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Induction of Apoptosis by Ursolic Acid in F9 Teratocarcinoma Cells

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Abstract

The apoptosis-inducing activity of ursolic acid (UA) was examined in mouse F9 teratocarcinoma cells on the bases of biochemical and morphological characteristics. UA, pentacyclic triterpene acid, exhibits antitumor activities including inhibition of skin tumorigenesis, induction of tumor cell differentiation and antitumor promotion. Treatment with UA showed that the decrease of cell viability was dose-dependent. UA also induced genomic DNA fragmentation, a hallmark of apoptosis, indicating that the mechanism of UA-induced F9 cell death was through apoptosis. When the morphology of the F9 cells was examined by electron microscopy, the cells treated with UA showed the characteristic morphological features of apoptosis such as chromatin condensation and nuclear fragmentation. DNA fragmentations by UA were inhibited by cycloheximide, which suggest that *de novo* protein synthesis was required for DNA fragmentation by UA. In addition, the expression of c-*jun* was increased, but those of c-*myc* and laminin B1 were decreased during apoptosis induced by UA in F9 cells. These results suggest that UA causes an apoptosis in F9 cells. Further, the increased expression of c-*jun* may be involved in the UA-induced apoptosis of F9 cells.

Key words : *Apoptosis, Ursolic Acid, F9 Teratocarcinoma*

Introduction

Recent approaches of therapy for various types of cancer have focused on drugs that induce the apoptosis or differentiation of maturation resistant cells causing the disease[1,2]. The finding that drugs (glucocorticoids, topoisomerase inhibitors, alkylating agents, antimetabolites, and antihormones) with different targets induced what appears to be final common pathway of cell death has shifted the focus of recent studies of the molecular pharmacology of these agents from their immediate targets to the events that they trigger in the cell. A variety of anti-cancer drugs have been shown to induce extensive apoptosis in rapidly proliferating normal cell populations, lymphoid tissues, and tumors[1,3-5]. The fact that disparate agents, which interact with different targets, induce cell death with some common features (endonucleolytic cleavage of DNA, changes in chromatin condensation) suggests that cytotoxicity is determined by the ability of the cell to engage the cell death. Recent reports have shown that a high level of c-*myc* expression is correlated with DNA damage in the presence of etoposide, a cytotoxic drug. The expression of c-*fos* and c-*jun* has been found to be induced during apoptosis of lymphoid cells[6]. In addition, some of the extracellular matrix (ECM) components, except fibronectin and collagen, have been shown to suppress apoptosis of mammary epithelial cells both in tissue culture and *in vivo*[1].

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Triterpenoid compounds are similar to glucocorticoid hormone in structure. Certain glucocorticoids strongly inhibit TPA-induced skin inflammation and tumor initiation[8]. Some triterpenoid compounds were also reported to antagonize tumor promoter[9-12] and to induce differentiation of F9 cells same as retinoic acid (RA)[13]. Ursolic acid (UA) is one of triterpenoid compounds and can be extracted from plants, *Eriobotrya japonica*[9], *Glechoma hederacea*[11], *Rosmarinus officinalis*[14], and *Calluna vulgaris*[15], *etc.* The UA was shown to inhibit TPA-induced inflammation and tumor promotion in mouse skin[11, 12], to inhibit TPA-induced Epstein-Barr virus activation in Raji cells[16], and to inhibit 12-O-hexa-decanoyl-16-hydroxyphorbol-13-acetate-induced edema of mouse ears[17]. When the inhibitory activity of UA was compared to that of RA in Raji cells, UA is less toxic than RA [10,12]. In addition, UA was also shown to possess an antiangiogenic effect in chick chorioallantoic membrane[18] and an anti-invasive activity in HT1080 human fibrosarcoma cells[19].

Based on the above facts, the possibility of whether UA could induce apoptosis was studied by employing several techniques, using F9 cells as a model system. In addition, the effect of CHX and the gene expression of c-*myc*, c-*jun* and laminin were examined to determine the possible interrelationship between the expression of these genes and the induced apoptosis in F9 cells.

Materials and Methods

Materials

Fetal calf serum (FCS), Dulbecco's Modified Eagle's Medium (DMEM), and other chemicals for cell culture were purchased from Gibco BRL (Grand Island, NY). Cell culture flasks and plates were purchased from Corning (Corning, NY). Agarose was purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, MD). UA, CHX, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Line and Cultured Conditions

The mouse F9 teratocarcinoma cell line utilized in these studies was obtained from the American Type Culture Collection (Rockville, MA). F9 cells were maintained in DMEM medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 g/ml streptomycin at 37 ℃ in a humidified atmosphere containing 5% CO_2/95% air. All cell culture flasks and plates were coated with 0.3% gelatin for more 2 hr at 4℃[20]. Stock cultures were maintained as monolayer cultures and passed by trypsinization every 2 to 3 days. The cells used for experiments were between passages of 5 and 15. The cell numbers were calculated with hemocytometer.

Cell viability test (MTT assay)

Cell viability was assessed as described by Mosmann[21]. Briefly, the cells were incubated in 100 μl of media in 96 well plate at an initial cell density of 3×10^5 cells/ml for 24 hr. An appropriate volume of drug vehicle was then added to cells. After each period of incubation, 100 μl of MTT solution (5 mg MTT/ml in H_2O) was added and the cells were further incubated for 4 hr at 37℃. After washing out the MTT-containing media, 200 μl of acid-isopropanol (0.04 N HCl in isopropanol) were added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. The relative cell viability was obtained by scanning with an ELISA reader (Molecular Devices Corp., USA) with 570 nm filter. All experiments were performed more then three times. All data were displayed as % of control condition. All the control experiments were carried out in the same media containing drug-free vehicle. Data were expressed as mean ± standard error of the mean (S.E.M.) and were analyzed using one way analysis of variance and Student Newman Keul's test for individual comparisons. P values less than 0.05 are considered to be statistically significant.

Agarose Gel Analysis of DNA Fragmentation

For qualitative analysis, F9 cells were harvested and

washed with cold phosphate buffered saline (PBS), and lysed with 500 mM Tris-Cl, pH 8.0, 2 mM EDTA, 10 mM NaCl, 1% (w/v) SDS, and proteinase K (500 μg /ml) at 50℃ for 24 hr. Samples were then centrifuged at 27,000 xg at 18℃ for 2 hr. Supernatants were extracted twice with phenol extraction, precipitated with 0. 1 volume of 5 M NaCl and 1 volume of isopropanol, and resuspended in distilled water. Samples were then treated with 100 μg/ml DNase-free RNase A for 1 hr at 37℃. Loading buffer (2.5% Ficoll, 0.025% bromophenol blue, 0.025% xylene cyanol) was added to samples and electrophoresis was performed on 1.5% agarose at 50 V in TAE buffer (40 mM Tris-acetate and 1 mM EDTA) for 3 hr and the agarose gel was stained with 0.5 μg/ml ethidium bromide.

Transmission Electron Microscopy

F9 stem and UA-treated cells were harvested and washed with cold PBS. Cell pellets were fixed at 4℃ for 30 min in freshly prepared mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer. Cell pellets were then mixed with 2% agarose gel, cut to 1 mm^3, and fixed at 4℃ overnight in freshly prepared mixture of 2 % paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer. Cell blocks were post-fixed in 1% osmium tetraoxide solution in 0.1 M phosphate buffer. The samples were finally dehydrated with Epon 812 and embedded in polar Epon mix. Sections were cut in 60−90 nm thickness with LKB ultramicrotome and stained with uranyl acetate and lead citrate, then examined by a transmission electron microscope (JOEL 1200EXII) at 80 KV.

Northern Analysis

Total cellular RNAs were isolated by the acid-guanidinium thiocyanate-phenol-chloroform extraction method [22]. The Northern blotted nylon membrane was hybridized with ^{32}P-labeled probes. The filter was dried and exposed to X-ray film for 2 to 5 days.

Results

Effects of UA on F9 Cell Proliferation.

Survival of F9 cells following 3-day continuous exposure to 1−12.5 μM of UA was determined by MTT assay. According to the increase of concentration of UA (Fig. 1), the proliferation of F9 cells was suppressed. In UA-treated cells, 10 and 12.5 μM UA decreased survival to 37.8% and 6.76%, respectively, compared with control. These results indicate that the growth of F9 cells is inhibited in a dose-dependent manner in case of the treatment with UA.

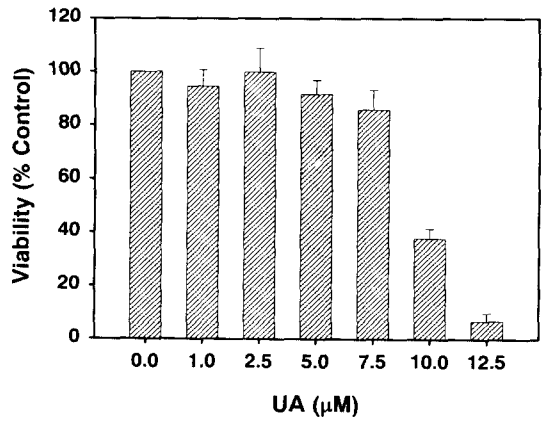

Fig. 1. Effects of UA on the viability of F9 cells. The relative viability of F9 cells was assessed by MTT assay. F9 cells were treated with various concentrations of UA for 72 hrs. *p<0.05 compared to control.

DNA Fragmentation by UA in F9 Cells.

To assess the pattern of DNA fragmentation caused by UA, agarose gel electrophoresis was performed (Fig. 2). F9 cells were treated with 0−20 μM of UA for 24 hr, respectively. The DNA fragment bands with a characteristic pattern of internucleosomal ladder suggesting of apoptosis were observed in cells treated with more than 5 μM of UA for 24 hr. On the contrary, no or little DNA fragment bands were observed in stem cells and the cells treated with less than 1 μM of UA for 24 hr.

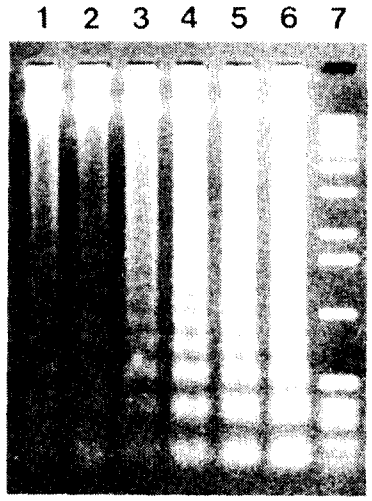

Fig. 2. DNA fragmentation in UA-treated F9 cells. *Lane 1,* DNA isolated from F9 stem cells ; *Lane 2, 3, 4, 5,* and *6* represent DNA isolated from cells treated with 1, 5, 10, 15, and 20 μM of UA for 24 h, respectively ; *Lane 7,* 1 Kb DNA ladder as a molecular weight marker.

Morphological Changes by UA in F9 cells.

F9 cell treated with 10 μM of UA for 24 hr showed the distinct morphological features of apoptosis as chromatin condensation and aggregation at the nuclear membrane, and several condensed nuclear fragments as shown in Figure 3*B*.

Effects of Cycloheximide on DNA Fragmentation by UA in F9 Cells.

Because apoptosis is an active process, it requires the synthesis of new proteins. We determined whether this inhibitor could cause DNA fragmentation by itself and prevent UA-induced DNA fragmentation in F9 cells. The cells were treated with 0, 0.5, 1, 5, or 10 μM of cycloheximide (CHX) for 28 hr. As shown in Figure 4A, no or little DNA fragment bands were observed in either stem cells or cells treated with less than 1 μM of CHX, whereas DNA fragment bands were observed in cells treated with more than 5 μM of CHX. When F9 cells

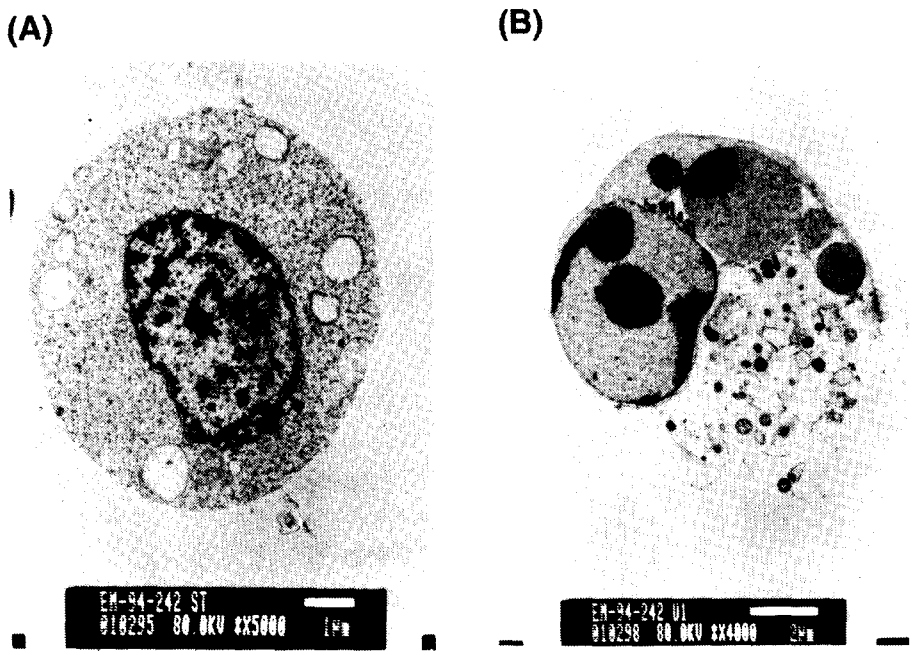

Fig. 3. Electron micrographs of apoptotic F9 cells. A, Stem cell (original magnification X5,000). B, 10 M of UA-treated cell (original magnification X4,000).

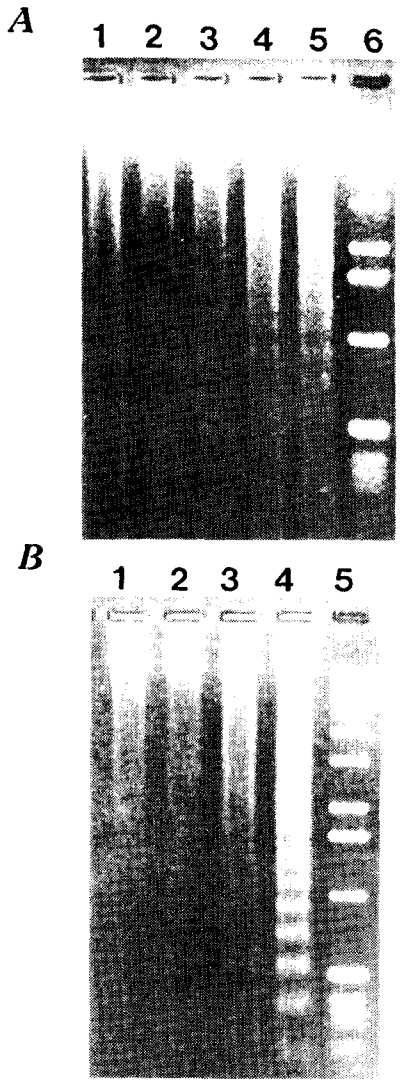

Fig. 4. DNA fragmentation by CHX and effects of CHX on UA-induced DNA fragmentation in F9 cells. *A, Lane 1,* control F9 cells ; *Lane 2, 3, 4,* and *5* represent DNA isolated from cells treated with 0.5, 1, 5, and 10 μM of CHX for 28 h, respectively ; *Lane 6,* 1 Kb DNA ladder. B, *Lane 1,* 2, and 3 represent DNA isolated from cells pretreated with 0.5 μM of CHX for 4 hr and treated with same concentration of CHX and 5, 10, and 20 μM of UA for 24 hr, respectively ; *Lane 4,* pretreated with CHX for 4 hr and treated only 10 M of UA for 24 hr ; *Lane 5,* 1 Kb DNA ladder as a molecular weight marker.

were pretreated with 0.5 μM of CHX for 4 hr and 5, 10, and 20 μM of UA with 0.5 μM of CHX for 24 hr, the DNA fragmentation by UA was blocked. DNA fragment bands were, however, observed in F9 cells pretreated with 0.5 μM of CHX for 4 hr, washed out the CHX, and treated with 10 μM of UA for 24 hr alone (Fig. 4*B*). These mean that *de novo* protein synthesis was necessary for DNA fragmentation by UA in F9 cells.

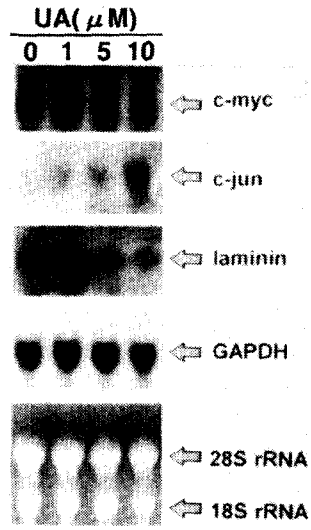

Fig. 5. Alteration of gene expression of UA-treated F9 cells. Human c-*myc*, human c-*jun*, mouse laminin B1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were labelled with α^{32}P-dCTP, and then used for northern blot analysis. For RNA loading control, GAPDH and rRNA were used.

Alteration of gene expression in apoptotic process induced by UA.

Northern blot analysis was used to determine the expression of genes in the F9 cells. The expression of c-*myc* proto-oncogene was decreased during apoptosis induced by UA in a dose dependent manner as shown in Fig. 5. In addition, the expression of laminin was sligh-

tly decreased, depending on the concentration of UA. In contrast, it was also found that the expression of c-*jun* was increased. This result indicated that the expression of c-*jun* may be related to apoptosis induced by UA.

Discussion

We have demonstrated that UA, known as a differentiation agent[13] and anticancer drug[8,9,11,12], causes apoptosis in F9 cells. These cells, which resemble the inner cell mass of the early mouse embryo, have the capacity to differentiate into multiple types of cell products[23,24] and have been used as a model to study differentiation in the mouse embryo. In a view of increasing evidences that a variety of chemotherapeutic agents can trigger programmed cell death, it is very interesting to investigate whether UA can cause DNA fragmentation and apoptosis in F9 cells.

In the present work, we attempted to determine whether UA could induce internucleosomal DNA fragmentation and trigger the process of apoptosis in F9 cells. F9 cells treated with UA were shown the morphological changes to rounded form (data not shown) and the decrease in the growth of the cells (Fig. 1). A DNA fragmentation assay by agarose gel electrophoresis and the rate of DNA fragmentation showed that UA at appropriate concentrations induced DNA fragmentation in a dose-dependent fashion (Fig. 2). These results mean that UA, have both antitumor and differentiation inducing activity, could cause DNA fragmentation. The ultrastructural morphology of F9 cell treated with UA (Fig. 3*B*) showed that the chromatin was condensed and aggregated at the nuclear membrane, and the cell contained several condensed nuclear fragments as described by other studies[25,26].

The finding that glucocorticoid-induced thymocyte apoptosis was prevented by treatment with protein synthesis inhibitors[27,28] demonstrates that apoptosis is an active, energy-requiring process and that the increments of protein are most likely required for apoptosis to occur. However, the requirement for macromolecule synthesis has not been a universal finding, as it has been found for other cell types, including S49 mouse lymphoma cells[29] and macrophages[30]. The effect of CHX, protein synthesis inhibitor, was tested on UA-induced DNA fragmentation in F9 cells. As results, F9 cells, pretreated with 0.5 μM of CHX for 4 hr and then treated with 10 μM of UA with CHX for 24 hr, did not show DNA fragmentation (Fig. 4B). However, DNA fragment bands were observed in F9 cells pretreated with 0.5 μM of CHX for 4 hr, washed out the CHX, and treated with 10 μM of UA alone for 24 hr (Fig. 4B). These mean that *de novo* protein synthesis was necessary for DNA fragmentation by UA in F9 cells. There are some possible explanations for the prevention of drug-induced DNA fragmentation and apoptosis by CHX. One possibility could be the inhibition of some critical proteins that may act as cofactors in triggering apoptosis. A second possibility might be an inhibition in the synthesis of repair enzymes[31].

In this study, we found that c-*jun* expression was increased depending on the concentration of UA in F9 cells (Fig. 5). The expression of c-*jun* may be related to apoptosis induced by UA. Previously, it was reported that AP-1 was induced in mouse mammary epithelial cells undergoing apoptosis[32] and that consists of dimeric complexes of different Fos and Jun family members [33]. Induced AP-1 transcription factor can express several genes which are related to apoptosis[34].

Recent reports have shown that a high level of c-*myc* expression is correlated with DNA damage in the presence of etoposide, a cytotoxic drug that inhibits topoisomerase activity[35]. In addition, when transfected with a constitutively expressed c-*myc* gene, Rat-1 fibroblasts enter apoptosis[36]. However, in this study we found the down-regulation of c-*myc* gene by UA, but the exact role of the c-*myc* down-regulation in the UA-induced apoptosis is not known (Fig. 5).

It also reported that apoptosis was induced by disruption of epithelial cell-matrix interactions[37]. Hence inhibitor of ICE whose activation gives rise to the disruption of ECM prevented apoptosis[38]. In this study the expression of laminin that is a major constituent of ECM, was slightly decreased by UA (Fig. 5), indicating that the structure of ECM in the cells may be somewhat disorganized during the apoptotic process. Taken together, these results suggest that apoptosis induced by UA may be related to the activation of AP-1 transcription factor and disorganization of ECM.

In the effort to screen naturally occurring substances which may be utilized as apoptosis inducing agents, UA was found to possess the ability to induce the apoptosis of F9 stem cells. The UA was reported to have anti-inflammatory and antitumor activity in some reports[8-12,15,16]. The UA is similar to glucocorticoid hormone in chemical structure. Glucocorticoids exert their physiological effects on target tissues by binding to specific receptor proteins that are present in glucocorticoid-responsive cells. The sequences to which glucocorticoid receptor (GR) binds are known as glucocorticoid responsive element (GRE) and typified by a region of the long terminal repeat (LTR) in the DNA of the glucocorticoid-regulated mouse mammary tumor virus (MMTV)[39]. Our previous study[13] showed that a high molecular weight protein (94 KDa) and a low molecular weight protein (45 KDa) band were present in the protein extracts of F9 cells incubated in the medium containing dexamethasone or UA. Therefore, it might be hypothesized that the UA-induced DNA fragmentation in F9 cells is occurred via the GR. From above results, it can be suggested that UA induce the apoptosis and regulate the gene expression by interaction with steroid receptor or orphan receptor of F9 cells. Therefore, the role of steroid receptors or a related orphan receptors in the apoptosis of F9 cells induced by UA is likely to be of great interest in the future.

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초록 : F9 기형암종세포에서 Ursolic acid의 apoptosis 유도기작

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Ursolic acid (UA)의 apoptosis 유도능을 F9 생쥐 기형암종세포를 대상으로 조사하였다. UA는 피부암화의 억제, tumor의 분화유도, tumor promotion의 억제 등의 항암 효과를 나타내는 pentacyclic triterpene acid로 물질로 알려져 있다. UA를 F9세포에 처리하였을 때 농도비례로 성장억제를 나타냈다. 또한 apoptosis과정에서 관찰되는 전형적인 DNA 분절을 관찰할 수 있었고, 전자현미경을 이용한 세포의 미세구조를 관찰한 결과, 역시 apoptosis과정에서 관찰되는 전형적인 형태인 염색질 응축, 핵의 분절들을 관찰할 수 있어, UA에 의한 항암 효과는 apoptosis에 의한 것임을 시사한다. UA에 의한 apoptosis는 단백질 합성 저해제인 cycloheximide에 의해 저해됨을 관찰할 수 있었다. UA를 F9세포에 처리한 후 oncogene의 발현양상을 조사한 결과, c-myc과 laminin B1은 apoptosis과정동안 점점 감소하고 c-*jun*은 증가함을 관찰할 수 있었다. 이상의 결과로서 UA에 의한 F9 세포의 apoptosis에는 새로운 단백질의 합성이 요구되며, c-*myc* 및 laminin의 발현 감소와 c-*jun*의 발현 증가가 관여하고 있음을 시사한다.