In Vivo and *Ex Vivo* UV-Induced Analysis of Pigmentation Gene Expressions

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TO THE EDITOR

Skin exposure to solar radiation initiates complex molecular processes. These include the protective tanning response, local inflammation, immune suppression, and DNA damage that can lead to skin carcinogenesis (De Fabo *et al.*, 2004). The tanning response protects against UV-mediated DNA damage, with UV irradiation triggering production of melanin by melanocytes, which is then transferred to the neighboring keratinocytes. To investigate the pigmentation process, numerous *in vitro* models have been developed, including cultured cell lines, melanocyte–keratinocyte co-culture, reconstructed epidermis, and skin biopsies (Duval *et al.*, 2001, 2003; Scott *et al.*, 2002; Corre *et al.*, 2004; Hofmann-Wellenhof *et al.*, 2004). However, gene expression levels involved in the UV response have been determined using cell tissue culture models (Chakraborty *et al.*, 1996; Gilchrest *et al.*, 1996; Nishioka *et al.*, 1999; Galibert *et al.*, 2001; Corre *et al.*, 2004). In order to examine UV-induced gene expression with respect to cell organization and cooperation, we investigated the *in vivo* UV response, using irradiated skin biopsies and irradiated cultured skin explants. For this purpose, six women (mean age 48 years) were included in a photobiological protocol, following ethical approval. We classified patients as phototype II (two patients) and III (four patients), following a detailed interview about skin response to sunlight (Fitzpatrick, 1988). Solar-simulated radiation was generated by a UV polychromatic light source (Dermolum UM-W1, Müller Elektronik®, Moosinning, Germany), equipped with a Schott WG 305 filter, resulting in 5% UVB- and 95% UVA-containing spectrum. The simulator irradiance was 100 mW/cm^2 (Müller Elektronik® dosimeter). UV irradiation was delivered at 2 and 4 J/cm^2 on the abdomen 5 hours before plastic surgery to allow significant UV-induced gene transcription. The value of 2 J/cm^2 was chosen as it corresponds to the minimal erythemal dose of our population in Brittany

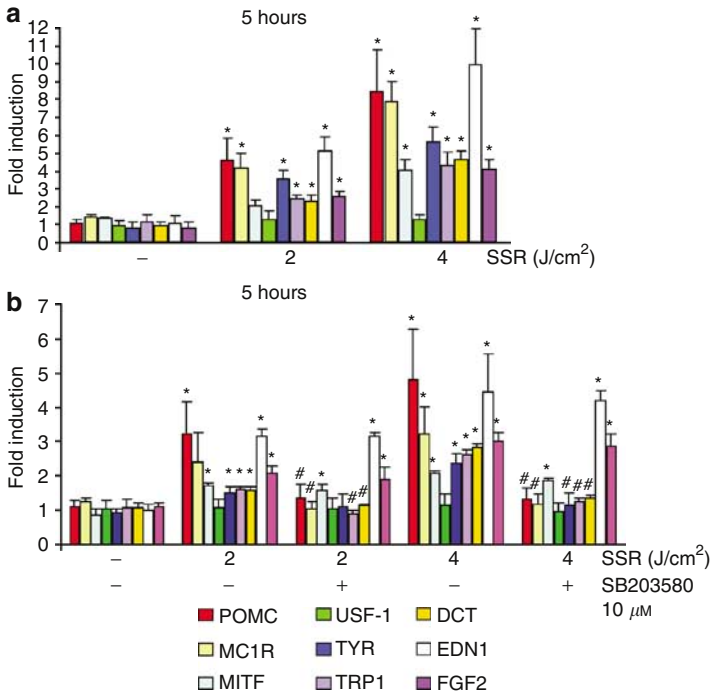

Figure 1. *In vivo* and *ex vivo* UV-induced gene expression analysis. UV-induced gene expression analyses were performed by real-time reverse transcription-PCR using the SYBER Green technology (Applied Biosystems) and specific forward and reverse primers. *POMC, MC1R, MITF, USF-1, TYR, TRP1, DCT, EDN1,* and *FGF2* relative transcript levels following UV stimulation (2–4 J/cm^2) are expressed as a fold increase compared to nontreated control skin biopsies using the C_t method (Livak and Schmittgen, 2001). Each PCR experiment was carried out at least twice and at each time point in duplicate. The data obtained from six healthy phototype II and III volunteers are presented as mean ± SEM, and are considered significant (*) if $P<0.05$, using the two-sample Wilcoxon's test (S-PLUS 6, Insightful™). (**a**) *In vivo* UV-induced gene expression analysis: six patients, referred for abdominal plastic surgery, were irradiated at 2 and 4 J/cm^2 using a UV polychromatic light source (solar-simulated radiation (SSR): 2–4 J/cm^2, Dermolum UM-W1, Müller Elektronik®, composed of a 1,000 W xenon lamp and a 1,000 W metal halide lamp) on the abdomen 5 hours before surgery. Skin biopsies of irradiated and non-irradiated areas were taken immediately after plastic surgery and processed for RNA extraction (Nucleospin® RNA II extraction kit, Macherey-Nagel) and quantitative real-time PCR. (**b**) *Ex vivo* UV-induced gene expression analysis: immediately after abdominal surgery, skin explants (0.8 cm diameter) of non-UV-irradiated skin were taken for tissue culture. After 24 hours of culture, skin explants were irradiated at 2 and 4 J/cm^2 (SSR: Dermolum UM-W1, Muller Elektronik®). When indicated, skin explants were pretreated either with p38-specific family kinase inhibitor (SB203580, 10 μM final concentration) or with a control solution (DMSO) before UV irradiation. Five hours after UV induction, the skin explants were recovered for RNA extraction and pigmentation gene expression analysis.

Abbreviation: MAPK, mitogen-activated protein kinase

(France), composed essentially of phototype II and III skins. Cultured skin explants were prepared from non-UV-irradiated skin biopsies, immediately after surgery. After removal of the hypodermis, epidermal/dermal skin explants (diameter: 0.8 cm; thickness: 5 mm) were placed, dermal side down, in 24-well culture plates. The dermis of skin explants was thus in contact with the medium (Biopredic International®, Rennes, France), whereas the epidermis was facing the atmosphere (5% CO_2, 37°C).

Keratinocytes and melanocytes, most critical pigmentation genes (Imokawa, 2004; Vance and Goding, 2004), include the *pro-opiomelanocortine (POMC)* and its receptor *(MC1R),* the *basic-fibroblast growth factor (FGF2)* and the *endothelin (EDN1)* paracrine factors genes, the *microphtalmia*-encoded transcription factor gene *(MITF),* and genes implicated in pigment manufacture *(TYR, TRP1, DCT).* Their relative gene expressions were analyzed using real-time reverse transcription-PCR (SYBR™ Green PCR Master Mix, Applied Biosystems, Foster City, CA) (Corre *et al.*, 2004), the most accurate technology for mRNA quantification (Livak and Schmittgen, 2001). Solar-simulated radiation (2 and 4 J/cm^2) delivered *in vivo* and *ex vivo* to phototype II and III skins caused a significant dose-dependent upregulation (Figure 1), in agreement with the previously reported *in vitro* tissue culture data (Galibert *et al.*, 2001; Corre *et al.*, 2004) and with *in vivo* fold increases being about two times higher than the *ex vivo* fold increases. Although phototype II and III are considered as poor and mild tanners, respectively, no significant difference could be observed between these skin phototypes, neither in basal pigmentation gene expression level nor in the UV-induced gene expression response, 5 hours post-irradiation, allowing us to present gene expression results as the mean of data from all six biopsies (Figure 1). However, patients did not respond equally in terms of local inflammation, and one phototype II patient presented a slight erythema on the 4 J/cm^2 irradiated area 5 hours after irradiation. Although one cannot draw any conclusion from one volunteer, these data are in accordance with type II and III specific UV response skins, leading in the end to distinct tan. Indeed, tanning is a complex growing process, which is not limited to gene expression regulation and includes modulation of protein activities (MC1R, tyrosinase, etc.). Tanning is thus time and dose dependent, requiring secondary stimuli that include additional UV hits and paracrine factors, justifying the need for an adequate and flexible protocol represented by our *ex vivo* approach. Indeed, incubation of cultured skin explants with α-melanocyte-stimulating hormone, FGF2, and endothelin activates signaling pathways (Halaban *et al.*, 1988; Imokawa, 2004), leading to a dose-dependant increase of *POMC, MC1R, MITF*, and *TYR* gene expressions (data not shown) mediating finally, specific skin responses.

Preincubation of the specific p38 kinase inhibitor with skin explants confirmed that the UV-induced expression of *POMC, MC1R,* and *TYR* was dependent on the p38 pathway (Galibert *et al.*, 2001; Corre *et al.*, 2004) and showed that *TRP1* and *DCT* had comparable gene expression modulation, in accordance with the presence of E-box regulatory elements within their promoter (Figure 1b). Indeed, the p38 stress-activated upstream stimulating factor-1 (USF-1) and critical E-box motifs have been shown to be key elements of UV-induced gene expression. In contrast, UV-induced FGF2 and EDN1 gene expressions proved to be independent of the p38 and mitogen-activated protein kinase (MAPK) pathways (Figure 1b and data not shown). However, both p38 and MAPK-specific inhibitors slightly reduced UV-induced *MITF* expression (Figure 1b and data

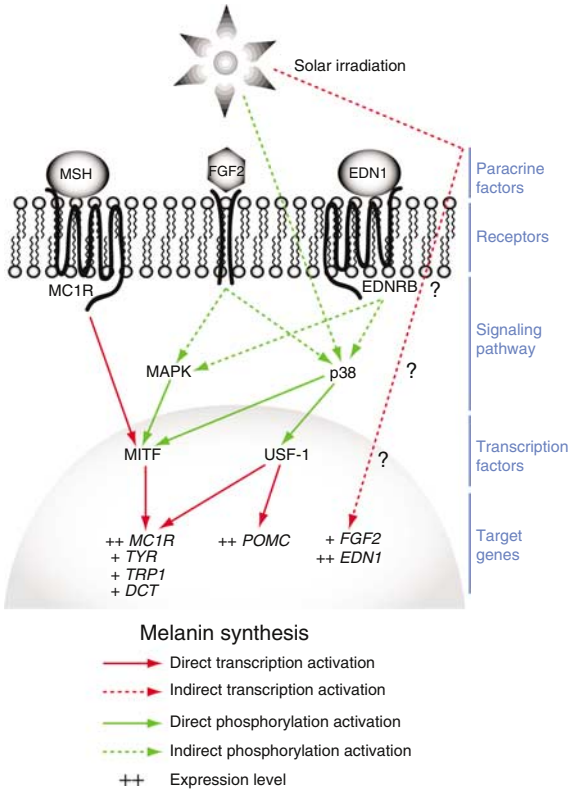

Figure 2. UV-induced pigmentation gene regulation process. Following UV exposure, the stress-specific p38 kinase pathway is activated, targeting direct phosphorylation of the USF-1 transcription factor. Phosphorylated USF-1 form activates gene expression of E-box-containing promoters, inducing direct upregulation of distinct pigmentation genes (*POMC, MC1R, TYR, TRP1, DCT*) following UV irradiation. *EDN1* and *FGF2* UV-induced gene expression mechanism is independent from the p38 and MAPK pathways, remaining unknown. Crossregulation involving paracrine factors (α-melanocyte-stimulating hormone, FGF2, EDN1) and signaling pathways (p38 and MAPK) leads to late induction of *MITF* expression, inducing complete tanning response.

not shown). Regulation of *MITF* occurs downstream of POMC, FGF2, and EDN1 signaling. Also, there are no E-box regulatory elements present within the *MITF* core promoter. Therefore, a slight decrease of *MITF* expression in the presence of either p38 or MAPK inhibitors is likely owing to indirect and complementary processes (Figure 2). In addition, our results highlight that UV activation of the USF-1 transcription factor is dependant only on the p38 kinase, leading to the appearance of a phosphorylated form of USF-1 (Galibert *et al.*, 2001), as no modification of the *USF-1* gene expression could be observed with UV or paracrine stimulations (Figure 1 and data not shown), indicating that post-translational modifications and active signaling pathways are crucial to the pigmentation regulation process (Figure 2).

In conclusion, gene expression analysis of the pigmentation process revealed that cultured skin explants lead to robust results, with reproducible levels of gene expression (SD < 1,2). Indeed, the preservation of cell interactions, with no alteration of spatial structure skin, and the short culture time guarantee accurate results in reproducing the *in vivo* response. Gene expression analysis, using *in vivo* and *ex vivo* data, thus allows us to define more precisely the molecular pathways induced in response to UV (Figure 2). In addition, cultured skin explants are an adapted approach to studying specific skin pigmentation disorders, inflammation, local immune response (Brink *et al.*, 2000; Beattie *et al.*, 2005), and DNA damage (Snellman *et al.*, 2003) following UV irradiation as well as pharmacological compounds implicated in skin molecular processes.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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