INTRODUCTION

The effect of Ultraviolet B (UVB) irradiation on human skin is a double-edged sword. The beneficial aspect has been obtained in the clinic to treat some skin diseases, such as psoriasis and vitiligo [1-3]. One mechanism of UVB phototherapy for psoriasis is by inducing apoptosis of T2 cells and epidermal keratinocytes within psoriatic lesions [4,5]. On the other hand, the over or chronic exposure to UV irradiation, particularly sun damaged by UVB (280—320nm) can lead photaging and photocarcinogenesis. On the cellular level, along with UV-caused DNA damage, the cell death of epidermal keratinocytes induced by sunburn is presented in two forms, necrosis and apoptosis. Apoptosis, a morphologically distinct form of cell death is characterized by cell shrinkage, condensation of chromatin and the cytoplasm, fragmentation of the nucleus, membrane blebbing and the formation of membrane-bound apoptotic bodies containing cell fragmentation [6,7]. In contrast with apoptosis, necrosis is unprogrammed death of cells and living tissue. The intracellular content was released after cellular membrane damage in necrosis [7]. A HaCaT cell is a cell type belonging to an immortalized human keratinocyte line used in scientific research [8]. Up to now, the immortalized keratinocyte cell line (HaCaT) has been used in experiments as a convenient substitute for cultured normal human keratinocytes.

Tumor necrosis factor α (TNF-α), in addition to being cytotoxic for certain tumor cells, has been confirmed as a multifunctional cytokine that is involved in innate and adaptive immunity [9-11]. Human epidermal keratinocytes can produce TNF-α and the level of TNF-α has shown to increase after exposure to UVB [12]. And TNF-α initiate its effect on apoptosis by binding to keratinocyte membrane receptor, TNFR1 (p55/60) [13]. Then some signal pathways/molecules such as p38 MAPK and Caspases(such as Caspase-3, Caspase-2, Caspase-8 and Caspase-9) can be activated to promote cells apoptosis [14-16]. Interestingly, although Schwarz A. [17] and other people [12,18] speculated that TNF-α play an role in UVB induced apoptosis, induction of apoptosis of keratinocytes in vivo or in vitro after application of TNF-α alone was not observed [17,19]. And the Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) can induce apoptosis of tumor cells but has no effect on normal cells and inflammatory...
cells either in vivo or in vitro [19]. Especially in the molecular mechanism, the relationship between TNF-α and apoptosis of HaCaT after UVB irradiation has not yet been stated clearly.

This research is to explore the relationship between TNF-α and UVB-induced keratinocyte death (apoptosis and necrosis) and its possible mechanism. In this experiment, we use different doses of UVB radiation in HaCaT cells and then tested viability of cells by MTT assay 24h after UVB irradiation, by flow cytometry to measure apoptosis rate, using transmission electron microscopy to observe morphological changes of cell death, the secretion of TNF-α in the supernatant was measured by ELISA after 24h exposure, and using statistical methods to explore the relationship between apoptosis rate and HaCaT cells viability, apoptosis rate and TNF-α.

RESULTS AND DISCUSSION

Cell viability of HaCaT exposed UVB (MTT assay)

HaCaT cells were treated with SS-03 UVB phototherapy instrument (10 bulbs, Shanghai Sigma High-tech Co., Ltd., China); (radiation intensity: 0.3 mw/cm² at 0mJ/cm², 10mJ/cm², 15 mJ/cm², 20mJ/cm², 25mJ/cm² and 30mJ/cm², respectively. And cell viability was estimated by MTT assay, as described below. The optical density value of HaCaT cells after UVB irradiation significantly decreased with the UVB dose increasing. Table 1, Figure 1 OD492 (optical density, 492nm). There were statistically significant differences between Those groups (Control group and various UVB-irradiated groups).*P﹤0.01

Apoptosis rate of HaCaT cells by flow cytometry

The percentage of apoptotic cells gradually increased with the increase of radiation dose at the extent of 0-30mJ/cm² UVB exposure. The results of UVB-irradiated groups were respectively 16.69%, 22.74%, 26.50%, 28.22% and 31.82%, while the control group was only 3.17%. (Figure 1, 2).

The morphological changes of HaCaT cells after UVB irradiation

The presence of apoptosis and necrosis was further confirmed by means of electron microscopy in harvested cells after a treatment with UVB (Figure 3. b, c, d, e, f). In apoptosis, these cells showed cell shrinkage, condensation of chromatin and the cytoplasm, chromatin marginalisation, fragmentation of the nucleus, darkening of euchromatin, increasing in electron density of the cytoplasm, and formation of dense bodies containing cell fragmentation. Control cells showed normal structures of cytomembrane, cytoplasm and chromatin (Figure 3a).

The effect of UVB on the secretion of TNF-α in HaCaT cells

Using a ELISA method for the determination of the concentration of TNF-α in the cultural supernatants. The untreated HaCaT cells secreted a small amount of TNF-α (2.86 ± 0.46 pg /ml), whereas the level of TNF-α produced by the irradiated HaCaT cells significantly rose with increasing UVB dose(0-30mJ/cm²), as shown in Table 2, Figure 4.

There were statistically significant differences between the groups (Control group and various UVB-irradiated groups). *P<0.01.

A analysis of correlation between HaCaT apoptosis rate and Cell viability after UVB irradiation

Figure 5, with apoptosis rate on the vertical and cell viability on the horizontal axis, showed that HaCaT apoptosis rate was negatively correlated with cell viability by linear correlation analysis (the correlation coefficient: r = -0.976, the hypothesis test of correlation coefficient: P=0.006<0.01).

The correlation between apoptosis rate and TNF-α in treated HaCaT cells

As depicted in Figure 6, between apoptosis rate and the concentration of TNF-α secreted by irradiated HaCaT cells were positive correlation by linear correlation analysis (the correlation coefficient: r = 0.931, the hypothesis test of correlation coefficient: P=0.007<0.01).

DISCUSSION

UVB-induced cell death is a subject of interest, due to the potential link between this process and UVB-related skin diseases. Acute, low-dose UVB impairs the function of epidermal APCs (Langerhans cells) and regulates the expression of
Figure 2 The assay of HaCaT cells apoptosis induced by UVB at various doses (0-30mJ/cm²) and incubated for 24 hours with flow cytometry.

Figure 3 The ultrastructure of HaCaT cells (a) left untreated and (b, c, d, e, f) treated with 30mJ/cm² UVB. Cells exposed with 30mJ/cm² UVB demonstrated chromatin marginalization (g, h), fragmentation of the nucleus (arrow, b), formation of electron-lucent membrane-bound apoptotic bodies (arrow, c) phagocytosed by neighbouring cells (arrow, d). e, f, diagram reflected the ultrastructure of HaCaT cells of necrosis after 30mJ/cm² UVB irradiation. The morphology HaCaT cells of necrosis was characterized by swelling and vacuolization of organelles (arrow, f), increased membrane permeability, and appearance of flocculent densities in the matrix of organelle, clumping of chromatin.
immunomodulatory cytokines secreted by epidermal cells and T cells, leading to impaired induction of contact hypersensitivity [20,21]. Chronic exposure of the skin to UVB radiation is relevant to the development of certain skin cancers. Tumor necrosis factor-α is a proinflammatory cytokine produced in the skin in response to ultraviolet B radiation [21]. It was delineated that TNF-α can regulated gene expression for all these processes ranging from immune response and inflammation to cell migration, epidermal differentiation, and tissue repair in the epidermal Keratinocytes [22]. And the responses of keratinocytes to UV light were slow, with just a handful of genes regulated in the first hour [22, 23]. Thus, it is well worth exploring the role of TNF-α in cell death of keratinocytes, too. In this study, we found that there is a steady increasing of apoptotic rate of HaCaT cells with increasing UVB (in the range of 0-30mJ/cm²). It suggested that UVB induced HaCaT cells apoptosis is a dose-dependent manner in the range of 0-30mJ/cm² doses UVB exposure. Similar result that UVB triggered HaCaT cells apoptosis is a dose-dependent manner was reported in some studies in which the immortalized keratinocytes were irradiated following doses UVB different from ours [24,25]. UVB can be directly absorbed by DNA and induces DNA damage. A previous study on HaCaT cells has demonstrated that increasing UVB exposures yielded dose-dependent increases in DNA fragmentation [26]. The major DNA lesions are covalent bond formation between two adjacent pyrimidines of the same DNA strand leading to Cyclobutane Pyrimidine Dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts ((6-4)-PP) [27]. And in a suberythemal dose of UVB radiation, the (6-4)-PP was repaired [27] while CPDs seemed to be more difficult in repair and accumulated over time [28,29]. This may be the reason that UVB induced HaCaT cells apoptosis is a dose-dependent manner. A previous study delineated that the apoptotic HaCaT cells were observed to double at UVB doses of 2.5-10 ml/cm². On the contrary, the fraction of apoptotic cells was decreasing in the 10-25 ml/cm² UVB dose range [30]. Nevertheless, a literature stated that the doses of UVB from 0-15 ml/cm² induced no significant apoptosis. However, UVB in the range 20-50 ml/cm² leads to significant apoptosis increasing to 80% 24 hours after exposure to 50 ml/cm²[31].

Cytokines/chemokines play pivotal roles in immunologic regulation in the human body and are involved in the induction of different immune responses. TNF-α, a proinflammatory cytokine, plays a critical role in the regulation of immune responses. In this study, we explored the effect of UVB on the secretion of TNF-α in HaCaT cells. The results showed that the concentration of TNF-α produced by the irradiated HaCaT cells increased with increasing UVB dose (0-30mJ/cm²). Data were expressed as mean ± standard deviation.

Table 2: The effect of UVB on the secretion of TNF-α in HaCaT cells.

<table>
<thead>
<tr>
<th>Dose of UVB (mJ/cm²)</th>
<th>TNF-α (pg/ml)</th>
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<tbody>
<tr>
<td>0 (Control group)</td>
<td>2.8±0.46</td>
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<tr>
<td>10</td>
<td>4.7±0.92</td>
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<tr>
<td>15</td>
<td>7.5±0.76</td>
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<tr>
<td>20</td>
<td>9.4±0.69</td>
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<td>25</td>
<td>11.6±0.59</td>
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<tr>
<td>30</td>
<td>14.1±2.37</td>
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Figure 4 The concentration of TNF-α produced by the irradiated HaCaT cells increased with increasing UVB dose (0-30mJ/cm²). Data were expressed as mean ± standard deviation.

Figure 5 The linear relationship between HaCaT apoptosis rate and Cell viability after UVB exposure.
of proliferation, differentiation, and cell death in many cell types, including keratinocytes [22,29,32]. For instance, TNF-α regulates not only immune and inflammatory responses but also tissue remodeling, cell motility, cell cycle and apoptosis [22]. In keratinocytes, apoptosis directly and initially triggered by UVB can be via several signal pathways/cytokines: upregulating expression of TNF-α and Fas ligand (FasL), and accumulation of the tumor suppressor gene p53 [33]. Furthermore, UV-induced apoptosis was divided into three types: immediate, intermediate and delayed [34]. Ultraviolet A1 (UVA1) induces immediate apoptosis while intermediate and delayed apoptosis are triggered by UVB [34]. Intermediate apoptosis is induced by the activation of cell surface receptors such as TNFR1 (p55/60), whereas delayed apoptosis is mediated via DNA damage which activates p53 and AP-1 pathways [35-37]. It is entirely possible that these signal pathways containing TNFR1 dependent pathway are involved in the induction of keratinocyte apoptosis by UVB and TNF-α. In other words, TNF-α mediates the apoptosis of HaCaT cells not only by TNFR1 dependent pathway but also by other apoptotic pathways such as promoting the expression of Fas receptor and pro-apoptotic Bax [33,37]. There is no doubt that numerous cellular signals are involved in the progress of HaCaT apoptosis triggered by UVB. In this study, the level of TNF-α produced by the irradiated HaCaT cells increased with increasing UVB doses (0-30mJ/cm²). We analysed the linear correlation between apoptosis rate and TNF-α in treated HaCaT cells regardless of the detailed mechanism of apoptosis (the correlation coefficient: $r = 0.931$). We found that TNF-α is very closely related to apoptosis of HaCaT in the UVB doses range of 10-30mJ/cm². There are two possible explanation to the role of TNF-α in HaCaT cells apoptosis: (1) TNF-α may be by other siganal pathways other than TNFR1 dependent pathway to induce apoptosis of HaCaT; (2) other pathways (singaling by FasL or p53) promoting apoptosis can upregulate expression of TNF-α via cascade amplification response and increased TNF-α induces the cell apoptosis. On the basis of theoretical model of the relationship between TNF-α and apoptosis rate, and previously recognized studies in the mechanism of apoptosis, it indicated that TNF-α may play a crucial role in UVB-induced apoptosis in keratinocytes.

Apoptosis was initially defined by ultrastructure changes in cells. The morphology diagnosis is considered as a dependable standard for cells apoptosis. To observe the morphology of apoptotic cells, electron microscopy appear to be indispensable [38]. In this experiment, we confirmed that UVB can induce apoptosis of HaCaT cells by transmission electron microscopy. And we also observed a small amount of cells dying by necrosis with the morphology of the permeable cell membrane, leakage of cellular contents, and dilated organelles necrosis. Previously, necrosis (oncosis) has been considered merely as an accidental uncontrolled form of cell death, but recently it was shown that the elicitation of necrotic cell death may be mediated by some signal transduction pathways [39]. Death domain receptors (e.g., TNFR1, Fas and TRAIL-R) have been demonstrated to regulate necrosis [40,41]. So TNF-α inducing cell death by means of binding death domain receptors can take the shape of either apoptosis or necrosis, which suggests that between apoptosis and necrosis mediated by some signals share a common pathway that can finely regulate the cell death. And there is an increase in the fraction of necrotic cells with increasing UVB in the doses range of 10-25mJ/cm² [30]. The level of TNF-α in the cultural supernatants is a UVB-dose-dependent increase at extent of 10-30mJ/cm² doses UVB. Thus, we cautiously speculated that the concentration of TNF-α did not inhibit cells necrosis, on the contrary, higher concentration of TNF-α might facilitate the HaCaT cells necrosis with the doses range of 10-25mJ/cm² UVB.

To give a further quantification to cell apoptosis and viability, we tested the HaCaT apoptosis rate by flow cytometry (Propidium Iodide (PI) staining) and cell viability with MTT assay, respectively. And a analysis of correlation between HaCaT apoptosis rate and cell viability after UVB irradiation was performed (the correlation coefficient: $r = -0.976$). It was found that the HaCaT apoptosis rate was negative consistent with cell viability for the quantification of cell apoptosis. Theoretical model of the linear correlation indicated that UVB mostly induced cell apoptosis not necrosis at the extent of 0-30 mJ/cm² irradiation. With flow cytometry (Propidium Iodide (PI) staining) apoptotic cells display a broad hypodiploid (sub-G1) peak, which can be distinguished from the narrow peak of cells with normal (diploid)
DNA content. However, necrotic cells sometimes display some extent of DNA degradation that may generate hypodiploid nuclei [42]. Thus, the apoptosis assay may result in a certain number of false positives that influence accurate assessment of apoptosis. It is a possible interference factor which may influence the result of the analysis of correlation between HaCaT apoptosis rate and cell viability for necrotic HaCaT cells displaying some degree of DNA degradation. But for all that, it is not certain and is still necessary to produce some very solid evidences as to some necrotic HaCaT cells interfering the apoptosis assay by flow cytometry (Propidium Iodide (PI) staining).

**EXPERIMENTAL SECTION**

**Reagents**

MTT (3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazo- liumromide) was obtained from Huamei Biotechnology Reagent Ltd, Chengdu, China. Tumor necrosis factor-α kit was from Jing-meit Biotech Ltd, Shenzhen, China. Propidium Iodide (PI), DMSO and Rnase A was purchased from Sigma-Aldrich, USA. Osmium and EPON-812 were from SERVA, U S A.

**Cell and cell culture**

The immortalized human keratinocytes cell line, HaCaT was purchased from China Center for Type Culture Collection, College of Life Sciences at Wuhan university and grown in 10% Dulbecco's modified Eagle medium (Gibco Life Technologies, U.S.) containing penicillin (100 U/ml), streptomycin (100 mg/ml) and 10% Fetal Bovine Serum (FBS) (Zhejiang Tianhang Biological Technology Co., Ltd) at 37 ° C in a humidified atmosphere containing 5% CO₂.

**UVB irradiation**

HaCaT cells were planted in 6-well plates (Corning Co., Ltd, NY, U.S.) at a density of 1×10⁵/ml with 100µL culture medium per well and cultured until 80% confluence. Cells were gently washed three times with Phosphate-Buffered Saline (PBS) and covered with a thin layer of PBS, followed by treatment washed three times with Phosphate-Buffered Saline (PBS) and cultured until 80% confluence. Cells were gently trypsinized and added to PBS and pelleted by centrifugation (1500r/min, 10min) and glutaraldehyde was added to each well and incubated at 37 ° C for 4 h. The medium was gently aspirated, and then 150 mL DMSO (dimethylsulfoxide) was added to each well to solubilize the formazan crystals. The Optical Density (OD) of each sample was immediately measured using an Automatic microplate reader (EL × 800, BIO-TEK Instrument, U.S.) at 492 nm. Nonirradiated cells underwent the identical procedure without UVB treatment [43]. The formula to calculate the cell viability (%): The cell viability (%) = the OD value of treated group / OD value of control group × 100%

**Apoptosis analysis by flow cytometry**

HaCaT cells were treated as described above in six-well plates. At 24 hours after UVB exposure, the culture medium (UVB-irradiated and nonirradiated cells) was collected. The cells were gently trypsinized and added to PBS and pelleted by centrifugation (1000r/m, 5min). The pellet was washed with PBS, and was resuspended in 100 µl of PBS. The cells were fixed in 900µl of 70% cold ethanol (-20°C) keeping the tubes on ice at 30 minutes. Then wash cells in 5 ml of PBS and centrifuge at 40g for 5 min. Remove the supernatant and resuspended cells followed by counterstaining with 50mg/L propidium iodide and 50mg/L Rnase A(Ambion) for at least 30 min at room temperature in the dark. Cells were analyzed using a flow cytometry (FACS Vantage type, Becton Dickison, U.S.) (488-nm laser line for excitation) .The analyses were done by Cell Quest software (BD Biosciences) for apoptosis [42,44].

**Using Transmission electron microscope to observe the ultrastructure of HaCaT cells**

Transmission electron microscopy (JEM21200-EX type, JEOL, Japan): Cultures of UVB-irradiated and nonirradiated keratinocytes cells after 24h were detached by 0.25% trypsin solution (containing 0.02%EDTA(Ethylene Diamine Tetraacetic Acid)(Hongheng chemical plant Shantou Guangdong China)) and centrifuged(800r/min, 5min). Then the supernatant was centrifuged (1500r/min, 10min) and glutaraldehyde was added to the pellet for 30 min. It was post-fixed in osmium 1% (SERVA, U.S.), and dehydrated in a graded series of ethanol (50%, 70%, 90%, 100%). Then samples were immersed in embedding agent EPON-812(SERVA, U.S.) and then sliced, uranyl acetate and lead citrate stained. Using JEM21200-EX transmission electron microscope to examine cells morphology and structure. Control cells were subjected to identical procedure without UVB treatment.

**ELISA method for the determination of the concentration of TNF-α**

Cells were treated as described above in 96-well plates (Corning Co., Ltd, NY, U.S.). The culture supernatants from UVB-irradiated and nonirradiated (control) cells were collected after 24h for TNF-α assays (method of operation in accordance with ELISA kit instruction.). The absorbance of each sample was tested via an Automatic microplate reader (primary wavelength 490nm, reference wavelength 650nm). Referring to the standard TNF-α concentration of 32 ± 0.5pg/ml, standard curve was performed.

**Statistical analysis**

Statistical analysis is via statistical software SPSS 11.0(Statistical Product and Service Solutions). All numeric data
are presented as mean ± Standard Deviation (SD) and analyzed for significance using one-way ANOVA and Student-Newman-Keuls (SNK). Between apoptosis rate and cell viability, apoptosis rate and the concentration of TNF-α was tested by the linear correlation analysis. Statistical significance was established at p < 0.05.

CONCLUSION

In summary, we found that UVB induced HaCaT cells apoptosis is a dose-dependent manner in the doses range of 0-30mJ/cm² by the analysis of flow cytometry (propidium iodide staining). And we also observed some necrotic HaCaT cells with the morphology of necrosis. The level of TNF-α produced by the irradiated HaCaT cells increased with increasing UVB doses (0-30mJ/cm²). According to the above analysis (the linear correlation analysis and experiment researches), we speculated that TNF-α plays a significant role in promoting HaCaT apoptosis at the extent of 10-30mJ/cm² UVB exposure. And we also cautiously speculated that the concentration of TNF-α might promote the HaCaT cells necrosis with the extent of 10-30mJ/cm² doses UVB. With the increasing of the concentration of TNF-α secreted by the immortalized keratinocytes in the culture supernatants the fraction of apoptotic HaCaT cells is increasing and the fraction of HaCaT necrotic cells may be also increasing in the doses range of 10-30mJ/cm² UVB.

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Cite this article