Skin Specific Cells and UVB Damage
An experimental assessment

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The alarming increased incidence of skin cancers is closely related to ultraviolet radiation exposure, known to induce cellular transformation in epidermal and dermal layers. The current study describes the behavior of normal (human keratinocytes – HaCat and fibroblasts -1BR3) and tumor cells (human – A375 and murine – B16A45 and B16-F0 - melanoma) after UVB irradiation. A dose of 40 mJ/cm² (312 nm) led to a decrease of both normal and tumor cells viability in a post-exposure time dependent manner, the most statistically significant reduction being recorded at 24h post-exposure. Moreover, some changes in the shape of cells exposed to UVB were detected. Our results confirm the cytotoxic effects associated with UVB exposure and could be further employed to evaluate the photo-protective effects of different agents.

Keywords: skin malignancies, irradiation, cells, viability

Currently, both classes of skin cancer, melanoma and non-melanoma, respectively are the most common skin diseases in the white population [1-3]. Due to the fact that skin, the largest organ of human body, is continuously exposed to a number of risk factors, such as: toxic substances (carcinogens, mutagens, and teratogens), various moles, ultraviolet radiation, white skin etc., all over the globe are reported alarming increases of these malignancies [4]. Increasing incidence rates of non-melanoma skin cancers (NMSC) have been notified in several European countries, like Scotland, United Kingdom, and Germany albeit from 5.4 million new cases diagnosed each year more than a half are founded in United States [5]. Regarding melanoma skin cancers (MSC) in the last three decades the incidence has increased at least three times, especially in Australia and USA [6].

NMSC, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), impart fundamental features based on the fact that both disorders result from normal keratinocytes founded in epidermis, by a multistage process which implies activation/inactivation of some genes (e.g. oncogenes, tumor suppressor genes) being related mainly with UV radiation and immunosuppression [7-10]. The major difference between the two classes of NMSC consist in the ability to metastasize: BCC seldom metastasize and derive from normal/healthy skin while SCC often metastasize and derive from attendant lesions, like actinic keratosis which occur after repeated sun exposure [8]. MSC, is the most aggressive type of skin cancer, arises from melanocytes, cells liable for skin pigmentation and photoprotection, during malignant alteration. Molecular tumorigenesis plays a crucial role, thereby depending on it is classified as follows: skin melanoma not associated with chronic sun-induced damage, skin melanoma with sun-induced damage, acral melanoma and mucosal melanoma [7].

The increasing incidence of cutaneous malignancies closely related to UV exposure denotes the necessity of different research studies to assess the mechanisms involved in the pathology of these disorders. There are three types of UV light from electromagnetic radiations spectrum, namely UVA (320-400 nm), UVB (290-320 nm) and UVC (100-290 nm). UVB radiation is directly absorbed in epidermis by a series of cellular constituents such as nucleic acids, proteins, etc., is capable to penetrate the dermis and to exhibit mutagenic effects, becoming one of the main factors responsible for skin malignancies [11,12].

Therefore, the manner in which cellular transformations occur in normal human keratinocytes and dermal fibroblasts cells after UVB irradiation continue to be unclear. In vitro studies performed on normal and tumoral cell lines are important tools in understanding the mechanisms involved in carcinogenesis. The purpose of the present study was to assess the behavior of two healthy cell lines (HaCaT - normal human keratinocytes and 1BR3 - normal dermal fibroblasts) and three tumor cell lines (human melanoma – A375 and murine melanoma – B16F0 and B16A45) after UVB exposure.

Experimental part

Materials and methods

Cell lines culturing

The cell lines used in the present study were: human keratinocytes - HaCat (a gift from Debrecen University), skin human fibroblasts - 1BR3 (ECACC - 90011801), human melanoma - A375 (ATCC - CRL-1619™), and two lines of murine melanoma - B16-F0 (ATCC - CRL-6322™) and B16A45 (Sigma Aldrich). HaCat, A375, B16-F0 and B16A45 were cultured in high glucose (4.5 g/L) medium - DMEM (Dulbecco’s Modified Eagle’s Medium) supplemented with 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin mixture. For 1BR3 (human fibroblasts) it was used as culture medium – EMEM (Eagle’s Minimum Essential Medium) supplemented with 15% FBS. During the experiment the cells were kept into an incubator according with standard conditions: 37°C temperature and 5% CO₂. The cells were counted using a hemocytometer (Neubauer chamber) in the presence of Trypan blue. All the media, supplements and reagents specific for cell culture were purchased from ATCC and Sigma Aldrich, Germany.

Cells irradiation

The cell lines were cultured in 12-well/96-well plates and were let to grow until it was reached a confluence of 80-85%. After the cells reached the appropriate confluence,
were prepared for irradiation, as follows: the old media was removed, the cells were washed with PBS (phosphate saline buffer) and a volume of 0.5 mL/well and 50 µL/well PBS, respectively, was added before exposure [13]. UVB exposure was performed at 312 nm wavelength, at a dose of 40 mJ/cm² by the means of Biospectra system (Vilber Lourmat, France).

**Determination of viable cell numbers**

**Trypan blue assay**

After UVB exposure it was determined the number of viable cells at 30 min and 1h as an early assessment marker. The number of viable cells was determined by applying Trypan blue assay. Addition of Trypan blue to cells suspension led to the identification of viable (bright) versus dead (blue) cells, this dye having the property to penetrate the membrane of dead cells.

The number of viable/dead cells was calculated according to the following formula:

Viable/dead cells/mL = average of the cells counted x 6 (dilution factor) x 10⁴

The total number of cells present into the well is the sum between the viable and dead cells.

The percentage of viable/dead cells was calculated as follows:

Viable cells (%) = number of viable cells/total number of cells x100.

Dead cells (%) = 100 – viable cells (%).

**Alamar blue assay**

Another assay applied to determine the number of viable cells after UVB exposure was Alamar blue technique. In brief, the cells were culture in 96-well plates (1x10⁴ cells/well) 200 µL culture medium), let to adhere and when the appropriate confluence was reached (80-90%), the old medium was removed and the cells were washed with PBS. In the presence of 50 µL PBS, the cells were exposed to UVB radiation (312 nm wavelength) to a dose of 40 mJ/cm² by the means of Biospectra system (Vilber Lourmat, France). After exposure it was added a volume of 150 µL culture media. For cell viability assessment at 3, 6 and 24h, the cells were incubated with 20 µL Alamar blue for 3h and the absorbance was measured by the means of xMark Microplate Spectrophotometer (Biorad) at 570 and 600 nm.

The reduction in viability of UVB-exposed cells was expressed as a percentage compared to non-exposed (control) cells that were considered to be 100% viable. The experiments were performed in triplicate.

**Scratch assay**

For scratch assay tests a number of 2x10⁵ cells/well were seeded in 12-well culture plates and were allowed to adhere to the plate for one day, in order to reach a confluence between 80-90%. Scratches were made on the middle of each well using a 10 µL pipette tip. The detached cells were discarded, washed with PBS (phosphate saline buffer) and observed for 24 h. Optika Microscopes Optikam Pro Cool 5 and Optika View software were used to take pictures at 0, 3 and 24h. These experiments were made at least in triplicate.

**Statistical analysis**

The results were expressed as the mean ± SD. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). One way ANOVA and Tukey’s multiple comparison test were performed to evaluate significant (p<0.05) differences between samples and controls.

**Results and discussions**

Of late years, an alarming worldwide increase of both type of skin cancer, NMSC and MSC, has been reported, while only mucosal and ocular melanoma, seems to be abiding [14]. From all the factors involved in these disorders, UV radiation seems to be the main responsible and the most deleterious. UV radiation, used especially in tanning beds is considered by different organizations, a human carcinogen and represents a major risk of melanoma development [15]. Instead, total or intermittent exposure to UVB radiation results in the appearance of certain types of lesions which in some cases evolve towards NMSC, but these types of radiation are considered an initiation agent in carcinogenesis while type A radiation a promoter agent [16].

Keratinocytes, fibroblasts and melanocytes respond to UV radiation through a complex defense mechanism which involves cell cycle delay, apoptosis, DNA repair etc., however secretion and activation of certain factors cannot be completely stopped and the cells react with these activators causing a series of genetic mutations [17,18]. For instance, apoptosis of keratinocytes involving both intrinsic and extrinsic pro-apoptotic pathways in a manner dependent on the activation of specific caspasases leads to formation of sunburn cells in the epidermal layer, cells with a damaged DNA [19]. A serious consequence of radiation is the formation of reactive oxygen species. Liu-Smith et al. highlight the three types of ROS founded in melanocytes, but also in melanoma cells, namely mitochondria, melanin/ melanosome and NOX-generated ROS, which increase oxidative stress and have a crucial role in genesis and progression of melanoma [20]. Some studies discuss the involvement of sex differences in appearance and evolution of SC but until now the processes involved are not fully described [21,22]. Both type hormones, estrogens and androgens, can regulate the proliferation and differentiation of keratinocytes and melanocytes (e.g. hyperpigmentation during pregnancy) and influences activity of fibroblasts being closely related to UV exposure [22]. In order to evaluate the impact of UVB radiation at skin level and on melanoma development, the experiments were performed on human normal cell lines, HaCat - keratinocytes and IBR3 – dermal fibroblasts that originate in the most upper skin layers, layers that are damaged in a considerable manner by UVB exposure. Pigmented and non-pigmented murine and human melanoma cells were also used in the present study.

**Scratch assay**

A group of the experimental images are exhibited in Figure 1 for the assessments of tumor cell lines initiated with 2x10⁵ cells/well. Scratches are easily identified in each of the images and the initial distinction regarding abundance and density of each type of cell is visually different in the marked area. The evolution over time of the aspects related to cell migration and proliferation are also presented. It can be noticed that a part of cells move into the empty space and cell density grows over time, slowly in the first three hours and considerable after 24 h. The microscopic interpretation of the images express the capacity of tumor cells to re-colonise.

The degree of occupation depends on the confluence of the cells at the beginning of the experiment, and at this stage the dissimilarity in the rate of migration and proliferation is generated by the differences in denseness.
The effect of UVB radiation on cells morphology was verified at different time points: before UVB exposure, at 3h and 24h post-exposure.

In figure 2 are presented representative images of the changes related to keratinocytes and fibroblasts shape after UVB exposure. Observable modifications of cells shape were detected at 24h post-exposure (fig. 2c), some of the cells became detached of the culture plate and their shape became round, a shape characteristic for apoptotic cells. These changes were visible in a higher degree to keratinocytes, results that are in agreement with the data recorded for viability assay.

Similar shape changes were observed for human and murine melanoma cells after UVB exposure, the most prominent effects being detected at A375 cells (fig. 3).

**Cell viability**

The effect of UVB radiation on cells viability was verified at different time points: at 30 min, 1h (by the means of Trypan blue – early assessment), and at 3, 6 and 24h post-exposure (by Alamar blue assay).

There were observed the following: human keratinocytes - HaCat viability decreased to aprox. 80% at 30 min, whereas a significant decrease was observed at 1h, the percentage of viable cells being around 50%, what indicates the cytotoxic effect of UVB radiation (table 1); in the case of human fibroblasts - 1BR3, the effect was opposite to the one observed in the case of HaCat cells, these type being more affected in the first 30 min post-exposure.

Similar results with the ones observed for 1BR3 were observed in human melanoma cells - A375, the number of viable cells increasing with the period post-exposure. In the case of murine melanoma cells - B164A5 and B16F0, the cytotoxic effect of UVB radiation was not so

![Fig. 1. Images of melanoma cell lines, A375, B164A5 and B16F0, from the experiment initiated with 2x10^5 cells/well at time indicated in the figure: 0, 3, and 24h](image1)

![Fig. 2. Keratinocytes and fibroblasts morphology - a) before UVB exposure, b) at 3h after UVB exposure, and c) at 24h after UVB exposure](image2)

![Fig. 3. Human and murine melanoma cells morphology - a) before UVB exposure, b) at 3h after UVB exposure, and c) at 24h after UVB exposure](image3)
pronounced in the first 30 min post-exposure as compared to the one observed in A375 cells (table 1).

In order to observe the behavior of the cell lines used in the study for longer periods after exposure to UVB radiation (3, 6 and 24h), the number of viable cells was assessed by Alamar blue technique. As control cells were used the cells non-exposed to UVB radiation. The results obtained indicate that the number of viable normal human cells (HaCat – keratinocytes and 1BR3 - fibroblasts) decreased in time, the most significant decrease being observed at 24h post-exposure. According to our results, the keratinocytes were more sensitive to UVB radiation effect as compared to fibroblasts (fig. 4), what could be asserted by the fact that UVB deleterious effects occur especially at epidermis level, the amount of radiation that penetrate the dermis being more reduced.

In the case of non-pigmented human melanoma cells - A375, the tendency was similar with the one described for the healthy cells with the difference that the number of viable cells decreased significantly starting at 3h post-exposure and continuing in the same manner until 24h, when the percentage of viable cells was lower than 40% (fig. 4).

The murine melanoma cells - B16A45 and B16-F0 are pigmented, melanin-producing cells and UVB exposure determined a decrease of their viability, the most significant decrease being observed at 24h for B16A45, whereas in the case of B16-F0 at 24h, the cells seemed to recover the percentage of viable cells being bigger that at 6h post-exposure (fig. 4).

Conclusions

In these experiments, were examined how normal/healthy cell lines (human keratinocytes and dermal fibroblast) and tumor (melanoma) cell lines behave to UVB exposure. Irradiation of cells in monolayers consist into a decrease of viability in all cases with the mention that after 24h post-exposure the reduction is the most significant. Also, changes in the cells morphology were reported, part of the cells were detached of the culture plate and their shapes are specific for apoptotic cells, data in agreement with the ones recorded for viability assay. These results certified the cytotoxic effects related to UVB irradiation and are useful tools in preliminary assessments of various protective and curative agents.

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References


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