Nuclear translocation of PDCD5 (TFAR19): an early signal for apoptosis?

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Abstract The programmed cell death 5 (PDCD5) protein is a novel protein related to regulation of cell apoptosis. In this report, we demonstrate that the level of PDCD5 protein expressed in cells undergoing apoptosis is significantly increased compared with normal cells, then the protein translocates rapidly from the cytoplasm to the nucleus of cells. The appearance of PDCD5 in the nuclei of apoptotic cells precedes the externalization of phosphatidylserine and fragmentation of chromosome DNA. This phenomenon is parallel to the loss of mitochondrial membrane potential, independent of the feature of apoptosis-inducing stimuli and also independent of the cell types and the apoptosis modality. In conclusion, the nuclear translocation of PDCD5 is a universal earlier event of the apoptotic process, and may be a novel early marker for apoptosis.

Key words: TF-1 cell apoptosis related gene-19; Programmed cell death 5; Nuclear translocation; Apoptosis

1. Introduction

Apoptosis is a form of cellular suicide that is widely observed in nature. This process can be triggered by a variety of physiological and pathological stimuli, including radiation, drugs, growth factor deprivation, hormones and stress [1–3]. Many apoptosis-related genes and their products have been found which take part in the regulation of the apoptosis process, including the caspase, BcL-2, tumor necrosis factor (TNF)/FasL, and oncogenes families [4].

TF-1 cell apoptosis related gene-19, also designated PDCD5 (programmed cell death 5) by the International Human Gene Nomination Committee, is a novel gene cloned from TF-1 cells undergoing apoptosis [5]. PDCD5 protein is conservative in the process of evolution and has significant homology to the corresponding proteins of species ranging from yeast to mice [5,6].

Early studies indicated that recombinant human PDCD5 could accelerate apoptosis of some tumor cells (HeLa, TF-1, HL60, MCG-803, MCF-7) [5,7]. In order to further study the biological activities of PDCD5, we successfully produced specific monoclonal antibodies against PDCD5. Then, we used these monoclonal antibodies as probes to observe the expression and localization of PDCD5 in cell apoptosis process. We demonstrate that PDCD5 protein can translocate to the nucleus rapidly in cells undergoing apoptosis and the accumulation of PDCD5 in the nucleus precedes the chromosome DNA fragmentation and phosphatidylserine (PS) externalization.

2. Materials and methods

2.1. Reagents

Etoposide (VP16), staurosporin, camptothecin, propidium iodide (PI), Rhodamine123 and 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI) were purchased from Sigma. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was from Promega. Enhanced luminol chemiluminescence reagent was obtained from NEN™ Life Science Products. Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 were obtained from Gibco BRL. Fetal calf serum (FCS) was from Hyclone. Complete protease inhibitor cocktail was procured from Boehringer Mannheim.

2.2. Cell culture and apoptosis induction

TF-1 cells were cultured in RPMI 1640 medium supplemented with 10% FCS in the presence or absence of 40 ng/ml of recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF). HeLa, HEK293, 293T (SV40 large T antigen) and HEC-1a cells were cultured in DMEM containing 10% FCS. K562 cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. For induction of apoptosis, HEK293, HeLa, HEC-1a and K562 cells were cultured in the presence of VP16; HeLa cells were also cultured in the presence of staurosporin, camptothecin, cisplatin and UV-irradiation.

2.3. Preparation of monoclonal antibodies against PDCD5

Balb/c mice were immunized with recombinant human PDCD5 protein [7], and spleen cells were fused with the mouse myeloma Sp2/0 in the presence of polyethylene glycerol as previously described [8,9]. Hybridoma cell lines producing antibodies were screened by indirect ELISA. One clone, named 3A3, was subcloned by limiting dilution and ascites fluid prepared using pristine primed Balb/c mice. 3A3 IgG was purified with Hitrap™ proteinG (Amersham Pharmacia Biotech). FITC-labeling of the purified IgG was prepared as previously described [10].

2.4. Immunofluorescence

At indicated time points, cells were washed with cold phosphate-buffered saline (PBS), fixed with freshly prepared 3% paraformaldehyde (10 min on ice), followed by the addition of PBS containing 0.2% Tween 20 for 15 min at 37°C. Cells were blocked with FCS and incubated with FITC-3A3 IgG for 30 min at 4°C. DAPI

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Abbreviations: PDCD5, programmed cell death 5; PS, phosphatidylserine; FCS, fetal calf serum; CLSM, confocal laser scan microscope; DAPI, 4,6-diamidino-2-phenylindole-dihydrochloride; GM-CSF, granulocyte macrophage colony-stimulating factor

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(0.5 μg/ml) was added for 15 min to visualize the cell nuclei. Washed cells were subsequently analyzed using FACScan flow cytometer (Becton Dickinson), Leica fluorescence microscope or Leica TCS NT confocal laser scan microscope (CLSM).

2.5. Western blotting

Cells were washed in ice-cold PBS and resuspended in lysis buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.2 mM sodium orthovanadate and the protease inhibitor cocktail for 30 min on ice. The lysates were then centrifuged at $14\,000\times g$ for 15 min and the supernatant was quantified by protein assay kit. An equal amount of extracted protein was separated by 15% SDS–PAGE and then transferred to nitrocellulose membrane. The membrane was blocked overnight in TBS containing 5% non-fat dried milk and 0.05% Tween 20 and then incubated for 1 h with monoclonal antibody against PDCD5 (3A3). After washing, the membrane was incubated with an HRP-conjugated secondary antibody and the bound antibody was visualized using enhanced chemiluminescence detection system.

2.6. Detection of DNA fragmentation

TF-1 cells ($5–10\times10^6$) were cultured as described above. Nuclear DNA from lysed cells, treated with protease K and RNase according to standard protocols, was subjected to conventional horizontal agarose gel electrophoresis (1.2%) followed by ethidium bromide staining [11].

2.7. Detection of PS externalization

Treated TF-1 cells (0.5×10^6) were washed and resuspended in binding buffer containing annexin-V-FITC (25 μg/ml) and PI (5 μg/ml) for 15 min prior to analysis. Ten thousand cells were collected on a FACScan flow cytometer equipped with a 488 nm argon laser and analyzed using the CellQuest software (Becton Dickinson).

2.8. Transient transfection

The construction of mammalian expression vector pEGFPC3–PDCD5 containing EGFP–human PDCD5 (full-length open reading frame) fusion gene has been described previously [5]. HeLa cells and 293T cells grown on 12 mm glass coverslips in 6-well plates were transfected at 60–80% confluence with pEGFPC3–PDCD5 and pEGFPC3 plasmids by Superfect Transfection Reagent according to the manufacturer's directions. After 24 h, 85 μm of VP16 was added to the 293T cell medium and cell localization of EGFP–PDCD5 was observed at different time points with fluorescence microscope. In addition, the nuclear transportation of PDCD5 in a single HeLa cell treated with staurosporin (1 μM) was analyzed under the CLSM. At the same time, for in situ analysis of apoptosis, cells were stained with R-PE annexin-V (Pharmingen) and PI.

2.9. Detection of mitochondrial membrane potential

The mitochondrial membrane potential was determined by the retention of fluorescent dye Rhodamine123 [12]. After the drug treatment, 5×10^5 cells in 1 ml complete DMEM were stained with the Rhodamine123 (1 μM) for 30 min at 37°C prior to the flow cytometric analysis. The same incubation time was applied to the untreated cells.

3. Results

3.1. TF-1 cell apoptosis is concomitant with nuclear translocation of PDCD5

TF-1 cell line is a human premyeloid cell established from a

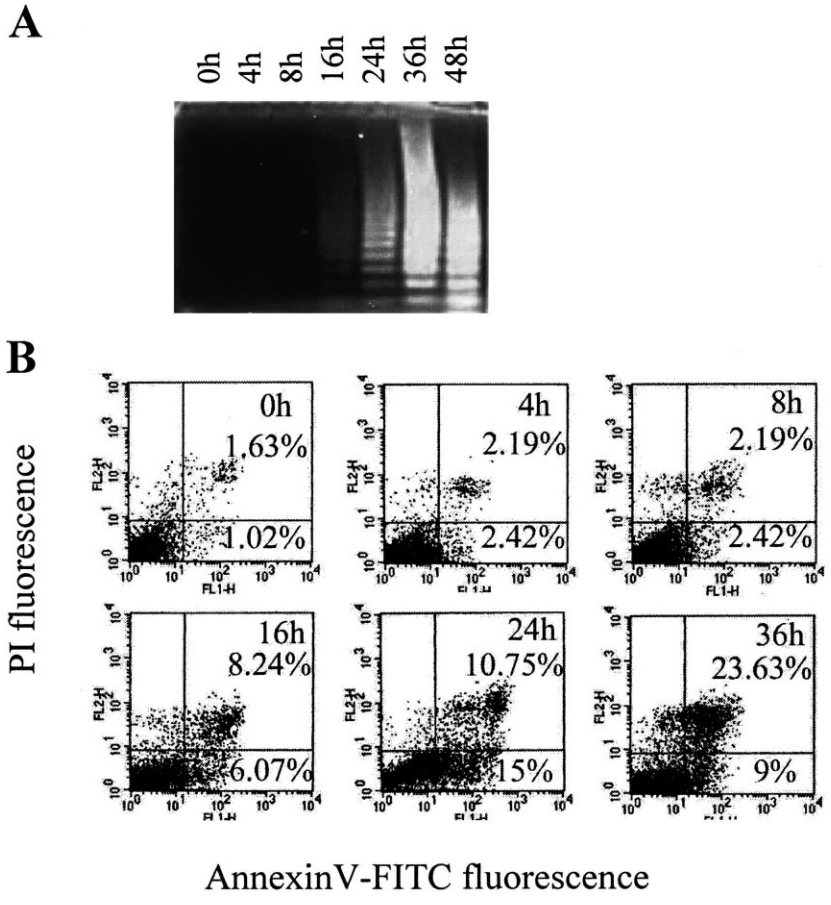

Fig. 1. Apoptotic features in TF-1 cells after GM-CSF deprivation. A: Detection of DNA fragmentation in TF-1 cells cultured in GM-CSF-free medium for the indicated times. B: Time course of PS externalization and PI uptake. Cells were cultured in GM-CSF-free medium for the indicated periods and PS externalization and PI uptake were determined by flow cytometry (see Section 2). Similar experiments were done in duplicate.

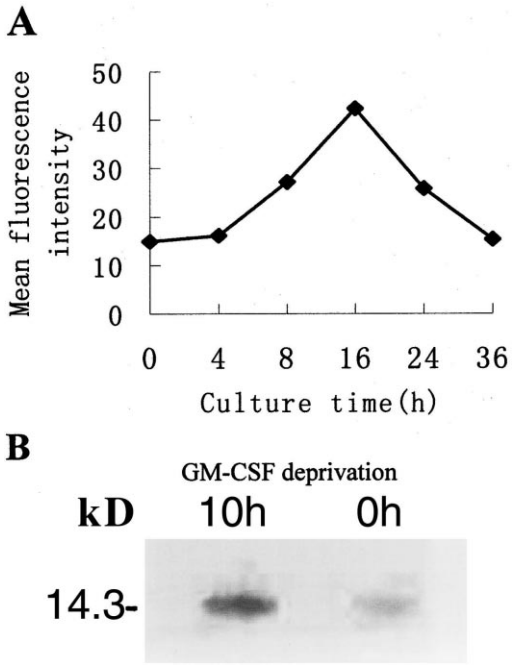

Fig. 2. Expression of PDCD5 in TF-1 cells after GM-CSF deprivation. A: Time course of mean fluorescence intensity of PDCD5 analyzed by flow cytometry. B: Western blot analysis of expression of PDCD5 in TF-1 cells after GM-CSF withdrawal for 10 h and 0 h. Equal amounts of cell lysates were separated on 15% SDS–PAGE gels and probed with antibody specific to PDCD5. Similar experiments were done in duplicate.

patient with erythroleukemia. Their survival and proliferation are dependent on interleukin-3, GM-CSF, EPO or other cytokines [5,13]. After GM-CSF deprivation, TF-1 cells were found to exhibit the features of apoptosis, including condensation of chromatin, blebbing of the cytoplasmic membrane, cell shrinking, externalization of PS and the formation of DNA fragmentation (Fig. 1A,B). Flow cytometric detection of PDCD5 protein using FITC-labeled anti-PDCD5 antibody showed that PDCD5-positive cells and fluorescence intension increased rapidly in TF-1 cells after GM-CSF deprivation, exhibited a time-dependent increase and then declined (Fig. 2A). Western blot analysis showed that the PDCD5 protein expressed in apoptotic TF-1 cells was higher than that expressed in normal TF-1 cells (Fig. 2B). Using the Leica fluorescence microscope and CLSM, significant nuclear translocation of PDCD5 was observed in TF-1 cells after GM-CSF deprivation (Fig. 3). In untreated TF-1 cells, PDCD5 protein was weakly expressed and localized in both the cytoplasm and nucleus. After GM-CSF deprivation for 8 h, more than 75% of the cells displayed a nuclear accumulation of PDCD5 protein, here in a condensed form. PDCD5 was also observed in the apoptotic small bodies after GM-CSF deprivation for 36 h. Interestingly, accumulation of PDCD5 in the nucleus of TF-1 cells precedes DNA fragmentation and PS externalization (Figs. 1 and 3). It is therefore suggested that the phenomenon may be an earlier phase event during cell apoptosis.

3.2. Nuclear translocation of PDCD5 is a widespread phenomenon during the apoptosis of cells

To determine whether the nuclear translocation of PDCD5 is a general accompaniment to apoptosis, we extended our study to investigate other cell types. Interestingly, similar results were also observed in other cell lines such as HEK293, HEC-1a, K562, and HeLa cells treated with VP16 (Fig. 4A). This was confirmed by pEGFPC3–PDCD5 plasmid transiently transfected 293T cells treated with VP16 (Fig. 4B). In addition, a dynamic nuclear transportation of EGFP–PDCD5 fusion protein in staurosporin-treated transfected HeLa cells was observed by CLSM. Before treatment, pEGFP–PDCD5 fluorescence showed a uniform distribution through the whole cell. After 30 min of treatment with staurosporin (1 μM), a nuclear accumulation of EGFP–PDCD5 was observed that later decreased (Fig. 5). However, there was no annexin-V-R-PE on its surface or PI uptake after 1 h treatment. These finding support a conclusion that nuclear translocation of PDCD5 in cells undergoing apoptosis may be considered a universal phenomenon preceding PS externalization and PI uptake.

3.3. Nuclear translocation of PDCD5 during apoptosis is stimulus-independent

To determine whether these changes could occur regardless of the initiating stimulus. We exposed HeLa cells to a range of apoptosis-inducing agents such as the chemotherapeutic agent etoposide, camptothecin, cisplatin, a protein kinase C inhibitor staurosporin, growth factor deprivation and UV light. Figure 6A demonstrates that nuclear translocation of PDCD5 accompanies apoptosis of HeLa cells in response to all of the agents tested. However, the rate of nuclear transportation of PDCD5 is different. This is accounted for by the fact that HeLa cells utilize different stress mechanisms in response to various stimuli. Taken together, these data provide compelling evidence for a tight association between apoptosis and nuclear translocation of PDCD5, regardless of the cell

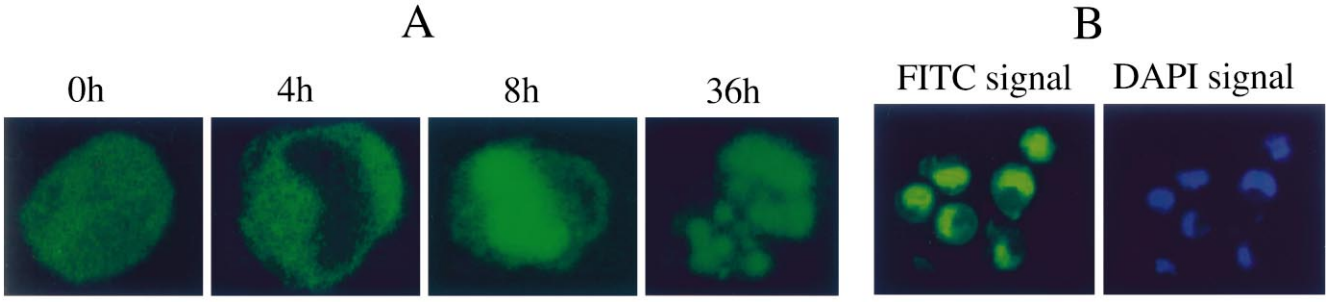

Fig. 3. CLSM evidence for the nuclear translocation of PDCD5 in TF-1 cells after GM-CSF deprivation. A: Cells were stained with FITC-labeled 3A3 antibody at different time points, and observed using CLSM. B: Cells were cultured in GM-CSF-free medium for 16 h, then stained with FITC-3A3 and DAPI. Nuclear accumulation of PDCD5 and condensed nuclei were obviously observed.

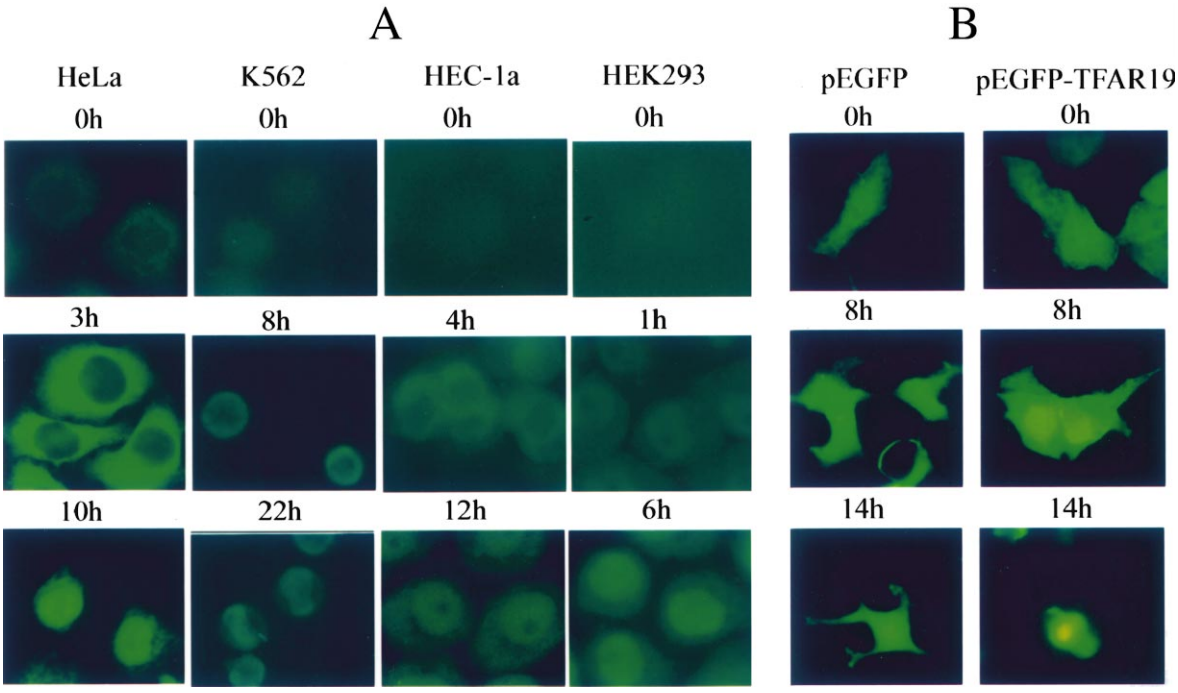

Fig. 4. Fluorescence microscope evidence for nuclear translocation of PDCD5 in different cell types. A: HeLa and HEC-1a cells were treated with 85 μM of VP16; K562 cells treated with 170 μm of VP16; HEK293 cells treated with 68 μM of VP16. B: 293T cells were transiently transfected with pEGFP and pEGFP–PDCD5 for 24 h, then treated with 85 μM of VP16 at different time points. (488 nm, ×400).

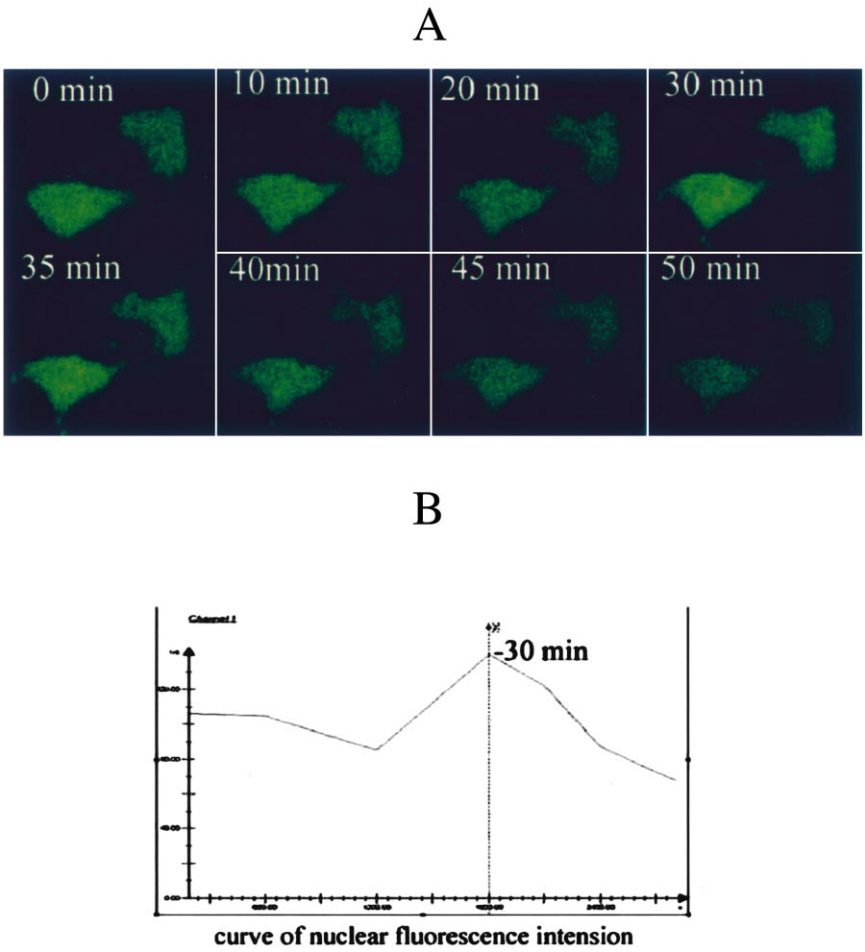

Fig. 5. Kinetic analysis of localization of EGFP–PDCD5 fusion protein in HeLa cells treated with staurosporin (1 μm) for indicated time points (A and B). HeLa cells were transiently transfected with pEGFP–PDCD5 plasmid for 24 h, then 1 μm of staurosporin was added to cultures. After 30 min of treatment, accumulation of EGFP–PDCD5 fusion protein in cell nuclei was clearly observed.

type or apoptosis-inducing stimulus. In addition, when HeLa cells were treated with staurosporin (0.15 μM), a transient cytoplasmic accumulation of PDCD5 was observed after 2 h and was translocated rapidly to the nucleus. In contrast, annexin-V-binding cells were apparent at 5 h and later (Fig. 6B).

3.4. PDCD5 translocation and mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi_{mt}$) was monitored by cationic fluorescent probe Rhodamine123. HeLa cells were treated with staurosporin (0.5 μM) at several time points, after which, they were stained with 1 μm Rhodamine123. A marked decrease of fluorescence intensity was observed after treatment for 3 h. This reflects the drug-induced depolarization of the inner mitochondrial membrane. At this time point, more than 60% of HeLa cells treated with staurosporin had nuclear accumulation of PDCD5 (Fig. 7), which indicates that PDCD5 translocation is parallel to the decrease of $\Delta\Psi_{mt}$.

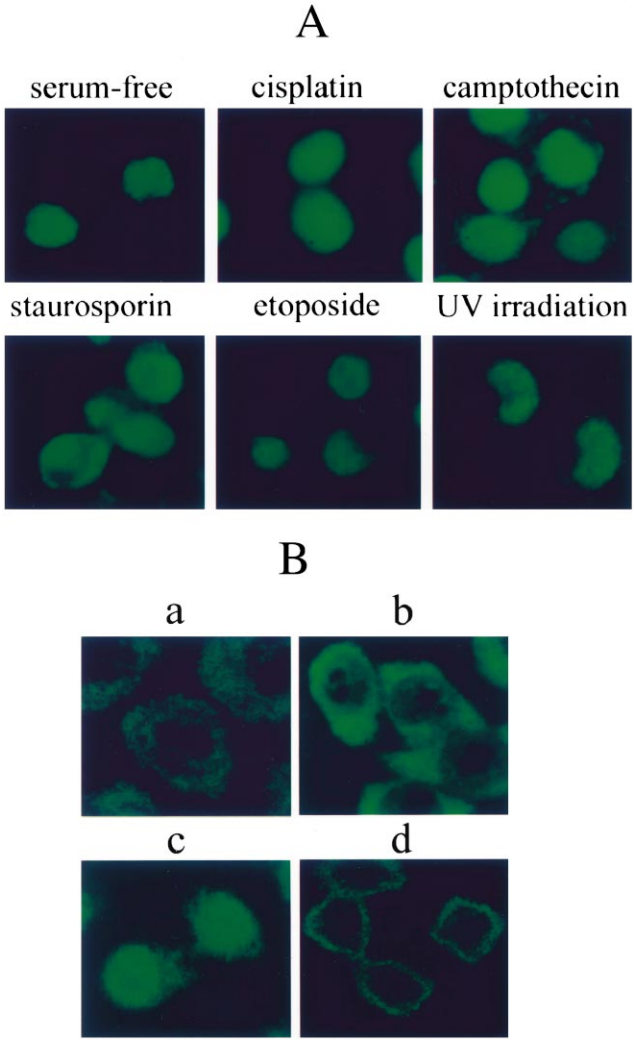

Fig. 6. Nuclear translocation of PDCD5 in HeLa cells treated with a variety of stimuli. A: Cells were cultured in serum-free medium for 48 h; exposed to cisplatin (33 μM, 16 h), camptothecin (2.8 μm, 16 h), staurosporin (0.5 μM, 4 h) and etoposide (68 μM, 8 h); UV irradiation for 9 h. B: Comparison of nuclear translocation of PDCD5 and PS externalization. HeLa cells were treated by staurosporin (0.15 μM) at 0 (a), 2 (b), 3 (c), and 5 h (d). At these time points, cells that were stained with FITC-3A3 (a–c) and annexin-V-FITC (d) indicated that nuclear translocation of PDCD5 precedes the externalization of PS.

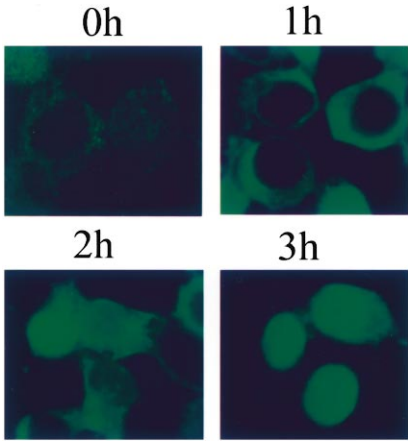

Fig. 7. Analysis of the nuclear translocation of PDCD5 in HeLa cells treated with staurosporin (0.5 μm) for indicated time points.

4. Discussion

PDCD5 is a novel protein related to the apoptosis process of cells. In this report, we first demonstrated that PDCD5, normally uniformly distributed among the cells, was rapidly translocated to the nucleus of target cells undergoing apoptosis. In addition, we found that the nuclear accumulation of PDCD5 preceded the externalization of PS and the formation of DNA fragmentation. This phenomenon is perhaps a very early common event of cell apoptosis, parallel to the loss of mitochondrial membrane potential, independent of the apoptosis-inducing stimuli (protein kinase inhibitor, topoisomerase I or II inhibitors, UV light and growth factor deprivation), and also independent of the cell types and the apoptosis modality (from early apoptosis to later apoptosis) (Figs. 3–6). The mechanism that results in nuclear translocation of PDCD5 is unclear at present. Since PDCD5 does not contain a linear or bipartite nuclear localization signal [14,15], there remains a distinct possibility that some carrier proteins facilitate the nuclear transportation of PDCD5 [16] or alternatively, perhaps there is an uncovered nuclear localization signal within the PDCD5 protein. Recent reports in apoptosis research have focused on the nuclear morphological changes characteristic of apoptosis [17,18]. Nuclear changes are distinct hallmarks during the cell apoptosis process. Several stages of nuclear apoptosis can be distinguished: stage I with rippled nuclear contours and a rather partial chromatin condensation; stage IIa having marked peripheral chromatin condensation; and, stage IIb showing the formation of nuclear small bodies [19]. Kinetic studies reveal that these stages actually reflect successive steps in the apoptotic process. Stage I is also called pre-apoptotic nuclei, and happens in the early stage of cell apoptosis [20]. Ultra-structure analysis reveals that this pre-apoptotic nucleus is characterized by heavily convoluted nuclei with numerous cavitations. It is closely associated with high molecular size DNA fragmentation. However, there is little evidence of molecular changes in this stage. Our studies hint that an interesting link might be established between the nuclear transportation of PDCD5 and the formation of pre-apoptotic nuclear morphological changes.

The binding of annexin-V on cells undergoing apoptosis has been proposed to be an early event based on correlative studies with cell populations [21]. Recent studies have found that annexin-V-binding cells have a high degree of DNA fragmentation [22]. Therefore, these two markers of apoptosis do not explain the much earlier changes of the cell apoptosis process. It is important to note that once PDCD5 enters the nucleus, it runs through the whole apoptotic process for cells (from early apoptosis to later apoptosis or death). Perhaps this would be a useful marker to account for an early event in the process of cell apoptosis and initial apoptotic changes in the nucleus. Combined with other conventional apoptotic markers (such as PS externalization, DNA fragmentation), it will be also helpful to surveillance the whole process of cells apoptosis.

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