Removal of proteasomes from the nucleus and their accumulation in apoptotic blebs during programmed cell death

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Received 9 August 1996

Abstract Apoptosis can be initiated in immortalized cAMP-stimulated rat ovarian granulosa cells by induction of wild-type p53 activity. Immunocytochemical studies using confocal microscopy reveal that in apoptotic, unlike in normal growing cells, the proteasomes are removed from the nucleus and accumulate within the apoptotic blebs at the periphery of the cell. In parallel, a striking reorganization of the actin cytoskeleton is observed which forms a spherical network separating the apoptotic blebs from the cytoplasmic organelles, such as mitochondria and lipid droplets which remain in the perinuclear region. The reorganization of the actin cytoskeleton as well as disappearance of proteasomes from the nucleus suggest possible function of proteasomes in apoptotic regulation.

Key words: Granulosa cell; Proteasome; Actin cytoskeleton; p53; Apoptosis

1. Introduction

Programmed cell death refers to a process in which a specific cell population is eliminated from the living organism in response to a variety of extracellular and intracellular stimuli [1–5]. Some of the morphological and molecular events leading to cell death have been characterized in a variety of cell types. It is believed that endonucleases [1–5], DNAase [6] and cysteine proteases of the ICE (Interleukin-1β converting enzymes) family [7,8] are critically involved in this process. It seems that molecular events, such as activation of the genes coding for the ICE proteases, have been conserved during evolution [9]. Yet it is not clear which proteins are actually degraded during apoptosis and which proteases beside the ICE-like proteases are involved in protein degradation during this process.

One of the most abundant intracellular proteases, which is not compartmentalized in lysosomes but found both in the nucleus and in the cytoplasm of cells, is the proteasome [10,11]. In its 26S form it is the central protease of the ubiquitin-dependent pathway of protein degradation and has a highly conserved structure from yeast to man [12,13]. The proteasome is known to degrade a large number of key regulatory proteins [14], but its role in apoptosis is as yet unknown. It was recently reported that ubiquitin gene expression is up-regulated during apoptosis [15]. However, there is no direct evidence for an involvement of the ubiquitin-proteasome-dependent degradation in apoptosis.

Apoptosis can be induced by overexpression of a temperature-sensitive (ts) mutant of the p53 tumor suppressor in cAMP-stimulated, *ras*-SV40 transformed, granulosa cell lines [16]. In such cells, shifting the temperature of growth from 37°C to 32°C allows the manifestation of the wild-type p53 phenotype, as evident by up-regulation of the WAF-1/CIP-1 gene and arrest of cell growth.

We found that while most of the proteasomes were accumulated in the apoptotic blebs during programmed cell death, the organelles such as mitochondria and lipid droplets remain intact in the perinucomiclear region during this process. Interestingly, confocal microscopy shows that concomitant with the sequestering of the proteasomes, the cytoskeleton is rearranged creating a barrier which separates organelles from the apoptotic blebs.

2. Materials and methods

2.1. Cell cultures

Immortalized granulosa cell line (GTS5), transfected by Simian virus (SV40) DNA, plus Ha-*RAS* oncogene and by the temperature-sensitive mutant of p53 (p53 val 135), were used for these studies [16]. This mutant was found to be temperature-sensitive: at 37°C it behaves like other p53 oncogenic mutants, while at 32°C it possesses a wild-type activity. Cells were maintained on Nunc petri dishes containing DMEM:F12 medium (1:1) supplemented with penicillin (100 IU/ml) streptomycin (100 μg/ml) and 5% fetal calf serum (FCS) for 24 h at 37°C and the medium was replaced by serum-free medium in the presence or absence of 50 μM of forskolin for another 24 h and subsequently cells were cultured at 37°C or at 32°C.

2.2. Biochemical assays

Cell protein, progesterone production and chymotrypsin-like proteolytic activity of proteasomes assayed with succinyl-Ala-Ala-Phe-7-aminomethylcoumarin were described by us elsewhere [11,16].

2.3. Preparation of polyclonal antibodies to proteasomes; gel electrophoresis and Western blots

Polyclonal antibodies to granulosa cell proteasomes were raised in rabbits following immunizing the rabbits with pure fraction of 20S proteasomes isolated from POGRS1 rat granulosa cells. For isolation and purification of the proteasomes, gel-electrophoresis and Western blots, see Ref. [11].

2.4. Fixation of cells and staining

Cells were fixed with 3% paraformaldehyde and permeabilized with Triton X-100. The detailed procedure of staining the cells with anti-proteasomes and anti-human adrenodoxin antibodies is described by us elsewhere [11,17]. Staining the actin cytoskeleton by rhodamine phalloidin is described by us elsewhere [18]. Staining of DNA with 4,6-diamido-2-phenylindol hydrochloride (DAPI) was described elsewhere [16].

2.5. Fluorescent microscopy

Fixed and stained cells were inspected in a Zeiss photoIII microscope in a fluorescent mode. For the visualization of DAPI, BP436 filter was used.

2.6. Laser confocal microscopy

Optical sections of the cells were taken with a LSM 400 Zeiss confocal microscope (Zeiss, Oberkochen, Germany). Fields of 256×256 pixels were scanned using the plan neofluor 40× objective

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PII S0014-5793(96)00920-9

and a pinhole size of 8. Due to the resolution limit, optical serial sections of a thickness of 0.4 μm were recorded. FITC and rhodamine signals were collected in parallel or subsequently using the appropriate lasers and filter sets (FITC: laser 488 nm, filter BP 510-525 beamsplitter FT 510; rhodamine: laser 543 nm, filter LP 570, beamsplitter FT 560). In parallel, the transmission picture was recorded in pseudo Nomarsky interference contrast. All pictures were transferred to an Indigo 2 (Silicon Graphics, Mt. View, CA, USA) and galleries of the 256 grayscale pictures were obtained with the program Advanced Visual System (Advanced Visual System Inc., Waltham, MA, USA).

2.7. Three-dimensional reconstruction

Picture stacks of serial optic section, recorded via the rhodamine and FITC channels, were retraced and assembled using the AVS program. Stereo-pairs were obtained by tilting each individual reconstituted image (of the pair) by 5° towards the twin image.

3. Results and discussion

GTS5 cells expressing the temperature-sensitive mutant of p53 were used in this study to examine the distribution of proteasomes and mitochondria as well as the organization of the actin cytoskeleton during progression of apoptosis. If apoptosis is initiated by shifting the incubation temperature from 37°C to 32°C, one can follow the progression of typical apoptotic events such as formation of apoptotic blebs and fragmentation of chromatin [16]. In order to visualize the precise location of proteasomes in relationship to the actin cytoskeleton and to the mitochondria, we examined the cells by confocal microscopy. In non-apoptotic cells double-stained with rhodamine phalloidin and antibodies against either proteasomes or the mitochondrial steroidogenic enzyme adrenodoxin we found, in accordance with previous observations, that proteasomes were located both in the nucleus and the entire cytoplasm (Fig. 1). In apoptotic cells, proteasomes were almost totally confined to the blebs as shown clearly by scanning the entire cell volume and 3D reconstruction (Fig. 2). The change in the intracellular distribution of the proteasomes correlated with rearrangement of the actin cytoskeleton which early in apoptosis formed a spherical network surrounding the perinuclear volume (Figs. 1 and 2), rather than following cell extensions as observed in non-stimulated cells. This organization of the actin network was evident through the entire cell depth, suggesting the formation of an efficient barrier between the apoptotic blebs loaded with proteasomes and the residual body of the cell including the nucleus. Most of the nuclei of apoptotic cells remain essentially intact, in spite of the intensive formation of numerous apoptotic blebs at the circumference of the cell.

In non-apoptotic cells, mitochondria were scattered throughout the cytoplasm. The actin filaments were organized along the entire length of the cell processes (Fig. 1). In contrast, in cells stimulated to undergo apoptosis, mitochondria were confined to the perinuclear region and were essentially absent from the apoptotic blebs. Here again, the actin cytoskeleton did not penetrate the apoptotic blebs, but assumed a spherical organization, sending short extensions towards the basis of the apoptotic blebs. In most cells stimulated for apoptosis for 24 h, apoptotic blebs loaded with proteasomes were still clearly visible. During further progression of apoptosis, the actin cytoskeleton collapses, but most of the mitochondria remained in the perinuclear space (not shown).

The actin cytoskeleton seems to be involved in the compartmentalization of proteasomes, since it was found to reorganize itself in the space bordering the main body of the apoptotic cell and the apoptotic blebs. The actin cytoskeleton appears not to interact directly with the membrane of the apoptotic blebs; it rather seems that the inner face of the plasma membrane changes physically and biochemically during apoptosis. It is not clear what causes the actin cytoskeleton to detach from the inner face of the plasma membrane. One hypothesis is that the proteasome itself is involved in the degradation of the actin binding proteins responsible for the association of the actin network with the plasma membrane. Recent observations suggest that fodrin, an analog of spectrin, which is a major component of the cortical cytoskeleton of most eukaryotic cells, is degraded during apoptosis in a variety of cells, including T-lymphoma cells [19]. Moreover, it is known that α-spectrin can be ubiquitinated [20], and that cells deficient in spectrin tend to show a blebbing reminiscent of the morphology of apoptotic cells [21]. In addition, partial degradation of actin during apoptosis was observed recently in human leukemia U93H cells [22]. Therefore a partial degradation of the cortical cytoskeleton may be responsible for the blebbing of the plasma membrane in apoptosis.

Up-regulation of ubiquitin gene expression was recently observed in normal circulating human lymphocytes during apoptosis [15]. Thus, it is likely that the proteolytic activity of proteasomes during apoptosis is regulated by increased formation and availability of ubiquitinated protein substrates. The proteasomes inside the apoptotic blebs may trigger the shedding of the apoptotic blebs during programmed cell

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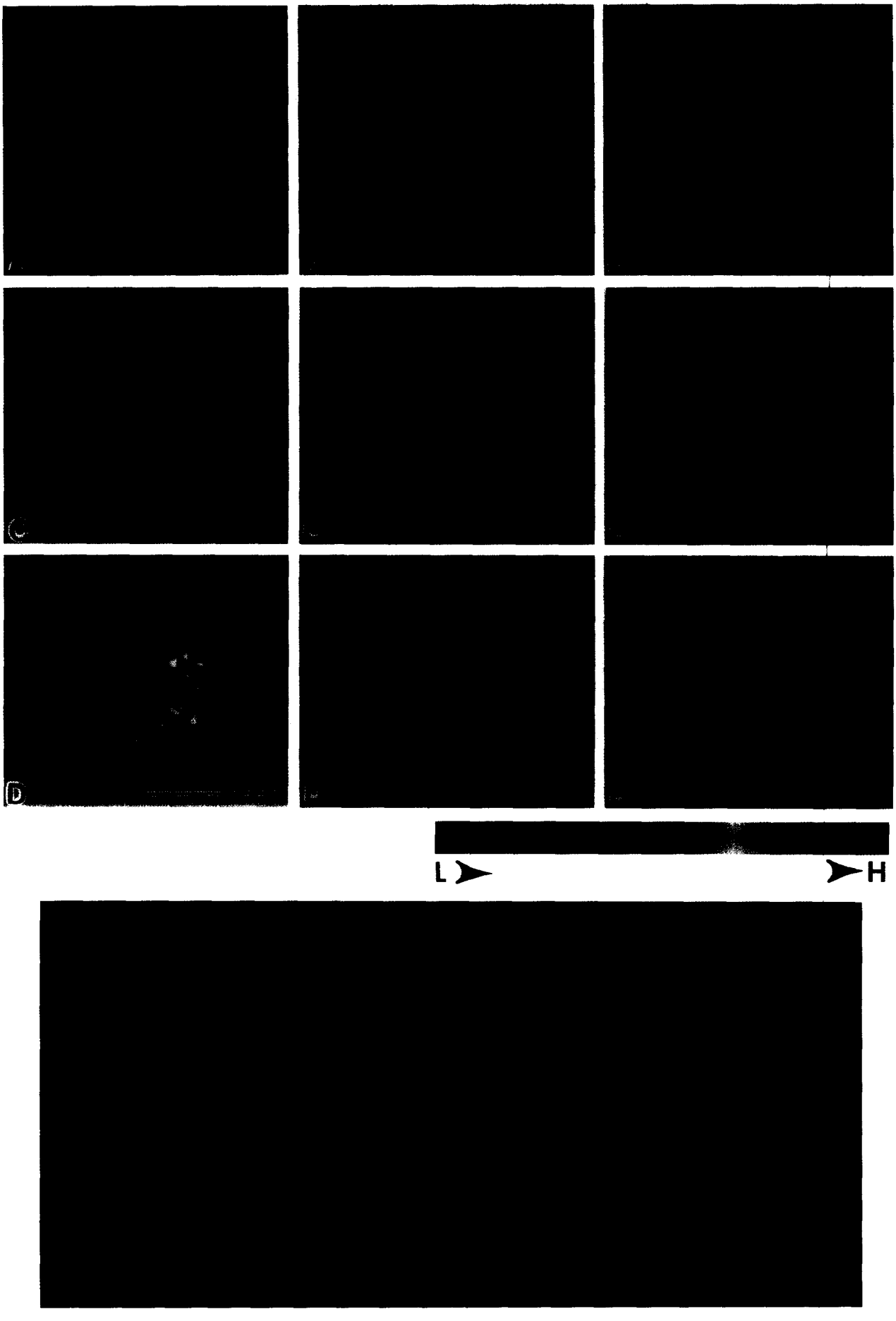
Fig. 1. Translocation of proteasome, mitochondria and actin cytoskeleton during apoptosis. Non-apoptotic cells stained with rabbit antibodies to proteasomes (A) or rabbit antibodies to adrenodoxin (a marker for steroidogenic mitochondria) (B) and with rhodamine phalloidin (B′). Proteasomes are distributed both in the nucleus and the cytoplasm while mitochondria are distributed in the cytoplasm and cell processes delineated by actin cables. Apoptotic cell (C) stained with antiproteasome antibodies (C′) show a high concentration of proteasomes in apoptotic blebs leaving the nucleus free of proteasomes. Reorganization of actin cytoskeleton to a condensed ring is evident (C″). Redistribution of mitochondria in the center of an apoptotic cell (D, D′) is characterized by reorganization of the actin cytoskeleton in a ring shape (D″). Apoptotic cells (C,D) were pretreated with 50 μM forskolin for 20 h at 37°C and stimulated for 5 h at 32°C in serum-free medium in the presence of forskolin. Control and apoptotic cells were fixed with 3% formaldehyde and permeabilized with Triton X-100. A,B,B′,C′,C″,D′,D″ fluorescent images in a laser confocal microscope (Ziess, 410; optical sections of 0.4 μm. L → → H, false colour intensity gradient; blue, lowest level; red, highest level). C,D, interference optic. Bar, 10 μm.

Fig. 2. Stereo-pair of three-dimensional reconstitution of optical sections of a cell stained with rhodamine phalloidine for actin filaments and rabbit antibodies to proteasomes followed by FITC goat anti-rabbit IgG. Apoptotic cell preteated with forskolin at 37°C and stimulated for 5 h at 32°C in serum-free medium in the presence of 50 μM forskolin. Proteasomes are almost exclusively located in apoptotic blebs and actin cytoskeleton reorganized in a spherical basket which seems to separate the apoptotic blebs from the main cell body. Picture size 25 μm.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death. On the other hand, preservation of the integrity of the steroidogenic mitochondria and lipid droplets and their clustering in the perinuclear region may explain the preservation and even enhancement of steroidogenesis during early stages of apoptosis [16].

It is also worth considering that p53 plays an important role in the induction and probably in the maintenance of apoptosis in GTS cells as in other cell types. p53 has been shown to be degraded via ubiquitination [23]. It was recently demonstrated that shifting the growth temperature from 37°C to 32°C in cells expressing the p53Val135 leads to its translocation to the nucleus and enhancement of its degradation, probably via ubiquitin-p53 and 26S proteasome interaction [24–26]. Translocation of proteasomes from the nucleus to the apoptotic blebs, as evident from our studies, may rescue the nuclear p53 from further degradation and thus play a role in the triggering of apoptosis. It remains to be seen whether turnover of nuclear p53 is indeed significantly reduced during initial steps of apoptosis.

Acknowledgements: We thank Malka Kopelowitz and Vivienne Laufer for excellent secretarial assistance, R. Albrecht, R. Sutterlin and R. Haering for excellent technical assistance, Dr. G. Gerisch for providing us with the confocal microscope facilities and Drs. U. Aebi, Y. Citri, A.M. Kaye, M. Oren and Y. Yarden for helpful discussions. A.A. is the incumbent of the Joyce and Ben B. Eisenberg Professorial Chair in Molecular Endocrinology and Cancer Research.

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