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Biological Effects Induced by Ultraviolet Radiation in Human Fibroblasts

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1. Introduction

As the most superficial body organ, skin plays an important role in protecting the body from environmental damage. The skin is composed of three layers: the epidermis, dermis and subcutaneous tissue. The epidermis, the outermost layer, has as main functions to protect the body against harmful environmental stimuli and to reduce fluid loss. It is a stratified squamous epithelium with several layers and its major cell type is the keratinocyte. This tissue is constantly being renewed by keratinization, a process of detachment of cornified cells (Blumenberg & Tomic-Canic, 1997). Located under the epidermis are the dermis and the dermal connective tissue, with extracellular matrix proteins such as collagen, elastic fibers, fibronectin, glycosaminoglycans and proteoglycans, which are produced and secreted into the extracellular space by fibroblasts, the major cell type found in this tissue (Makrantonaki & Zouboulis, 2007). The extracellular matrix proteins in the dermal connective tissue contribute for maintaining skin preservation and integrity (Hwang *et al.*, 2011). Stromal fibroblasts play an important role in tissue homeostasis regulation and wound repair via protein synthesis and secretion of growth factors or cytokines of paracrine action with direct effect on proliferation and differentiation of adjacent epithelial tissues (Andriani *et al.*, 2011). Solar ultraviolet (UV) radiation is a predictable epidemiologic risk factor for melanoma and non-melanoma skin cancers. (Katiyar *et al.*, 2011). UV irradiation can impair cellular functions by directly damaging DNA to induce apoptosis (Wäster & Ollinger, 2009). Among other things, longer UV wavelengths (UVB, UVA) induce oxidative stress and protein denaturation whereas short wavelength UV radiation (UVC) causes predominantly DNA damage to cells in the form of pyrimidine dimers, 6-4 photoproducts and apoptosis (Armstrong & Kricker, 2001; Gruijl *et al.*, 2001). UVB irradiation damages skin cells by the formation of ROS (Reactive Oxygen Species) resulting in oxidative stress, an important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA (Wäster & Ollinger, 2009). However, it has less penetrating power than UVA and acts mainly on the epidermal basal layer of the skin. UVC, on the other hand, is extremely damaging to the skin because its wavelengths have enormous energy and induce genotoxic

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stress. Fortunately, UVC is prevented from reaching the earth, as it is largely absorbed by atmospheric ozone layer (Afag, 2011). It has already been proposed that programmed cell death (apoptosis) can be induced by UV light in various cell types (reviewed in Schwarz, 1998). The cellular responses to injuries or stresses are important in determining cell fate (Aylon & Oren, 2007). Many signaling pathways participate in this process, with the mitogen-activated protein kinase (MAPK) cascades and p53 pathway being two of the major pathways implicated (Aylon & Oren, 2007; Li *et al*., 2009). The cellular response to DNA damage is focused on p53, which can induce the cell to apoptosis by the protein PUMA (p53 up-regulated modulator of apoptosis), a member of the Bcl-2 homology (BH)3-only Bcl-2 family proteins. Recent studies suggest that Bcl-2 family members play an essential role in regulating apoptosis initiation through the mitochondria (Zhang *et al*., 2009). UV irradiation induces permeabilization of the lysosomal membrane with release of cathepsin B and D to the cytosol, translocation of the proapoptotic Bcl-2 proteins Bax and Bid to mitochondrial-like structures. Subsequently, there is cytochrome c release and activation of caspase-3 (Bivik *et al*., 2006). p38 MAPK, one of the four MAPK subfamilies in mammalian cells, is activated by proinflammatory cytokines and environmental stress (Brown & Benchimol, 2006; Johnson & Lapadat, 2002). p38 is not only reported to be phosphorylated and activated to mediate cell apoptosis and the differentiation process (Thornton & Rincon, 2009), but also to have cell protective effects under certain circumstances (Chouinard *et al*., 2002). MAPK pathways mediate cellular responses to many different extracellular signaling molecules such as the ones involved in differentiation, gene expression, regulation of proliferation, apoptosis, development, motility or metabolism. The typical MAPK pathways, characterized by the ERK1/2, ERK5, JNK, and $p38^{MAPK}$ components, comprise a cascade of three successive phosphorylation events exerted by a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK (Kostenko *et al*., 2011).

Ultraviolet UVA light absorption after solar exposure is responsible for photoactivation of DNA and other biomolecules. Additionally, UVA radiation (320-400 nm) induces photoaddition, oxidative stress and DNA damage, which may be continuous. The cell is also unable to replicate in case of severe DNA damage. This way, DNA repair must be considered essential for genetic information preservation and transmission in any life form. UVA light generates mutagenic DNA lesions in the skin. Exposure to solar UVB radiation is responsible for skin inflammation and tumorigenesis.

Besides that, oxidative stress induced by solar radiation could be responsible, as well, for the increased frequency of DNA mutations in photoaged human skin. Genomic DNA damage triggers the activation of a network of pathways that rapidly modulate several cellular activities. ROS and hydrogen peroxide can damage DNA. Furthermore, it has been recently shown that increased oxidative stress is correlated to DNA alterations. ROS are deleterious to DNA, membranes and proteins although their exact role in mutagenesis and lethality is still unclear in the many skin cell types. In addition, repair ability and defense mechanisms may differ a lot from one cellular type to another.

Epidermal and dermal cells are targets for UVA oxidative stress and their antioxidant defenses can be defeated. Keratinocytes and fibroblasts may respond differently to UV radiation depending on their localization in the body or their functional and metabolic characteristics. Cell culture models have helped to describe the cytotoxic action of UVA and the role of ROS in UVA-induced cellular damage (Tyrrell, 1990). p53 stabilization and

activation, an essential outcome of the DNA damage response pathway, leads to cell cycle arrest and DNA repair, apoptosis, or cellular senescence. More specifically, initial genomic insults lead to p53 stabilization and nuclear localization where transient cell cycle arrest can be quickly activated, allowing damaged DNA repair prior to replication. A signaling cascade can be activated by p53 in case of extreme and irreversible DNA damage to induce programmed cell death through transcription of different proapoptotic factors.

UV radiation induces phospholipids peroxidation in cellular membrane. Lipid peroxidation is a consequence of free primary radicals (ROS). This, in turn, leads to the generation of polar products and increase the membrane dielectric constant and capacitance. An important consequence of this phenomenon is the alteration of transport particles across the membrane (Strässle *et al.*, 1991)

During the Fenton reaction, singlet oxygen directly initiates lipid peroxides and hydrogen peroxides indirectly initiate hydroxyl radicals (Halliwell & Gutteridge, 1999).

Cellular responses that lead to cell cycle arrest, DNA repair, apoptosis or senescence are induced by the p53 tumor suppressor pathway upon activation by genotoxic stress. This pathway works mostly through transactivation of different downstream targets, for example, p21 cell cycle inhibitor, required for short-term cell cycle arrest or long-term cellular senescence, or other proapoptotic genes such as p53 upregulated modulator of apoptosis (PUMA) (Tavana *et al.*, 2010). Yet, the mechanism that regulates the switching from cell cycle arrest to apoptosis is still unknown. In case of extreme or irreversible damage, p53 can additionally activate a signaling cascade to induce apoptosis through transcription of pro-apoptotic genes, most particularly p53-upregulated modulator of apoptosis (PUMA) and trans-repression of anti-apoptotic genes including Bcl-2. Programmed cell death directly protects cells against the accumulation of genomic instability that could lead to tumorigenesis.

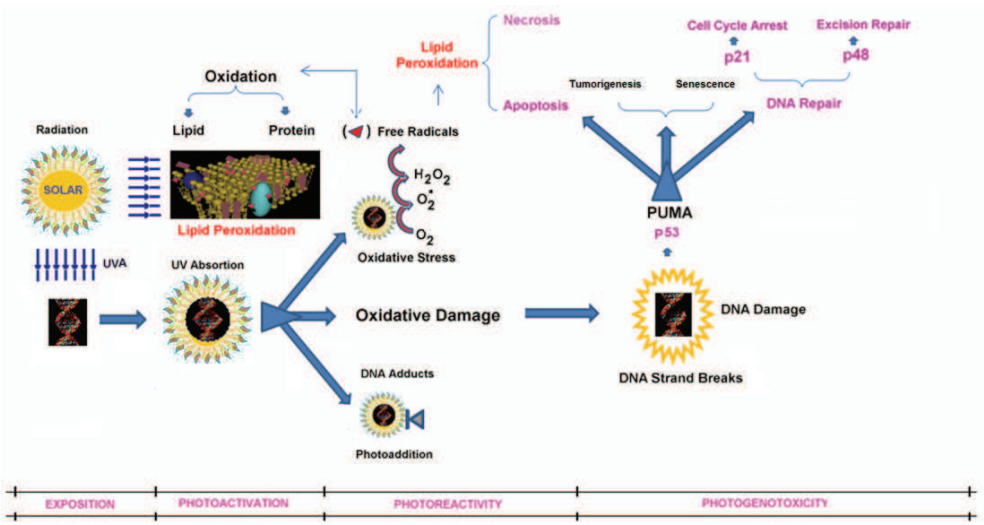

Fig. 1. Schematic representation. Photobiological effects of ultraviolet radiation on human skin cells

Senescence, an irreversible cell cycle arrest, can also be induced by DNA damage. p21, the cyclin-dependent kinase inhibitor, plays an important role in cell cycle checkpoint regulation and induction of cellular senescence, thus being one key p53 target. After DNA damage, p21 is commonly transactivated and induces G1arrest by inhibiting the cyclinE/CDK2 complex. (Campisi, 2009). Many different stimuli can induce cellular senescence including telomere shortening (replicative senescence), oncogenic signaling (oncogene-induced senescence), or stress/DNA damage irrespectively of the two previous signaling pathways (premature senescence) (Campisi, 2009). Despite the stimuli, cellular senescence and apoptosis are somewhat equivalent in preventing genomic instability and consequently inhibiting tumor formation (Van Nguyen, 2007). Upon UV exposure, p48 mRNA levels strongly depended on basal p53 expression and increased even more after DNA damage in a p53-dependent manner thus pointing as the link between p53 and the nucleotide excision repair apparatus (Hwang *et al.*, 1999).

2. Objective

The objective of this study was to investigate modifications in cytoskeleton through the formation of blebs and apoptosis in cultured human fibroblasts by confocal microscopy and flow cytometry.

3. Methods

This study was performed in accordance with the ethical standards laid down in the updated version of the 1964 Declaration of Helsinki and was approved by the Research Ethics Committee of the Federal University of São Paulo. All patients signed a free and informed consent form. Samples of normal adult human skin (6 women, 18-50 years, skin phototype Fitzpatrick class. III-IV) were obtained as discarded tissue from trunk cosmetic surgery.

3.1 Fibroblast culture

Primary human skin fibroblast culture was done by explant. Fragments were placed in 15 ml conic tubes and exhaustively rinsed (six times) with 10 ml PBS (Phosphate-Buffered Saline, Cultilab, Campinas, SP, Brazil) containing penicillin (100 Ul/ml, Gibco, Carlsbad, CA, USA) and streptomycin (100µg/ml, Gibco) under vigorous agitation, changing tubes and PBS at each repetition. Then, fragments were transferred to 60 mm^2 diameter Petri dishes, in grid areas scratched with a scalpel. Dishes were left semi-opened in the laminar flow for 20 min, for the fragments to adhere to its surface. Then, 6 ml of DMEM (Dulbecco's Modified Eagle's Medium, Cultilab) supplemented with 10% FBS (Fetal Bovine Serum, Cultilab), 1% glutamine, penicillin (100 UI/ml, Gibco) and streptomycin (100 µg/ml, Gibco) were carefully added to each plate. Plates were kept in humidified incubator (37ºC, 95% O_2, 5%CO_2).

Culture medium was changed every two days and a few days after establishing the primary culture, spindle-like cells were seen proliferating from the edges of the explanted tissue, regarded as culturing fibroblasts. Fibroblast satisfactory proliferation was observed in approximately 7-14 days and subculturing (passage) was performed when cellular confluence reached approximately 80% at the Petri dish. For all experiments, cells from passages one to five (Figure 2) were used after harvesting by trypsinization [0.025% trypsin,

0.02% ethylene diamine tetra acetic acid (EDTA; Sigma Chemical Co., Saint Louis, MO, USA) in PBS].

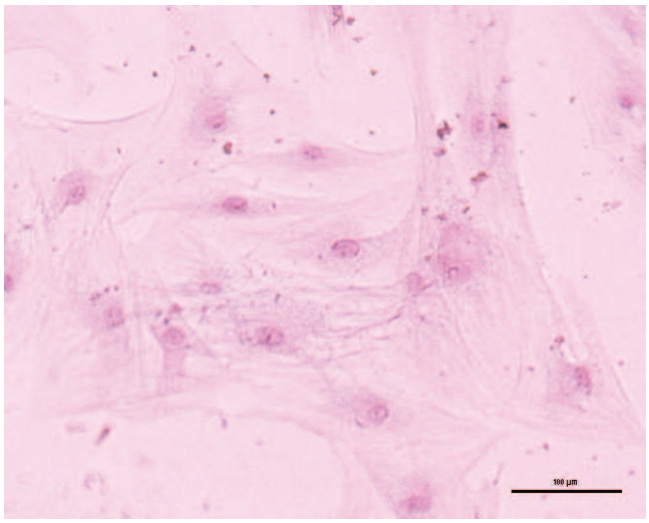

Fig. 2. Optical microscopy. Primary human skin fibroblast culture. Hematoxilin and eosin staining

3.2 Ultraviolet irradiation

Cells were rinsed in PBS. The PBS was then removed and a thin layer of buffer was left on top of the coverslip. Fibroblasts were irradiated in culture dishes in a 10cm^2 field using a UV chamber (with 6 UV F40 Philips lamps) in exposure times of 30 and 60 minutes.

3.3 Immunofluorescence labeling

Primary human skin fibroblast culture were used after harvesting by trypsinization [0.025% trypsin, 0.02% ethylene diamine tetra acetic acid (EDTA; Sigma Chemical Co., Saint Louis, MO, USA) in PBS]. The cells were washed 3 times with phosphate-buffered saline (PBS). Human fibroblasts were plated on glass coverslips, fixed in 2% paraformaldehyde for 10 minutes at 4°C, washed 3 times in PBS, and washed twice in PBS with 50 mmol/L NH_4Cl. Cells were permeabilized with 0.1% saponin in PBS containing 10% normal bovine serum for 30 minutes at 22°C and stained with a combination of fluorescent dyes. Filaments of cytoskeleton immunostained with phalloidin conjugated fluorescent with Alexa Fluor 594 (red) - Molecular Probe, were used to identify actin filaments F inside the cells. Phalloidin (1:500) incubation was performed in PBS containing 10% normal bovine serum and 0.1% saponin. Nuclei were counter stained with blue - fluorescent DNA stain DAPI (4_6-diamidino-2-phenylindole) 1:10000 (catalog #D1036; Molecular Probes, Invitrogen, Carlsbad, CA), and excited using a 750nm multiphoton source (two simultaneous photon excitations at 375nm). The images are a composite of three images acquired using filter sets appropriate for blue and red fluorescence, on a Zeiss confocal microscope (LSM 510, Germany).

3.4 Determination of MDA-TBA levels

Taking the 1h time-point, which proved to be optimal for the determination of MDA increase, we then studied dose kinetics. Fibroblasts were exposed to a series of single doses UV irradiation in exposure times of 30 and 60 minutes. Markedly elevated MDA concentrations in the UV and TBARs–MDA complex concentrations were determined by high-performance liquid chromatography (HPLC) as described by Gueguen *et al*., 2002. The MDA-TBA test, which is the colorimetric reaction of malondialdehyde and thiobarbituric acid in acid solution, was used to determine the MDA levels. HPLC was used after the formation of the MDA-TBA complex (Figure 3) to assess the concentration of the complex based on a known standard curve. After heating at 95 ºC for 60 min, the MDA-TBA chromogen was fluorometrically analyzed using a reversed-phase C18 column HPLC and a wavelength of 532 nm. The MDA-TBA method was previously described by Chirico *et al*. (1993). MDA levels were expressed in relation to the total cellular lysate protein amount, which was assessed using Bradford's method (Bradford, 1976).

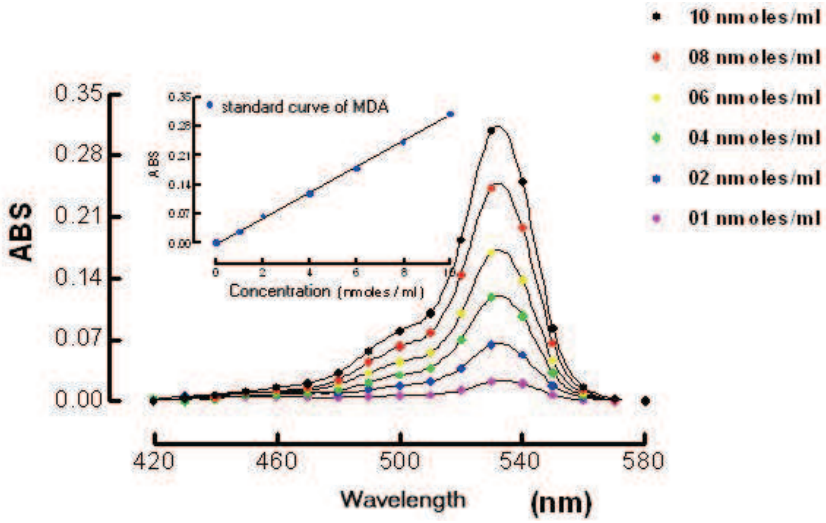

Fig. 3. Absorbance spectra for MDA – TBA chromogen complex standards in Thiobarbituric Acid Reactions (TBARs). Malondialdehyde (MDA) is a very effective method for determining lipid peroxidation levels in fibroblasts exposed to ultraviolet radiation. The standards absorption peaks of the inserted curve were highly linear in the range of 0 to 10nmoles/mL with maximum absorption at 532nm

3.5 Apoptosis assay

Flow cytometry technique, using propidium iodide, was used to detect apoptosis in fibroblast culture of human skin exposed to UV radiation (Nicoletti *et al*., 1991).

Human fibroblasts were labeled with annexin V-FITC (Roche), which bind to phosphatidylserine at the cell surface of apoptotic cells, and propidium iodide (PI; Sigma Aldrich), was used as a marker of cell membrane permeability according to manufacturer's

directions. Samples were examined by fluorescence-activated cell sorter (FACS) analysis, and the results were analyzed using Cell-Quest software (Becton Dickinson, San Jose, CA) (Vermes *et al*., 1995).

3.6 Flow cytometric analysis of caspase 3 and p53

Briefly, normal human fibroblast cells from cultures with increasing passage number were collected and re-suspended in a buffer saline (PBS) containing 0.1% sodium azide (Sigma) containing 20 mM HEPES (pH 7.5), the cells were homogenized and centrifuged at 10,000 x g for 5 min. For analysis of caspase 3 and p53 expression, cells were fixed in 2% paraformaldehyde for 10 minutes at 4°C, washed 3 times in PBS, then washed twice in PBS with 50 mmol/L NH_4Cl. Cells were permeabilized with 0.1% saponin in PBS containing 10% normal bovine serum for 30 minutes at 22°C. The first primary antibody incubation (anti-p53 (SER 15) or anti–cleaved caspase 3) was performed in PBS containing 10% normal bovine serum and 0.1% saponin. Aliquots were then incubated for 60 minutes with anti-caspase 3 and p53 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), final dilution 1:800, or rabbit IgG as a control, followed by washing in PBS containing 0.1% saponin 3 times for 5 minutes each at 22°C. Cells were then incubated with the first fluorochrome-conjugated secondary antibodies Alexa 488 and 594 diluted 1:1600, and incubation was performed for 40 minutes at 37°C in the dark (Danova *et al*., 1990).

3.7 Statistical analysis

The results obtained were analyzed using a one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls Multiple Range Test. Data were analyzed by GraphPad Prism v.3.0 software.

4. Results and discussion

Skin cells exposure to solar radiation may result in biological consequences, one of the most important being skin DNA photodamage due to sunlight ultraviolet (UV) radiation. Wavelengths in the UVB range are absorbed by DNA and can induce mutagenesis. It has been suggested that p53-independent mechanisms of killing tumor cells may not involve programmed cell death and could be a result of induced mechanical damage, rather than apoptosis (Funkel, 1999).

Ultraviolet A radiation (UVA, 320–400 nm), an oxidizing component of sunlight, exerts its biological effect mainly by producing reactive oxygen species (ROS) which cause biological damage in exposed tissues, including the lipid bilayer, via iron-catalyzed oxidative reactions (Halliwell & Gutteridge, 1999; Tyrrell, 1990). Membrane alterations induced by UV irradiation were determined, such as MDA concentration increase, which indicates lipid peroxidation levels (methods previously described -Figure 3). The UV radiation effects in the cellular production of ROS were indirectly determined by the ratio: (MDA concentration / total amount of fibroblasts) at the sample. Analyses of the lipid peroxidation by measuring the products that react with Tiobarbituric Acids (TBARs) normalizing the obtained MDA (malondialdehyde) results by the number of cells in the sample (Figure 4). A significant MDA increase was observed, of about 45.0 % after 30 min of UV exposure and 130% after 60 min of UV exposure.

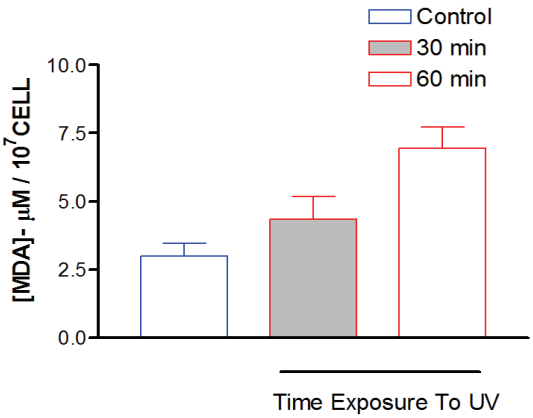

Fig. 4. Effect of UV radiation in the cellular production of oxygen reactive species measured by ratio: MDA concentration by total number of fibroblast in the sample. Histograms values differ significantly from each other. *Data analyzed with one-way ANOVA followed by Newman Keuls (significance level $p < 0.05$). Values represent the mean ± SEM of at least four different experiments

Similar results are also described by several studies demonstrating that low UVA radiation doses can induce lipid peroxidation in membranes of both human fibroblasts and keratinocytes via pathways involving singlet oxygen and iron (Morliere *et al*, 1991).

Looking from a different angle, cells also have repair mechanisms to respond to DNA damage, and at least two different mechanisms are responsible for UVA-induced DNA damage repair. The primary process that removes bulky damage is the nucleotide excision repair pathway. Small lesions induced by ROS are mostly processed by base excision repair pathway. On the other hand, highly damaged cells may undergo cell cycle arrest, apoptosis and senescence (Hazane *et al*., 2006). Our results are consistent with those of Shindo *et al.* (1994) who investigated antioxidant molecules in crude extracts of human epidermis and dermis. In addition, Moysan *et al.* (1995), using cells from the same biopsy, found no link between UVA cytotoxicity and antioxidant capacity since SOD, catalase and GSH were identical in both cells and GSH-Px was higher in fibroblasts (Degterev *et al*., 2008). Other authors, however, have found more antioxidant molecules in fibroblasts than in keratinocytes. Yohn *et al.* (1991), using cells from different donors, found increased GSH-Px, SOD and catalase in fibroblasts compared to keratinocytes, and in keratinocytes compared to melanocytes (Huang *et al*., 2008).

Several *in vitro* and *in vivo* studies on skin cells have demonstrated that UV radiation can damage many molecules and structures (Matsumura & Ananthaswamy, 2004). Corroborating these results, morphological analysis by confocal fluorescence microscopy of fibroblasts group control showed characteristics of nuclear and cytoskeleton integrity. High cellularity was also observed (Figure 5). In contrast, exposed to UV for 30 and 60 minutes showed changes in the actin filaments arrangement of the cellular cytoskeleton. Groups irradiated for 30 and 60 min presented disruption of the actin filaments, with the formation of blebbing and nuclear fragmentation as a consequence of the ultraviolet radiation (Figure 6).

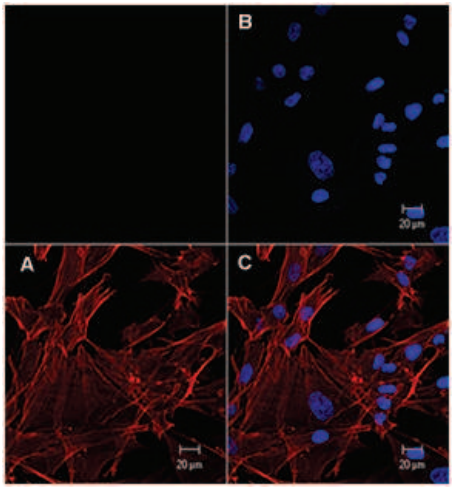

Fig. 5. Confocal microscopy. Cultured human skin fibroblasts. Control group. Cellular localization of actin filaments and nuclei. A) Actin filaments immunostained with phalloidin conjugated with Alexa Fluor 594 (red). B) Cell nuclei stained with DAPI (blue), showing characteristics of nuclear and cytoskeleton integrity. High cellularity was also observed. C) Overlapped images A and B

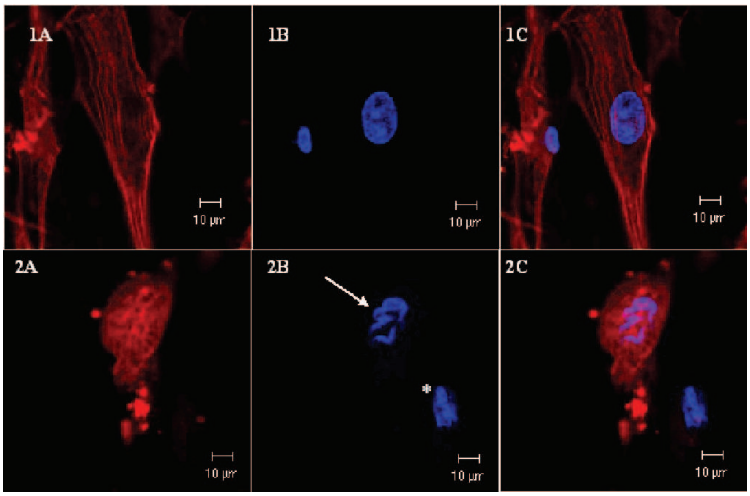

Fig. 6. Confocal microscopy. Cultured human skin fibroblasts. Cellular localization of actin filaments and nuclei. Cells exposed to UV radiation for 30 min (1A, 1B, 1C) or 60 min (2A, 2B, 2C). 1A) Actin filaments immunostained with phalloidin conjugated with Alexa Fluor 594 (red). The occurrence of blebbing can be observed. 1B) Cell nuclei stained with DAPI (blue), showing characteristics of nuclear and cytoskeleton integrity. 1C) Overlapped images A and B. 2A) Actin filaments immunostained with phalloidin conjugated with Alexa Fluor 594 (red). 2B) Pyknotic nuclei (*) and nuclear fragmentation (arrow) were observed. 2C) Overlapped images A and B

In addition, skin fibroblasts viability, stained by propidium iodide (PI), was analyzed by flow cytometry. Viable cells were characterized by a structurally intact cell membrane and no PI uptake. In contrast, dead cells (necrosis or late apoptotic cells) were characterized by loss of the integrity of their membranes and were stained by PI. At all UV radiation tested doses, the amount of viable cells was reduced, as verified by PI staining. The amount of viable fibroblasts was dramatically reduced by UV radiation at all tested doses/exposure times, about 80% after 30 min of exposure and 30% after 60 min of exposure (Figures 7A and 8A).

There are strong evidences that skin cancer can be developed as a result of ultraviolet radiation, which is directly associated to the TP53-gene tumor mutation.

To further investigate whether p53 is involved in the apoptosis induced by UV, cells were first stained for membrane-exposed phosphatidylserine using annexin-V conjugated to fluorescein (FITC). There was a significant increase of the number of apoptotic cells: about 21.0 % (30 min) and 50% (60 min) after irradiation (Figures 7B and 8B) and (Figures 7C and 8C), respectively.

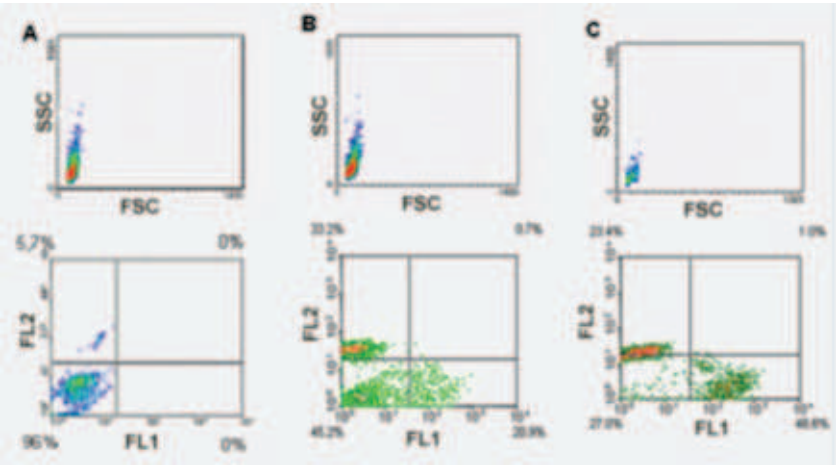

Fig. 7. Contour diagram of PI flow cytometry of cultured fibroblasts for groups: A) Control; B) UV irradiated for 30 min and C) UV irradiated for 60 min. The lower left quadrant of the cytograms shows the viable cells, which excluded PI. The upper right quadrants represent the apoptotic cells showing PI uptake. Panel (B) shows cells number (%) for apoptosis and necrosis 30 minutes after exposure to ultraviolet radiation. Panel (C) shows cells number (%) for apoptosis and necrosis 60 minutes after exposure to ultraviolet radiation. Data are representative of 04 independent experiments

Ultraviolet radiation is a carcinogenic agent for the skin. Even though being a tumor suppressor gene, details are still needed in order to understand the signaling mechanisms of skin cell death induced by UV radiations, which can lead to cancer and/or cell aging.

DNA alteration can ultimately lead to the development of skin cancer, so DNA itself is a critical target (Matsumura *et al.*, 2004). Skin DNA photodamage activates the signaling pathway of cell death by apoptosis. Apoptosis is a crucial mechanism in eliminating cells with unrepaired DNA damage and preventing carcinogenesis.

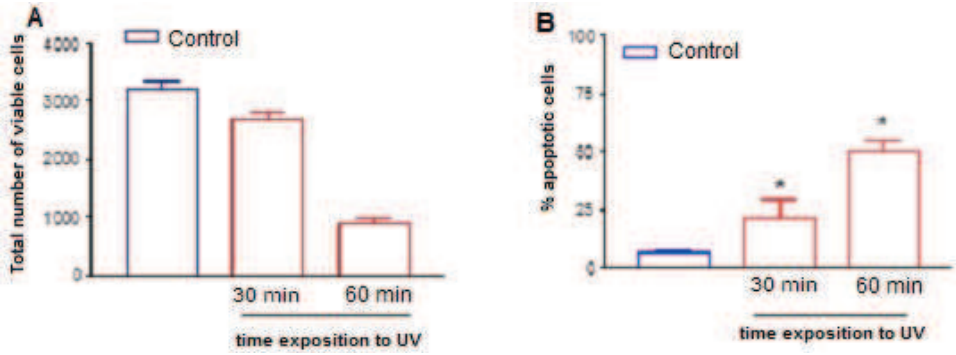

Fig. 8. Mean percentage ± of apoptotic cells in groups control and 30min and 60min after exposure to UV radiation. Data are the means of triplicate assays of one experiment representative of three that gave similar results. A) Total number of viable cells and B) Percentage of apoptotic cells. *Data analyzed with one-way **ANOVA** followed by Newman Keuls (significance level $p < 0.05$). Values represent the mean ± SEM of at least four different experiments

DNA is a critical target because its alteration can ultimately lead to the development of skin cancer (Matsumura *et al*., 2004). In addition, Skin DNA photodamage activates the signaling pathway of cell death by apoptosis. Apoptosis is a crucial mechanism in eliminating cells with unrepaired DNA damage and preventing carcinogenesis (or preventing the formation of malignant tumors).

Apoptosis is characterized by a p53-dependent induction of pro-apoptotic proteins, leading to permeabilization of the outer mitochondrial membrane, release of apoptogenic factors into the cytoplasm, activation of caspases (cysteine-aspartic proteases) and subsequent cleavage of various cellular proteins. Apoptogenic effects include chromatin condensation and exposure of phosphatidylserine on the cell membrane surface (Meier *et al*., 2007).

p53 levels increased about 40% after 30 min of UV exposure and about 60% after 60 min of UV exposure (Figure 9).

Previous studies indicated that BimL was involved in UV-induced apoptosis, but it remains unclear whether Bim directly activates Bax or if this activation occurs via the release of pro-survival factors (antiapoptotic) such as Bcl-xL. In recent studies, Wang *et al*. (2009) determined the interactions between BimL and Bax/Bcl-xL during UV-induced apoptosis.

Caspases have a major role in apoptosis. They are synthesized as inactive proenzymes that become activated by cleavage. Procaspase 3 is a constitutive proenzyme activated by cleavage during apoptosis. (Cohen, 1997). Caspase-3 is the most important protease in the caspase-dependent apoptosis pathway, as it is required for chromatin condensation and fragmentation (Porter & Jänicke, 1999). Poly-ADP ribose polymerase (PARP-1) is a major target of caspase-3, since cleavage-mediated inactivation of PARP-1 preserves cellular ATP that is required for apoptosis (Bouchard *et al*., 2003).

Regarding the caspases, the resulting enzyme is able to cleave several aspartate residues of many target proteins, after a DEVD sequence common to all caspases 3 and 7 substrates

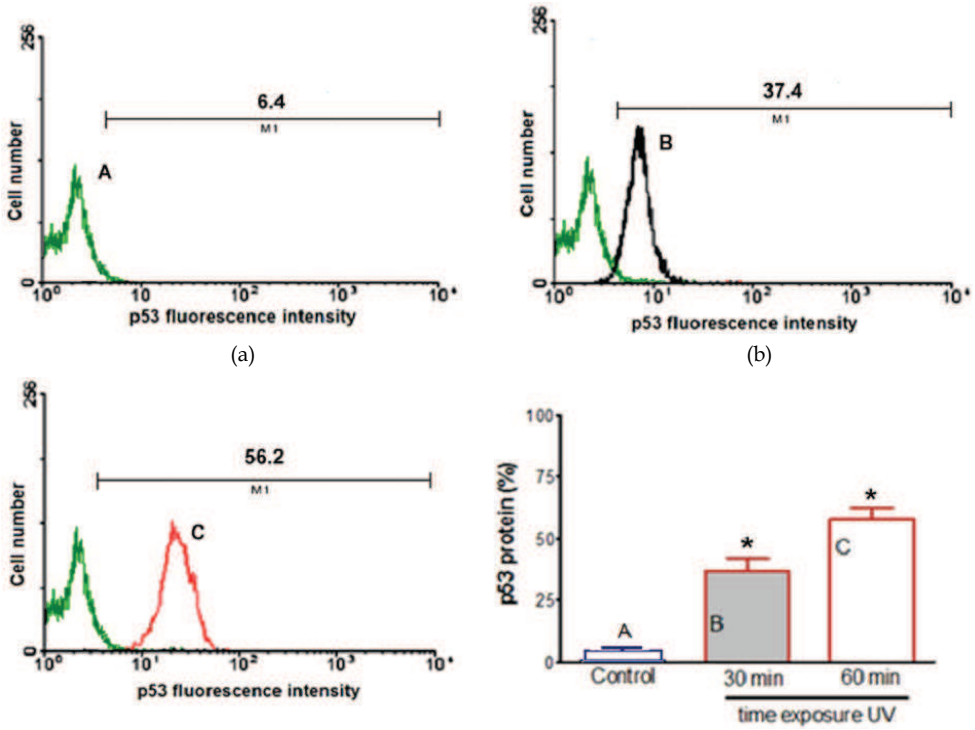

Fig. 9. Flow cytometry (FCM) analysis of p53 protein accumulation control (upper left set of panel – figure 9A – green line) and / or activation by UV can be followed of cultured fibroblasts for groups: UV irradiated for 30 min (right set of figure 9B – Black line) and UV irradiated for 60 min (right set of figure 9C – red line). The fibroblasts treated in 2% paraformaldehyde are the same as those shown in Figure 05 (control group) and figure 06 (UV irradiated groups). Cells were permeabilized with 0.1% saponin in PBS containing 10% normal bovine serum for 30 minutes at 22°C and stained with anti-p53 (SER 15) antibodies at figures (9A), (9B), and (9C) after the beginning of the experiment and analyzed by FCM. A control performed with an irrelevant antibody is shown figure 9A. The percentage of cells exhibiting active p53 conjugated with FITC is indicated on each histogram. The results from one representative experiment of four experiments performed are shown. The numbers indicate the percentages of positive cells and fluorescence intensity. Histogram overlays show the FL1 (green fluorescence) intensity corresponding to a given p53 (black line – UV irradiated for 30min and red line – UV irradiated for 60min) compared to the intensity for the control (green line). 9D Mean percentage ± of cells exhibiting active p53 in groups control (figure 9A – green line) and groups UV irradiate for 30 min (figure 9B – Black line) and UV irradiated for 60 min (figure 9C – red line). Data are the means of triplicate assays of one experiment representative of three that gave similar results. A) Total number cells fibroblasts exhibiting active p53 antibodies at figures (9A), (9B), and (9C). Histograms values differ significantly from each other. *Data analyzed with one-way ANOVA followed by Newman Keuls (significance level $p < 0.05$). Values represent the mean ± SEM of at least four different experiments

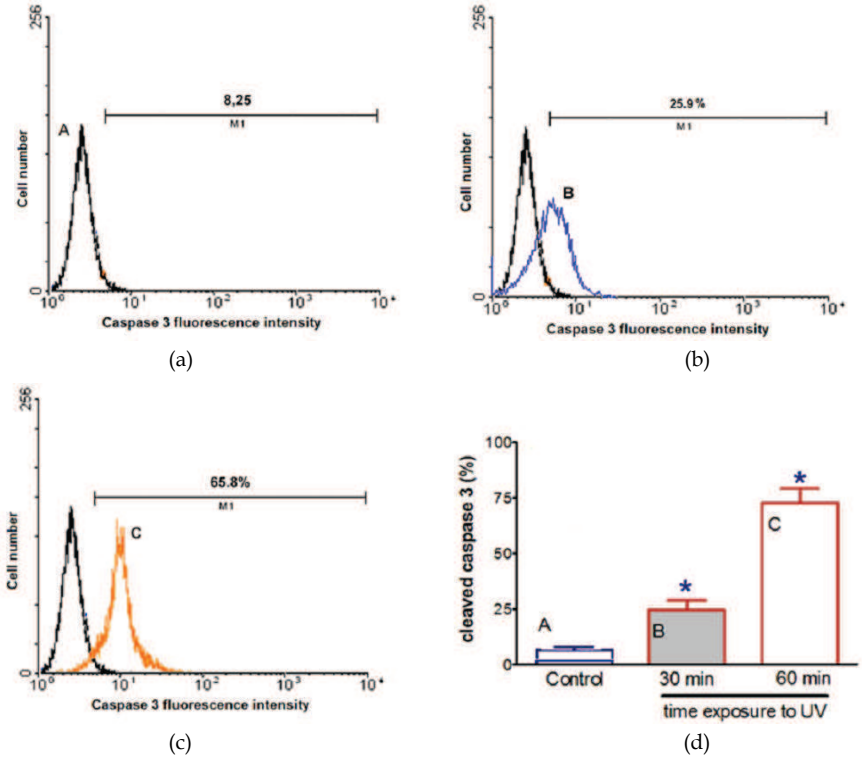

Fig. 10. Activation of caspase 3 by UV can be followed by flow cytometry (FCM) of cultured fibroblasts for groups: A) control (upper left set of panel – figure 10A - Black line) and UV irradiated for 30 min (right set of figure 10B – Blue line) and UV irradiated for 60 min (right set of 10C – orange line). The fibroblasts treated in 2% paraformaldehyde are the same as those shown in figure 05 (control group) and figure 06 (UV irradiated groups). Cells were permeabilized with 0.1% saponin in PBS containing 10% normal bovine serum for 30 minutes at 22°C and stained with anti–cleaved caspase 3 antibodies at figures (10A), (10B), and (10C) after the beginning of the experiment and analyzed by FCM. A control performed with an irrelevant antibody is shown 10A. The percentage of cells exhibiting active caspase 3 conjugated with FITC is indicated on each histogram. The results from one representative experiment of four experiments performed are shown. The numbers indicate the percentages of positive cells and fluorescence intensity. Histogram overlays show the FL1 (green fluorescence) intensity corresponding to a given caspase 3:(blue line – UV irradiated for 30min and red line – UV irradiated for 60min compared to the intensity for the control (black line). 10D Mean percentage ± of cells exhibiting active caspase 3 in groups control (figure 9A – green line) and groups UV irradiated for 30 min (figure 9B – Black line) and UV irradiated for 60 min (figure 9C – red line). Data are the means of triplicate assays of one experiment representative of three that gave similar results. A) Total number cells fibroblasts exhibiting active caspase 3 antibodies at figures (10A), (10B), and (10C). Histograms values differ significantly from each other. *Data analyzed with one-way ANOVA followed by Newman Keuls (significance level $p < 0.05$). Values represent the mean ± SEM of at least four different experiments

(DEVDase). Thus, active caspase 3 is a common effector protein in several apoptotic pathways, and it may be a good marker to detect (pre-) apoptotic cells by flow cytometry (Porter & Jänicke, 1999). Taking this into consideration, apoptosis was confirmed by determining the increased expression of cleaved caspase 3 after fibroblasts exposure to UV radiation. In this work we could verify a significant increase of cleaved caspase 3 levels, about 25.0 % after 30 min of UV exposure and 75% after 60 min of UV exposure (Figure 10).

Although caspases represent a significant component of the apoptotic pathway, there is indication that a caspase-independent apoptosis pathway also exists (Broker *et al*., 2005). This pathway involves the Apoptosis-Inducing Factor (AIF), which translocates from the mitochondria to the nucleus to cause chromatin condensation (Daugas *et al*., 2000).

Then again, genotoxic effects of solar UVA are mediated essentially by the activation of endogenous photosensitizers which generate a local oxidative stress. Depending on the dose and duration of exposure, UV-induced effects may occur, and DNA damage can lead to mutations and genetic instability. This is one of the reasons why sunlight overexposure increases the risk of skin cancer and DNA photolesions can also be involved in other skin-specific responses to UV radiation: erythema, immunosuppression, and melanogenesis (Matsumura & Ananthaswamy, 2004).

5. Conclusion

Damages occurring on DNA molecules not always induce mutagenesis. We should take in consideration many strong scientific evidences showing that specific activation molecular signaling pathways promote several different answers. Both the prolonged exposure time and the increase in the UV radiation dose were able to induce lipid peroxidation and cell death by apoptosis. Our results suggest that the major part of UV induced apoptosis cell death is caspase-dependent, although a minority of cells may die by a caspase-independent pathway, presumably apoptotic. In this work we also showed that p53 levels increased after UV exposure. In these circumstances, the action of UV radiation on skin cells still involves many issues depending on the cell type and on different cellular response pathways induced by phototoxic stress. Skin fibroblasts are surely sensitive to UV radiation, thus, from a better understanding of the molecular mechanisms triggered by the action of UV radiation on skin cells, it will be possible to work on improving skin radioprotection and attenuating the effects of sunlight exposure.

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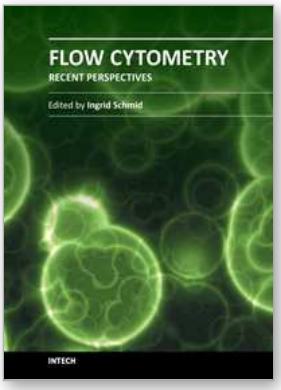

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"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

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