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Apoptosis induced by uterine 24p3 protein in endometrial carcinoma cell line

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Abstract

The biological functions and reaction pathways of lipocalins in mammalian system were sought. Mouse uterine 24p3 protein is a secreted lipocalin from mouse uterus. To evaluate the effect of uterine 24p3 protein on the reproductive system, endometrial carcinoma cell line (RL95-2) was an experimental target for achieving the *in vitro* study. The cells were treated with 0.75 μM dexamethasone (DEX) or under serum-free medium to mimic the stress environment for various time periods, then employing Western blot to measure the 24p3 protein secretion. It showed the time-dependent induction effect on 24p3 protein and suggested the level of protein secretion correlating to environmental stress. Furthermore, the supplementation of 24p3 protein to the medium accompanied the reduction of cell viability. It showed that the 24p3 protein may be a death factor under conditional media via PI and annexinV-FITC assay. Based on the autocrine hypothesis, we investigated the effect of 24p3 protein on cultured RL95-2 cells upon the 24p3 protein interaction. We have demonstrated significant increase in intracellular reactive oxygen species upon 24p3 protein interaction. While the changes of mitochondrial membrane potential and cytochrome *c* release from mitochondria occurred, the activities of caspase-8, -9 and -3 were found to have increased. The condensation of DNA was occurred suggesting that 24p3 protein induced irreparable DNA damage, which in turn triggered the process of apoptosis. It shows evidence for the direct effect of this protein on endometrial cells. These findings suggest that 24p3 protein creates an intracellular oxidative environment that induces apoptosis in RL95-2 cells.

Keywords: Cell death; Endometrial cell; Lipocalin; Uterine secretory protein

1. Introduction

Lipocalins are a large and ever expanding group of proteins exhibiting considerable structural and functional variation, both within and between species (Garibotti et al., 1997; Flower, 1994; Lopez-Boado et al., 1996). However, the functions of many lipocalins remain unclear to date. Human neutrophil gelatinase-associated lipocalin (hNGAL/24p3) is a 25 kDa glycoprotein, which has the alternative name of 24p3 protein like a lipocalin from mice (Akerstrom et al., 2000). It belongs to the lipocalin super-family with a hydrophobic binding site (Flower, 1996), first found in the granules of the human neutrophil (Kjeldsen et al., 1994); homologous protein has been identified in mouse (24p3/uterocalin) (Akerstrom et al., 2000). NGAL/24p3 is an acute phase protein expressed in several tissues and organs, and identified in human, mouse, and rat (SIP24) genomes as

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lipocalin2 (lcn2) (Kjeldsen et al., 2000). Expression of the mouse NGAL homologue, 24p3 protein, is also up-regulated in response to inflammatory stimuli (Liu and Nilsen-Hamilton, 1995) and in the uterus at the time of preimplantation or parturition, where it is believed to be part of a local inflammatory response associated with birth (Huang et al., 1999; Liu et al., 1997). Besides neutrophil, 24p3 protein is expressed in most tissues, human and mouse, and its synthesis is induced in epithelial cells during inflammation (Kjeldsen et al., 2000). Using purified mouse 24p3 protein from mouse uterine fluid, we attempt to figure out the possible effect on endometrial cells.

According to a report, a stress-induced increase in serum glucocorticoid concentration has an effect on Leydig cells in the male reproductive system and plays a role as a stress hormone (Hardy et al., 2005), many stress molecules being induced under such circumstance, but the downstream regulation of molecules is unclear. In our previous study, regarding the sexual hormones' effect on the mouse 24p3 protein expression in murine uterus, the results have documented that the progesterone showed an antagonistic effect on the estrogen-stimulated 24p3 expression in mouse uterus (Liu and Nilsen-Hamilton, 1995). A study of Pollar et al. also demonstrated that the estrogen status of the female rat may affect its endocrine response to stress. They showed that the glucocorticoid levels are highest on proestrus when estrogen levels are the highest in a control rat (Pollar et al., 1975), suggesting the estrogen led to the formation of endometrial carcinoma, and that glucocorticoid may control the powerful action of estrogen (Bigsby, 1993). The glucocorticoid induced gene expression may correlate with the diminishing estrogen effect on the endometrial cells. A human endometrial cell line, RL95-2, derived from an adenosquamous carcinoma of the endometrium and estrogen receptor is present (Way et al., 1983). The cell line may provide a useful *in vitro* system for the investigation of the 24p3 protein regulation of endometrial growth. Regulating the cell growth may have an effect on the endometrial carcinoma formation. According to the results of Garay-Rojas et al. (1996), two overlapping glucocorticoid response elements (GRE) but no ERE, have been mapped in the 5′-flanking region of a 24p3 genomic clone. It also found that there is one GRE and no ERE in the NGAL genomic clone (Kjeldsen et al., 2000). It may indicate that glucocorticoid directly plays a role in the regulation of NGAL/24p3 expression.

In addition, lipocalin 24p3, reported to be induced in hematopoietic cells by interleukin-3 (IL-3) depletion, induces hematopoietic cell apoptosis (Kamezaki et al., 2003). Expression of rat SIP24 in peripartum and postpartum suggested that this protein was involved in cell death (Elkhalil et al., 2005). The mass of uterus increases rapidly during pregnancy and then quickly regresses after parturition by the process of tissue involution. The involution of mouse mammary epithelial cells involves apoptosis of resident cells and also triggers the 24p3 expression (Bong et al., 2004). Besides that, we also found 24p3 expression during the proestrous and estrous phases in the mouse estrous cycle (Huang et al., 1999). These observations implied that the 24p3 protein is involved in the cell death. Mammalian endometrium is one of the few tissues in which proliferation and apoptosis take place cyclically in a hormone-dependent process during estrous cycle. Abnormalities in the regulation of the apoptosis pattern may contribute to neoplastic transformation (Bozdogan et al., 2002). The regulation of this cyclical cell growth is unclear. It prompted us to clarify whether 24p3 protein plays a role in this process. Using purified mouse 24p3 protein, which includes the native glycan constituting the difference from the recombinant one, we attempt to get more information regarding the 24p3 protein function and further to elucidate the above issues. Here, for the first time, we give evidence that 24p3 protein can be induced in a time-dependent manner by glucocorticoid stimulation or under serum-free conditions on the endometrial carcinoma cell line (RL95-2). As previously mentioned, 24p3 protein has recently been considered to be related with cell death (Kamezaki et al., 2003); we identified the cell death process of this protein via the 24p3 protein supplemented endometrial cell line (RL95-2). We also provide evidence for the direct effect of 24p3 protein on the viability of endometrial carcinoma cells.

2. Materials and methods

2.1. Cell culture and treatment conditions

The human endometrial cell line (RL95-2) used in this study was obtained from American Type culture, and cultured as recommended. The cell line was cultured in MEM medium containing 15% (v/v) heat-inactivated Fetal Bovine Serum, 5 μg/ml insulin (Gibco). 100 i.u./ml penicillin and 0.1 mg/ml streptomycin were employed in culture dishes under air/CO_2 (19:1). After incubation, the media were collected and subjected to Western blot. For experimental considerations, cells were treated with 24p3 protein for the indicated time periods. The 24p3 protein was purified from mouse uterine fluid and 24p3 protein antibody was induced against rabbits, using the previous methods (Chu et al., 1996).

2.2. Trypanblue assay for cell viability

The trypanblue assay assesses cell viability by measuring the integrity of cell membrane. RL 95-2 cells were seeded into 96 well plates. Cells were incubated with different media. After incubation, cells were washed with serum free medium and then incubated with 0.2% trypanblue in PBS (150 mM NaCl in 5 mM Phosphate buffer, pH 7.4) for 2 min at room temperature. After the reaction, we removed the trypanblue solution and wash once with PBS. Then, counting the number of blue-color cells indicated a loss of cell viability of cell growth.

2.3. Western blot analysis of culture medium supernatant

A 500 μl sample of media was centrifuged with CentriconYM50 (Millipore, USA) to remove the excessive serum in medium. Then, a 100 μg protein sample was subjected to electrophoresis on pre-cast 4–20% -polyacrylamide Tris/glycine gels (Novex, Groningen, the Netherlands); the proteins were electroblotted to nitrocellulose membranes. Blots were incubated with an antiserum raised against mouse 24p3 protein in a rabbit, then horseradish peroxidase-conjugated secondary antibodies diluted to 1:2000 (Sigma) for detection. The reactive bands were visualized by enhanced chemiluminescence (ECL) (Amershan Pharmacia Biotech UK Limited) with exposure to X-ray film.

2.4. Protein labeling

FITC-labeled 24p3 protein (FITC-24p3) was prepared by incubating purified 24p3 protein (5 mg/ml) with fluorescein isothiocyanate (FITC; 5 mg/ml) in a sodium bicarbonate buffer (200 mM), pH 9.0, for a period of 20 h at 10 °C. Unconjugated FITC was removed by gel filtration on a PD-10 column (Amersham Bioscience, Germany).

2.5. Detection of FITC-24p3 protein interaction with RL95-2 cells and HeLa cells

Cells at a concentration of 1×10^5 were cultured on the coverslips for 48 h, washed twice with PBS and fixed with methanol, and again washed with PBS to remove the organic solvent. The cells were overlaid separately with 100 μl PBS, 100 μl PBS containing 5 μg FITC-labeled 24p3 protein, and 100 μl PBS containing 5 μg FITC-labeled 24p3 and 50 μg unlabeled 24p3 protein for competitive assay. The cells were incubated in the dark, for 1 h, at 37 °C, washed twice with PBS. The protein internalization was detected after 1.0 μg FITC-labeled 24p3 protein incubation at 10 or 37 °C. The cells were again washed twice with PBS, and observed under a fluorescence microscope. The HeLa cells were as a negative control assay.

2.6. Measurement of intracellular reactive oxygen species (ROS) production

The intracellular ROS were measured essentially as described by Hallowell and Whiteman (2004). Cells (2×10^4) were cultured on 96 well plates for 48 h and prepared for use. For the measurement of hydrogen peroxide, following incubation in 10 μM 24p3 protein containing medium for various time intervals, RL95-2 cells were treated with 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) for 15 min. The dichlorodihydrofluorescein (DCFH), the deacetylated product of DCFH-DA by intracellular esterases, reacts with H_2O_2 to form dichlorofluorescein (DCF), which is an oxidized fluorescent compound. The amount of intracellular hydrogen peroxide can be quantified by detection of DCF using a microfluorometer (Berthold Twinkle LB970) with excitation and emission wavelengths set at 485 and 535 nm, respectively. Diphenyleneiodonium chloride (DPI) used as a ROS inhibitor was purchased from Sigma.

2.7. Detection of cytochrome c release by antibodies

Cells (2×10^4) cultured on cover glasses for 36 h, and then supplemented with 10 μM 24p3 protein for 24 h are washed once with PBS and fixed with the fixative solution (70% alcohol, precooled at −70 °C) for 5 min. Thereafter, cells are rinsed three times with PBS. Gentle pipetting is recommended to avoid cell detachment. 0.1% TritonX-100 in PBST (PBS containing 0.05% Tween 20) is used to permeabilize the cells for 10 min. Wash three times with PBS and block nonspecific sites with 3% BSA in PBST for 30 min. Subsequently, wash once with PBST. For staining, incubate the cells with the primary antibody (anti-cytochrome *c* and a mitochondrial marker: anti-Hsp60) in 3% BSA-PBST for 2 h. For Hsp60 staining, a mouse antiserum was used at 1/1000 dilution. For cytochrome *c* detection, a mouse monoclonal antibody from Santa Cruz Biotechnology (sc-13156) was used (diluted 1/200). Thereafter, cells were washed three times with PBST and incubated with the fluorescein isothiocyanate (FITC) conjugated second antibody diluted in 3% BSA-PBST for 1 h. From this step onwards, samples should be protected from light, washed three times with PBST and observed under a fluorescence microscope (AH3-RFCA, Olympus, Tokyo, Japan).

2.8. Analysis of membrane potential with JC-1 by flow cytometry

The integrity of the inner mitochondrial membrane may be measured by observing the potential gradient across this membrane. Loss of the membrane potential ($\Delta\Psi$) is a hallmark of cellular apoptosis and was measured with a mitochondrial membrane potential detection kit (Mitochondria staining kit, Sigma, USA). It contains a cationic dye (5,5′, 6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolo-carbocyanine iodide). JC-1 forms J-aggregates (red fluorescence, FL2) in direct proportion to the mitochondrial potential. As the membrane potential

declines, JC-1 converts to a monomeric form (green fluorescence, FL1). Cells exposed to various conditions were incubated in 10 μg/ml JC-1 for 10 min at 37 °C in a humidified atmosphere containing 5% CO_2. After a wash with PBS, cells were harvested by trypsinization. The fluorescence of JC-1 was measured by flow cytometry with an excitation wavelength of 488 nm. Emission wavelengths of 527 and 590 nm were used for detecting green and red fluorescence, respectively. Based on the intensity of FL1, we calculated the percentage of mitochondrial membrane potential of 24p3 protein treated-RL95-2 cells by Eq. (1), compared to the control cells.

$$\frac{FL1_{24p3\,protein} - FL1_{control}}{FL1_{valinomycin} - FL1_{control}} \times 100\% = \Delta\psi_{24p3\ protein}\% \quad (1)$$

Valinomycin permeabilizes the mitochondrial membrane for K^+ ions, dissipates the mitochondrial electrochemical potential and is used as a positive control that prevents JC-1 aggregation (Inai et al., 1997).

2.9. Analysis of caspases activities by using fluorescent peptide substrates

Caspase-8, -9, and -3 activities were determined using PhiPhiLux G1D2, a fluorophore labeled caspase specific substrates (OncoImmunin Inc., USA). The main peptide amino acid sequences are IETDGI, LEHDGI and DEVDGI, respectively. The cleaved substrate has the following excitation and emission peaks: $\lambda_{ex} = 505$ nm and $\lambda_{em} = 530$ nm. The caspases activities were measured as fluorescence intensity. Cells were placed in a 48-well plate with different treatment, and incubated with 50 μl of substrate solution (10 μM PhiPhiLux G_1D_2 with 10% FBS) for 1 h at 37°C. After a wash with PBS, cells were harvested by trypsinization and detected by flow cytometry.

2.10. Flow cytometry and fluorescence microscopy for DNA damage

Flow cytometry data was collected using a COULTER EPICS XL flow cytometer (Beckman-Coulter, FL, USA). Fluorescence was initiated by excitation at a wavelength of 488 nm and measured via emission filters. The fluorescence intensity of FITC was quantified for 10,000 individual cells. For calculating the percentages of apoptotic cells, we established a control of noninduced RL95-2 cells and another cell treated with or without 10 μM 24p3 in serum-free medium. Incubated all of them in 37 °C for 24 h then wash the cell with PBS and detached by 0.25% trypsin–EDTA. Resuspended the cell in 400 μl medium containing 2 μg/ml propidium iodide (PI) and 0.5 μg/ml FITC labeled annexin-V to each and incubated at room temperature for 10 min under dark. Determine the fluorescence of cells immediately with a flow cytometer by excitation at a wavelength of 488 nm and measured via 525 and 625 nm emission filter. We analyzed the cell cycle by determining the DNA content with the use of PI after with or without the 10 μM 24p3 protein for 24 h culture. An apoptotic process, with cleavage of the DNA, was indicated by a sub-G1 peak that separated from G1/G0 peak. For the examination of nuclear morphology, cells were stained with 2 μg/ml of 4′, 6-diamidino-2-phenylindole (DAPI) to stain DNA, and observed under a fluorescence microscope (AH3-RFCA, Olympus, Tokyo, Japan).

2.11. Statistical analysis

The statistical analysis was conducted using One-way ANOVA with Dunnett's post test using GraphPad InStat version 3.00 for Windows, GraphPad Software, San Diego California USA.

3. Results

3.1. Induction of 24p3 protein secretion by glucocorticoid

In previous report (Garay-Rojas et al., 1996), they mentioned that dexamethansone (DEX) can induce the 24p3 gene expression in L929 cell line. Stress, such as under glucocorticoid supplement or starvation, are known to induce harmful stress in cells. Because 24p3 protein is a typical secreted lipocalin, we performed an immunoblot analysis of the cell culture supernatant. To test whether the 24p3 protein is a stress-related protein in RL95-2 cells, we treated the cells with 0.75 μM dexamethasone under serum supplement medium (Fig. 1, *S + D*) and also cultured in the media with or without 10% FBS for different time periods (Fig. 1, *S* and NS). In Fig. 1, dexamethasone induced 24p3 protein secretion from RL95-2 cells, showed a time-dependent manner, the 24p3 protein production initiated at 24 h incubation and has a great effect on 24p3 protein production at 72 h. We also found the decrease of cell viability after 72 h incubation (data not shown). Even without dexamethasone, 24p3 protein production would appear significantly

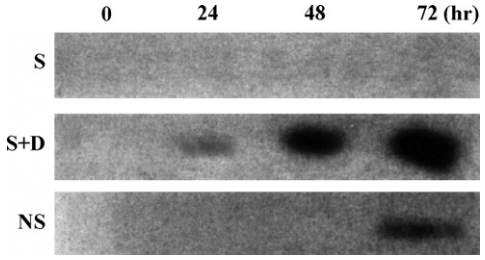

Fig. 1. Time-dependent effect of dexamethasone on 24p3 protein secretion. RL95-2 cells were cultured for 24 h, different fresh media were changed and the cultures were continued in with (S) or without (NS) 10% FBS or 10% FBS medium containing 0.75 mM DEX (S + D) for various periods of time. The secretion proteins (300 μg) were extracted from media and analyzed in 15% SDS-PAGE and were followed by Western analysis. The procedures are in materials and methods.

at 72 h serum-free incubation (Fig. 1, NS), but no 24p3 protein secretion in 10% FBS medium. Based on these data, we supposed that the RL95-2 cells would be responsive to 24p3 protein interaction under protein supplement serum-free medium. Before biological response evaluation, we had to confirm the 24p3 protein interaction with the endometrial cells. Because the 24p3 protein production on serum-free medium is initiated at 72 h (Fig. 1, NS), we measured all the parameters regarding the cell death before 24 h in the later experiments, to avoid the endogenous 24p3 protein. To eliminate the contamination of glucocorticoid in serum medium, using serum-free medium to study the 24p3 protein effect on RL95-2 cells should be the better way.

3.2. FITC-labeled 24p3 protein bound and internalized to the cells

In the previous report (Garay-Rojas et al., 1996), it was mentioned that 24p3 protein could be expressed and secreted via dexamethasone induction in L929 cells and was further amplified by the autocrine mechanism. It implied that the 24p3 protein should act on the cells. In order to clarify this issue, we used fluorescein isothiocyanate (FITC) labeled-24p3 protein to confirm the interaction of this secreted 24p3 protein with RL95-2 cells. To study the binding process, RL95-2 cells (1.2×10^5) were grown on a cover slip, in MEM medium for 24 h, and these cells were analyzed using fluorescence microscopy (Fig. 2). Efficient binding of the RL95-2 cell with 5 μg FITC-24p3 protein was observed after 1 h of incubation, followed by the removal of unbound protein by two PBS washings. After fixing with cold acetone, FITC-24p3 protein was found to bind to the cell membrane (Fig. 2b). In the competition assay, RL95-2 cells were incubated for 1 h with 5 μg FITC-24p3 protein and 50 μg unlabeled 24p3 protein (Fig. 2c). The efficiency of FITC-24p3 protein binding was inhibited by 10-fold unlabeled 24p3 protein, whereas in the control, the RL95-2 cell devoid of FITC-24p3 protein (Fig. 2a and d) showed no fluorescence on cells. Thus, in our competition study, we confirm that 24p3 is specifically bound to the cell surface. Based on our data (23), we proposed that protein internalization would occur on this cell line. To investigate whether the internalization of 24p3 protein occurs as happening in L929 cells, we tested the internalization of 24p3 protein at different temperatures. Significant inhibition of FITC-24p3 protein internalization was observed at 10 °C incubation RL95-2 cells (Fig. 2e) when compared with 37 °C incubation one (Fig. 2f). The data were similar to our previous findings in L929 cells (Chou et al., 2006). The data confirmed the 24p3 protein interacting with RL95-2 cells. Based on the results of Devireddy et al. (2005), we used HeLa cells as our negative control assay (Fig. 2g–i). We did not observe any binding or internalization of 24p3 protein to HeLa cells and coincided with our previous data for the negative interaction with A31 cells (embryo fibroblast cell line) (data not shown). It indicates a kind of cell type specificity of 24p3 protein; even it can bind with L929 cells. Results like these tend to reinforce our prediction of biological response of 24p3 protein on RL95-2 cells.

3.3. Detection of cell viability in 24p3 protein supplemented medium

Using 24p3 protein supplemented medium, we proceeded to clarify the directed effect of 24p3 protein on the RL95-2 cell viability under serum-free condition. In our previous experiment, we found that the concentration of 24p3 protein in mature mouse uterine fluid was around 5–10 μM (data not shown). Based on that, we used 10 μM 24p3 protein for our protein effect study in RL95-2 cells. The data in Fig. 3 showed that the cell would maintain in better condition ($93 \pm 3\%$) under serum medium, than that had $83 \pm 5\%$ viable cells under serum-free medium. In a 24p3 protein supplemented serum-free medium, the viability of the cells would be diminished significantly to $54 \pm 4\%$ (comparison of control cells and serum-free medium cultured cells; $p < 0.001$). The effect of this protein also showed dose-dependence on the RL95-2 cell viability under both cultured conditions (data not shown). The supplement of 24p3 protein would reduce the cell viability in these cultured conditions, suggesting 24p3 protein as a death-promoting protein. For clarify the cell death, we identified the cell status via double staining assay. After 24 h incubation of RL95-2 cells with 24p3 protein, AnnexinV-FITC and PI double staining showed a 4.5–5-fold increase in the number of cells undergoing early apoptosis and 7.5–10-fold increase in the number of cells undergoing late apoptosis after 24 h of 24p3 protein treatment comparing to the serum and serum-free cultured cells (Fig. 4). RL95-2 cells cultured in serum and serum-free media showed the similar apoptotic percentage as each other, even the less viable cells in serum-free cultured cells showing in Fig. 3. It suggested the cell died via apoptotic pathway.

3.4. Induction of ROS by 24p 3 protein

Increasing intracellular ROS may lead to stress and resultant induction of apoptosis, so we attempted to evaluate the ROS in RL95-2 cells. With this purpose, the cultured cells were incubated with the membrane

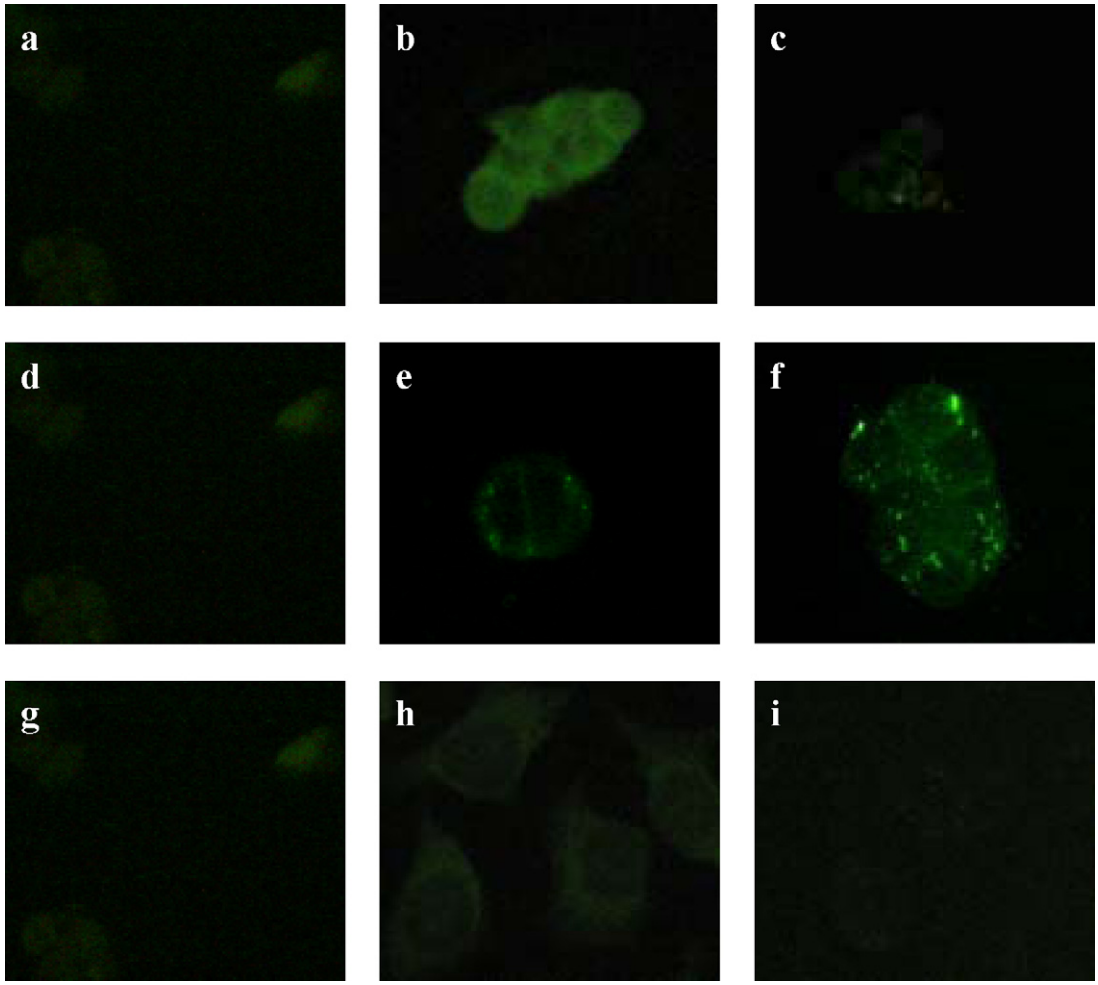

Fig. 2. 24p3 protein specific binding assay and internalization. 1.2×10^5 RL95-2 cells and HeLa cells were grown on coverslips in MEM medium. After 24 h, cells were washed twice with PBS buffer then fixed with methanol for 2 min. FITC-labeled 24p3 protein was supplemented and incubated for 1 h at 37 °C under dark conditions. Before observation under the fluorescent microscope, the free protein was removed from the cells with PBS (upper panel). (a) Control cells without 24p3 protein incubation; (b) with 5 μg FITC-labeled 24p3 protein; (c) with 5 μg FITC-labeled 24p3 protein and 50 μg unlabeled 24p3 protein. The internalization detections were incubated with 1.0 μg of FITC-labeled 24p3 protein for 30 min at different temperatures (middle panel). After incubation, free proteins were removed by 50 mM glycine and 150 mM NaCl (pH 3.0) buffer for 1 min. Washed twice with PBS and fixed with methanol:acetone (1:1, v/v) for 5 min and observed under the fluorescent microscope. (d) Cells without FITC-labeled 24p3 protein; (e) incubated with FITC-labeled 24p3 protein at 10 °C; (f) incubated with FITC-labeled 24p3 protein at 37 °C. The lower panel is negative control of HeLa cells. (g) HeLa cells without 24p3 protein; (h) HeLa cells incubated with FTIC-labeled 24p3 protein at 37 °C for internalization test; (i) fixed HeLa cells were incubated with FITC-labeled 24p3 protein for protein binding measurement.

permeable probe DCFH/DA. Inside cells, the oxidation of DCFH to fluorescent DCF resulted in high cellular fluorescence intensities. We tested ROS production in RL95-2 at various time intervals, in 24p3 protein supplemented medium. The data is shown in Fig. 5A. After exposure to 24p3 protein for 2 h, although no distinct morphological changes were observed, ROS increased almost 5-fold. The increase of intracellular ROS would maintain high levels for at least 10 h. The elevation of intracellular ROS may initiate the intracellular response. Meanwhile, there has no significant increase of intracellular ROS in control cells without 24p3 protein supplement within 10 h (data not shown). Even the 24p3 protein treatment of RL95-2 cells for short period, we also found that the ROS have risen to 1.2-fold and 1.7-fold under 30 and 60 min incubation, respectively (Fig. 5B). Using 20 μM DPI could prevent the 24p3 protein from inducing ROS production to 50% under 60 min incubation ($p < 0.001$, comparing to the absence of DPI). The data indicated that 24p3 protein could induce the

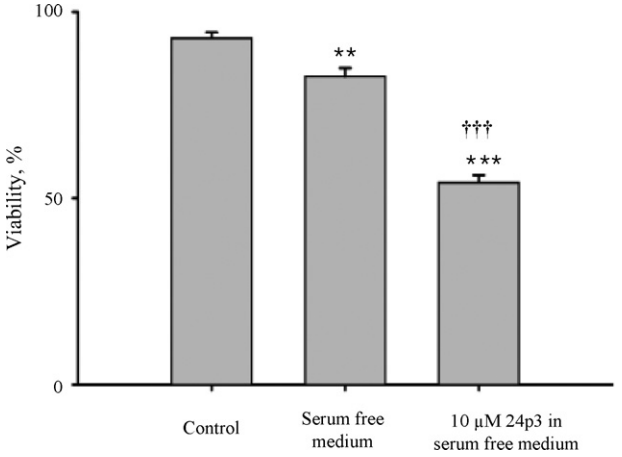

Fig. 3. Cell viability under 24p3 protein supplemented media. RL95-2 cells were cultured for 36 h. Fresh media were changed and the cultures were continued in with or without 10 μM 24p3 protein for 24 h under serum-free medium. The viabilities were compared to that of RL95-2 cells cultured in 10%FBS medium for 24 h as control after trypan-blue staining. The results were confirmed in multiple experiments and presented as the mean ± S.D., (**) $p < 0.01$, (***) $p < 0.001$, $n \geqq 4$.

increase of intracellular ROS significantly within a short time, upon the protein interaction with the cells and suppressed by ROS inhibitor.

3.5. Effect of 24p3 protein on the mitochondrial membrane potential

Apoptosis involves a disruption of mitochondrial membrane integrity that is decisive in the process of cell death (Zamzami et al., 1995). Based on that, we measured the mitochondrial membrane potential changes via JC-1; cells were labeled with JC-1 reagent for 15 min after 24p3 protein incubation. After washing to remove the free proteins, cells were analyzed on a flow cytometer and we calculated the decrease of mitochondrial membrane potential via Eq. (1). Upon the 24p3 protein reacting with the cells for 12 h, the mitochondrial membrane potential would decrease to 48 ± 2.2%, as compared with the control cells (Fig. 6). Valinomycin permeabilized the mitochondrial membrane for K^+ ions, dissipates the mitochondrial electrochemical potential, and is used as a positive control that prevents JC-1 aggregation and with the green fluorescence. The results revealed a suppressing effect of 24p3 protein on mitochondrial potential in 10 μM for 12 h.

3.6. 24p3 protein induced cytochrome c release from mitochondria

Disruption of mitochondrial membrane potential, results from the opening of permeability transition pores, causing a local disruption of the outer mitochondrial membrane, and leading to the release of soluble intermembrane proteins, including cytochrome *c* release, which contributes to the caspase activation (Slee et al., 1999). Since, Fig. 6 showed a decrease of mitochondrial membrane potential ($\Delta\Psi$m) in 24p3 protein-exposed RL95-2 cells, it was important to establish the relationship between $\Delta\Psi$m and cytochrome *c* release individual cells. We exposed the cells to 24p3 protein, to induce the change of $\Delta\Psi$m for 12 h and then observed the distribution of cytochrome *c* and mitotracker. Fig. 7A shows that only a mitotracker, Hsp60, distributed in the outer membrane surface of mitochondria and shows the filamentous structure. It indicates the integrity of mitochondria in the control cells. As for the incubation of normal RL95-2 cells with anti-cytochrome *c* antibody, the data indicated that cytochrome *c* resided on the mitochondria

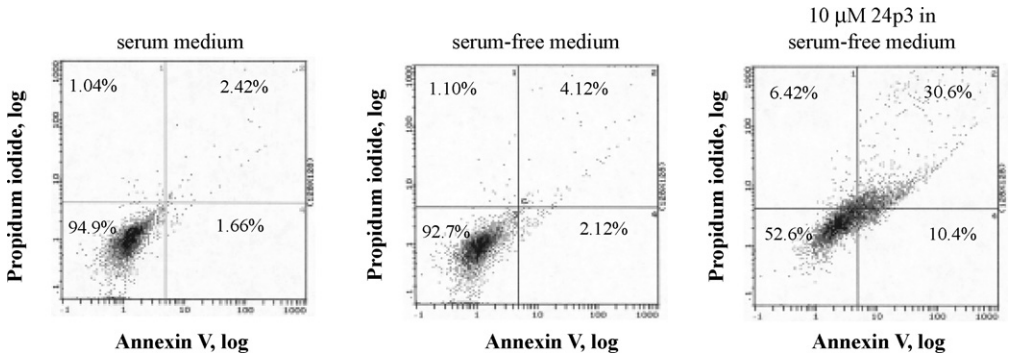

Fig. 4. Effect of 24p3 protein on RL95-2 cells apoptosis. RL95-2 cells were cultured for 36 h. Fresh media were changed and the cultures were continued in with or without 10 μM 24p3 protein for 24 h under serum-free medium. The cell death was compared to that of the control cells with double staining method. The percentage of apoptotic cells detected by annexinV-FITC/PI double-staining was determined by flow cytometry. We calculated the percentages of apoptotic cell in comparison to the untreated cells.

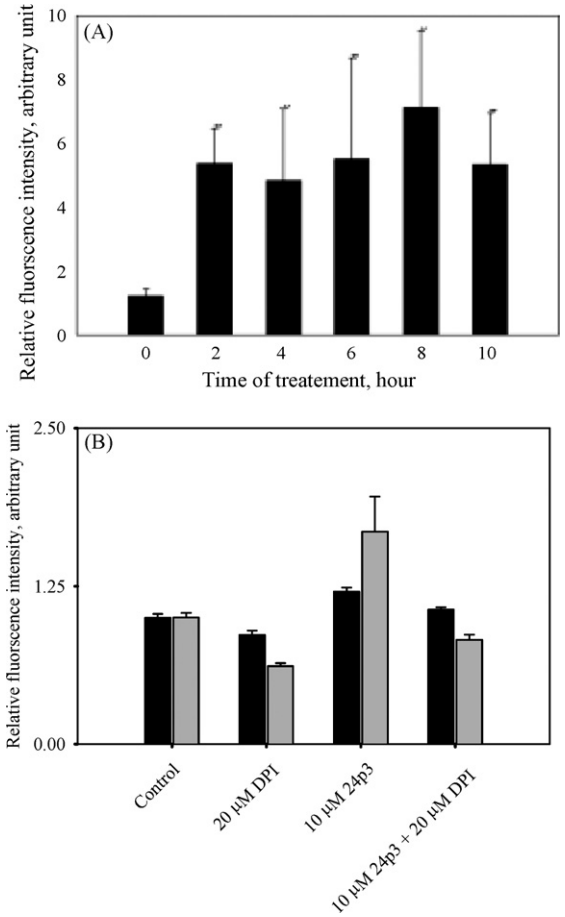

Fig. 5. Induction of ROS by 24p3 protein treatment. RL95-2 cells were treated with 5 μM 24p3 protein for various time intervals, followed by incubation with 20 μM DCFH-DA for 15 min. The amount of intracellular ROS can be quantified by detection via a microfluorometer with excitation and emission wavelengths at 485 and 535 nm, respectively. The results were confirmed in multiple experiments and presented as the mean ± S.D., (**) $p < 0.01$, (***) $p < 0.001$, $n = 5$. (A) The time course of 24p3 protein effect on RL95 cells. (B) The DPI prevents the 24p3 protein from inducing ROS in RL95-2 cells. The black bar indicates the 30 min incubation and the gray bar indicates 60 min incubation of 24p3 protein with the cells.

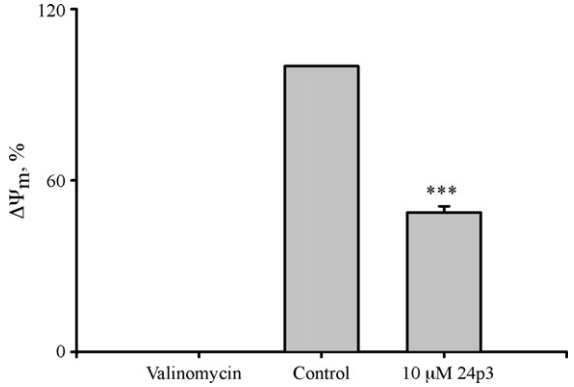

Fig. 6. Effect of 24p3 protein on the mitochondrial membrane potential. Cells were incubated with 10 μM 24p3 protein for 12 h, after which they were subjected to cell membrane potential assays along with the control cells. Valinomycin, a potassium ionophore, was added to decrease the membrane potential as a positive control. Using the flow cytometric method, the mitochondrial membrane potential was measured after 24p3 protein treatment. Based on the intensity of FL1, we calculated the percentage of mitochondrial membrane potential of 24p3 protein treated-RL95-2 cells. The results were confirmed in multiple experiments and presented as the mean SD, (***) $p < 0.001$, $n = 3$.

(Fig. 7B). Before cytochrome *c* was released from mitochondria, the distribution pattern of both mitotracker and cytochrome *c* was similar to that of mitochondria in the normal cells, i.e., they appeared as filamentous structures. To determine whether 24p3 protein plays a role in regulating the release of cytochrome *c* from mitochondria into cytosol, the location of cytochrome *c* was examined. Immunoassaying showed that 24p3 protein caused the disappearance of mitochondria filamentous structure, the smeared and aggregated cytochrome *c* molecules could be observed and indicated the release of the cytochrome *c* (Fig. 7C). In addition, treatment with 24p3 protein and 20 μM DPI (ROS inhibitor) led to the reduction of the cytochrome *c* release and a part of the mitochondrial filamentous structure reappeared in RL95-2 cells (Fig. 7D). The reduction of ROS accompanied the reduction of cytochrome *c* release, suggesting that the 24p3 protein caused ROS and triggered cytochrome *c* release from mitochondria.

3.7. Involvement of caspases activation in 24p3 protein-induced cell death

Because activation of the caspase-9 is necessary for the processing and activation of caspase cascades, we evaluated whether the processing and activation of caspase-3, is considered to play a central role in many types of stimuli-induced apoptosis (Nicholson and Thronberry, 1997). Caspase-8 is associated with receptor-mediated cell death pathway (Nagata, 1997); the activation of caspase-8 was detected also after 24p3 protein treatment. The involvement of caspases in 24p3 protein-induced cell death was tested using specific fluorogenic assays for caspase-8, -9 and -3. Results of these studies are shown in Fig. 8A, where it can be seen that exposure to 24p3 protein led to a significant enhancement in caspase-8, -9 and -3 activity up to 4–8-fold after 12 h incubation, compared with control values measured in untreated cells. Stimulation of the initiator-caspase-9 activity was much higher compared to initiator-caspase-8. The caspase 9 may play an important role in effector caspase 3. According to data of Fig. 5B, using 20 μM

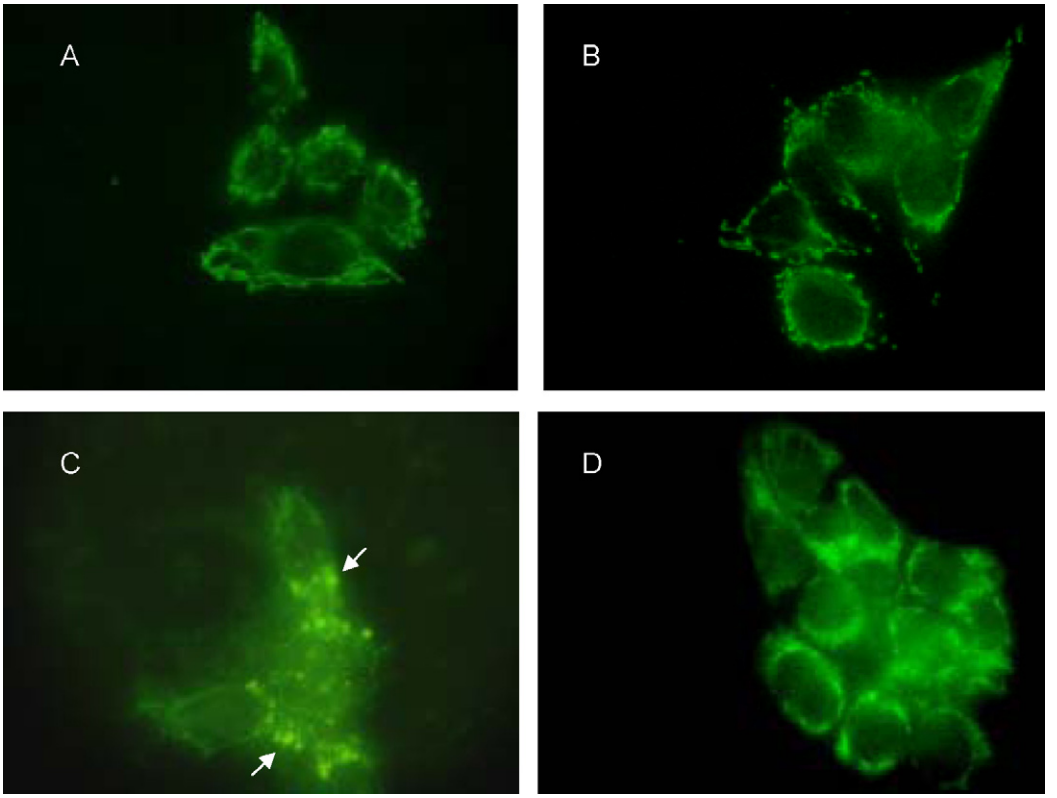

Fig. 7. Immunofluorescence detection of mitochondrial cytochrome *c* release from RL95-2 cells. RL95-2 cells were incubated with or without 10 μM 24p3 protein for 12 h in a serum free medium and processed for cytochrome *c* and Hsp60 immunostaining as described in the text. Hsp60 as mitochondrial marker indicates the integrity of the mitochondria. (A) Control cells were stained with anti-Hsp60 antibody; (B) Control cells were stained with cytochrome *c* antibody; (C) 24p3 protein treated cells were stained with cytochrome *c* antibody; (D) 24p3 protein and 20 μM DPI (ROS inhibitor) treated cells were stained with cytochrome *c* antibody. Arrows indicate the aggregation of cytosolic cytochrome *c*.

DPI as a suppressor of ROS production, we found the suppression of effector caspase 3 activity could be inhibited for about 40% (Fig. 8B) ($p < 0.01$, comparing to the absence of DPI). It's coincided with the reduction of ROS production in the presence of DPI in 24p3 protein supplement medium. The data suggested that the suppression of RL95-2 cell apoptosis would appear upon the caspase 3 activity reduction.

3.8. DNA abnormality and cell cycle changes induced by 24p3 protein

Oxidative stress is associated with induction of DNA damage or DNA abnormality by caspases activated DNase, triggering the changes of cell cycle progression (Enari et al., 1998; Wiseman, 2006). Upon the caspases activation by 24p3 protein, we should identify the DNA status and cell cycle progression in RL95-2 cells. Fig. 9 shows the effects of 24p3 protein at 10 μM for 24 h on the DNA damage based on the DAPI and PI staining. After 24p3 protein treatment, nuclear condensation was noted and the small DNA particles appeared in intracellular of the RL95-2 cells, comparing to the control cells (Fig. 9A and B). Cell cycle analysis yielded a significant difference between control cells (Fig. 9C) and protein treated cells (Fig. 9D) in the proportion of each phase. The data showed the distribution of cell cycle of 24p3 protein treated cells at G0/G1, S, and G2/M were lower than that in control cells, but the subG1 (25–30%) was higher than that in control cells. It suggested that 24p3 protein induces subG1 phase and that this result coincides with DAPI observation and the data in Fig. 4, and that it was associated with a marked induction of the apoptotic response to 24p3 protein supplement, which exhibited DNA damage and interfered with the normal cell cycle in RL95-2 cells.

4. Discussion

24p3 protein has been mentioned as mainly expressed in tissues that may be exposed to microorganisms (Friedl et al., 1999), or detected in acute inflammatory response

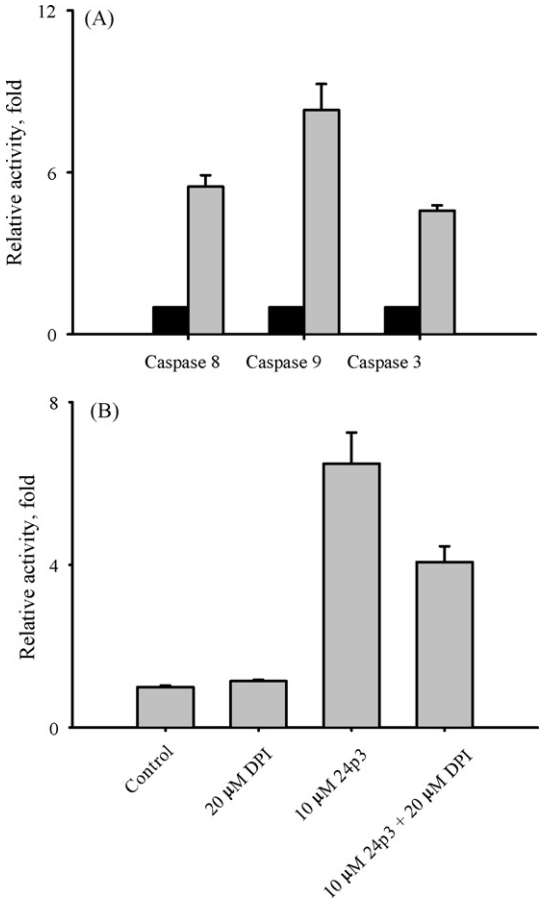

Fig. 8. Effect of 24p3 protein on activities of caspases. (A) The caspases activities for caspase 8, -9 and -3. (B) The effect of ROS inhibitor, 20 μM DPI, on caspase 3 activity. Cells were cultured in 48-well plates with 10 μM 24p3 protein supplement for 12 h, then incubated with 50 μl of substrate solution (10 μM PhiPhiLux G1D2 with 10% FBS) for 1 h at 37 °C. After a wash with PBS, cell were harvested by trypsinization and detected by flow cytometry. The cleaved substrate has the following excitation and emission peaks: $\lambda_{ex} = 505$ nm and $\lambda_{em} = 530$ nm. The black bars indicate the caspases activities control cells and the gray bars indicate that of the 24p3 protein-treated cells. The results are expressed as the ratio of fluorescent intensity and are given as the mean ± S.D. for five experiments $p < 0.001$ as compared with control ($n \geqq 1$).

(Xu et al., 1995), regarding these conditions' stress status. Besides, Kjeldsen et al. (2000) noted that while the 24p3 promoter was processed as a glucocorticoid responding element, its functional significance is still not clear. In the present study, we have demonstrated that RL95-2 cells produce 24p3 protein and are up-regulated both in dexamethasone stimulation and starvation condition. Moreover, it suggested that the protein caused the reduction of cell viability. These findings are in agreement with other reports (Garay-Rojas et al., 1996; Kamezaki et al., 2003). Cowland et al. (2003) showed that during lung inflammation, the human 24p3 protein synthesis increases in bronchial epithelial cells, so it was considered as a disease activity marker (Kjeldsen et al., 2000; Hemdahl et al., 2006). All of these imply that the 24p3 protein correlates with environmental stress and tissue damage.

Mouse 24p3 protein in uterine fluid is related to estrogen stimulation and fluctuation during the estrous cycle of mice (Chu et al., 1996), in fact, estrogen can not stimulate the 24p3 protein expression directly on RL95-2 cells (data not shown). Actually, two GREs being identified in 24p3 promotion suggested that 24p3 synthesis was indirectly regulated by estrogen *in vivo*. In our laboratory, we have characterized mouse 24p3 protein expression during the proestrous and estrous phases of estrous cycle (Huang et al., 1999). During this period, a lot of molecules are highly expressed in response to endometrial regeneration (Yousefi and Simon, 2002). 24p3 protein may be involved in the proceedings regarding the balance between cell proliferation and cell death. From the data, it appears that 24p3 protein expression is activated in stress conditions of different nature, which is either physiological or pathological. Glucocorticoid has been designated as the stress hormone because its levels in circulation rise in response to stress and show the changes of specific physiological stimuli (Hardy et al., 2005). In cultured cells, 24p3 protein increment is observed in response to glucocorticoid stimulation, and also in other conditions such as serum deprivation. Elevating the glucocorticoid level in circulation during stress, may trigger the 24p3 protein highly expression under this stress condition and exert an autocrine control (Bigsby, 1993) during stress thus playing a role in cell death (Sivridis and Giatromanolaki, 2004). Our data coincided with their hypothesis. There is a report (Cancedda et al., 2002) that lipocalin EX-FABP represents stress protein expressed in tissues where active remodeling is taking place, and also is expressed in stress response. The 24p3 protein suggested the similar function of this protein in uterus.

Over the years, ROS has been perceived as a biological hazard, causing oxidative damage to the cellular components, and leading to cancer, cell degeneration and disorders related to aging (Molavi and Mehta, 2004). The uterus undergoes cellular remodeling during the estrous cycle, and the uterine cellular changes are regulated by the levels of sexual hormones during the cycle. One of the basic events of uterine cellular change is systematic cell turnover that consists of cell death by apoptosis and cell renewal by proliferation (Joswig et al., 2003; Zhang and Paria, 2006). During the estrous cycle, the estrogen (E) level is significant higher in the proestrous

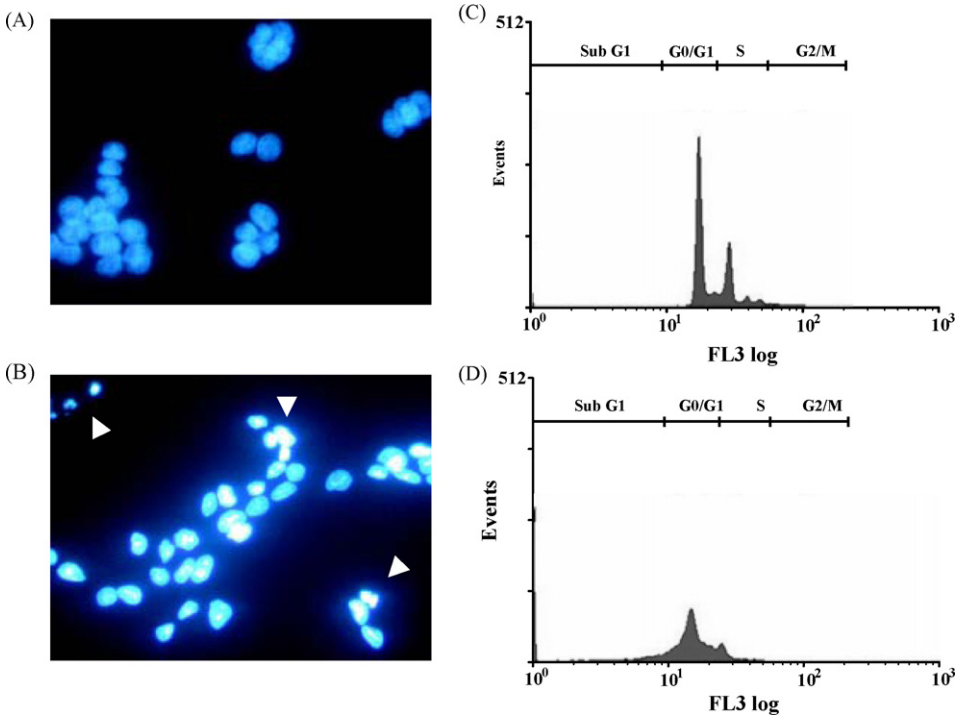

Fig. 9. Detection of 24p3 protein inducedRL95-2 cells DNA damage. Cells were cultured in cover slips with 10 μM 24p3 protein supplement for 24 h; the formation of DNA damage can be observed under a fluoromicroscope after DAPI staining. (A) Control; (B) 24p3 protein treated cells, the condensed DNA and fragmented DNA indicated with arrows. After treatment with 24p3 protein, cells were detached by trypsinization and stained with PI then determined cell cycle by flow cytometry. (C) Control; (D) 24p3 protein treatment.

phase than that in the estrous phase, whereas the progesterone (P) level is higher in the estrous phase and lower in the proestrous phase. Elevated E and P levels would be expected to be associated with an increase in the level of glucocorticoid (Smith et al., 1994) and raised a physiological stress-like condition. In our previous study, 24p3 protein appeared intensively in proestrus and estrus, and then declined from metestrus and diestrus (Huang et al., 1999). It suggested that 24p3 protein secretion closely overlapped with the remodeling of the endometrial cells. At this moment, a stress-like situation may exist, with 24p3 protein secretion playing a role in cell death. The present study is undertaken to examine the tenet that intracellular ROS level, which rises by 24p3 protein, plays a role in determining events like apoptosis in RL95-2 cells. It is commonly hypothesized that the balance between cell proliferation and cell death is important in epithelial cell homeostasis (Mori et al., 1999), such as during the estrous cycle. In this study, 24p3 protein was implied in the process potential to generate cellular apoptosis. It seems to provide a clue for the cell death occurring in the estrous cycle. According to our data, 24p3 protein should be a death promoting factor rather than an anti-stress protein. 24p3 protein, as a uterine protein, may be involved in the uterine remodeling for balancing the cell status during menstrual cycle. The data initiate another interesting issue in female reproductive system.

Previous studies from our laboratory (Chou et al., 2006; Elangovan et al., 2004) as well as from other investigations (Devireddy et al., 2005) have provided evidences that 24p3 protein internalizes via the 24p3 protein receptor, but the function is unclear. Richardson (2005) mentioned that both apo- and holo-24p3 protein can be bound and internalized by their receptor, but with each resulting in different biological functions. Our results showed that supplementation of 24p3 protein in RL95-2 cells can causes the elevation of intracellular ROS, changes of mitochondrial membrane potential, release of cytochrome *c* and activation of caspases, resulting in apoptosis. At present, we figure that the apo-24p3 protein causes the cell death via the apoptosis pathway in RL95-2 cells. We proposed that glucocorticoid induced the 24p3 protein expression and triggered an autocrine pathway to affect the RL95-2 cells. Thereafter, apoptosis is mediated by interaction between 24p3 protein and its receptor, which leads to a cascade of activation of caspases via ROS production (Fig. 10).

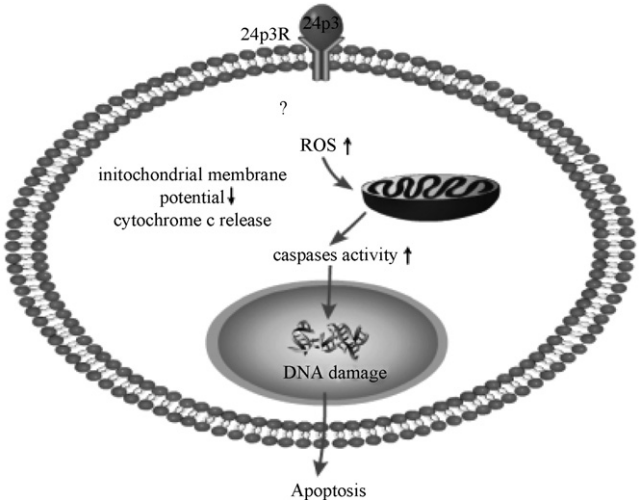

Fig. 10. Proposed the way of 24p3 protein-induced apoptosis. This schematic depicts the caspase pathway involved in apoptosis. The mitochondrial-mediating pathway involves loss of mitochondrial membrane potential and cytochrome *c* release leading to activation of caspases and resultant cell death. Reactive oxygen species (ROS) may act as an extracellular intermediate directly stimulating the mitochondrial cell death pathway.

The caspases cascade is activated by two distinct pathways and regulates apoptosis: the intrinsic pathway, in which mitochondria play a central role, and the extrinsic pathway, in which cell plasma membrane receptors act as the initiation point of the apoptotic process (Beere, 2004). In the extrinsic pathway, caspase-8 is activated and subsequently cleaves and activates down-stream caspases, such as caspase-3 (Peter and Krammer, 2003). In the intrinsic pathway, various apoptosis inducing signals directly or indirectly change mitochondrial membrane potential and cause cytochrome *c* release. In cytoplasm, caspase-9 activation leads to the activation of the down-stream caspases, such as caspase-3 (Li et al., 1997). Caspase-3, known as the executioner caspase, is located downstream of the caspase-8 or -9 activation cascade, and represents a point where the intrinsic and extrinsic apoptosis pathways converge. The intrinsic and extrinsic pathways can interact and regulate each other, forming a complex network through apoptotic related proteins (Zhang et al., 2003). According to our data, caspase-8 and -9 have been activated upon 24p3 protein's interaction with RL95-2 cells. The apoptotic pathway related proteins and relationship between ROS production and caspases require further study.

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