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Debacq-Chainiaux, Florence; Leduc, Cedric; Verbeke, Alix; Toussaint, Olivier

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# UV, stress and aging

Florence Debacq-Chainiaux,\* Cedric Leduc, Alix Verbeke and Olivier Toussaint

Research Unit of Cellular Biology (URBC); NARILIS; University of Namur (FUNDP); Namur, Belgium

**Key words:** UVB, fibroblasts, keratinocytes, senescence

**Abbreviations:** CPD, cumulative population doubling; HDF, human diploid fibroblast; NHEK, normal human epidermal keratinocyte; ROS, reactive oxygen species; SA- $\beta$ gal, senescence-associated  $\beta$ -galactosidase; UV, ultraviolet

Skin is a model of choice in studies on aging. Indeed, skin aging can be modulated by internal and external factors, reflecting its complexity. Two types of skin aging have been identified: intrinsic, mainly genetically determined and extrinsic—also called “photo-aging”—resulting on the impact of environmental stress and more precisely of UV rays. Simplified *in vitro* models, based on cellular senescence, have been developed to study the relationship between UV and aging. These models vary on the cell type (fibroblasts or keratinocytes, normal or immortalized) and the type of UV used (UVA or UVB).

## Introduction

Skin is a model of choice in studies on aging. Indeed, aging of the skin can be modulated by internal and external factors, reflecting its complexity. Two different types of skin aging are identified: intrinsic aging, mainly genetically determined and similar to aging of other organs, and extrinsic aging, resulting on the impact of environmental stress and more precisely of UV rays (for a review see refs. 1 and 2). If these two types of skin aging present differences at the morphological and at the histological levels,<sup>1,2</sup> they share molecular similarities (for a review see ref. 3) as the induction of matrix metalloproteinases. In order to better understand skin photo-aging, and more precisely, the relation between UV and aging, several simplified *in vitro* models have been developed, based on *in vitro* models of cellular senescence.

## Skin Aging

Skin aging can be modulated by external factors. This extrinsic aging is superimposed on intrinsic one, and is also referred as “photo-aging.” Indeed, if several exogenous factors as tobacco smoke, infrared radiation, pollution, malnutrition etc. can interfere with skin aging, the factor having the greatest impact is clearly UV rays. Naturally, intrinsic and extrinsic aging of the

skin are observable in the same individual depending on whether the parts of skin were protected from the sun or not. The face and the backside of the hands are usually the most photoaged affected areas. Clinically, intrinsically aged skin is thin, smooth and presents only light wrinkles.<sup>2,4</sup> Different subtypes can characterize extrinsic aging of the skin. Classically, it is distinguished by a thicker skin (leathery aspect), with deeply marked wrinkles and an irregular pigmentation (age spots).<sup>2,4</sup> Histologically, both types of skin aging are characterized by change in the organization of structural components of the connective tissue. Intrinsically aged skin is marked by a decrease in epidermal and dermal thickness. The interstitial collagen and elastin content are reduced while collagen cross-links fibers content is increased.<sup>2,4</sup> Extrinsically aged skin shows hyperplasia, with an increase of the thickness of the epidermis and dermis. There is a complete perturbation of the structural content (reduced interstitial collagen, increased elastic fibers) associated with damaged fibers leading to a severe disorganization of the connective tissue structure.<sup>2,4</sup> Aging of the individuals appears to be linked to internal factors as genetic predispositions (as shown for longevity<sup>5–7</sup>), hormonal status<sup>8</sup> and to environmental factors. The degree of influence of these genetic and environmental factors has not been clearly described in aging of the skin.<sup>9</sup> However, several studies of cohorts of twins helped highlight the importance of these two factors.<sup>10,11</sup> Despite their differences, evidence shows that intrinsic and extrinsic aging of the skin are probably driven by similar biological, biochemical and molecular mechanisms.<sup>12</sup> Thus, the formation of reactive oxygen species (ROS) and the induction of matrix metalloproteinases are shown to be common factors of both types of skin aging.<sup>3</sup> It is assumed that ROS accumulation detected in intrinsic and extrinsic aging leads to the activation of MAPK (mitogen-activated protein kinases) pathways. ERK (extracellular signal-regulated kinases), JNK (c-Jun N-terminal kinase) and p38<sup>MAPK</sup> once activated induce the activation of AP-1 (activator protein-1) transcription factor. AP-1 induces collagen degradation by promoting the expression of matrix metalloproteinases MMP-1, MMP-3 and MMP-9<sup>12,13</sup> and by preventing the expression of procollagen-1.<sup>14</sup>

## UV and Photo-Aging

UV are essential components of sunlight. *In vivo*, skin is exposed to UVB and UVA as UVC are stopped by the ozone layer. UVB

\*Correspondence to: Florence Debacq-Chainiaux;  
Email: florence.chainiaux@fundp.ac.be  
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(290–320 nm) and UVA (320–400 nm) are able to cross the epidermis and to reach the dermis.<sup>15</sup> UVB and UVA can interact with endogenous chromophores and photosensitizers resulting in the generation of ROS causing damage to DNA, proteins and lipids. Moreover, UVB can directly interact with DNA and generate dipyrimidine photoproducts such as cyclobutane pyrimidine dimers and pyrimidine (6–4) pyrimidone photoproducts (for a review see refs. 16 and 17). UVB are therefore considered as the most harmful UV rays. UV radiation activates several signal transduction pathways related to growth, differentiation, senescence and connective tissue degradation<sup>18</sup> by the activation of several cell surface receptors. This includes cytokines or growth factors receptors as the receptors for epidermal growth factor (EGF), tumor necrosis factor (TNF), interleukin-1 (IL-1)<sup>15</sup> and keratinocyte growth factor (KGF).<sup>19</sup> The biological responses to UV can be immediate and transient (inflammation, sunburn cell formation, pruritus) or delayed and chronic (photo-aging, immunosuppression, carcinogenesis). Exposures to UV rays are also used in dermatology to treat many skin diseases including psoriasis, atopic dermatitis, vitiligo, etc.<sup>20</sup>

In most cases, studies on photo-aging require the participation of human volunteers. This implies ethical constraints and limits sample size. In order to investigate in vitro photo-aging, various models have been developed, based on cellular senescence.

### Replicative Senescence and Stress Induced Premature Senescence

In vitro, proliferative somatic cells, as human diploid fibroblasts (HDF), have a limited capacity of cellular divisions. Replicative senescence is defined as an irreversible growth arrest. This was first described by Hayflick in the early 1960s on HDFs<sup>21</sup> and was later extended to most proliferative cell types (for a review see ref. 22). This growth arrest is established and maintained by the p53/p21<sup>WAF-1</sup> and the p16<sup>INK-4A</sup>/pRb pathways.<sup>22</sup> Thirty years later, Harley et al.<sup>23</sup> showed that telomeres shorten during aging of HDFs, and replicative senescence was associated to a “critical” shortening of the telomeres. Cells can remain alive for several months after the onset of replicative senescence<sup>24</sup> and show apoptosis resistance.<sup>25</sup> Several markers can identify senescent cells in vitro and in vivo, however, none is exclusive to the senescent state.<sup>22</sup> Among these biomarkers are: typical enlarged and flattened morphology,<sup>24</sup> senescence-associated  $\beta$ -galactosidase activity (SA- $\beta$ gal),<sup>26</sup> senescence-associated DNA-damage foci (SDFs),<sup>27,28</sup> altered gene expression<sup>29,30</sup> and the common mitochondrial DNA deletion of 4,977 bp.<sup>31</sup> More recently, it was shown that senescent cells secrete growth factors, proteases, chemokines and inflammatory cytokines allowing them to interact with their cellular environment. This was termed senescence-associated secretory phenotype (SASP).<sup>32,33</sup>

Some of these biomarkers are also detected in vivo. For instance, in the skin, some of these biomarkers are also detected during aging: typical senescent morphology,<sup>2</sup> SA- $\beta$ gal activity,<sup>26</sup> p16<sup>INK-4A</sup> overexpression<sup>34</sup> and the “common” mitochondrial DNA deletion.<sup>35</sup>

In vitro, it is possible to induce the appearance of these biomarkers by exposing human proliferative cell types, such as HDFs, endothelial cells, melanocytes, etc. to acute stress at sublethal doses of stressing agents inducing oxidative stress and/or DNA damage. This was defined as “Stress Induced Premature Senescence” (SIPS) and is detectable at 3 d after the stress,<sup>36</sup> long before the cells reach the critical telomere length observed in replicative senescence. Cells in SIPS induced by subcytotoxic concentrations of oxidative agents such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)<sup>37</sup> or *tert*-butylhydroperoxide (*t*-BHP)<sup>31</sup> remain alive for months and display several features of replicative senescence. These features include senescent morphology,<sup>24,31</sup> SA- $\beta$ gal activity,<sup>31,38</sup> common mitochondrial deletion<sup>31</sup> and altered gene and protein expression.<sup>39,40</sup>

In order to study the long-term effect of UV on skin cell types, several in vitro models were developed. Indeed, if multiple studies dealt with the effects of UV very few investigate the long-term effects of UV. Here we present different models that were developed in order to study long-term effects of subcytotoxic doses of UV. Several models were set up varying on the cell type (fibroblasts or keratinocytes, normal or immortalized) and the type of UV used (UVA or UVB).

### UV-Stress Induced Premature Senescence in Fibroblasts

Fibroblasts constitute the classical cell type for studies on aging. Historically, it was the first strain on which replicative senescence was detected<sup>21</sup> and constitutes since the cell type of reference for studies of aging.<sup>22</sup> In the skin, fibroblasts constitute the main cell type of the dermis and are responsible of the production of the different extracellular matrix (ECM) components.<sup>41</sup>

Models of premature senescence were developed by exposing dermal HDFs to sublethal doses of UVB.<sup>42,43</sup> After ten repeated exposures to sublethal dose of UVB, HDFs display biomarkers of senescence as typical senescent morphology, SA- $\beta$ gal activity, altered gene expression and the “common” mitochondrial DNA deletion.<sup>42,43</sup> As the UV dose used must not induce mortality or apoptosis, caspase-3 activity and PARP cleavage were checked as negative in these conditions. The decreased proliferative potential observed after these UVB stresses are correlated with overexpression of p53, p21<sup>WAF-1</sup> and p16<sup>INK-4A</sup>, involved in the growth arrest in replicative senescence. Concerning gene expression change observed after these repeated UVB exposures, the relative steady-state mRNA level of c-jun, c-fos, MMP-1 and MMP-2 was found to be increased. C-fos and c-jun are known to be components of the c-jun:c-fos AP-1 transcription factor. A transient increase of extracellular release of H<sub>2</sub>O<sub>2</sub> was detected after repeated UVB exposures.<sup>44</sup> This model has been accepted as an in vitro simplified model of dermal aging. It was used to identify potential protective effect of marine algal components.<sup>45</sup> Moreover, it was shown that telomerase activity did not prevent premature senescence induced by UVB<sup>46</sup> as telomerase-immortalized human foreskin fibroblasts (hTERT-BJ1) developed biomarkers of senescence after UVB exposures.

At the mechanistic level, the pathways inducing senescence are still unknown. In SIPS, the role of Transforming Growth

Factor- $\beta$ 1 (TGF- $\beta$ 1) in the appearance of some of the biomarkers of senescence has been described. TGF- $\beta$ 1 is a multifunctional cytokine involved in many cellular functions like cellular division, differentiation and connective tissue synthesis.<sup>47</sup> TGF- $\beta$ 1 is secreted in a latent form (LTGF- $\beta$ ), which consists of TGF- $\beta$ 1 noncovalently associated with its N-terminal propeptide called latency associated peptide (LAP) (for a review see ref. 48). The abundance of TGF- $\beta$ 1 mRNA is increased in premature senescence induced by H<sub>2</sub>O<sub>2</sub>,<sup>38</sup> *t*-BHP,<sup>49</sup> ethanol<sup>49</sup> and UVB.<sup>43</sup> By using neutralizing antibodies, it was shown that TGF- $\beta$ 1 is responsible for the appearance of several biomarkers of senescence induced by these stresses.<sup>38,43,49</sup> In premature senescence induced by H<sub>2</sub>O<sub>2</sub>, it was shown that H<sub>2</sub>O<sub>2</sub> induces a first phase of activation of p38<sup>MAPK</sup> (ref. 50). This activation triggers an overexpression of TGF- $\beta$ 1, which starts a positive feedback loop allowing sustained activation of p38<sup>MAPK</sup>. P38<sup>MAPK</sup> phosphorylates and activates the transcription factor ATF-2 that interacts with hypophosphorylated pRb. This complex induces the appearance of features of replicative senescence. This regulatory loop probably also triggers UVB-induced premature senescence with concomitant activation of p53. Indeed, UVB-induced premature senescence is associated with a transient increase of p53 protein abundance and DNA-binding activity. Silencing p53 expression with small interfering RNA (siRNA) affected the basal level of SA- $\beta$ gal and proliferative potential, as the expression of genes differentially expressed after repeated exposures to UVB.<sup>51</sup>

Concerning long-term effects of UVA exposures on HDFs, Herrmann et al. showed that treatment of HDFs with 8-methoxypsoralen and subsequent UVA irradiation resulted in a long-term growth arrest, alterations in cell morphology (post mitotic phenotypes) and increased expression of SA- $\beta$ gal.<sup>52</sup> Combined treatment of psoralen and UVA, also known as pUVA therapy, is widely used in the treatment of different skin disorders as psoriasis. Psoralens act as photosensitizers via the generation of ROS.<sup>53</sup> The long-term growth arrest of pUVA-treated HDFs is associated to increased protein levels of p53, p21<sup>WAF-1</sup> and p16<sup>INK-4A</sup> (ref. 54) and change of expression of genes involved in growth arrest, stress response, modification of the extracellular matrix and senescence.<sup>55</sup> However, pUVA-induced growth arrest, senescent morphology, SA- $\beta$ gal increased activity and MMP-1 overexpression are fully reversible at days 100 to 130 post pUVA treatment. This suggests that pUVA-induced changes do not fully reflect replicative senescence in HDFs but rather represent a long-term transient phenocopy of senescence<sup>56</sup> and was therefore presented as a SIPS model.<sup>57</sup>

For UVA irradiation alone, Berneburg et al. showed that the common mitochondrial deletion was detectable in HDFs exposed to 36 repeated sublethal doses of UVA radiation.<sup>58</sup> This deletion was found to be mediated by singlet oxygen.

### UVB-Stress Induced Premature Senescence or Alternative Differentiation in Keratinocytes

Normal human epidermal keratinocytes (NHEK) constitute the main cell type of the epidermis and are the first line of skin's defense against environmental stresses. They proliferate

in the basal layer before moving upwards to the suprabasal layers through a complex differentiation program that culminates in fully differentiated dead cells in the cornified superficial layer, maintaining a strong impermeable barrier.<sup>59</sup> If it has been detected that keratinocytes show characteristics of replicative senescence *in vivo*<sup>26</sup> very little was devoted to study the aging of keratinocytes *in vitro*. Moreover, very few are known on the long-term effect of subcytotoxic UV exposures on NHEKs. NHEKs progressively show proliferation arrest and reach a senescence plateau after about 15–25 population doublings (according to the donor) in culture.<sup>60</sup> This plateau lasted only a few days to 2–3 weeks and is followed by a massive detachment of almost all cells. Some remaining cells with partially transformed and tumorigenic traits will then spontaneously emerge from senescent cultures, linked to the accumulation of ROS during senescence.<sup>61</sup> Senescent keratinocytes display morphological changes as increase of cytoplasm size and of perinuclear organelle contents.<sup>62</sup> They also display increased SA- $\beta$ gal activity<sup>26,60</sup> and p16<sup>INK-4A</sup> expression.<sup>62</sup>

Lewis et al.<sup>63</sup> demonstrated that low doses of UVB irradiation induce cellular senescence in NHEKs with increased SA- $\beta$ gal activity and increased p21<sup>WAF-1</sup> and p53 protein abundance. Activation of IGF-1R promotes this UVB-premature senescence through increased generation of ROS and by maintaining the expression of p21<sup>WAF-1</sup>.<sup>63</sup>

Telomerase expression alone is not sufficient to immortalize human keratinocytes. Indeed, normal human oral keratinocytes (NHOK) expressing telomerase enter in replicative senescence after several population doublings in culture.<sup>64,65</sup> To be immortalized, keratinocytes should express telomerase and lack p16<sup>INK-4A</sup>. As described earlier, p16<sup>INK-4A</sup> is a strong biomarker of skin aging.<sup>34</sup> Immortalized keratinocytes lacking p16<sup>INK-4A</sup> and expressing telomerase retain other growth controls and keep the ability to differentiate in reconstructed epidermis *in vitro*.<sup>66</sup> Repeated exposures to UVB of immortalized keratinocytes induce an alternative state of differentiation rather than stress-induced premature senescence.<sup>67</sup> This alternative differentiation state is characterized namely by an increased abundance of involucrin, a late marker of differentiation, and cytokeratins (K) K6, K16 and K17, phosphorylation of p38<sup>MAPK</sup> and HSP27, and elevated secretion of active MMP-9, as observed in primary keratinocytes and *in vivo* in the epidermis.<sup>67</sup> Expressions of proteins involved in keratinocyte differentiation and survival were shown to be changed after UVB exposures. Among them, TRIM 29 (TRIPartite Motif Protein 29), a survival factor, is dependent on PKC $\delta$  signaling pathway.<sup>68</sup> These results suggest that p16<sup>INK-4A</sup> is essential for keratinocytes to enter into senescence.

### Conclusions

In conclusion, several models were developed in which human fibroblasts or keratinocytes, exposed to subcytotoxic doses of UV rays, show characteristics of cellular senescence. These models can be used to better understand the relationship between UV stress and aging. Since the skin is a complex organ, consisting of different compartments with connections between them,

it would be interesting to develop in the future more complex models allowing to take into account interactions between the different cell types of the skin. In addition, aging of the skin is modulated by multiple internal (genetic, hormonal) and external (oxidative stress, UV, pollution) factors, which makes it complex to study. However, this complexity makes it an interesting model to use the skin as a picture of the overall aging of the individual.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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