Development of an in vitro immunosuppression model

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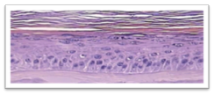
INTRODUCTION

Ultraviolet Radiation (UVR), particularly UVB (280–320 nm), initiates inflammation, local and systemic immunosuppression and skin cancer. Substantial evidence, primarily from animal models and succesively on human volunteers (Young et al. 1996), suggests that UVR-induced cytokines are key mediators of these interrelated effects. Immunosuppressive effects of excessive ultraviolet radiation in humans include exacerbation of infectious diseases, skin cancer and skin aging. Relatively low doses of UVR causes an early local immunosuppression mainly mediated by a functional inhibition of Langerhans cells and a delayed systemic immunosuppression in which pro-inflammatory soluble factors as cytokines play a crucial role (Schwarz T et al. 2005). **TNFα** and IL-1α are recognized as responsible for the onset of the inflammation and the induction of chemotaxis of the neutrophils and macrophages into the skin after UVR and in particular **TNFα** is an important mediator in local UVB-induced immunosuppression on human skin. In a kinetics study on human skin (suction blisters) Barr et al. have demonstrated that at epidermal level, UV induces the release of the immunosuppressive cytokine **IL-10,** responsible for a shift from Th-1 to Th-2 immunoresponse involving the TNF-α. **IL-12** promotes Th-1 response establishing a protective mechanism against UV induced immunosuppression. **IL-10** is a cytokine that has a profound impact on skin immunity and tumour immunosurveillance: invasive basal cell carcinomas have been shown to secrete high levels of this cytokine (Kim, J. et al.1995) and IL-10 levels in melanoma are directly correlated with a bad prognosis (Kruger-Krasagakes, S. 1994). In particular IL-10 possesses immunosuppressive activity and inhibits antigen presentation in in vitro and in vivo systems (Fiorentino DF et al. 1991). In UVB-irradiated skin, IL-10 is primarily secreted by keratinocytes and activated macrophages, thereby IL-10 mediates down-regulating contact hypersensitivity (CHS) delayed responses (Howard M. et al. 1992). **IL-12** regulates the growth and functions of T cells and especially augments the development of Th1-type cells by stimulating the production of IFN-γ. It has been shown that intraperitoneal injection of recombinant IL-12 in mice prevents UV-induced immune suppression. These studies imply that a cytokine's imbalance between Th1 and Th2 may be responsible for the development of UVB-induced immunosuppression (Trinchieri, G 1997). IL-12 immuno-potentiating role seems also justified by its activity in inducing DNA repair. Since UV-induced DNA damage is an important molecular trigger for UV-mediated immunosuppression it has been proposed that IL-12 could act as a protective molecule (Schwarz, A. 2005).

METHODS

The **EPISKIN Model™** (EPISKIN-LM™) is manufactured by SkinEthic, Lyon (F).
It is a reconstructed organotypic culture of human adult keratinocytes reproducing a multilayered and differentiated human epidermis. Cells are grown on a collagen matrix for 13 days using the air-lift technique.

The Episkin Phototoxicity Assay, **EPA** has demostrated the relevance of the Episkin 3D model in photobiology investigation.
It has been able to discriminate between phototoxic and non phototoxic chemicals in compairison with the 3T3 Balb-NRU Phototoxicity assay (OECD TG 432) (Lelievre et al. 2007) and to identify photo-protective formulations in a modified protocol (Meloni et al. 2011).



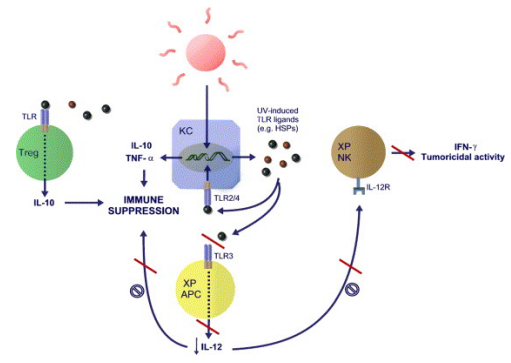
Episkin® Model

TRANSCRIPTIONAL STUDY: mRNA by qRT-PCR
Expression levels of the selected biomarkers were monitored by mRNA quantification using qRT-PCR (Taqman® assay) in a thermal cycler (Applied Biosystems ABI FAST 7500 Real Time PCR System) with GAPDH, as an internally validated house-keeping gene. Calibrator sample was the not irradiated control at each time point (control =1).

UV SOURCE: **1 kW Oriel solar simulator** equipped with Spectra-Physics Lamp (Xenon 1000W, WG 320 Shott 1,3 mm, emitting UVA+UVB. Flux uniformity 10x10 cm). Irradiance: UVA (69,9 W/m²) and UVB (5,4 W/m²). 1 MED corresponding to 0,025 J/cm².
Episkin tissues (triplicate) were placed in a 12-well plate with 0,75 mL of PBS. Non-irradiated tissues were used as control. After irradiation the PBS was replaced by Episkin culture medium.

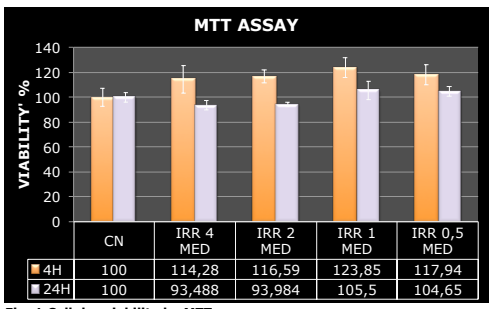
UV DOSES and POST IRRADIATION TIMES SELECTION
Literature reports (Young, AR 1996) that 2-3 MED on humans are sufficient to determine an immunosuppressive response and that a specific kinetics corresponds to the post irradiation periods: between 14-16h the maximum of cytokines was released, describing an immunosuppressive response and after 24h a prevalent DNA damage was established.

Therefore tissues were irradiated with UVR doses correspondant to 0.5, 1, and 2 and 4 MED (0,0125 to 0,1 J/cm²). After irradiation, skin tissues were let in the incubator for 4, 6, 16 and 24h post irradiation periods. Cell viability (MTT test) and adenilate kinase release (Toxilight®-Roche Diagnostics) have been monitored in parallel.



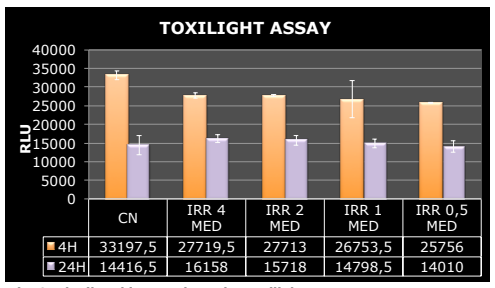
Photoimmunosuppression. UV radiation can up-regulate endogenous Toll-like receptor *(TLR)* ligands. By ligating TLR, these stress signals activate the production of immunosuppressive IL-10 and TNF-α. The immunosuppressive cytokine milieu is further supported by the production of IL-10 by regulatory T cells *(Treg)*. Defective Toll-like receptor 3 signaling may be linked to the vulnerability of XP (xeroderma pigmentosum) patients to UV-induced skin cancers. As stress signals cannot properly activate Toll-IL-1 receptor pathways to produce IL-12 (an important antagonist of IL-10), the effects of immunosuppressive cytokines would predominate (adapted from Kang S. et al. 2006).

RESULTS



	CN	IRR 4 MED	IRR 2 MED	IRR 1 MED	IRR 0,5 MED
4H	100	114,28	116,59	123,85	117,94
24H	100	93,488	93,984	105,5	104,65

Fig. 1 Cellular viability by MTT assay



	CN	IRR 4 MED	IRR 2 MED	IRR 1 MED	IRR 0,5 MED
4H	33197,5	27719,5	27713	26753,5	25756
24H	14416,5	16158	15718	14798,5	14010

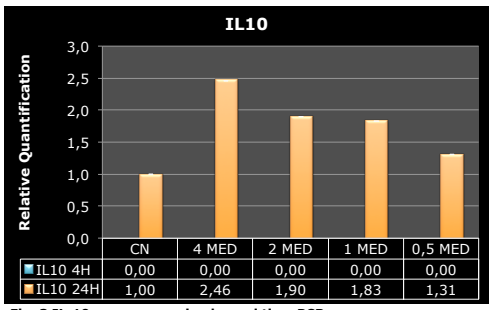
Fig. 2 Adenilate kinase release by Toxilight assay

UV DOSES SCREENING: CELL VIABILITY

All the doses tested (ranging from 0,5 to 4 MED) have induced no significant modification of the viability at 4h and 24h (minimum and maximum post irradiation time defined in the study) (Fig. 1). According to previous data (Meloni et al 2003) not toxic effect at mitochondrial level has been associated to increasing UVR doses and on the contrary a metabolic activation should be installed and responsible for the observed increased viability values. The adenilate kinase release compared to the non irradiated control response and absolute values have confirmed the MTT results (Fig. 2).

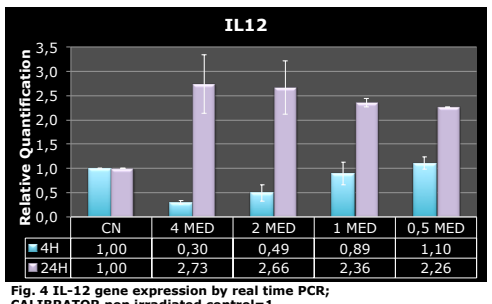
UV DOSES SCREENING: IL-10 and IL-12 GENES EXPRESSION

The first trial (Fig. 3-4) was conducted to monitor the Episkin response in term of IL-10 and IL-12 molecular modifications at the UV doses selected for the citotoxicity assay: fixed post irradiation times of 4h and 24h were first tested.
The IL-10 gene transcriptional activity was modulated by the UVR in the delayed read out, at 24h after UV exposure: 4h after irradiation the gene was not at all modulated. A significant up regulation was quantified at the dose of 4 MED and near to the significancy value of RQ=2 at 2 MED and 1 MED underlying a immunosuppressive Th- 1 response (Fig. 3). These results confirm literature data (Barr et al. 1999) on human volunteers where a 2 fold increase of IL-10 release was quantified between 16h and 24h post irradiation (3 MED). The IL-12 gene was not expressed at early recovery time indicating a null contribution and thus interference of the gene in the immuno-protective response at this time (Fig. 4). On the contrary IL-12 has been significantly up regulated independently from the UV dose applied at 24h post irradiation confirming literature data of an involvement of IL-12 in the immunoprotecting response and suggesting at this time the establishment of self-protective response of the living tissue to reach an homoeostasis balance (Fig. 4).



	CN	4 MED	2 MED	1 MED	0,5 MED
IL10 4H	0,00	0,00	0,00	0,00	0,00
IL10 24H	1,00	2,46	1,90	1,83	1,31

Fig. 3 IL-10 gene expression by real time PCR; CALIBRATOR non irradiated control=1



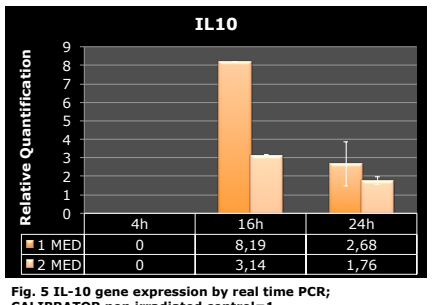
	CN	4 MED	2 MED	1 MED	0,5 MED
4H	1,00	0,30	0,49	0,89	1,10
24H	1,00	2,73	2,66	2,36	2,26

Fig. 4 IL-12 gene expression by real time PCR; CALIBRATOR non irradiated control=1

Based on these results the doses of 1MED and 2 MED have been applied in a second trial (Fig. 5-6-7-8) in order to assess the kinetics of non toxic and biologically relevant UV dose inducing the expression of the relevant biomarkers of the immunosuppressive mechanism. In order to take into account the dynamic of the immunosuppresive imbalance mediated by both cytokines the post irradiation times of 4h, 16h (overnight) and 24h were selected to establish an experimental window with a prevalent Th-1 response and a minimal or null IL-12 expression.

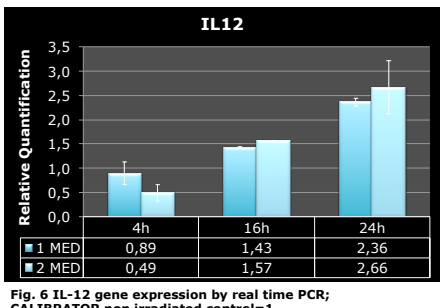
KINETICS OF THE IMMUNOSUPPRESSIVE RESPONSE

The already observed kinetics of IL-10 gene at 4h has been confirmed: a null contribution to the immunosuppressive pathway at this time (Fig. 5). IL-10 gene was significantly over-expressed at the end of 16h post irradiation period suggesting a strong immunosuppressive pathway in particular after 1 MED (8 fold increase) and lower after 2MED (3 fold): it seems interesting to observe that low UVR doses are more effective in inducing IL-10 mediated immunosuppression. In the longer post incubation period, 24h results, the immunosuppressive response was reduced and partially lost (Fig. 5). The results obtained with IL-12 gene are consistent with the study rational: IL-12 was not modulated in the early recovery confirming first trial results and it was significantly modulated in the delayed recovery period at 24h indicating a physiological defensive and protective response exclusively at this time (Fig. 6). On the contrary in the 16h time point the IL-12 transcriptional activity was still not significant indicating that at this time the Th-1 response was definetely installed and prevalent (Fig. 6). A time-dependent increase in TNFα gene expression has been observed: this is an important finding since TNFα is essential for the formation of sunburn cells, inflammatory response and mobilization of Langerhans cells. The TNF-α gene was upregulated at the 16h timepoint confirming this post irradiation period as the most interesting in terms of inflammatory and molecular modification underlying the immunosuppressive TH-1 response (Fig. 7).
24h after irradiation the full recovery of IL-10 and IL-12 balance described in the literature has been observed in our experimental conditions. It is well known that DNA damage plays a major role in UVB-induced immune suppression. The 24h results (overexpression of IL-12 and reduced expression of IL-10 and TNF-α compared to 16h time) suggest a DNA damage determing a physiological tissue response in terms of immunoprotective pathway consistent with the IL-12 role. In order to confirme this hypothesis the expression level of OGG1 gene (that encodes the enzyme responsible for the excision of 8-oxoguanine, a mutagenic base byproduct which occurs as a result of exposure to reactive oxygen) has been monitored as reported in fig. 8: after 2 MED the biomarker was not modulated neither after 4h nor 16h and it was finally significantly overexpressed at 24h thus confirming that a damage at DNA level was present involving the OGG1 and IL-12 response in restauring tissue's homeoastasis and counteracting the immunosuppressive Th-1 pathway.



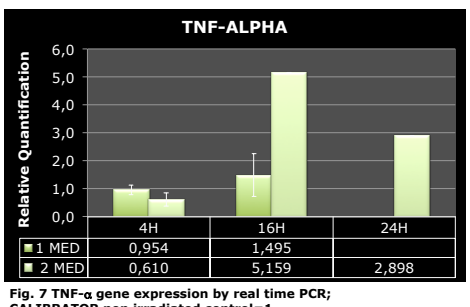
	4h	16h	24h
1 MED	0	8,19	2,68
2 MED	0	3,14	1,76

Fig. 5 IL-10 gene expression by real time PCR; CALIBRATOR non irradiated control=1



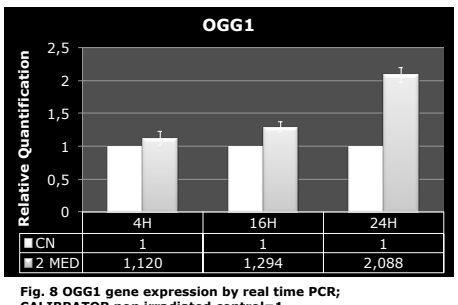
	4h	16h	24h
1 MED	0,89	1,43	2,36
2 MED	0,49	1,57	2,66

Fig. 6 IL-12 gene expression by real time PCR; CALIBRATOR non irradiated control=1



	4H	16H	24H
1 MED	0,954	1,495	
2 MED	0,610	5,159	2,898

Fig. 7 TNF-α gene expression by real time PCR; CALIBRATOR non irradiated control=1



	4H	16H	24H
CN	1	1	1
2 MED	1,120	1,294	2,088

Fig. 8 OGG1 gene expression by real time PCR; CALIBRATOR non irradiated control=1

DISCUSSION AND CONCLUSION

The biological relevance of Episkin epidermis model has been investigated and its predictive capacity for photobiology has been demonstrated confirming literature data on humans. The kinetics of the immunosuppressive pathway has been monitored confirming in vivo literature human data either for the UV doses and for post irradiation periods. It has been possible to define for the in vitro immunosuppression model where the TNF-α gene expression levels were consistent with a pro-inflammatory mechanism, the IL-10 was up regulated and IL-12 expression was not modified.
The response of Episkin epidermis model to the dose of 1-2 MED at 16h post irradiation describes a dynamic response where a pure immunosoppressive Th-1 response (IL-10 and TNF-a over-expression and not regulation of IL-12) is observed in a transient but reproducible experimental window. Then at 24h the physiological tissue defence mechanism to inflammation and oxidative stress has been higlighted: the damage at DNA has been demostrated and quantified by the OGG1 and IL-12 genes exression levels. The first hypothesis to understand the early tissue response (4h results) is based on the following data: according to previous finding (Meloni et al. 2003) on epidermis in vitro model, an impairment of Gluthatione redox status have been quantified at 4h –max 6h after UV radiation suggesting at this time the prevalent establishment of an oxidative stress status without involvment of skin immuno-system
Glutathione plays a pivotal role in the biological antioxidant systems and it is involved not only in the "first line " defence systems but also in repairing and recycling. The glutathione redox status model (relative amount of reduced (GSH) an oxidized form (GSSG), offers a suitable approach and describe the cellular redox status: GSH depletion has been observed immediately after irradiation and remained between 1 and 5 hours after irradiation. In parallel the enzymatic balance investigated at molecular level and quantified as mRNA expression of GPX1 and GSR (glutathione peroxidase and glutathione reductase) was modified in irradiated control compared to non irradiated tissues confirming the oxidative damage induced by the UV. (internal data not shown). It is possible to conclude that this final optimized protocol corresponds to a new in vitro model that seems useful and valid in determining the immunoprotective capacity of ingredients and formulations claiming a protective mechanism against UV induced immunosuppression.

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