jaggi, R. (2000) Eur. J. Cell Biol. 79, 358–364.
[8] Faleiro, L. and Lazebnik, Y. (2000) J. Cell Biol. 151, 951–960.
[9] Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W.T. and Wang, X. (1998) Proc. Natl. Acad. Sci. USA 95, 8461–8466.
[10] Sakahira, H., Enari, M. and Nagata, S. (1998) Nature 391, 96–99.
[11] Rodríguez, J. and Lazebnik, Y. (1999) Genes Dev. 13, 3179–3184.
[12] Clavería, C., Albar, J.P., Serrano, A., Buesa, J.M., Barbero, J.L. and Martínez-A, C. (1998) EMBO J. 17, 7199–7208.
[13] Ruiz-Vela, A., Albar, J.P. and Martinez-A, C. (2001) FEBS Lett. 501, 79–83.
[14] Ruiz-Vela, A., González de Buitrago, G. and Martínez-A, C. (1999) EMBO J. 18, 4988–4998.

[15] Núñez, G., Benedict, M.A., Hu, Y. and Inohara, N. (1998) Oncogene 17, 3237–3245.
[16] Qin, H., Srinivasula, S.M., Wu, G., Fernandes-Alnemri, T., Alnemri, E.S. and Shi, Y. (1999) Nature 399, 549–554.
[17] Perkins, C., Kim, C.N., Fang, G. and Bhalla, K.N. (1998) Cancer Res. 58, 4561–4566.
[18] Kamarajan, P., Sun, N.K., Sun, C.L. and Chao, C.C.K. (2001) FEBS Lett. 505, 206–212.
[19] Moroni, M.C., Hickman, E.S., Denchi, E.L., Caprara, G., Colli, E., Cecconi, F., Müller, H. and Helin, K. (2001) Nature Cell Biol. 3, 552–558.
[20] Chen, F., Hersh, B.M., Conradt, B., Zhou, Z., Riemer, D., Gruenbaum, Y. and Horvitz, H.R. (2000) Science 287, 1485–1494.