行政院國家科學委員會專題研究計畫 成果報告

果酸對培養的人類角質細胞與纖維母細胞的生物效應及果酸對無毛鼠皮膚作用的研究

研究成果報告(精簡版)

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中 華 民 國 98 年 11 月 02 日

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中文摘要

果酸(α-hydroxy acid)被廣泛應用於化妝品與皮膚淺層換膚之臨床治療，我們之前的研究證實甘醇酸及乳酸會經由引發人類皮膚角質細胞 (HaCaT cells)的凋亡作用而抑制細胞生長，而臨床上也觀察到陽光曝曬常會加重皮膚的光敏感性，但是果酸與紫外線對於人類皮膚角質細胞的生物效應仍有待進一步釐清。

材料及方法：

本研究選擇人類皮膚角質細胞株 HaCaT 及培養的人類角質細胞，分別進行甘醇酸(GA)、紫外線 B 光 (UVB)、以及甘醇酸加上 UVB 的試驗，以研究 GA 與 UVB 對人類皮膚細胞凋亡相互之影響。我們使用顯微鏡觀察細胞型態的改變、運用流式細胞儀技術來偵測細胞存活率，細胞週期及粒線體膜電位變化、利用 DAPI 染色偵測細胞受損的情形、以西方墨點法偵測細胞凋亡相關的蛋白質如: Bax, p53, p21, cytochrome *c*, 凋亡相關的蛋白水解酵素 caspases 3, AIF, ER stress 路徑的 GADD153 等的活性，並測定細胞內 ROS 濃度的變化。

研究結果：

GA 與 UVB 在不同的條件下作用於 HaCaT 細胞，我們的研究發現：

(1) 抑制細胞增生的作用：無論是 GA 或是 UVB 本身均可以抑制 HaCaT 細胞增生，並呈現劑量-依賴性 (dose-dependent effects)，然而 GA 前處理後再照射 UVB 比單純 UVB 照射，更明顯抑制 HaCaT 細胞增生的能力。

(2) 促進細胞週期停滯：DNA 細胞週期分析顯示甘醇酸並未引發明顯的細胞週期停滯，而 UVB 可以使細胞週期停滯在 S 期，然而經 GA＋UVB 的處理，細胞週期停滯在 S 期的比例更爲明顯 。

(3)GA、UVB、GA＋UVB 會造成粒線體膜電位下降、ROS 釋出，GA 前處理後照射 UVB，會造成粒線體膜電位下降、ROS 釋出更明顯

(4) 引發細胞凋亡：GA、UVB、GA＋UVB 處理的 HaCaT 細胞均會引發細胞凋

亡，但均無劑量-依賴性。GA 處理產生的細胞凋亡與粒線體路徑較爲相關，而 UVB 照射後產生的細胞凋亡與 ER 路徑較相關。此外，發現 GA＋UVB 處理的細胞 caspses 3 與 Bax, AIF, cytochrome c 等蛋白均有明顯上升的現象，ER stress 路徑 GADD153 也顯著上升，顯示 UVB+GA 處理的 HaCaT 細胞凋亡的發生與內質網及粒線體路徑都有密切的關係。另外，GA＋UVB 處理 HaCaT 細胞時發現更加乘 MMP 的下降，也會釋出更多的 ROS。GA 會加強 UVB 誘發角質細胞凋亡的發生。

結論：

我們發現 GA、UVB 皆會抑制角質細胞生長及引起細胞凋亡，而 GA 合併加上 UVB 處理 HaCaT 細胞時，則更明顯抑制角質細胞生長，並誘使 S 期細胞週期停滯。研究顯示 GA＋UVB 引發 HaCaT 細胞凋亡的機轉，乃是經由粒線體及 ER stress 等多種路徑。我們發現甘醇酸會增加 UVB 對人類皮膚角質細胞 HaCaT 的凋亡，值得我們進一步探究與釐清臨床上甘醇酸的使用與皮膚光敏感之間的關係。

關鍵字：果酸(α-hydroxy acid，AHA)、甘醇酸(glycolic acid)、紫外線B光(UVB)、HaCaT細胞、細胞週期(cell cycle)、細胞凋亡(apoptosis)、粒線體、內質網

Abstract

α-hydroxy acids (AHAs) has been widely used in cosmetic agents and superficial chemical peeling in recent years. We have found that glycolic acid (GA) and lactic acid (LA) have anti-proliferative and apoptotic effects on human keratinocyte cell line (HaCaT) in our previous studies. It has long been concerned that UV irradiation would enhance the photosensitivity of AHAs on human skin. It is mandatory to explore the biologic effects of GA and UV on human keratinocyte.

Materials and methods:

We used HaCaT and primary cultured human keratinocytes to investigate the effects of GA, UVB, and GA co-treated with UVB (GA+UVB) on human keratinocytes. We used phase microscope to observe morphological changes of the cells, flow cytometry to detect cell viability, cell cycle, and mitochondrial membrane potential (MMP), and intra-cytoplasmic reactive oxygen species (ROS) levels. DAPI stain and comet assay were used to detect cell damage, and Western blot to detect the activities of apoptosis-related protein such as Bax, p53, p21, cytochrome *c* and the activities of caspase 3, AIF, and GADD153.

Results:

(1) Inhibition of cell proliferation: Both GA and UVB inhibited cells proliferation in a dose-dependent manner. However, pre-treatment with GA caused a significant enhancement in the antiproliferative response of UVB in HaCaT cells.

(2) Induction of cell cycle arrest: DNA cell cycle analysis revealed that GA didn't cause cell cycle arrest; UVB induced HaCaT cells accumulated at S phase; co-treatment with GA＋UVB induced cell at S phase more prominently.

(3) Induction of apoptosis: All of the treatment of GA, UVB, or GA＋UVB in HaCaT cells could induce apoptosis in a dose-independent pathway. GA induced apoptosis through mitochondrial pathway, and UVB induced apoptosis through ER

pathway. GA+UVB had synergistic effect on apoptosis through the over-expressions of caspase 3, Bax, AIF, cytochrom c, and GADD153. In addition, GA and UVB had synergistic effects on the decline of MMP and increase of ROS release which may contribute to enhance the occurrence of apoptosis in HaCaT cells. The occurrence of apoptosis induced by co-treatment of UVB and GA was via multiple pathways including mitochondria- and ER-dependent, and caspase-dependent and capase–independent pathways.

In conclusion, we demonstrated that GA, UVB, GA+UVB inhibited cell growth and induced apoptosis in HaCaT cells. We demonstrated that GA had a synergistic effect on cytotoxicity and apoptosis. Moreover, co-treatment with GA＋UVB enhanced the cell cycle arrest at S phase. We explored the molecular mechanisms of apoptosis and disclosed the molecular mechanisms of apoptosis induced by co-treatment of GA＋UVB were via multiple pathways including mitochondria- and ER-dependent, and caspase-dependent and capase–independent pathways in a human keratinocyte cell line HaCaT.

Key words: Glycolic acid (GA), Alpha-hydroxyacids (AHAs), Ultraviolet B (UVB), HaCaT cell, Cell cycle, Apoptosis, Caspase, Mitochondrium, Endoplasmic reticulum

Introduction

AHAs (α–hydroxy acid) are widely used skin care products, and glycolic acid (GA) and lactic acid (LA) are well-known for being most widely used as cosmetic ingredients and superficial peeling agents in dermatology. However, information regarding the biological effects of AHAs in human keratinocytes is very limited. We have demonstrated that GA blocked cell cycle at G2/M phase, and induced apoptosis via activation of caspase-9 and -3 in HL-60 cells (Yang *et al.*, 2004) and found that LA has antiproliferative effects via the induction of apoptosis and cell cycle arrest in a human keratinocyte cell line (HaCaT) (Yang *et al.*, 2009). Thus, exploration of the biological effects and long-term application of GA in human skin is warranted.

Photo-protective and anti-inflammatory effects and anti-oxidant effects of topical GA treatment on UVB-irradiated skin has been reported (Perricone and Dinardo, 1996; Morreale and Livrea, 1997). It has been of some concern that topical application of GA can increase photosensitivity of skin to ultraviolet radiation (Anderson, 1998; Kaidbey et al, 2003). It was demonstrated that topical application of 10% GA may enhance photodamage by UV light in human subjects, which led the speculation that a subclinical irritation may contribute to photosensitivity induced by topical application of GA (Kaidbey et al., 2003). It is still obscure whether GA enhance or diminish photodamage of the skin. Thus, we tried to elucidate the mechanisms of cytotoxic effects of GA or/and UVB in human keratinocytes.

Materials and methods

Chemicals and reagents

L(+)-Glycolic acid was obtained from Fluka (BioChemika, Switzerland). Trypan blue, Tris–HCl, triton X-100, propidium iodide (PI) and ribonuclease A were obtained from

Sigma Chemical Co (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), potassium phosphates and TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA) were purchased from Merck Co (Darmstadt, Germany). Fetal bovine serum, penicillin–streptomycin, trypsin–EDTA and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Caspase-3 activity assay kit was purchased from Roche Diagnostics (Mannheim, Germany). All of the chemicals used were reagent grade.

Human immortalized keratinocytes (HaCaT) cell line

Cultures of HaCaT cells were kindly provided from Dr. Norbert E. Fusenig (Institute of Biochemistry, Germn Cancer Research Center) and were grown on Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1 L-glutamine, 25mM HEPES, 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco, Carlsbad, CA, USA) at 37℃ in a humidified incubator with 5% CO_2 atmosphere.

UVB Irradiation

Immediately before UVB irradiation, the medium were replaced by phosphate-buffered saline (PBS). UVB were supplied by a closely spaced array of seven Westinghouse FS-40 sunlamps, which delivered uniform irradiation at a distance of 90 cm. The energy output of UVB (290–320 nm) at 38 cm was measured with a UVB photometer (IL 1350 photometer, International Light, MA). The output of the FS-40 sunlamps is 0.07 mW/cm2. We treated HaCaT cells with various UVB irradiation doses (50, 100, 150, 200 mj/cm^2).

Morphological changes and viability of HaCaT cells treated with GA, UVB, GA+UVB

Cells were seeded in 12 well plates at a density of 2×10^5 cells per well and grown for 24 h until 80% confluency. Then continuously cultured for 24 h in DMEM with GA (1,

5, 10, 15, 20, 25 mM) until the cells were harvested. Cells were then washed with phosphate-buffered saline and exposed to UVB (50, 100, 150, 200 mj/cm^2). We observed the cell morphology under a phase-contrast microscope and photographed. HaCaT cells were collected and stained with PI and detected the viability with a flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm wavelength.

Flow cytometric analysis of cell cycle and apoptosis of HaCaT cells treated with GA, UVB, GA+UVB

HaCaT cells treated with UVB/GA were incubated in an incubator for different time periods before the cells were harvested by centrifugation. The cells were fixed gently by 70% ethanol at 4℃ for overnight and were then resuspended in PBS containing 40 mg ml^{-1} PI and 0・1 mg ml^{-1} RNase and 0・1% Triton X-100 in a dark room. After incubation at 37℃ for 30 min, the cell cycles and apoptosis were analyzed with a flow cytometer.

Examination of apoptosis by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining and DNA gel electrophoresis

An aliquot of 2×10^5 cells per well of HaCaT cells had been treated with UVB/GA for 24h before the cells were isolated by centrifugation for 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. Cells were fixed with 37% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and stained with 1 mg/ml DAPI at 37℃for 5 min. The cells were then washed with PBS and examined by fluorescence microscopy (Nikon Coolpix 4500, Tokyo, Japan, 200x). Approximately 5×10^6 HaCaT cells/ml were treated with UVB/GA for 48 h before isolating the cells to extract. DNA to be used in DNA gel electrophoresis, as described previously.

Detection of the changes of levels of reactive oxygen species (ROS), and mitochondrial membrane potential (MMP) by flow cytometry

The levels of ROS and MMP of the HaCaT cells were determined by flow cytometry. HaCaT cells were treated with UVB/GA for different time periods (0, 1, 6, 12, 24, and 48 h). The cells were harvested and washed twice, resuspended in 10 μM 2,7-dichlorodihydrofluorescein diacetate (Sigma-Aldrich) and incubated at 37°C for 30 min and the levels of ROS were analyzed by flow cytometry.

For detecting the changes of levels of MMP in HaCaT cells, HaCaT cells were treated with UVB/GA for different time periods (0, 1, 6, 12, 24, and 48 h), the cells were harvested and washed twice, resuspended in 500 μl of $DiOC_6$ and incubated at 37°C for 30 min, and subsequently analyzed by flow cytometry.

Protein extraction for Western blot analysis

Total proteins were collected from HaCaT cells treated with UVB/GA for different time period (0, 24, and 48 h). Western blotting was used to examine the expression levels of the apoptosis-related proteins including Fas, Bax, Bcl-2, Bcl-xl, caspases-3, -8, -9, Endo G, AIF, cytochrome *c*, caspase-12 by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Results and Discussion

Human keratinocytes (HaCaT cells) were treated with GA (5mM), UVB (50mj/cm^2), and GA+UVB for different incubation periods (24 and 48 h). The percentage of viability was approximately 70%, 80% and 50% at 24 h, and was approximately 90%, 70% and 50% at 48 h on HaCaT cells treated with GA, UVB and GA+UVB, respectively. A larger proportion of cells were swelling and round-out

indicating necrosis, and some revealed shrinkage of cells with unclear nuclei suggesting apoptosis (Fig. 1a). GA and UVB had a synergisitc anti-proliferative effect on HaCaT cells (Fig. 1b).

The flow cytometry revealed that GA didn't cause significant cell cycle arrest (Fig. 2a). UVB induced S phase arrest, and GA+UVB enhanced cell cycle arrest at S phase at 24 h (Fig. 2a). The proportions of apoptotic phases were also increased with the treatment of GA, UVB, and GA+UVB at 24 h (Fig. 2b) and 48 h (Fig. 2d). Apoptotic cells demonstrated with DAPI staining were higher in intensity than that in non-apoptotic live cells, and an increase in the number of fragmented nuclei during the treatment with GA, UVB and GA+UVB shown in Figure 3.

To explore the molecular mechanisms of apoptosis induced by GA, UVB and GA+UVB in HaCaT cells, we investigated the levels of ROS, MMP, apoptosis-related proteins including GADD153, AIF, p21, p53, Bax, caspase 3, and cytochrome *c*. We found that GA+UVB had a marked decrease in the level of MMP at 1 h, and subsequently demonstrated an increase in the levels of ROS and a decrease in the levels of MMP in a time-dependent manner in 1 to 12 h (Fig. 4). Our findings suggested that ROS and MMP may play a significant role in the early phase of apoptosis in treated cells.

In Figure 6, we demonstrated the expressions of apoptosis-related proteins to explore the molecular mechanisms of apoptosis. GA increased the expressions of p21, p53, cytochrome *c*, Bax and caspase 3, indicating the dominant role of caspase-dependent and mitochondria-dependent apoptotic pathways. UVB increased the expressions of GADD153, AIF, p21, p53, Bax, caspase 3, and cytochrome *c* which suggested the dominant role of ER-dependent, caspase-dependent and -independent apoptotic pathways. GA+UVB induced the higher expressions of p21, p53, GADD153, AIF, cytochrome *c*, Bax and caspase 3 than treated with GA or UVB

alone. The data indicated that GA+UVB had synergistic effect on the occurrence of apoptosis, and the apoptosis was related to multiple mechanisms including caspase-dependent and –independent pathways, and mitochondria- and ER-dependent pathways.

Ahn *et al*. (2002) claimed that GA inhibited UVB-induced cytotoxicity and attenuated apoptosis in HaCaT cells; However, our findings is contradictory to the findings of Ahn *et al*.. Therefore, it is imperative to further clarify the effects of glycolic acid and UVB in HaCaT cells, primary human keratinocytes, and in human skin in the future.

Conclusion

We demonstrated that GA, UVB, GA+UVB inhibited proliferation and induced apoptosis in HaCaT cells. Moreover, co-treatment with GA＋UVB enhanced the cell cycle arrest at S phase. The mechanisms of apoptosis induced by co-treatment of GA＋UVB were via caspase-dependent and –independent pathways, and mitochondria- and ER-dependent pathways. However, there are still contradictory findings among different investigators, and it is imperative to further clarify the effects of glycolic acid and UVB *in vitro* and in human skin in the future. We proposed the molecular mechanisms of cytotoxicity and apoptosis of GA, UVB and GA+UVB on a human keratinocyte cell line (HaCaT) (Fig. 7).

計畫成果自評

我們的研究顯示，甘醇酸與 UVB 照射均會抑制角質細胞生長，並且誘導細胞凋亡；甘醇酸前處理可以加強 UVB 抑制角質細胞生長的作用，並使 HaCaT 細

胞細胞週期改變停滯在 S 期。我們發現甘醇酸會增加 UVB 對人類皮膚角質細胞 HaCaT 的凋亡，值得我們進一步探究與釐清甘醇酸的使用與皮膚光敏感之間的關係。本研究雖然是 *in vitro* 之發現，但仍值得提醒臨床醫師與一般大眾在使用含有甘醇酸等產品時，更須審慎以對，留意果酸光敏感帶來長期對於皮膚的影響。

預估本研究內容與原計畫相符程度與預期目標情況約為 70%，目前將研究成果整理中，擬發表論文於台灣皮膚科醫學會年會暨國際學術期刊。目前執行之計畫為主持人多年來持續性之果酸研究計畫，未來擬在既有之基礎下，繼續進行包括纖維母細胞與無毛鼠動物實驗或人體之試驗計劃。

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黃芯蘭(指導教授：楊仁宏)。探討乳酸對人類皮膚角質細胞株(HaCaT)誘發細胞凋亡及細胞週期停滯的影響。中山醫學大學醫學研究所碩士論文，2006。

Legends to Figures

Fig. 1

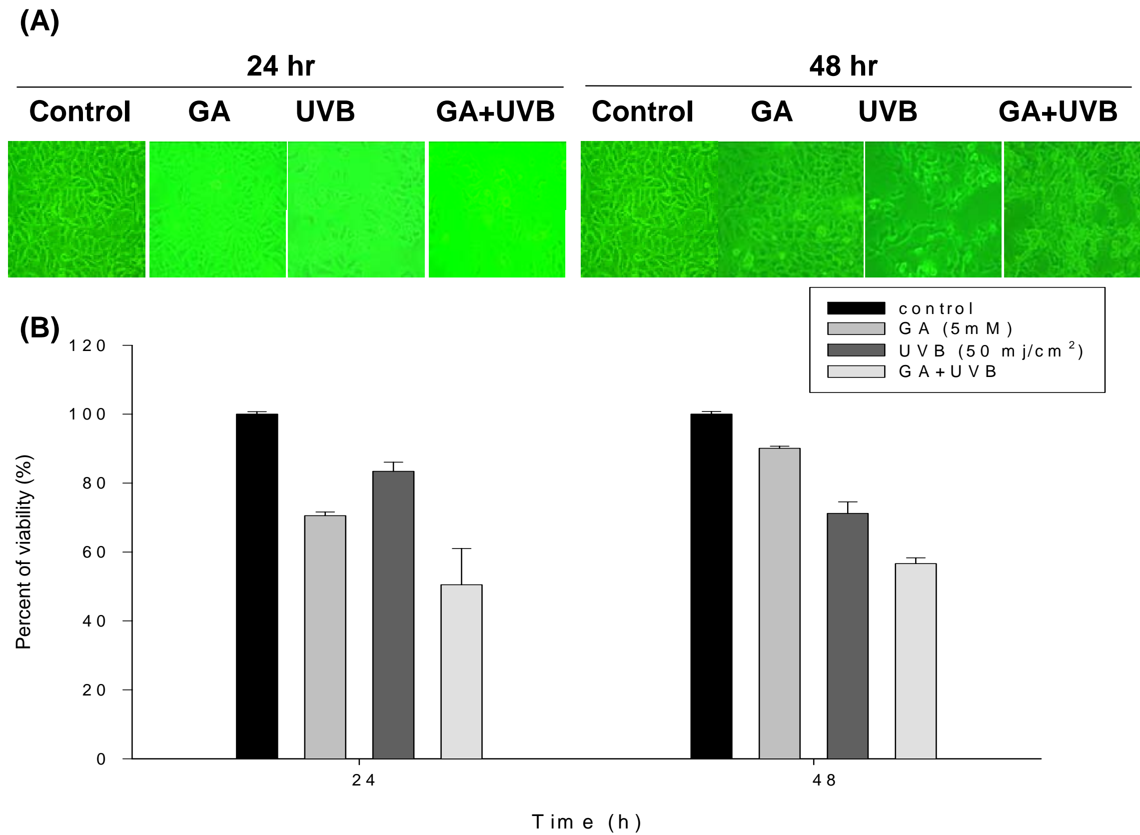

Fig. 1 The cell morphologies and viabilities of HaCaT cells treated with glycolic acid, UVB irradiation, and co-treatment with UVB irradiation and GA for 24 h and 48 h.

(a) The morphological changes of GA (5mM), UVB (50 mj/cm^2), and GA+UVB-treated cells (phase-contrast microscopy, x200).

(b) The viabilities analysis revealed that GA (5 mM) enhanced UVB (50 mj/cm^2)-induced inhibition of cell growth during 24 h and 48 h.

Fig.2(a)

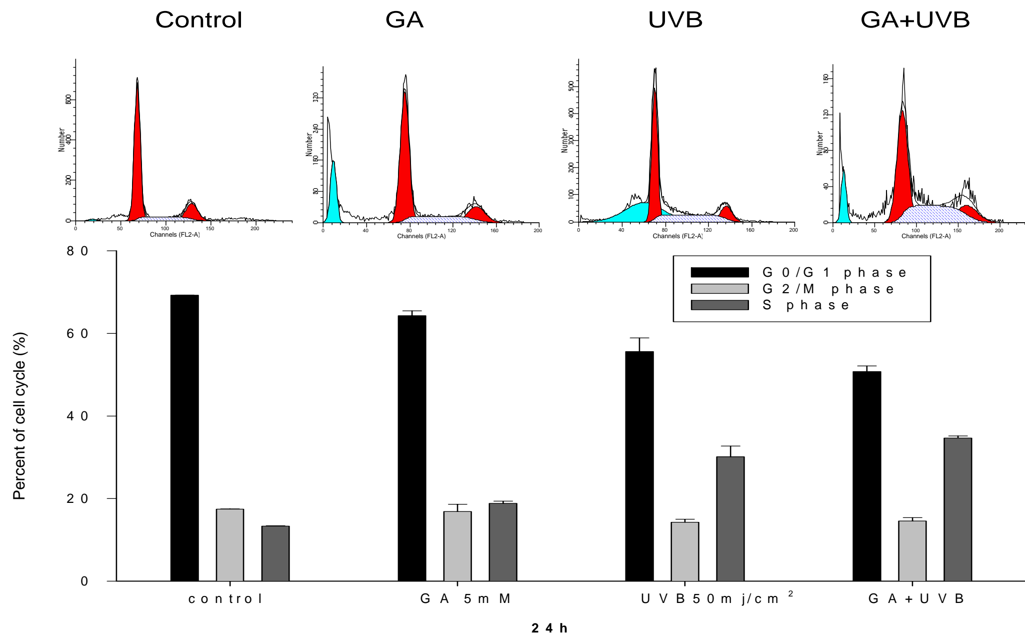

Fig.2(b)

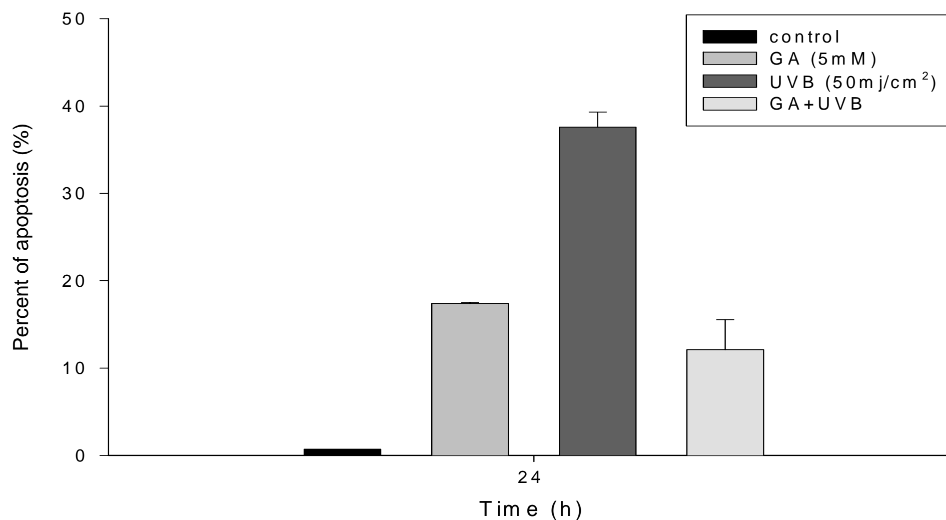

Fig. 2 Flow cytometric analysis of the effects of GA, UVB, and GA+UVB on the cell cycles and subG1 group (apoptosis phase) in HaCaT cells.

(a) The HaCaT cells treated with GA (5mM), UVB (50 mj/cm^2), and GA+UVB for 24 hour. GA enhanced UVB-induced cell cycle arrest at S phase.

(b) The proportions of apoptotic phases were also increased with the treatment of GA, UVB, and GA+UVB at 24 h.

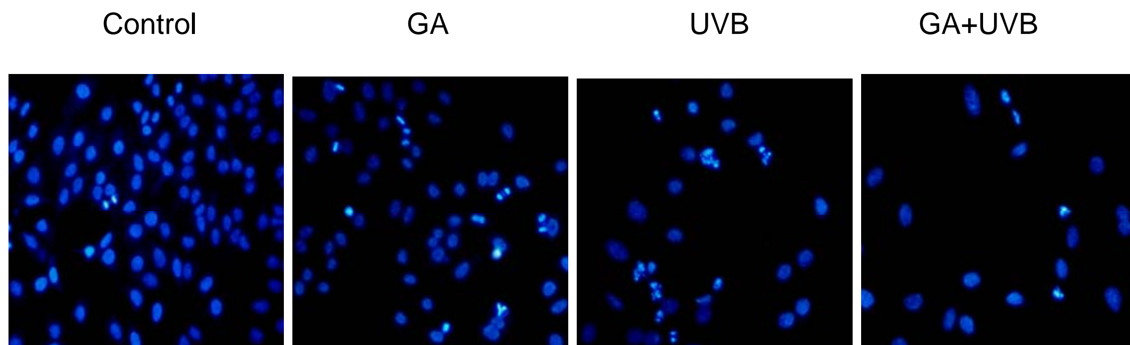

Fig.3

Fig. 3 HaCaT cells were incubated with GA, UVB, and GA+UVB for 24 h. There was an increase in the number of higher intensity DAPI-staining cells and fragmented nuclei of GA (5mM), UVB (50 mj/cm^2), and GA+UVB treatment (fluorescence microscope, x200).

Fig.4

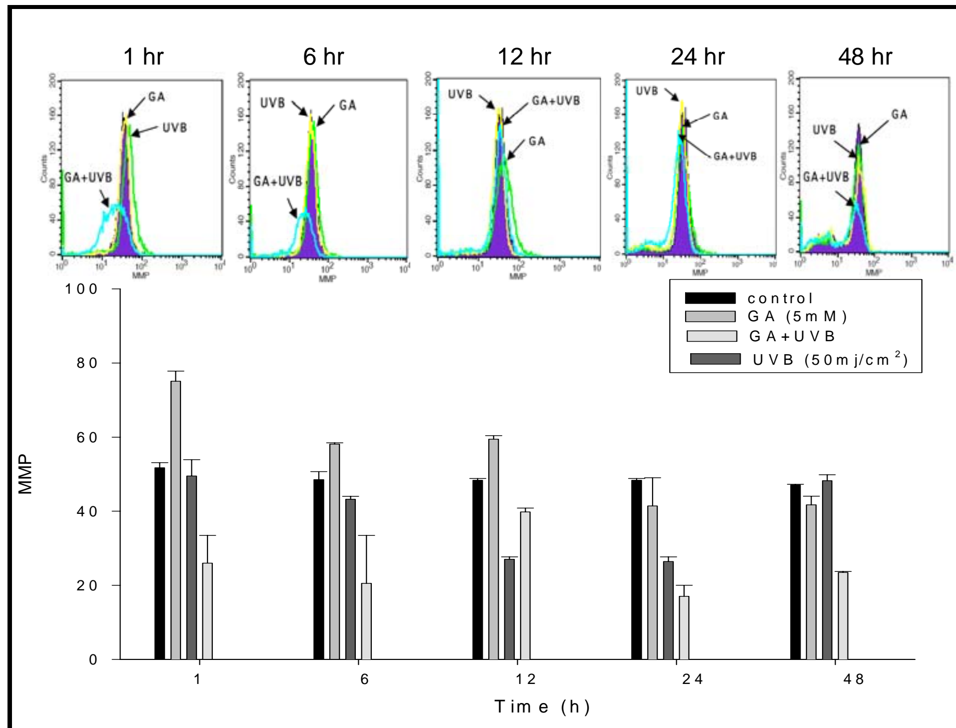

Fig. 4 Effect of GA, UVB, GA+UVB on mitochondrial membrane potential（MMP）in HaCaT cells for variable durations of 1, 6, 12, 24, and 48 h.

Fig. 5

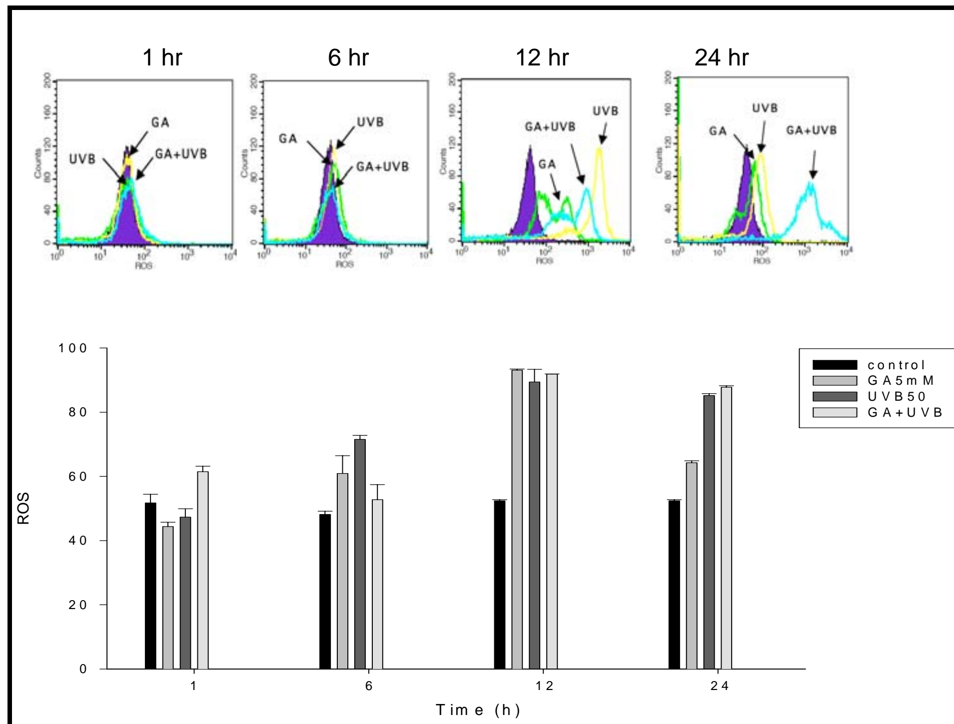

Fig. 5 Effect of GA, UVB, GA+UVB on ROS levels in HaCaT cells for variable durations of 1, 6, 12, and 24 h.

Fig. 6

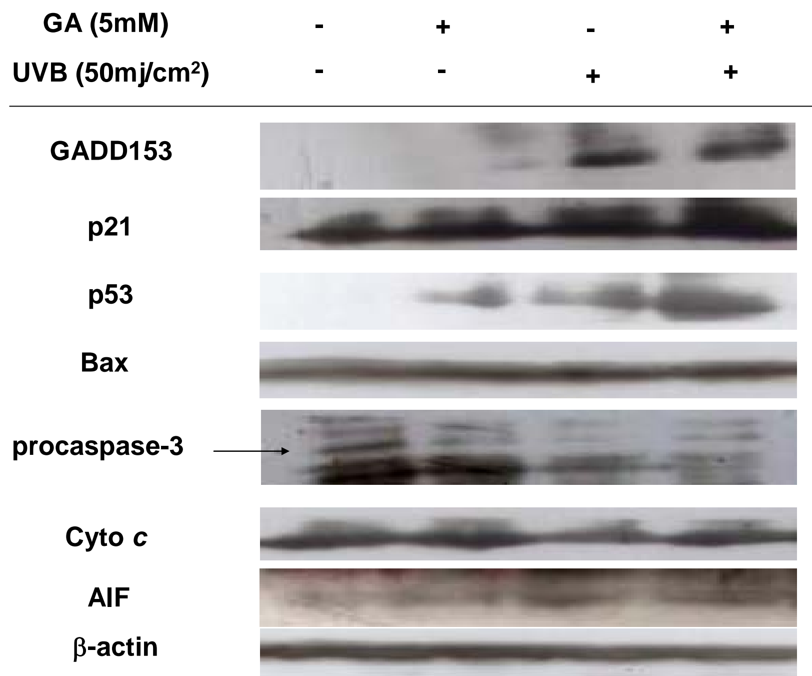

Fig. 6 Western blot demonstrated the expressions of apoptosis-related proteins with the treatment of GA, UVB, and GA+UVB in HaCaT cells at 24 h.

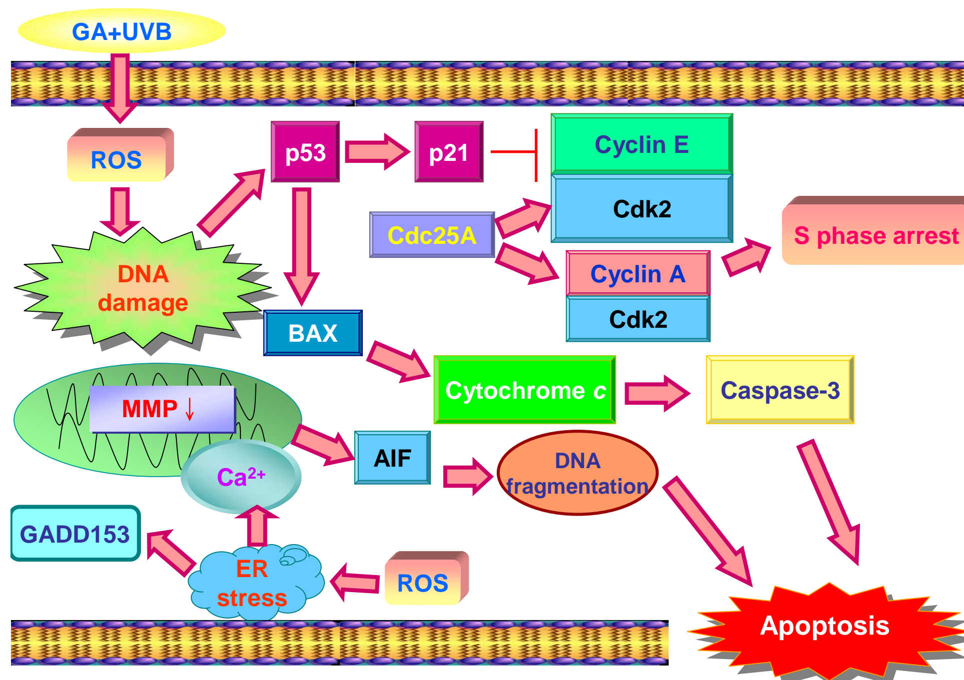

Fig. 7 Molecular mechanisms of cytotoxicity and apoptosis of GA+UVB in HaCaT cells.