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ACADEMIC DISSERTATION
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PASTILA Riikka. STUK-A216. Effect of long-wave UV radiation on mouse melanoma: An in vitro and in vivo study. Helsinki 2006. 125 pp + appendices 55 pp.

Keywords Ultraviolet A radiation, melanoma, cell adhesion, metastasis, immunosuppression, gene expression

Abstract

The skin cancer incidence has increased substantially over the past decades and the role of ultraviolet (UV) radiation in the etiology of skin cancer is well established. Ultraviolet B radiation (280–320 nm) is commonly considered as the more harmful part of the UV-spectrum due to its DNA-damaging potential and well-known carcinogenic effects. Ultraviolet A radiation (320–400 nm) is still regarded as a relatively low health hazard. However, UVA radiation is the predominant component in sunlight, constituting more than 90% of the environmentally relevant solar ultraviolet radiation. In the light of the recent scientific evidence, UVA has been shown to have genotoxic and immunologic effects, and it has been proposed that UVA plays a significant role in the development of skin cancer. Due to the popularity of skin tanning lamps, which emit high intensity UVA radiation and because of the prolonged sun tanning periods with the help of effective UVB blockers, the potential deleterious effects of UVA has emerged as a source of concern for public health.

The possibility that UV radiation may affect melanoma metastasis has not been addressed before. UVA radiation can modulate various cellular processes, some of which might affect the metastatic potential of melanoma cells. The aim of the present study was to investigate the possible role of UVA irradiation on the metastatic capacity of mouse melanoma both *in vitro* and *in vivo*. The *in vitro* part of the study dealt with the enhancement of the intercellular interactions occurring either between tumor cells or between tumor cells and endothelial cells after UVA irradiation. The use of the mouse melanoma/endothelium *in vitro* model showed that a single-dose of UVA to melanoma cells causes an increase in melanoma cell adhesiveness to non-irradiated endothelium after 24-h irradiation. Multiple-dose irradiation of melanoma cells already increased adhesion at a 1-h time-point, which suggests the possible cumulative effect of multiple doses of UVA irradiation. This enhancement of adhesiveness might lead to an increase in binding tumor cells to the endothelial lining of vasculature in various internal organs if occurring also *in vivo*. A further novel observation is that UVA induced both decline in the expression of E-cadherin adhesion molecule and increase in the expression of the N-cadherin adhesion molecule. In addition, a significant

decline in homotypic melanoma-melanoma adhesion (clustering) was observed, which might result in the reduction of E-cadherin expression. It appears that UVA irradiation might reduce melanoma-melanoma interaction through decreasing the expression of E-cadherin and simultaneously enhance the adhesiveness of melanoma cells to endothelium, which in part could be mediated by N-cadherin expression.

The aim of the *in vivo* animal study was to confirm the physiological significance of previously obtained *in vitro* results and to determine whether UVA radiation might increase melanoma metastasis *in vivo*. The use of C57BL/6 mice and syngeneic melanoma cell lines B16-F1 and B16-F10 showed that mice, which were *i.v.* injected with B16-F1 melanoma cells and thereafter exposed to UVA developed significantly more lung metastases when compared with the non-UVA-exposed group. To study the mechanism behind this phenomenon, the direct effect of UVA-induced lung colonization capacity was examined by the *in vitro* exposure of B16-F1 cells. Alternatively, the UVA-induced immunosuppression, which might be involved in increased melanoma metastasis, was measured by standard contact hypersensitivity assay (CHS). It appears that the UVA-induced increase of metastasis *in vivo* might be caused by a combination of UVA-induced systemic immunosuppression, and to the lesser extent, it might be caused by the increased adhesiveness of UVA irradiated melanoma cells.

Finally, the UVA effect on gene expression in mouse melanoma was determined by a cDNA array, which revealed UVA-induced changes in the 9 differentially expressed genes that are involved in angiogenesis, cell cycle, stress-response, and cell motility. These results suggest that observed genes might be involved in cellular response to UVA and a physiologically relevant UVA dose have previously unknown cellular implications.

The novel results presented in this thesis offer evidence that UVA exposure might increase the metastatic potential of the melanoma cells present in blood circulation. Considering the well-known UVA-induced deleterious effects on cellular level, this study further supports the notion that UVA radiation might have more potential impact on health than previously suggested. The possibility of the pro-metastatic effects of UVA exposure might not be of very high significance for daily exposures. However, UVA effects might gain physiological significance following extensive sunbathing or solaria tanning periods. Whether similar UVA-induced pro-metastatic effects occur in people sunbathing or using solaria remains to be determined. In the light of the results presented in this thesis, the avoidance of solaria use could be well justified.

PASTILA Riikka. STUK-A216. Pitkäaaltoisen UV-säteilyn vaikutus hiiren melanoomaan in vitro ja in vivo. Helsinki 2006. 125 s + liitteet 55 s (vain sidotussa versiossa)

Avainsanat Ultravioletti A-säteily, melanooma, solujen välinen adheesio, metastasia, immunosuppressio, geenien ilmentyminen

Tiivistelmä

Ihosyöpien ilmaantuvuus on lisääntynyt voimakkaasti viimeisten vuosikymmenten aikana ja ultraviolettisäteilyn (UV-säteilyn) vaikutus ihosyöpien ilmaantuvuuteen on kiistaton. Ultravioletti B-säteily (280–320 nm) on tunnetusti haitallista DNA:ta vaurioittavien ja karsinogeenisten vaikutustensa vuoksi. Ultravioletti A-säteilyn (320–400 nm) terveysvaikutuksia on puolestaan pidetty pitkään harmittomampana kuin UVB-säteilyn vaikutuksia. Maanpinnalle saapuvasta auringonvalosta kuitenkin yli 90 % on UVA-säteilyä. Viimeaikaisten tutkimustulosten perusteella UVA-säteilyllä on kuitenkin havaittu olevan perimää vaurioittavia ja immunologisia vaikutuksia ja lisäksi sillä saattaa olla myös merkittävä rooli ihosyöpien kehittymisessä. UVA-säteilyaltistuksen aiheuttamat epäsuotuisat terveysvaikutukset ovat nousseet huolenaiheeksi ihmisten ruskettaessa itseään solariumeissa, jotka säteilevät voimakasta UVA-säteilyä, tai ottaessa pitkiä aikoja aurinkoa tehokkaiden UVB-aurinkosuojien turvin, jotka suojelevat ihoa tehokkaasti UVB-säteilyn aiheuttamalta palamiselta suojaten vähemmän UVA- kuin UVB-säteilyltä.

UV-säteilyn vaikutuksia melanooman kykyyn lähettää etäpesäkkeitä ei ole tutkittu aikaisemmin. UVA-säteilyn on kuitenkin havaittu aiheuttavan soluissa monia sellaisia fysiologisia muutoksia, jotka saattavat vaikuttaa solujen kykyyn lähettää etäpesäkkeitä (metastasoida). Tämän väitöstutkimuksen tarkoituksena oli tarkastella UVA-säteilyn vaikutusta hiiren melanoomasolujen metastaattisiin ominaisuuksiin *in vitro* ja *in vivo*. *In vitro* -osiossa tutkittiin UVA-säteilyn vaikutusta melanoomasolujen ja endoteelisolujen väliseen sitoutumiseen sekä melanoomasolujen keskinäiseen sitoutumiseen. Yksittäisen UVA-säteilyannoksen havaittiin lisäävän melanoomasolujen adhesiivisuutta säteilyttämättömiin endoteelisoluihin 24 tuntia UVA-säteilytyksen jälkeen. Kun melanoomasolut altistettiin jakamalla säteilyannos useisiin pienempiin osiin, UVA-säteilyn adhesiivisuutta lisäävä vaikutus havaittiin jo 1 tunnin kuluttua. Tämä viittaa UVA-säteilyn mahdollisiin kumulatiivisiin vaikutuksiin sekä siihen, että UVA-säteily saattaa lisätä melanoomasolun adhesiivisuutta *in vivo* ja tällöin sen kiinnittyminen verisuonen endoteeliin kohde-elimessä saattaa lisääntyä. Uusi löytö oli myös UVA-säteilyn vaikutus kadheriini-adhessiomolekyylien ilmenemiseen

melanoomasolun pinnalla siten, että E-kadheriinin ilmeneminen laski mutta N-kadheriinin ilmeneminen nousi UVA-säteilyn vaikutuksesta. Samanaikaisesti havaittiin melanoomasolujen keskinäisessä sitoutumisessa vähenemistä UVA-säteilyn vaikutuksesta. UVA-säteily saattaa siis vähentää melanoomasolujen keskinäistä sitoutumista E-kadheriinin vähenemisen kautta, ja samalla lisätä melanoomasolun adheesiota endoteeliin lisäämällä N-kadheriinin ilmenemistä melanoomasolujen pinnalla.

Saatuja tuloksia tarkasteltiin myös *in vivo* -hiirimallissa, jossa tutkittiin, ovatko *in vitro*-havainnot melanoomasolujen adhesiivisuudessa fysiologisesti merkittäviä ja sitä, lisääkö UVA-säteily melanooman etäpesäkkeiden muodostumista C57BL/6-hiirissä. C57BL/6-hiiriin injektoitiin *i.v.* häntälaskimosta melanoomasoluja, jonka jälkeen hiiret altistettiin *in vivo* välittömästi UVA-säteilylle. UVA-annoksen havaittiin lisäävän keuhkometastaasien määrää UVA-käsitellyissä hiirissä verrattuna kontrolliryhmään, jotka eivät olleet saaneet säteilyä. Melanoomasoluja UVA-altistettiin myös *in vitro* ennen hiireen injektoimista tutkittaessa UVA-säteilyn suoraa vaikutusta melanoomasolujen metastaattiseen kapasiteettiin. Vaihtoehtoisesti mitattiin UVA-säteilyn aiheuttama immunosuppressio, joka saattaa myös vaikuttaa melanooman etäpesäkkeiden muodostamiskykyyn käyttämällä paikallista yliherkkyysreaktio-menetelmää (contact hypersensitivity, CHS-menetelmä). Tutkimusten mukaan UVA-säteilyn kyky lisätä hiiren melanoomasolujen metastaattista aktiivisuutta saattaa johtua sekä UVA-säteilyn suorista vaikutuksista melanoomasoluihin ja myös sen aiheuttamasta immunosuppressiosta.

Tutkimuksen viimeisessä osiossa tarkasteltiin UVA-säteilyn vaikutusta hiiren melanoomasolujen geenieksпressioon eli geenien ilmentymiseen cDNA-menetelmällä. UVA-säteilyn havaittiin muuttavan yhdeksän geenin ilmenemisprofiilia melanoomasoluissa säteilytyksen jälkeen. Havaittujen geenien tiedetään osallistuvan soluissa verisuonten uudismuodostukseen, solusykliin, stressivasteeseen ja solun liikkuvuuteen. Näiden tulosten perusteella havaittujen geenien voidaan arvella olevan osa UVA-säteilyn aiheuttamaa soluvastetta ja sen lisäksi osa UVA-säteilyn aiemmin tuntemattomia vaikutuksia soluissa.

Tässä väitöstutkimuksessa on osoitettu ensimmäistä kertaa, että UVA-säteily saattaa lisätä verenkierrossa olevien melanoomasolujen metastaattista kapasiteettia. Nämä tulokset tukevat aiempien tutkimustulosten näkemystä siitä, että UVA-säteilyllä saattaa olla enemmän terveysvaikutuksia kuin aiemmin on osattu epäillä. Jokapäiväisessä elämässä UVA-säteilyn vaikutukset ovat pieniä, mutta ne saattavat olla merkityksellisiä solariumia käytettäessä tai otettaessa pitkään aurinkoa, jolloin altistuminen UVA-säteilylle on voimakasta. UVA-säteilyn vaikutukset ihmisen melanooman kykyyn lähettää etäpesäkkeitä jää vielä selvitettäväksi. Näiden tutkimustulosten perusteella solariumin käytön välttäminen voi kuitenkin olla aiheellista.

Contents

Original publications

I Pastila R, Leszczynski, D (2005): Ultraviolet A exposure might increase metastasis of mouse melanoma: A pilot study. *Photodermatol Photoimmunol Photomed,* 21: 183-190.

II Pastila R, Leszczynski D (2005): Ultraviolet A Exposure Alters Adhesive Properties of Mouse Melanoma Cells. *Photodermatol Photoimmunol Photomed,* 21: 234-241.

III Pastila R, Ylianttila L, and Leszczynski D (2005): UVA-induced immunosuppression might play a role in UVA-induced melanoma metastasis in mice. *Revised version resubmitted.*

IV Pastila R, Leszczynski D (2005): UVA induces changes in cyclin G gene expression in mouse melanoma B16-F1 cells. *Submitted.*

List of abbreviations

AA	Arachidonic acid
AK	Actinic keratosis
AKT/PKB	AKR thymoma/Protein kinase B
ALCAM	Activated leukocyte cell adhesion molecule
AP-	Activating protein-
APC	Antigen presenting cell
α-MSH	α-melanocytic stimulating hormone
BCC	Basal cell carcinoma
bFGF	Basic fibroblast growth factor
CIE	Commission Internationale de l'Eclairage
CHO	Chinese hamster ovary cell line
CHS	Contact hypersensitivity
CDK	Cyclin dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CO	Carbon oxide
COX	Cyclo-oxygenase
CPD	Cyclobutane pyrimidine dimer
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
DAG	Diacyl glyserol
DNL	Draining lymph node
DT	Delayed tanning
ECM	Extracellular matrix
EGF	Epidermal growth factor
ET-1	Endothelin-1
HGF/SF	Hepatocyte growth factor/Scatter factor
HO-1	Hemeoxygenase-1
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
IL-	Interleukin-
IPD	Immediate pigment darkening
IPF	Immunoprotection factor
LC	Langerhans cell
nm	nanometer
NMSC	Non-melanoma skin cancer
NO	Nitric oxide
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin 1 receptor

MCP-1	Monocyte chemotactic protein-1
MED	Minimal erythemal dose
MGSA	Melanocyte growth stimulating antigen
MHCII	Major Histocompatibility Complex II
MM	Malignant melanoma
MMP	Matrixmetalloproteinase
8-OHdG	8-hydroxy-deoxyguanosine
PBS	Phosphate buffered saline
PG	Prostaglandin
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PPD	Persistent pigment darkening
RGP	Radial growth phase
ROS	Reactive oxygen species
SBC	Sunburn cell
SCC	Squamous cell carcinoma
SK	Solar keratosis
SPF	Sun protection factor
SED	Standard erythemal dose
TNF-α	Tumor necrosis factor-α
UCA	Urocanic acid
UVA	Ultraviolet A
UVB	Ultraviolet B
UVR	Ultraviolet radiation
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VGP	Vertical growth phase
XP	Xeroderma pigmentosum

Introduction

Ultraviolet radiation is the major environmental risk factor for non-melanoma and melanoma skin cancer formation due to its DNA-damaging potential and mutagenic and mitogenic actions. In addition, ultraviolet radiation-derived immunosuppression has been identified as a risk factor for skin cancer induction. The terrestrial spectrum of solar UV radiation consists, depending on latitude and season of the year, of 1–5% ultraviolet B radiation (280–320 nm), whereas the majority of the radiation reaching the Earth's surface belongs in the ultraviolet A (320–400 nm) region. Moreover, when reaching the skin, UVB is almost completely absorbed by the stratum corneum and top layers of the epidermis, whereas up to 50% of incident UVA radiation penetrates Caucasian skin deep into the dermis, and irradiates the full thickness of the skin. The carcinogenic potential of UVB is well known and understood but the role of ultraviolet-A radiation in photocarcinogenesis is obscure. The role of UVA as a risk factor has been elucidated over the past 10–15 years. It is known that UVA exerts its biological effect mainly by producing reactive oxygen species (ROS) that promote biological damage in exposed tissue. UVA radiation has been shown to participate in the pathogenesis of non-melanoma skin cancer in mouse models and in human skin carcinoma samples. The role of UVA in the development of melanoma has been elucidated mainly through animal studies, and some of these have indicated a positive association between the onset of melanoma and UVA exposure.

The most threatening characteristic of malignant melanoma is its extremely high potential to develop metastasis. Although most individuals with primary cutaneous melanoma can be cured surgically, the prognosis of patients with metastatic disease remains poor. Thus, there is a need to gain a better understanding of the mechanisms involved in the progression of melanoma to metastatic disease.

UVA radiation can modulate various cellular processes, some of which might affect tumor development. Some UVA-induced cellular effects, such as the upregulated expression of matrix degrading enzymes and certain interleukins and adhesion molecules, may enhance the metastatic potential of cancer cells. Thus, UVA is capable of inducing such biochemical responses in the skin and the microvasculature that could enhance tumor metastasis. However, the effect of UVA on melanoma metastasis has been not studied widely thus far. The possibility of the pro-metastatic effects of UVA/solar UV might not be of relevance for daily low exposure, but they may gain significance during sunbathing or tanning on sun-beds, when people are exposed to large doses of predominantly UVA radiation. So far, this possibility has not been investigated. If proven, it

would suggest that exposure to UVA radiation during sun and solarium tanning might have negative health effects.

This thesis places special emphasis on investigating the hypothesis that UVA radiation may enhance the metastatic potential of melanoma cells in the C57BL/6 mouse model. The overall objective of the work is to characterize the role of UVA as a possible metastatic enhancer in the *in vitro* and *in vivo* approaches. In order to achieve these aims, the focus in this work was

- to examine the UVA induced alterations in melanoma adhesiveness *in vitro*
- to determine the physiological significance of UVA exposure on melanoma metastasis *in vivo*
- to study the possible effect of UVA on melanoma gene expression

1. Review of the literature

1.1 Ultraviolet radiation

1.1.1 Definition and sources of ultraviolet radiation

UVR is located in the electromagnetic spectrum between the ionizing x-rays and the non-ionizing visible light (Figure 1). It spans a wavelength of 100–400 nanometers (nm), being non-ionizing and non-visible. Since the biological effects of UVR vary greatly with wavelength, it has further been divided into the three subclasses: shortwave ultraviolet-C (UVC), mid-wave ultraviolet-B (UVB) and long-wave ultraviolet-A (UVA) radiation. The wavelength definitions of the subclasses are arbitrary and differ depending on the field of interest (Diffey 2002). In the area of photobiology and photomedicine, the spectral distribution is often defined as follows: UVC ranges from 100/200 nm to 280/290 nm, UVB ranges from 280/290 nm to 315/320 nm and UVA ranges from 315/320 to 400 nm. Since the shorter wavelengths are photobiologically more active, wavelengths from 320 to 340 are defined as the UVAII region and 340–400 nm as the UVAI region (Urbach 1992).

The intensity of terrestrial UVR is attenuated largely in the atmosphere, where ozone, clouds, and pollutants scatter and absorb ultraviolet rays and thus modify the quality (spectrum) and quantity (intensity) of solar UVR (Diffey 2002). The spectrum and intensity of UVR vary largely with latitude and solar altitude, which depends on time of day, season and geographical location. The atmospheric ozone in the stratosphere absorbs all UVC and most UVB radiation. However, the ozone layer does not significantly attenuate UVA wavelengths and it has very little influence on the ambient UVA level: absorption by the ozone layer decreases to essentially zero in the region from 350–400 nm (Frederick & Alberts 1992). Thus, the terrestrial spectrum of ultraviolet radiation consists, depending on latitude and season of the year, of 1-5% UVB radiation and 95–99% of UVA radiation.

Sunlight is the most prominent source of ultraviolet radiation (UVR) for humans. However, several artificial UV sources (UV lamps) have been developed for use in tanning devices, for germicidal purposes and for the therapeutic use of UV radiation (phototherapy). Depending on the lamp type and filters used, UV sources can provide very different UV spectra from the broadband solar-simulated radiation spectrum to specific narrow-band applications.

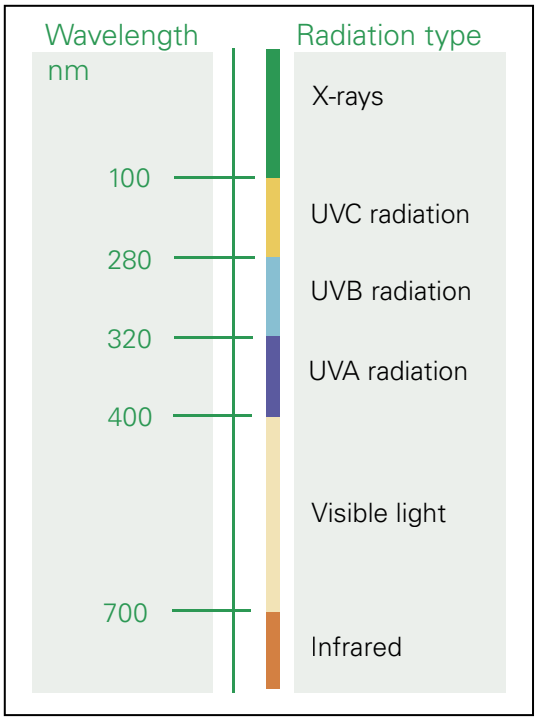

Figure 1. Ultraviolet radiation is located in the electromagnetic spectrum between the ionizing x-rays and the non-ionizing visible light, spanning in the wavelength region of 100–400 nanometers. Ultraviolet radiation has been divided into the three subclasses: shortwave ultraviolet-C (UVC), mid-wave ultraviolet-B (UVB) and long-wave ultraviolet-A (UVA) radiation.

1.1.2 Human exposure to UVA radiation

Human exposure to UVR, including UVA radiation, is on the rise not only because of the environmental factors, but also due to human activity and behavior. The human population is increasingly exposing itself to UVR due to changes in lifestyle, such as a decrease in body clothing and an increase in leisure time (Kricker *et al.* 1993). Diffey has estimated that one of the major contributors for the annual accumulation of UVA exposure is seasonal variation in behavior (Diffey 1998a). Autier *et al.* have shown that recreational exposure, sunbathing and the number of holiday weeks spent annually in sunny resorts was a significant risk factor for the development of melanoma (Autier *et al.* 1994b). The annual cumulative UVA dose from the sun has been estimated to be 7700 kJ/m^2, and for more avid tanners, it is as much as 19 250 kJ/m^2 (Wang *et al.* 2001). Recently, a lot of attention has been drawn to the fact that sunscreens, while efficiently

absorbing in the UVB range, are generally poor UVA absorbers (Wang *et al.* 2001). Through using effective UVB blocks during tanning, sunbathers protect themselves from developing painful erythema, which is mainly caused by UVB. However, this may lead to longer tanning periods and as a consequence, larger UVA doses up to as much as 100 J/cm^2 per day (Kimlin *et al.* 2002). Extensive tanning in solaria leads to increased UVA exposure. In the 1980s, growing scientific evidence for the genotoxic, immunotoxic and carcinogenic effects of UVB radiation encouraged solaria manufacturers to change the physical spectral properties of UV device towards the UVA region. As a result, solaria devices that may emit up to 5–10 times more UVA when compared to natural solar radiation came onto the market.

UVA and UVB radiation have different biological effects on skin. UVB radiation is more potent and effective in causing biological damage; photon energy grows along with the shorter wavelength. UVB wavelengths are estimated to contribute 80% of the harmful effects of exposure to the sun and UVA is estimated to contribute the remaining 20% (Diffey 1998b). The depth of penetration into human skin is also different for UVA and UVB (Figure 2). Predominantly, UVB radiation directly affects the epidermis, and only 10–20% of it penetrates to the dermis. However, UVA penetrates deeper when compared to UVB because of its longer wavelength, and ~50% of incident UVA penetrates into the papillary and reticular dermis (Parrish 1983;Bruls *et al.* 1984). This difference in penetration depth is likely to affect the biological effects induced by UVA and UVB. UVR has been shown to induce DNA damage in circulating blood cells in skin capillaries (Moller *et al.* 2002) and to cause the necrosis and loss of dermal endothelial cells in capillary venules, thus damaging dermal blood vessels (Margolis *et al.* 1989;Clydesdale *et al.* 2001). Finally, the stem cells in the basal layer of skin divide and are less differentiated and thus, they are more prone to the malignant changes induced by the deep penetration of UVA (Matsui & DeLeo 1991).

1.1.3 The visible effects of UV on human skin

Human skin types have been divided into the six categories based on the facultative skin color that reflects genetically determined sensitivity to sunburn and the ability to tan (Fitzpatrick 1988). UVR has both acute and chronic effects on human skin. The acute signs of UV exposure are pigmentation (tanning), erythema (sunburn) and the synthesis of vitamin D, which is one of the rare beneficial health effects of UVB radiation on the skin. Vitamin D is required for the proper absorption of calcium from the intestines, thus regulating calcium homeostasis and maintaining skeletal health. Chronic exposure to UVR causes premature skin aging and increases the risk of skin cancer. Pigmentation and

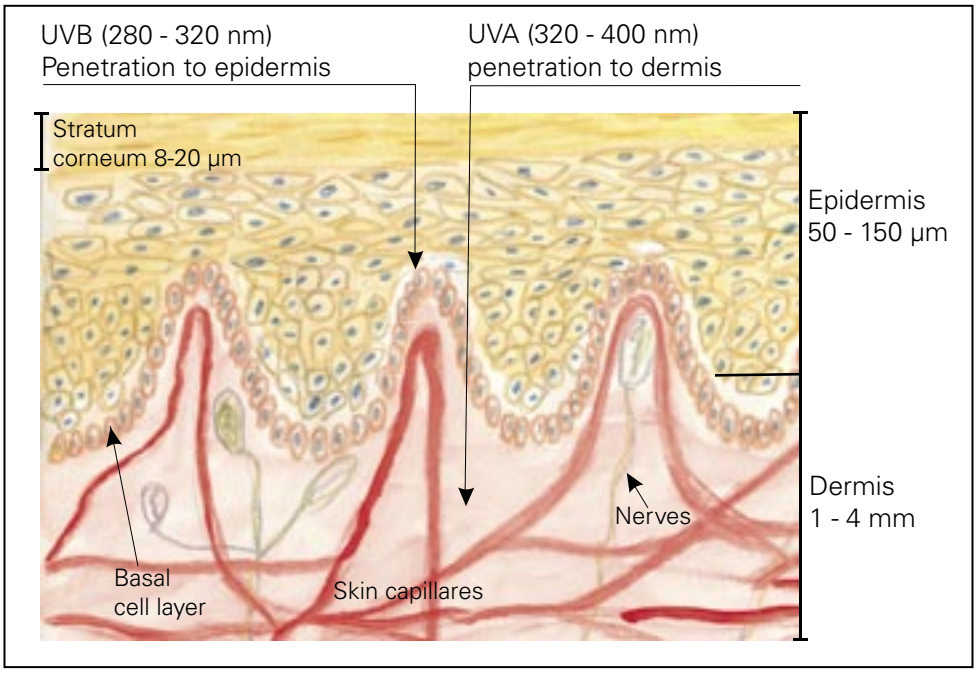

Figure 2. The penetration depth of UVB and UVA in skin. UVB radiation affects mainly in epidermis. UVA penetrates deeper as compared to UVB, because of its longer wavelength, and ~50% of incident UVA penetrates into the papillary and reticular dermis.

the production of vitamin D, though for different reasons, are viewed as desirable consequences of UV radiation. However, erythema and photoaging are widely recognized as the adverse effects of exposure, mainly because of their close association with malignant skin transformations such as actinic/solar keratosis (AK/SK) and, ultimately, skin cancer.

1.1.3.1 UV-induced pigmentation and hyperkeratosis

Melanin is the major pigment in the skin and it is a natural human photoprotector. Melanin is produced and stored by the neural-crest-derived melanocytes that are aligned along the basement membrane. Each melanocyte reaches with it dendrites into the upper layer of the epidermis and transports melanosome granules filled with melanin to approximately 36 keratinocytes (Jimbow 1995; Seiberg 2001). Melanin accumulates inside the cells as a nuclear cap and protects cells from the damaging effects of UVR by absorbing and scattering UV rays and by scavenging the UV-induced oxygen radicals. Two kinds of melanin exist in the epidermis: brown eumelanin and red pheomelanin. Eumelanin is believed

to be photoprotective, acting as a free radical scavenger, whereas pheomelanin can act as a photosensitizer, being potentially phototoxic due to the generation reactive oxyfgen species that might contribute to oxidative DNA damage (Krol & Liebler 1998; De Leeuw *et al.* 2001; Kadekaro *et al.* 2003).

Skin pigmentation response is biphasic following UV exposure. It comprises immediate tanning induced mainly by UVA radiation and the delayed formation of new pigment induced primarily by UVB radiation (Parrish *et al.* 1982). Immediate pigment darkening (IPD) is a transient change in skin color due to the oxidation of pre-existing melanin and melanin precursors (Honigsmann *et al.* 1986; Gilchrest *et al.* 1996). IPD is induced by the whole UVA spectrum up to visible light at 470 nm (Rosen *et al.* 1990; Irwin *et al.* 1993), with a threshold dose of 1-2 J/cm^2 (Honigsmann 2002). Interestingly, IPD has neither been found to be protective against UVB-induced erythema (Black *et al.* 1985) nor UVB induced DNA lesions (Honigsmann *et al.* 1986;Cesarini 1992). However, it may provide additional protection against further UVA-induced cutaneous injury, mainly the infiltration of mononuclear cells into skin (Margolis *et al.* 1989). With higher UVA doses (>20 J/cm^2) the change in skin color may persist for several days (Kollias *et al.* 1991). This type of pigmentation is called persistent pigment darkening (PPD). PPD is used to determine the UVA protection factor in sunscreens (Moyal *et al.* 2000a; Moyal *et al.* 2000b).

UVR-induced delayed tanning (DT) – melanin synthesis (melanogenesis) – is stimulated mainly by UVB radiation and to a lesser extend by UVA exposure (Agar & Young 2005). The production of new melanin is a photochemically initiated, tyrosinase-controlled production of melanin polymers from pre-existing monomers inside the melanocytes (Bech-Thomsen 1997;Honigsmann 2002). Other skin cells assist the melanogenesis by secreting paracrine factors such as the α-melanocytic stimulating hormone (α-MSH) and endothelin-1 (ET-1). These, in turn, stimulate melanin synthesis and melanocyte migration, enhance their dendricity and survival rate during subsequent UV exposure (Jimbow 1995; Agar & Young 2005). The newly synthesized pigment appears 3–5 days after exposure, peaking after one week (Kollias *et al.* 1991). UVAI produces an increased melanin density mostly on the basal cell layer, whereas the UVAII region and UVB increase the synthesis and transfer of melanosomes to the upper epidermis (Honigsmann 2002).

There is growing evidence that activation of the tyrosinase enzyme may be initiated by UV-induced DNA damage (Eller *et al.* 1996;Gilchrest *et al.* 1996), which suggests that erythema and melanogenesis may have the same chromophore, DNA (Young *et al.* 1998a). This is further supported by the notion that the action spectrum for delayed pigmentation and for erythema formation have been found to be similar in fair skin types I and II (Parrish *et al.* 1982; Gange

1986). The ability to tan has been considered photoprotective, since darker skin types are at a lower risk of sunburn formation and apparently of DNA damage. However, melanin is not an effective sunscreen in Caucasian skin, offering a sun protection factor of 1.5–3. Moreover, it has been shown that photoprotection against erythema and DNA damage is independent of induced tan in fair skin types, offering quite poor photoprotection (Sheehan *et al.* 1998). It has also been observed that skin type IV has faster DNA repair capacity after UV exposure than skin type II does (Sheehan *et al.* 2002). These results postulate the notion that skin pigmentation ability, i.e. skin type, is a measure of inducible DNA repair capacity (Agar & Young 2005) more than a marker of protection through the formation of pigment.

Skin hyperplasia occurs as a result of an increased proliferation of keratinocytes in the lower epidermis and from the increased thickness of the stratum corneum. The skin thickening increases the optical path length and thus leads to an increased protection of the underlying tissues against UV exposure by a sun protection factor of 5 or higher (Diffey 1998b), regardless of melanogenesis (Parrish 1983). This adaptive process does not involve genetic predisposition and it might be the major protective factor for poorly tanning individuals (Diffey 2004).

1.1.3.2 Erythema (sunburn)

Erythema, an acute skin injury following excessive UV exposure, is the most obvious and prevalent effect of UVR on human skin. Individual sensitivity, which varies greatly from one subject to another, is assessed by the minimal erythema dose (MED), which is defined as the minimal dose required in order to elicit an observable reddening (erythema) on previously unexposed skin. However, MED is subjective and does not represent a defined physical measure of irradiation. For this reason the standard erythema dose (SED) was proposed as an equivalent to an erythemal effective radiation of 100 J/m^2 (Diffey *et al.* 1997).

According to a reference erythema action spectrum by Commission Internationale de l'Eclairage (CIE) (McKinlay & Diffey 1987), erythemal effectiveness declines rapidly with the increasing UV wavelength. UVB radiation is approximately 1000 times more efficient in causing sunburn when compared to UVA radiation (Matsui & DeLeo 1991). UVA has been estimated to contribute approximately 14% of the erythemal UVR in sunlight (Parisi & Wong 2000). Following UVB exposure, there is a latent period of 2–4 hours before the UVB-induced erythema develops. The maximum intensity of erythema is reached within 8–24 hours after UVB exposure (Honigsmann 2002). The time course for UVA-induced erythema is biphasic, consisting of immediate and delayed erythema formation. Immediate erythema already develops during irradiation

and thereafter decreases within 4 hours. Delayed erythema develops 6–24 hours after UVA exposure (Gilchrest *et al.* 1983; Diffey *et al.* 1987; Ortel & Gange 1992).

The chromophores that initiate sunburn inflammation have not been precisely identified, but a comparison of erythema action spectra and epidermal DNA photodamage showed that DNA is the major chromophore for erythema formation (Young *et al.* 1998a). There are also some other chromophores in the UVAII region, the nature of which is yet unidentified, mediating the DNA damage via oxygen species (Anders *et al.* 1995). This suggests that a principal event in erythema formation would be the direct damage of DNA by UVB and UVAII wavelengths (Parrish *et al.* 1982). DNA photodamage has been shown to be the major determinant of sunburn cell (SBC) formation in the epidermis. It is one of the characteristic events in the epidermis following acute UVB exposure or after UVA irradiation in the presence of psoralens, at doses of around or above 1 MED (Murphy *et al.* 2001). SBCs have been identified as keratinocytes undergoing apoptosis in a process controlled by both p53-dependent and p53-independent manners (Murphy *et al.* 2001; Sheehan & Young 2002). SBCs can be distinguished on the basis of their distinctive appearance, i.e. pyknotic nuclei and a shrunken and eosinophilic cytoplasm. SBC formation is believed to be a protective mechanism that eliminates damaged cells that are at risk of developing malignant transformations.

The initial UV-induced inflammatory response is the vasodilatation of skin capillaries that leads to augmented blood flow and increased leukocyte infiltrates in the skin (Terui & Tagami 2000; Clydesdale *et al.* 2001; Rhodes *et al.* 2001). In UVB-induced erythema, vasoactive prostaglandins (PGs) and nitric oxide (NO) mediate erythema formation by diffusing from the epidermis to the dermal skin capillaries (Clydesdale *et al.* 2001). It is supposed that UVA induces erythemal response by affecting either directly the skin capillaries or via diffusible mediators (Kelfkens *et al.* 1990). Many cytokines, such as interleukins (IL) IL-1, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), are synthesized and released within the skin by keratinocytes and leukocyte infiltrates (Terui & Tagami 2000; Honigsmann 2002). They regulate the expression of adhesion molecules in keratinocytes and in vascular endothelium and thus, they are involved in recruiting of the polymorphonuclear leukocytes that play a pivotal role in the UV(B)-induced erythema.

1.1.3.3 Skin photoaging

Photodamage of the skin is the result of chronic exposure to UV radiation. Photoaging predisposes to the formation for solar keratosis, which is considered a precursor of squamous cell carcinoma (SCC). The clinical symptoms of photoaged

skin are dryness, roughness, deep wrinkles, irregular pigmentation, loss of elasticity (elastosis) and telangiectasia (chronic dilatation of blood vessels). These appear in areas exposed to the sun, such as the face, neck and the upper extremities. Histopathological alterations in photodamaged skin have been found in papillary dermis: the degradation and disorganization of type collagen I, an increase of the collagen III to collagen I ratio, an accumulation of abnormal elastin, as well as an increase of glycosaminoglycans and telangiectatic vessels (Seite *et al.* 1998). The histological hallmark of photoaging, called solar elastosis, is the massive accumulation of atypical elastotic material in the upper and middle dermis. It has been suggested that elastosis results from the effects of UVR on cytokine expression (Kondo 2000) and on mast cells (Gonzalez *et al.* 1999) that both stimulate elastin production from fibroblasts. UVR also accelerates the secretion of matrix metalloproteinase (MMP), which leads to the degeneration of the collagenous meshwork into the elastotic masses (Koivukangas *et al.* 1994; Fisher *et al.* 1997). UV-induced cytokines and growth factors promote the growth of keratinocyte, resulting in hyperplasia and epidermal thickening (Kondo 2000).

Although UVB radiation is mainly responsible for sunburn, UVA rather than UVB is considered a major factor in the process of skin photoaging due to the increased penetration of UVA into the dermis. Chronic UVA exposure has been shown to cause epidermal hyperplasia, collagen changes, stratum corneum thickening, Langerhans cell depletion and dermal inflammatory infiltrates (Kligman & Gebre 1991; Lavker *et al.* 1995a; Lavker *et al.* 1995b). UVA is known to induce a mitochondrial mutation called 'common deletion', which is present in high amounts in the dermis of photoaged skin (Krutmann 2000). In addition, UVA induces matrix metalloproteinases (Wlaschek *et al.* 1995; Wlaschek *et al.* 1997), leading to the formation of wrinkles.

1.1.4 UV-induced molecular changes

1.1.4.1 Pyrimidine dimer formation

UV has the capacity to initiate skin carcinogenesis through DNA damage. DNA absorbs UVR maximally at wavelengths of 245 to 290 (Matsumura & Ananthaswamy 2004). This induces covalent DNA lesions that occur most frequently in tandem pyrimidine bases (Tornaletti & Pfeifer 1996). Cyclobutane pyrimidine dimers (CPD) are formed between thymine (T) and cytosine (C) residues, whereas pyrimidine (6–4) pyrimidone photoproducts are formed among adjacent pyrimidine residues. CPDs are produced three times as often as (6–4) photoproducts (Pfeifer *et al.* 2005). Methylated CPDs rather than 6–4

photoproducts or other lesions, are considered responsible for the majority of mutations induced by UVB (You *et al.* 1999; You *et al.* 2001). The yield of (6–4) photoproducts is several folds lower than the yield of CPDs, and they are repaired more efficiently than CPDs are (Ichihashi *et al.* 2003).

At longer UV wavelengths, the yield of pyrimidine photoproducts decreases since DNA absorbs weakly wavelengths above 320 nm. However, UVA has been demonstrated to have the capability of causing pyrimidine dimers in cell cultures (Rochette *et al.* 2003;Douki *et al.* 2003;Courdavault *et al.* 2004) and in human skin (Freeman *et al.* 1987; Burren *et al.* 1998; Young *et al.* 1998b), as summarized in Table 1.

1.1.4.2 Oxidative DNA damage

Despite the ability of UVA to induce pyrimidine dimers to some extent, CPD formation is not the characteristic hallmark for UVA wavelengths (Berg *et al.* 1995). The genotoxicity of UVA occurs mainly through an indirect photosensitization process via the generation of reactive oxygen species (ROS) that are capable of inducing oxidative DNA damage (Cadet *et al.* 2005; Pfeifer *et al.* 2005). UVA-induced singlet oxygen ($^1O_2$) and hydrogen peroxide are generated through the photoactivation and excitation of endogenous chromophores such as riboflavin, phorphyrins, quinones and pheomelanin (Klotz *et al.* 2001; Marrot *et al.* 2005). The reactive oxidative compounds have been shown to target DNA base guanine, producing 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) within a strand of DNA (Kvam & Tyrrell 1997). Kvam and Tyrrell have demonstrated that 8-OHdG is induced in human skin fibroblasts by monochromatic radiation ranging from a UVB wavelength of 312 nm up to wavelengths in the near visible light (434 nm) (Kvam & Tyrrell 1997). UVA wavelengths above 334 nm were responsible for almost all of guanine oxidation and the authors concluded that especially UVA, rather than any other UV wavelength, contributed to the oxidative DNA damage observed in non-melanoma and melanoma skin cancers. UVA-induced oxidative DNA damage has also been shown by Zhang *et al.*, who have found that the relative yield of 8-OHdG to pyrimidine dimers increases nearly 1000-fold in UVA-irradiated cells when compared with cells subjected to either UVC or UVB exposure (Zhang *et al.* 1997). In addition to the *in vitro* models, UVAI and II have been shown to induce high levels the 8-OHdG in human skin) (Table 1).

UVR also causes other oxidative DNA damage, such as protein-DNA cross-links and single-strand breaks (Peak *et al.* 1991; Peak & Peak 1991). Interestingly, DNA strand breaks and DNA-protein cross-links are more characteristic for UVA radiation than for the UVB (Matsui & DeLeo 1991; Tyrrell 1996). Finally, both UVA and UVB have been found to induce chromosomal instability through oxidative damage (Phillipson *et al.* 2002; Dahle & Kvam 2003).

Table 1. UVA-induced genotoxic markers.

Examined DNA damage	Cells	Reference
Cyclobutane dimers	CHO cells, human fibroblasts and keratinocytes, human skin	Freeman et al 1987, Burren et al 1998, Young et al 1998a, Douki et al 2003, Rochette et al 2003, Courdavault et al 2004
8-OHdG	Human fibroblasts, Calf thymus DNA and HeLa cells, human skin	Kvam and Tyrrell 1997, Zhang et al 1997, Liardet et al 2001
Protein DNA-cross links, single-strand breaks	Human epithelioid cells and XP cells	Peak and Peak 1991, Peak et al 1991
Chromosomal instability	Keratinocytes and hamster fibroblasts	Phillipson et al 2002, Dahle and Kvam 2003
Mutations		
GC to AT [1]	Mouse SCC tumors	van Kranen et al 1997
GA to TC[2]	Mouse embryonic fibroblasts	Besaratinia et al 2004
AT to CG [3]	CHO cells, human AK and SCC tumors	Drobetsky et al 1995, Agar et al 2004
p53 effects[4]		
p53 increase	Mouse and human skin	de Laat et al 1997a, Burren 1998
cell cycle arrest	Fibroblasts, mouse skin	de Laat et al 1996 &1997a
p53 mutation	Mouse SCC, human keratinocytes, human AK and SCC	van Kranen et al 1997, Persson et al 2002, Agar et al 2004

[1] UVB fingerprint
[2] 8-OHdG related
[3] UVA fingerprint
[4] Discussed in the chapter 1.1.6.1

1.1.4.3 UV-induced signature mutations

Despite the ability of cells to repair UV-induced DNA lesions, some damage will remain unrepaired. UVB mutagenesis is characterized by a high frequency of mutations in the DNA sequences containing CPDs and (6–4) photoproducts leading to GC to AT transversions. These lesions, C to T point mutations/ transitions, and especially CC to TT tandem mutations, are considered as UVB signature mutations (Matsumura & Ananthaswamy 2004; Melnikova & Ananthaswamy 2005).

UVA has been found to induce mutations at AT base pairs more frequently than by UVB (Robert *et al.* 1996). The high incidence of AT to CG transversions established this base change as a molecular fingerprint or a signature mutation to UVA (Drobetsky *et al.* 1995). Recently, this transversion of AT to CG was found in the epidermal basal layer stem cells in the human squamous cell carcinoma and actinic keratose (Agar *et al.* 2004) (Table 1).

The mutational specificity of UVA is distinct from that of UVB because the initiating type of damage, i.e. ROS-mediated base injury, is different. UVA-induced oxidative base damage, 8-OHdG, has been shown to generate G to T and A to C transversions (Cheng *et al.* 1992; Besaratinia *et al.* 2004). However, when assessing the impact of ROS on the induction of oxidative base damage-related mutations, it is difficult to define which particular oxidative base change has been caused by a particular free radical. Consequently, it is not possible definitely to determine which mutation has been caused by UVA and which has been caused by UVB-induced ROS.

1.1.5 UVA-induced cellular effects

UVB radiation for long time has been considered the more harmful part of the UV spectrum due to its DNA-damaging potential and carcinogenic effects. Recently published studies have demonstrated that UVA radiation can modulate a variety of biochemical processes and cause severe oxidative damage via ROS generation. Besides an elicitation of oxidative DNA damage, oxidative stress also plays a crucial role in modulating gene and protein expression as well as in damaging membranes and inducing apoptosis. Although cells have the free radical scavenging systems, high levels of ROS and reactive oxygen intermediates can overwhelm normal defense mechanisms leading to permanent damage. UVA also induces the expression of several soluble factors such as proteins and lipid mediators that mediate inflammation; they may also promote tumorigenesis.

1.1.5.1 Oxidative stress and apoptosis

The deleterious effects of UVA on lipids and proteins are mediated through singlet oxygen formation (Bose *et al.* 1990; Vile & Tyrrell 1995) (Table 2). Low doses of UVA radiation have been shown to induce lipid peroxidation in the cell membranes (Bose *et al.* 1990; Punnonen *et al.* 1991; Gaboriau *et al.* 1995). The singlet oxygen upregulates the expression of heme oxygenase (HO-1) (Keyse *et al.* 1990; Basu-Modak & Tyrrell 1993), catalyzing heme breakdown into iron, CO and biliverdin. HO-1 is considered a marker of the oxidative stress in mammalian cells and it is believed to serve as a long-term protective and defense mechanism against oxidative damage (Applegate *et al.* 1991). However, cell

Table 2. UVA-induced ROS/singlet oxygen-mediated cellular effects.

Examined	Cells	Reference
Oxidative stress		
HO-1 gene upregulation	Fibroblasts, epidermoid carcinoma cell line	Keyse et al 1990, Soriani et al 1998
CL100 gene upregulation[1]	Fibroblasts	Keyse and Emslie 1992
Membrane damage	Fibroblasts, keratinocytes	Bose et al 1990, Punnonen et al 1991, Gaboriau et al 1995, Vile and Tyrrell 1995
Ferritin upregulation	Fibroblasts	Vile and Tyrrell 1993, Vile et al 1994
Iron release	Fibroblasts	Pourzand et al 1999, Kvam et al 2000
Apoptosis	Mouse lymphoma cells, human T and B lymphocytes, rat fibroblasts	Godar 1996 & 1999a, Pourzand et al 1997, Pourzand and Tyrrell 1999
FasL expression	T-cells	Morita et al 1997b
Bcl-2 expression	Rat fibroblasts, rat endothelial cells	Pourzand et al 1997, Pourzand and Tyrrell 1999
Adhesion molecules[1]		
ICAM-1 expression	Keratinocytes	Krutmann and Grewe 1995, Grether-Beck et al 1996
Soluble mediators[1]		
MMP-1 secretion	Fibroblasts	Wlaschek et al 1994, 1995 & 1997
Signal transduction pathways[2]		
MAPK activation	Fibroblasts	Klotz et al 1997 & 1999

[1]Discussed in chapter 1.1.5.2
[2]Discussed in chapter 1.1.5.3

overexpressing HO-1 have also been demonstrated to be hypersensitive to UVA, since temporarily increased HO-1 activity apparently releases iron from heme and sensitizes the cells to iron-derived-oxidative stress (Kvam *et al.* 2000). UVA also increases the level of ferritin, which is considered another adaptive response that protects cells by iron-catalyzed free radical reactions (Vile & Tyrrell 1993;

Vile *et al.* 1994). Interestingly, the free iron amount in human fibroblasts has been shown to increase as a result of the UVA-induced degradation of ferritin (Pourzand *et al.* 1999).

UVA-mediated apoptosis has been shown to be induced via singlet oxygen formation (Godar 1996; Pourzand *et al.* 1997; Pourzand & Tyrrell 1999; Godar 1999b) (Table 2). UVA triggers a mixed apoptotic mechanism including (i) an immediate apoptosis (0–4h) through mitochondrial membrane damage and (ii) a delayed apoptosis (>20h) that requires the expression of apoptotic proteins (Godar 1996; Godar 1999a). UVA-induced singlet oxygen has also been shown to trigger apoptosis in human skin, infiltrating T-cells via the upregulation of Fas-ligand (Morita *et al.* 1997b). UVA-induced apoptosis may mediate the effectiveness of UVA-phototherapy, as it was suggested for the treatment of T-cell mediated skin diseases such as T-cell lymphoma and atopic dermatitis (Morita *et al.* 1997b). UVA may also inhibit apoptosis through the induction of antiapoptotic proteins such as Bcl-2 (Pourzand *et al.* 1997; Pourzand & Tyrrell 1999). Authors postulate that Bcl-2 acts as an antioxidant against the effects mediated by UVA-induced ROS, however preventing apoptosis. This might lead to the survival of severely damaged cells that possibly further lead to the mutagenesis.

1.1.5.2 Soluble mediators and adhesion molecules

UVA enhances the generation and secretion of soluble mediators and adhesion molecules as shown in Table 3. UVA participates in the skin inflammation through the enhanced cytokine secretion by skin cells: TNF-α, IL-1α, IL-6, IL-8 and IL-12 (Wlaschek *et al.* 1993; Corsini *et al.* 1997; Morita *et al.* 1997a; Avalos-Diaz *et al.* 1999; Kondo 1999). UVA has been also shown to cause a time- and dose-dependent induction of immunosuppressor IL-10, thus possibly mediating the immunosuppression along with UVB (Grewe *et al.* 1995).

UVA plays an important role in photoaging and possibly in metastasis, modulating the release of the vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs). VEGF is essential in tumor neovascularization and thus, in tumor progression and metastasis. UVA is known to induce the expression of VEGF from fibroblasts and keratinocyte-derived cell lines (Mildner *et al.* 1999; Trompezinski *et al.* 2000). However, in normal keratinocytes UVA causes a decline in the generation of VEGF (Mildner *et al.* 1999) and an inhibition of UVB-induced VEGF production (Longuet-Perret *et al.* 1998). The differential response of primary keratinocytes and autonomously growing keratinocyte-derived cell lines suggest that VEGF secretion may offer tumor cells the advantage of growth over normal cells (Mildner *et al.* 1999).

Table 3. UVA effects on soluble mediators and adhesion molecules.

Examined	Effect	Source	Reference
Soluble mediators			
TNF-α	↑	Keratinocytes, fibroblasts, epidermis, epidermoid carcinoma cell line	Morita 1997a, Corsini 1997, Avalos-Diaz et al 1999
IL-1α and -β	↑	Keratinocytes, fibroblasts	Wlaschek et al 1994, Corsini 1997
IL-6	↑	Fibroblasts, epidermoid carcinoma cell line, keratinocytes	Wlaschek et al 1993, 1994 & 1997, Morita et al 1997a, Avalos-Diaz et al 1999
IL-8	↑	Epidermoid carcinoma cell line	Morita et al 1997a
IL-10	↑	Keratinocytes	Grewe et al 1995
IL-12	↑	Keratinocytes	Kondo 1999
VEGF	↑	Fibroblasts, keratinocyte-derived cell lines	Mildner et al 1999, Trompezinski et al 2000
VEGF	↓	Primary keratinocytes	Longue-Perret et al 1998, Mildner et al 1999
MMP-1	↑	Fibroblasts, SCC tumor cells	Wlaschek et al 1994, 1995 & 1997, Petersen et al 1995, Soriani et al 1998, Ramos et al 2004
MMP-10	↑	SCC tumor cells	Ramos et al 2004
Adhesion molecules			
ICAM-1	↑	Dermal endothelial cells, fibroblasts, vascular structures, keratinocytes	Heckmann et al 1994b, Krutmann and Grewe 1995, Treina et al 1996, Grether-Beck et al 1996
ICAM-1	↓	Epidermal keratinocytes	Treina et al 1996
E-selectin	↑	Dermal endothelial cells	Heckmann et al 1994
Integrin chains α1, α2, α5	↑	Fibroblasts	Tupet et al 1999
Integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$	↑	Melanocytes	Neitmann et al 1999
MHC I, II	↑	Rat myeloid leukemia cells	Leszczynski et al 1996

MMPs are known to be involved in tumor progression and metastasis by degrading extracellular matrix components (Matrisian 1992). UVA upregulates the expression of interstitial collagenase (MMP-1) (Wlaschek *et al.* 1994; Petersen *et al.* 1995;Wlaschek *et al.* 1995; Wlaschek *et al.* 1997; Ramos *et al.* 2004) and of stromelysin-2 (MMP-10) (Ramos *et al.* 2004). The MMP-induction is mediated via UVA-induced singlet oxygen, which activates the expression of IL-1α, IL-1β, and IL-6 that in turn induce MMP-1 synthesis via the autocrine loop (Wlaschek *et al.* 1994). Protein kinase C (PKC) (Petersen *et al.* 1995) and p38 stress kinase pathway (Ramos *et al.* 2004) have also been shown to mediate MMP-secretion after UVA exposure.

Besides cytokine release, adhesion molecule expression is also one of the characteristic features of inflammation. Adhesion molecules are responsible for the recruitment of blood leukocytes to the inflammation site. They are also involved in metastatic cascade, mediating tumor cell adhesion to the stromal cells of the host. UVA-induced singlet oxygen has been shown to upregulate intercellular adhesion molecule-1 (ICAM-1) expression (Krutmann & Grewe 1995; Grether-Beck *et al.* 1996; Krutmann 2000). A similar positive association between UVA and the ICAM-1 expression has also been demonstrated in cultured dermal fibroblasts and in the vascular structures of dermis (Treina *et al.* 1996). Heckmann *et al.* have demonstrated that dermal microvascular endothelial cells are the direct target of UVA by observing the upregulation in the expression of ICAM-1 and E-selectin in dermal endothelial cells after UVA exposure (Heckmann *et al.* 1994). However, the controversial result demonstrated the depleted ICAM-1 expression after UVA-exposure in human keratinocytes (Treina *et al.* 1996). Besides the adhesion molecule expression, UVA induces the expression of major histocompatibility class I and II on the cell surface with simultaneous PKC activation (Leszczynski *et al.* 1996) (Table 3).

Since adhesion molecules mediate intercellular interaction, it is not surprising that UVA has been shown to alter the adhesive properties of cells. UVA increases the adhesion of endothelial cells to peripheral blood derived granulocytes, lymphocytes, and monocytes (Heckmann *et al.* 1997). UVA has also been shown to stimulate fibroblast adhesion to collagen (Tupet *et al.* 1999) as well as melanocyte adhesion to fibronectin (Neitmann *et al.* 1999) through the expression of integrins.

1.1.5.3 Tumor promotion

Several proteins/enzymes, whose activity is increased following the UVA radiation, may play a role in uncontrolled cellular proliferation and hyperplasia (Table 4). UVA activates the phospholipases A and C (PLA, PLC) (Hanson & DeLeo 1989; Hanson & DeLeo 1990; Cohen & DeLeo 1993) that stimulate the release of

Table 4. UVA effects that may enhance tumor promotion.

Examined	Effect	Source	Reference
PLC, PLA	↑	Human and mouse fibroblasts, keratinocytes	Hanson and DeLeo 1989, Hanson and DeLeo 1990, Cohen and DeLeo 1993
AA	↑	Human and mouse fibroblasts, keratinocytes	Hanson and DeLeo 1989, Hanson and DeLeo 1990, Cohen and DeLeo 1993
COX	↑	Dendritic cells, keratinocytes, fibroblasts, epidermoid carcinoma cell line	Hanson and DeLeo 1989, Hanson and DeLeo 1990, Soriani et al 1998, Krutmann and Morita 1999, Bachelor et al 2002
Prostaglandin E	↑	Fibroblasts, keratinocytes	Hanson and DeLeo 1989, Hanson and DeLeo 1990
DAG	↑	Keratinocytes	Hanson et al 1989, Hanson and DeLeo 1989
PKC activity	↑	Fibroblasts, keratinocytes, rat chloroma cells	Matsui and DeLeo 1990, Matsui et al 1994, Leszczynski et al 1995 & 1996
EGF receptor binding	↓	Fibroblasts	Matsui and DeLeo 1990, Djavaheri-Mergny et al 1994
MAPK family members p38, JNK, ERK1/2	↑	Fibroblasts, keratinocytes, melanocytes	Klotz et al 1997 &1999, Yanase et al 2001, Bachelor et al 2002
AP-1, AP-2	↑	Keratinocytes	Djavaheri-Mergny et al 1996, Djavaheri-Mergny and Dupertret 2001, Grether-Beck et al 1996
c-Jun	↑	Fibroblasts	Bose et al 1999, Soriani et al 2000
c-Fos	↑	Fibroblasts, keratinocytes	Silvers and Bowden 2002, Silvers et al 2003
NF-κB	↑	Fibroblasts	Vile et al 1995, Reelfs et al 2004

choline compounds and arachidonic acid (AA) from the cell membranes (Hanson & DeLeo 1989). Arachidonic acid is further catabolized by cyclo-oxygenases and lipoxygenases to inflammatory mediators, such as prostaglandins and leukotrienes. UVA is also known to induce directly cyclo-oxygenase (COX) activity (Hanson & DeLeo 1989; Soriani *et al.* 1998; Krutmann & Morita 1999;

Bachelor *et al.* 2002). The above-mentioned changes are involved in UVA-induced inflammatory responses that might lead to cellular proliferation (Matsui & DeLeo 1991).

UVA causes a release of diacyl glyserol (DAG) in cultured cells (Hanson & DeLeo 1989; Hanson *et al.* 1989). This event mediates PKC activation, which further enhances the cancer promoting effect of UVA (Matsui & DeLeo 1991). UVA exposure has been shown to elevate directly PKC activity (Matsui *et al.* 1994; Leszczynski *et al.* 1995; Leszczynski *et al.* 1996). UVA exposure is also known to induce inhibition of the epidermal growth factor (EGF), binding to its receptor (Matsui & DeLeo 1990; Djavaheri-Mergny *et al.* 1994) via PKC-induced phosphorylation (Matsui & DeLeo 1991). Inhibition of EGF receptor binding is one of the earliest membrane events in the activation of the mitogenic signaling cascade. Taken together, PKC activation, which has been strongly linked to chemical tumor promotion by phorbol esters, can also be inducible by a low, physiologic dose of UVA, possibly promoting the development of skin cancer. Moreover, PKC activation and translocation to the plasma membrane has been shown to be involved in melanoma metastasis (Gopalakrishna & Barsky 1988; Rusciano 2000).

The oxidative stress evoked by UV can have far reaching implications on carcinogenesis by activating the intracellular signal transduction pathways, which might lead to enhanced cellular proliferation (Devary *et al.* 1992; Ichihashi *et al.* 2003). UVA-induced ROS generation has been shown to activate mitogen-activated protein kinase (MAPKs) pathway members: extracellular signal regulated kinases (ERK 1 and 2), c-Jun terminal kinases (JNK) and p38 MAPK (Klotz *et al.* 1997; Klotz *et al.* 1999; Yanase *et al.* 2001; Bachelor *et al.* 2002). UVA-related oxidative stress also upregulated CL100 protein tyrosine phosphatase (Keyse & Emslie 1992). In addition to the direct activation of protein kinases, UVA affected transcription factors AP-1 and AP-2 (Grether-Beck *et al.* 1996; Djavaheri-Mergny *et al.* 1996; Djavaheri-Mergny & Dubertret 2001) and c-fos and c-jun (Bose *et al.* 1999; Soriani *et al.* 2000; Silvers & Bowden 2002; Silvers *et al.* 2003). All these events might affect the proliferation, differentiation and progression of cells (Djavaheri-Mergny *et al.* 1996; Klotz *et al.* 1999). UVA is also known to activate transcription factor NFκB, which plays a pivotal role in the regulation of inflammation, infection and stress response, and it also acts as a proto-oncogene (Vile *et al.* 1995; Reelfs *et al.* 2004). The UVA radiation-dependent activation of NFκB correlates with the UVA radiation-dependent peroxidation of cell membrane lipids and membrane damage. NFκB expression is known to mediate of the survival pathway in many cells and this phenomenon is believed to prevent the apoptosis in UVA exposed cells (Pourzand & Tyrrell 1999).

1.1.6 Photocarcinogenesis of non-melanoma skin cancers

Ultraviolet radiation is the major environmental risk factor for non-melanoma skin cancer (NMSC) formation due to its DNA-damaging potential. SCC development is related to the total, life-long accumulated UV dose. SCC tumors occur most regularly in parts of the body exposed to the sun, such as the face, neck and hands. Basal cell carcinomas (BCC) does not share the same direct relation, but it does appear to be associated with childhood UV exposure as well as with intermittent, heavy exposure that leads to sunburn formation (Longstreth *et al.* 1998; Armstrong & Kricker 2001).

UVB is considered a complete carcinogen involved in the initiation, promotion and progression of NMSC. A direct correlation between p53 mutations – which occur in UVB-induced pyrimidine hot spots - and the onset of human and mice SCC and BCC provides direct evidence for the mutagenic role of UVB in skin carcinogenesis (Brash *et al.* 1991; Ziegler *et al.* 1994; Ziegler *et al.* 1996). UVA is 5000 times less effective as a complete carcinogen, when compared to UVB (Matsui & DeLeo 1991). However, UVA has been shown to alone, or in combination with UVB, induce papillomas (Talve *et al.* 1990; Kelfkens *et al.* 1991; Kelfkens *et al.* 1992). It has also been shown to induce SCC in hairless mice (van Weelden *et al.* 1990; de Laat *et al.* 1997b). UVA is also believed to promote UVB-induced carcinogenesis but the mechanism by which it could occur is unknown; possibly the mechanism is related to the UV-mediated inflammation (Matsui & DeLeo 1990; Matsui & DeLeo 1991).

An UV-action spectrum for squamous cell carcinomas has been derived from experiments with hairless mice (de Gruijl *et al.* 1993). The action spectrum for NMSC development reaches its maximum at 293 nm in the UVB region, after which it shows a steep drop for wavelengths over 300 nm. However, another peak of tumor formation is observed in the UVA range at 380 nm, being 5-fold higher than with 350 nm (Runger 1999). The UV-action spectrum for the formation of pyrimidine dimers and oxidative DNA base modifications has indicated that a second peak in oxidative DNA damage showed up at a UVA range extending from UVAI region to visible light (Kielbassa *et al.* 1997). This might explain the second peak of tumor formation in the NMSC-action spectrum and the capability of UVA to act as a complete carcinogen, inducing SCC in mice (Runger 1999).

1.1.6.1 UV-induced *p53* mutations in NMSC

The tumor suppressor protein p53 protein plays an important role in the protection of tissues against extracellular stress as well as in maintaining cellular integrity. Accumulation of the p53 protein arrests a cell cycle at the G1 phase via induction of the cyclin-dependent kinase inhibitor $p21^{Waf1/CIP1}$, which forms a complex with cyclin dependent kinases (CDKs). This allows DNA repair before its replication

into the S phase and it prevents the replication of such damage that might lead to mutagenesis and carcinogenesis. Both UVA and UVB radiation have been shown to induce p53 expression (Hall *et al.* 1993; Campbell *et al.* 1993a; de Laat *et al.* 1997a; Burren *et al.* 1998) and to inhibit DNA synthesis by arresting the cell cycle at the S phase (de Laat *et al.* 1996; de Laat *et al.* 1997a) (Table 1). In addition to cell cycle arrest, the p53 protein induces apoptosis and sunburn cell formation if the DNA damage is lethal (Matsumura & Ananthaswamy 2004).

Mutation in the tumor suppressor gene *p53* has been found to play a critical role in the development of NMSC (Melnikova & Ananthaswamy 2005). *p53* mutation appears to be an early genetic change in the development of NMSC (Campbell *et al.* 1993b; Ziegler *et al.* 1994), and it is found even in normal skin exposed to the sun (Nakazawa *et al.* 1994) as well as in UV-exposed mouse skin months before skin tumors appear (Ananthaswamy *et al.* 1997; Ananthaswamy *et al.* 1999). *p53* mutation has been detected at high frequency (50-90%) in SCC, BCC (Brash *et al.* 1996) and in actinic keratoses (Ziegler *et al.* 1994). The predominant alterations found in these lesions have been C to T and CC to TT transitions at pyrimidine sites, indicating the remarkable role of UVB radiation in carcinogenesis (Dumaz *et al.* 1993; Ziegler *et al.* 1996).

The causal relationship between UVA exposure, *p53* mutations and the onset of NMSC is not as clear when compared to UVB radiation (de Gruijl 2002a; de Gruijl 2002b). A physiological dose of UVAI has been shown to cause oxidative damage-derived *p53* mutations in human skin (Persson *et al.* 2002). Moreover, UVA-induced SCC in mice contains some *p53* mutations, however they occur at the UVB pyrimidine hot spots (van Kranen *et al.* 1997). Finally, Agar *et al.* have shown that UVA is able to induce UVA-specific signature mutation in the *p53* gene in the basal cell layer in human SCC and AK samples (Agar *et al.* 2004) (Table 1).

1.1.7 Ultraviolet radiation induced immunosuppression

The carcinogenic potential of UV radiation is associated with its ability to suppress the cell-mediated immune responses. Primarily, this prevents the development of excessive inflammation and thus, further damage to skin. However, at the same time UV-induced immunosuppression may comprise a major risk factor for the development of skin cancer by allowing cancer cells to escape from the immunosurveillance (Longstreth *et al.* 1998; Ullrich 2005).

The antigen presentation by antigen presenting cells (APCs) to T-cells forms the first step of a normal primary immune response. The contact hypersensitivity (CHS) reaction is initiated by epicutaneous application of a known allergen/ contact sensitizer. Langerhans cells (LCs) and macrophages catch foreign

material, e.g. hapten, migrate to the draining lymph nodes (DNLs) and activate naïve T lymphocytes, which elicits and initiates the final immune response. CHS elicitation can be used when studying and determining UV-mediated immunological responses (Schwarz 2002). Exposure to UV-radiation results in a significant decline in the number and morphology of cutaneous APCs, thereby impairing their antigen-presenting activity in the lymph nodes to the T-cells, leading to immunosuppression. UVR induces two kinds of immunosuppression, local and systemic (Kelly *et al.* 1998). Local immunosuppression refers to a situation where the sensitizing agent/hapten (e.g. allergen) is applied directly to the UV-exposed skin area after irradiation. In systemic immunosuppression, one area of skin is exposed to UV and the distant, non-exposed site is sensitized with hapten (Schwarz & Schwarz 2002; Schwarz 2002).

1.1.7.1 Local and systemic immunosuppression

The initial step in the UV-induced formation of the local immunosuppression is a change in the ability of Langerhans cells to present the antigen to T-cells. UV exposure decreases the number of LCs in the skin and downregulates the expression of the MHCII antigen and co-stimulatory molecules on the cell surface (Toews *et al.* 1980; Aberer *et al.* 1981; Weiss *et al.* 1995). Normal LCs present antigens equally for both T helper cell subclasses Th1 and Th2, but UV-irradiated LCs present antigen efficiently only to Th2 cells (Ullrich 1995). It enhances Th2 cell proliferation and increases the production of immunosuppressor cyokines, such as IL-4, IL-5, IL-6 and IL-10, by Th2 cells. However, at the same time it leads to the failure of the proliferation and secretion of inflammation-related, anti-immunosuppressive cytokines (IFN-γ, IL-12) by Th1 cells (Ullrich 1995).

UV-altered Langerhans cells have failed to generate the contact hypersensitivity response in mice after contact with allergen sensitization (Toews *et al.* 1980). Moreover, the resensitization of mice with the same hapten again caused failure in CHS response (Toews *et al.* 1980). The mechanism by which the unresponsiveness is formed in the resensitized mouse is called the tolerance induction. It involves the formation of the antigen-specific suppressor T-cells found in the lymph nodes of UV-irradiated, hapten sensitized mice (Elmets *et al.* 1983). The detailed mechanism by which these regulatory cells suppress the induction of immune responses remains to be determined. However, it is known that one class of these suppressor/regulatory T cells express on their surface the cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) that appears to be important: hapten-specific tolerance was abrogated and the transfer of immunosuppression was inhibited by using a (neutralizing) antibody against CTLA-4 (Schwarz *et al.* 2000; Schwarz 2002). Antigen-specific T-suppressor cells are known to secrete immunosuppressive cytokines, such as IL-10, upon antigenic

stimulation and to increase the immunosuppressive effect of UV (Schwarz & Schwarz 2002).

Cytokines released by UV-exposed keratinocytes, such as IL-10, TNF-α, calcitonin-gene related peptide α-MSH and NO, affect the APC functions in the skin, preventing inflammatory effects. This UV-related phenomenon is employed on UV-derived phototherapies (Krutmann 1998; Krutmann & Morita 1999). IL-10 is considered to have a major role in immunosuppression by decreasing the number of dendritic cells as well as by complicating the antigen presentation of APC to the Th1 cells (Shreedhar *et al.* 1998; Ullrich 2005). TNF-α decreases the dendricity of LCs, changing their appearance to a more globular form (Vermeer & Streilein 1990). This prevents LC migration to the lymph nodes and antigen presentation to T cells. Calcitonin gene-related peptide has been shown to suppress the antigen presentation capability of LCs after UV exposure, inducing IL-10 expression (Scholzen *et al.* 1999; Kitazawa & Streilein 2000). UV-induced α-MSH inhibits the release of proinflammatory cytokines by Th1-cells and activates the keratinocytes to release IL-10 (Grabbe *et al.* 1996). Nitric oxide (NO) might be responsible for solar-simulated radiation-induced immunosuppression, inducing a loss of dendritic cells in the epidermis (Kuchel *et al.* 2003).

In systemic immunosuppression, UV induces failure in the elicitation of CHS when an unexposed skin site is sensitized with hapten. This phenomenon is due to the keratinocyte-derived release of immunosuppressive soluble mediators into the circulation; for example, prostaglandin E_2 (Shreedhar *et al.* 1998), TNF-α (Rivas & Ullrich 1994), IL-10 (Rivas & Ullrich 1992; Ullrich 1994). IL-12 and IFN-γ have opposite effects in immunosuppression when compared to IL-10 (Schwarz & Schwarz 2002; Schwarz 2002). IL-12 injection before UV-exposure has been shown to prevent UV-induced local and systemic immunosuppression as well as hapten-specific tolerance (Schmitt *et al.* 1995; Schwarz *et al.* 1996). The mechanism mediating this event might be the decline of IL-10 and of TNF-α secretion by fibroblasts and keratinocytes after IL-12 application (Schmitt *et al.* 2000; Werth *et al.* 2003), as well as the IL-12-mediated DNA repair (Schwarz *et al.* 2002). UVR-induced cellular and molecular event that that mediate the formation of immunosuppression are summarized in Table 5.

1.1.7.2 Molecular targets mediating UV-induced immunosuppression

Urocanic acid (UCA) (Norval 1996) and DNA (Kripke *et al.* 1992) comprise the major molecular chromophores in the skin that mediate UV-induced immunosuppression (Table 5). UVB is found to isomerize *trans*-UCA in the epidermis to a more soluble cis-isomer, which has been found in UV-irradiated mice serum (Moodycliffe *et al.* 1993). *Cis*-UCA is able to mimic many of the effects

Table 5. UVR-induced cellular and molecular events that mediate the formation of immunosuppression.

Examined	Source	Reference
Langerhans cell depletion	-	Toews et al 1980, Aberer et al 1981
Suppressor T-Cell formation	-	Toews et al 1980, Elmets et al 1983
IL-10 secretion	Keratinocytes, suppressor T-cells	Rivas and Ullrich 1992, Ullrich 1994 &1995, Schwarz and Schwarz 2002
TNF- secretion	Keratinocytes	Vermeer and Streilein 1990, Rivas and Ullrich 1994
NO secretion	Human skin	Kuchel et al 2003
Calcitonin-related peptide secretion	Neurons	Scholzen et al 1999, Kitazawa and Streilein 2000
α-MSH secretion	Melanocytes	Grabbe et al 1996, Scholzen et al 1999
IL-12 decrease	Th2 cells	Ullrich 1995, Schwarz and Schwarz 2002, Schwarz 2002
IFN- decrease	Th2 cells	Ullrich 1995, Schwarz and Schwarz 2002, Schwarz 2002
cis-UCA formation	Epidermis	De Fabo and Noonan 1983, Norval 1995, Kondo et al 1995
DNA-damage	Mouse keratinocytes	Kripke 1992, Nishigori et al 1996 & 1998, O'Connor et al 1996

of UV exposure. It decreases the number of Langerhans cells in the skin and impairs their antigen presentation that is followed by the immunosuppression (De Fabo & Noonan 1983; Kondo *et al.* 1995).

DNA damage has been shown to decrease immune responses through keratinocyte-derived IL-10 and TNF-α secretion (Nishigori *et al.* 1996; O'Connor *et al.* 1996; Nishigori *et al.* 1998). However, when DNA damage was repaired, the UV-induced immunosuppressive reactions were abrogated in the mouse (Kripke *et al.* 1992; Yarosh *et al.* 1994) and opossum models (Applegate *et al.* 1989). One mechanism behind the restored immune capacity might be the decreased number of the DNA-damaged, CPD-containing Langerhans cells in the lymph nodes of the UV-irradiated mice that restored the APC function (Vink *et al.* 1996).

1.1.7.3 UVA-induced immunosuppression

UVA is known to suppress the local immune responses measured by CHS in mice and humans (LeVee *et al.* 1997; Damian *et al.* 1999; Damian *et al.* 2001). UVA has also induced systemic immunosuppression in the C57BL/6 mice strain after primary UVA exposure (Byrne *et al.* 2002). The mechanisms for how UVA mediates the immunosuppressive responses might be similar to those involved in UVB-induced suppression. UVA is known to induce a marked decrease in the number of Langerhans cells (Bestak & Halliday 1996; LeVee *et al.* 1997; Halliday *et al.* 1998). UVA has also been shown to decrease the co-stimulatory molecule expression on the LC surface (Iwai *et al.* 1999). ROS are suggested to be involved in UVA-induced immunosuppression, impairing the Langerhans cell functions, since the antioxidant treatments with glutathione or vitamin E mitigated UVA-induced immunosuppression (Iwai *et al.* 1999; Halliday *et al.* 2004; Halliday 2005). Langerhans cell depletion may also be involved in the UVA-radiation induced nitric-oxide release that mediates UVA-derived immunosuppression (Table 6).

Table 6. UVA[a] or SSR[b]-induced cellular and molecular events that affect immunological defence mechanisms.

UVA effects that mediate immunosuppression		
Examined	**Location**	**Reference**
Local immunosuppression	Mouse, human	Bestak and Halliday 1996, Le Vee et al 1997, Halliday et al 1998
Systemic immunosuppression	Mouse	Byrne et al 2002
Langerhans cell depletion	Human and mouse skin	Bestak and Halliday 1996, Le Vee et al 1997, Halliday 2005
Co-stimulatory molecule (B7-1, B7-2 and ICAM-1) downregulation on LC surface	Mouse keratinocytes	Iwai et al 1999
NO secretion[a,b]	Mouse[a] and human[b] skin	[a]Yuen et al 2002, [b]Kuchel et al 2003
ROS secretion	Mouse	Yuen et al 2002, Halliday 2004, 2005
UVA effects that abolish immunosuppression		
Examined	**Location**	**Reference**
IFN-γ expression	Mouse skin	Reeve and Tyrrell 1999
HO-1 expression	Mouse skin	Allanson and Reeve 2004 & 2005
HO-1 related CO-release	Mouse skin	Allanson and Reeve 2004 & 2005

However, controversial results concerning UVA-induced immunosuppression have been published. In contrast to UVA-induced primary immunosuppression, the re-exposure, resensitization and rechallenging of mice after 10 weeks from the first dose led to immunoprotection instead of to systemic immunosuppression (Byrne *et al.* 2002). Damian *et al.* showed that the low-dose of UVA applied within three days induced local immunosuppression, but when exposure time was increased to 4-5 days, UVA was no longer capable of inducing significant immunosuppression (Damian *et al.* 1999). Reeve *et al.* demonstrated that a single, suberythemic dose (37.5 J/cm^2) of UVA protected mice from UVB-induced immunosuppression (Reeve *et al.* 1998). They determined that the photoprotective effect depended on the presence of IFN-γ (Reeve *et al.* 1999), HO-1 expression (Reeve & Tyrrell 1999) and HO-1-related carbon monoxide release (Allanson & Reeve 2004;Allanson & Reeve 2005). In humans, UVA has been shown to offer partial protection from UVB-induced immunosuppression in CHS induction (Skov *et al.* 2000). These results suggest that UVA exposure prior the UVB-exposure or to consecutive UVA exposures that increase the total UVA dose might diminish UVA-induced immunosuppression. Halliday has postulated that low doses of UVA may initiate the ROS production that mediates immunosuppression, whereas higher UVA doses stimulate production of the protective antioxidant enzymes such as HO-1 that might reverse UVB-induced immunosuppression through the antioxidant effect (Halliday 2005).

1.2 Melanoma

Cutaneous malignant melanoma (MM), the most lethal form of skin cancer, arises from the malignant transformation of melanocytes. MM is known for its poor prognosis and high resistance to medical therapy, and its lethality is primarily related to its ability to metastasize. The incidence of melanoma has increased by 3–7% per year among the Caucasian population throughout the world (Armstrong & Kricker 1994). Melanoma accounts for ~4% of all cancer cases, but it causes about 79% of all cancer deaths (Rouzaud *et al.* 2005). The increasing incidence rate of melanoma fortunately seems to exceed the mortality rate, apparently because of the early detection of melanoma lesions, which are curable through surgery.

The development of melanoma is a multistep process. As in all cancers, genetic and environmental factors participate in the onset of MM. Studies with melanoma families have identified susceptibility genes predisposed to melanoma. The main etiological risk factor for the development of melanoma is UV radiation, although hereditary reasons also play a notable role in the progression of melanoma. As with NMSC, individuals sensitive to the sun who

do not tan and burn easily are at greatest risk. Pigmentary traits, such as red hair, fair complexion, and a tendency to freckle have been shown as risk factors for the development of melanoma. In addition, atypical nevi that indicates a general instability of cellular growth, predisposes an individual to melanoma. A history of previous melanoma, either individual of familial, also constitutes a risk factor for the development of MM (Tucker & Goldstein 2003).

1.2.1 Genetic alterations in melanoma

Approximately 50% of the melanoma prone families have been shown the linkage to 9p21-22 chromosome (Cannon-Albright et al. 1992; Gruis et al. 1999). The germline mutations in cyclin-dependent kinase inhibitor 2A gene *(CDKN2A, INK4A/ARF)* located in the chromosome 9p have been identified about in the 20% of melanoma prone families (Cannon-Albright et al. 1992; Hussussian et al. 1994; Gruis et al. 1999). In sporadic primary melanoma, only a few mutations (0-25%) and homozygous deletions (10%) are found in this gene (Ruas & Peters 1998). However, this locus was found to carry UVB signature transversions in the sporadic primary melanomas, suggesting that UVB may play a role in the etiology of melanoma development (Pollock et al. 1995; Peris et al. 1999). The *CDKN2A* locus encodes two different tumor suppressor proteins: $p16^{INK4}$ and $p14^{ARF}$. The $P16^{INK4}$ protein inactivates cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) and thus arrest cell cycle progression (Serrano et al. 1993). The majority of sporadic and metastatic melanomas have been shown to represent a lack in the expression of the gene product of *CDKN2A*, the tumor suppressor protein $p16^{INK4A}$ (Talve et al. 1997; Chin 2003; de Gruijl et al. 2005). If the $p16^{INK4A}$ protein is inactivated via mutation or deletion or it is not sufficiently expressed, a cell is incapable of arresting the cell cycle at the G1 checkpoint. Related to the mutations of *CDKN2*, a germline mutation in the cyclin-dependent kinases 4 gene in chromosome 12q13 has been found in individuals belonging to melanoma-prone families (Zuo et al. 1996). This mutation leads to the structural changes of CDK4. The mutated gene product is defective in binding to $p16^{INK4A}$ but holds the ability to bind cyclin D. As a result, CDK4 is constantly activated, thus promoting the cell cycle.

The other gene encoded by the *CDKN2* locus, $p14^{ARF}$, has also been shown to mutate in human melanomas as well in melanoma cell lines (Kumar *et al.* 1998; Chin 2003). The mutation of $p14^{ARF}$ causes degradation of the p53 protein, disrupting the G1 restriction point and DNA repair. Thus far, no direct link has been established between the mutation of the *p53* gene and melanomagenesis (Lubbe *et al.* 1994), which suggests that p53 is kept inactive via other mechanisms such as through the lack of the ARF protein that leads to p53 degradation (Omholt *et al.* 2003).

A significant relationship between the melanocortin 1 receptor (MC1R) genotype and the onset of melanoma has been identified (Palmer *et al.* 2000; Kennedy *et al.* 2001; Rouzaud *et al.* 2005). 3–4 non-functional allelic variants of the *MC1R* gene have been shown to predispose individuals to a higher risk of skin cancer (Sturm 2002; Sturm *et al.* 2003), increasing the melanoma risk over 2-fold (Palmer *et al.* 2000). MC1R functions as a primary regulator of eumelanin synthesis by binding the α-melanocyte stimulating hormone (α-MSH). Non-functional MC1R results in the inability of melanocytes to respond to α-MSH, which increases the pheomelanin synthesis and which in turn contributes to skin carcinogenesis by producing free radicals (Rouzaud *et al.* 2005). Moreover, MC1R variants appear to increase the susceptibility of $p16^{INK4A}$ mutations in families prone to melanoma (Rouzaud *et al.* 2005).

Finally, mutations in the *N-ras* and *B-raf* genes in the Ras-Raf-MAPK-signaling pathway have also been linked to the development of MM (Omholt *et al.* 2003; de Gruijl *et al.* 2005). *Ras* mutations in primary melanoma were located on sites regularly exposed to the sun, referring the UV-induction of this mutation ('t Veer *et al.* 1989; van Elsas *et al.* 1996). The activating mutation in the *B-raf* gene has been found to occur in melanomas from intermittently exposed skin and rarely from unexposed or chronically exposed skin (de Gruijl *et al.* 2005).

1.2.2 Development of melanoma from melanocyte to metastatic melanoma

Cutaneous melanoma can develop directly from melanocytes or from precursor lesions such as congenital or atypical dysplastic nevi (Herlyn *et al.* 1987). However, only approximately 50% of melanomas arise from a pre-existing nevus (Skender-Kalnenas *et al.* 1995). Five stages can be distinguished in the progression of melanoma (Li & Herlyn 2000; Bogenrieder *et al.* 2003). In step 1, there is a common acquired or congenital nevus without dysplastic changes. Melanocytes have a restricted lifespan; they do not carry any genetic abnormalities and they depend on exogenous growth factors secreted by neighboring keratinocytes. In step 2, called dysplastic nevus, melanocytes show structural atypia and are considered as precursors of melanoma. Dysplastic lesions progress to step 3, the radial growth pattern (RGP) phase. In RGP, cells grow laterally but remain confined to the epidermis, and they still depend on exogenous growth factors supplied by keratinocytes. In addition, cells are unable to achieve anchorage-independent growth and thus, they are not metastatic. In step 4, cells turn to the more aggressive and invasive phenotype. Melanoma cells grow vertically (vertical growth pattern, VGP), invading the dermis and subcutaneous tissue through the basement membrane. In this state, cells can be characterized by genetic instability, anchorage-independent growth and they represent a high potential

for metastasis. They totally escape the proliferation control of keratinocytes and form connections with fibroblasts, endothelial cells and the extracellular matrix. In VGP, melanoma cells begin to produce cytokines and growth factors in an autocrine manner, which enhances their autonomous growth and proliferation. Transition from RGP to VGP is believed to be the crucial step in the acquisition of metastatic phenotype and poor clinical outcome. Finally, in step 5, the melanoma has progressed as a fully metastatic melanoma.

1.2.3 Melanoma staging

Melanomas have been classified into four classes according to their growth patterns (Friedman & Heilman 2002; Balch *et al.* 2003): 1) Superficial spreading melanoma is the most common type with the radial growth phase. It occurs on the upper back of men and legs or on the back of women. It generally fulfills the ABCD rule for suspicious moles: A stands for asymmetry – one half is unlike the other half, B is for asymmetric borders that can be irregular, diffused and poorly constricted, C is for varying color and D for a diameter larger than 6 mm; 2) nodular melanoma is the second most common type of melanoma with the vertical growth phase. It occurs often in trunk, head and neck and has more serious prognosis and rapid onset possessing metastatic potential; 3) lentigo malignant melanoma arises in older age groups on areas exposed to the sun, such as on the face, head and neck region; 4) acral lentiginous melanoma occurs on the palms, soles and nail beds of older patients and individuals with darker skin types, demonstrating lateral growth.

Melanomas are classified histologically based upon their location and stage of progression. Clark *et al.* refined the microstaging of melanomas by classifying tumors according to the level of invasion from class I with intraepidermal location to the class V with invasion into subcutaneous fat (Clark, Jr. *et al.* 1969). Breslow postulated further that vertical tumor thickness might be a better indicator and predictor of prognosis and of metastatic capacity than the level of invasion is because patients with thin tumors had a longer survival rate than those with thick lesions did (Breslow 1970). Besides tumor depth and invasion, ulceration and the high vascularity of primary melanoma are poor prognostic markers.

A clinical staging system is important in order to define patients with metastatic risk, to predict survival rates and to select the appropriate medical treatment (Lang 2002; Balch *et al.* 2003). The revised melanoma staging system by the American Joint Committee of Cancer (AJCC) categorizes melanoma into local (I-II), regional (III), and distant types (IV) using TNM (Tumor, Node, Metastasis) classification (Balch *et al.* 2001). Patients without lymph node or distant metastases are categorized to have stage I or II disease. Class T

characterizes the local stage of primary melanoma and it is based on microscopic evaluation of the invasion and the thickness of the tumor. Patients with regional diseases with lymph node metastases are categorized to have a stage III disease, which is further subdivided into N classes based on the number of melanoma positive lymph nodes that are found. Patients with systemic metastatic melanoma at a distant body site have stage IV disease and they have been grouped into M classes according to the location of the metastases.

1.2.4 Initial growth and angiogenesis

While normal melanocytes totally depend on exogenous growth stimuli, melanoma cells produce a large repertoire of autocrine growth factors and their receptors (Lazar-Molnar *et al.* 2000). Thus, melanoma is able to proliferate autonomously and to stimulate its own migration. Several paracrine growth factors produced by melanoma cells are targeted to host stroma. Upon activation, endothelial cells, fibroblasts, keratinocytes and inflammatory cells start to secrete feedback substances (Lazar-Molnar *et al.* 2000; Li *et al.* 2003). All these factors act in concert by modulating the microenvironment to benefit tumor growth, angiogenesis, invasion and eventually metastasis.

One critical aspect in the progression of melanoma to the more aggressive phenotype is neovascularization. In the absence of neoangiogenesis, a primary tumor cannot grow beyond the size of 1-2 mm^3 because the normal stromal vasculature is incapable of supporting tumor growth due to the lack of supply of oxygen and nutrients (Timar *et al.* 2001; Streit & Detmar 2003). Neovascularization is necessary not only for tumor development but also for invasion and metastasis. The rich vascularity in primary melanoma has been associated with poor prognosis (Graham *et al.* 1994).

In angiogenesis, new capillary blood vessels are generated from pre-existing vessels. Microvascular endothelial cells are stimulated to migrate from their parent vessels to the perivascular stroma and to initiate a capillary sprout that expands to capillary structures (Liotta 1986). The direction of the migration is determined by angiogenic stimulus. Melanoma cells constitutively overexpress the three major angiogenic factors of VEGF, basic fibroblasts growth factor (bFGF) and IL-8 when compared to normal melanocytes (Lazar-Molnar *et al.* 2000). bFGF is obligatory for melanoma growth and survival and it has been detected in primary invasive and metastatic lesions, whereas it is not expressed by normal melanocytes. It also acts as a strong mitogenic factor for fibroblasts and endothelial cells (Li *et al.* 2003), inducing VEGF expression from endothelium (Srivastava *et al.* 2003). VEGF, also known as a vascular permeability factor, is regarded as the major angiogenic stimulator under physiological and pathological

conditions. It promotes melanoma growth and invasiveness. The increase in the VEGF expression correlates with melanoma thickness and indicates the transition from radial to vertical growth (Velasco & Lange-Asschenfeldt 2002; Streit & Detmar 2003). bFGF and VEGF secretion from melanoma cells can also induce MMP secretion by endothelial cells, which further enhances tumor invasion to the surrounding tissue (Velasco & Lange-Asschenfeldt 2002). IL-8 has been shown to play an important role as a multifunctional cytokine in the development of melanoma in that it stimulates melanoma proliferation and correlates with the metastatic potential (Singh & Varney 2000), possibly through the IL-8-mediated upregulation and the activation of MMP-2 (Luca *et al.* 1997).

Although many primary tumors are highly vascular, the vessels are not exactly identical to normal vessels, having differences in cellular composition and permeability. The concept of vasculogenic mimicry describes the ability of aggressive melanoma cells to mimic the capability of the embryonic cell to form tubular structures that facilitate tumor perfusion (Maniotis *et al.* 1999; Seftor *et al.* 2002; Hendrix *et al.* 2002; Hendrix *et al.* 2003). In vasculogenic mimicry, highly patterned vascular networks are composed by extracellular matrix and tumor cells that line the venules instead of the endothelial cells. Moreover, the activation and generation of endothelial associated genes, proteins and signaling pathways, such as vascular endothelial-cadherin (VE-cadherin), laminin and epithelial cell kinase , underlies the capability of melanoma to form these tubular structures (Seftor *et al.* 2001; Hendrix *et al.* 2001).

1.2.5 Melanoma metastasis

Melanoma is characterized by high risk of hematogeneous metastases in the early stages of disease and it is the major reason for melanoma mortality. The risk of metastasis formation increases with the thickness and ulceration of a primary tumor (Gershenwald *et al.* 2003). Metastasis may have occurred at the time of initial diagnosis and thus, melanoma might have been disseminated widely and metastatic lesions can be fairly large (Fidler & Hart 1982). Melanoma metastases are often located on the skin and in lymph nodes, in the visceral organs (e.g. lungs and liver), and in the brains. The biological heterogeneity of primary and the metastatic lesions may impair medical treatment (Fidler & Hart 1982; Fidler 1990). Due to the different genetic, biochemical and immunological phenotypes of tumor cells, the sensitivity and response to therapeutic agents differ within the primary and metastatic neoplasm and even within secondary neoplasms (Fidler & Hart 1982; Fidler 1990). Furthermore, the host organ environment modifies the response of a tumor cell to systemic therapy, thus altering the efficiency of medical treatment.

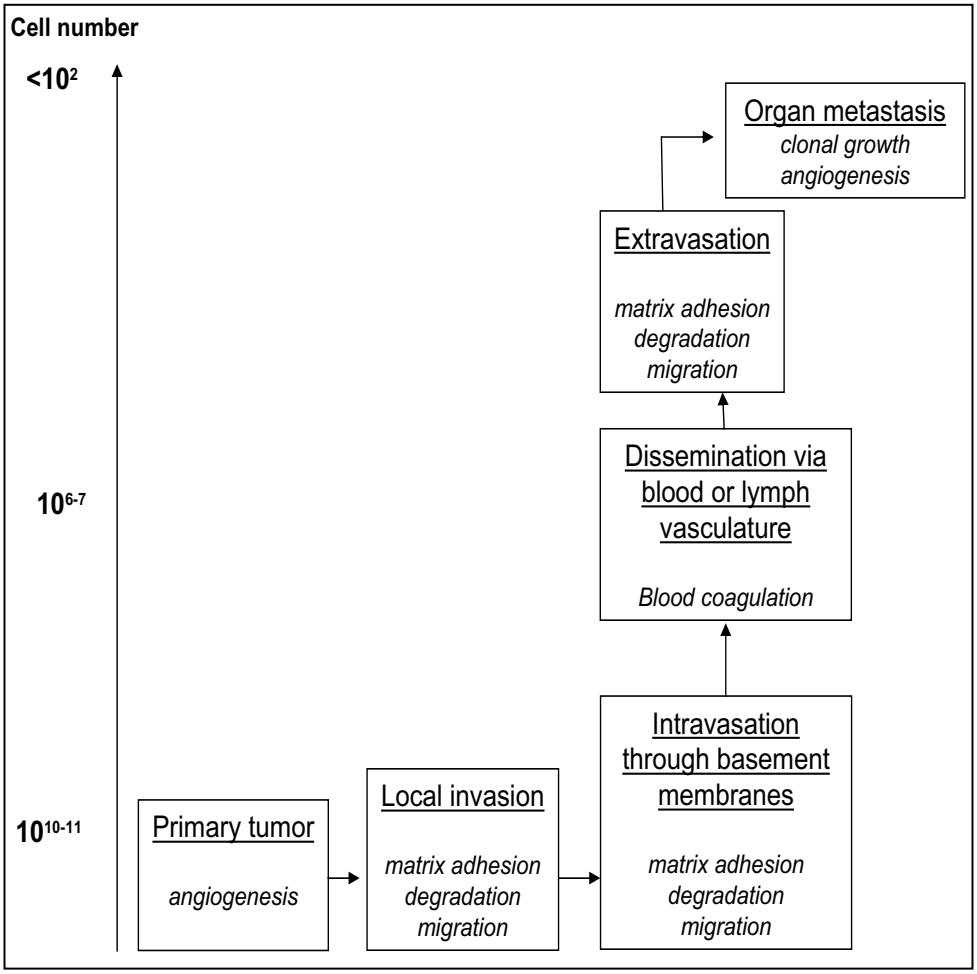

Figure 3. Metastatic cascade (adapted from Timar et al 2001).

The metastatic process is a cascade of interrelated steps between tumor cell and host (Fidler & Hart 1982; Liotta & Stetler-Stevenson 1991). Tumor cells must dissociate from the primary tumor, invade the host stroma, intravasate to local microvessels and disseminate in circulation. They have to arrest at the capillary bed of the host organ, invade through the vessel wall and extravasate into the parenchyma, and finally to proliferate to form a new colony (Figure 3). Metastasizing tumor cells must overcome the immunological defense mechanisms of the host. The whole cascade makes metastatic dissemination a highly selective process and only a small subpopulation of cells is capable of completing all the steps necessary for colonization to distant sites (Figure 3) (Fidler & Hart 1982; Liotta & Stetler-Stevenson 1991).

1.2.5.1 Invasion

Invasion begins with the escape of single tumor cells or small cell clusters from the primary tumor mass. After dissociation, tumor cells invade the surrounding extracellular matrix (ECM) and form interactions with the ECM components using specific cell surface adhesion molecules (Liotta 1986; Liotta & Stetler-Stevenson 1991; Liotta *et al.* 1991). After anchorage, tumor cells start to secrete proteases by themselves or agitate the stromal cells to produce them. Highly controlled degradation of the matrix component occurs in a localized region close to the tumor cell surface. Tumor cell locomotion in partially degraded ECM is a tightly regulated process of repeated detachment and adhesion to the matrix components and the rearrangement of the cytoskeleton. Melanoma motility is regulated by motility and scatter factors secreted by melanoma cells, but the direction is also influenced by host-derived chemoattractants. The invasion across the basement membrane at the dermo-epidermal junction and in the vessel wall enables the melanoma cell access to lymph and blood vessels for further dissemination.

The invasion involves a tightly controlled balance between substrate-specific proteases and their inhibitors. The different proteolytic enzyme families in this process comprises MMPs, the plasminogen activator system, and aspartyl and cysteine proteases (Kurschat & Mauch 2000). Proteases degrade a broad spectrum of matrix components, such as collagens, gelatins, fibronectin, proteoglycans and laminin (Edward & MacKie 1993). Melanoma cells express several MMPs, such as MMP-1,-2,-9,-13, membrane type 1-MMP (MT1-MMP) as well as their tissue inhibitors TIMP-1, -2, and -3 (Hofmann *et al.* 2000b). In melanoma metastasis, increased expression of MMP-1, MMP-2, MMP-9 and MT1-MMP have been shown to correlate with an invasive phenotype (Hofmann *et al.* 2000b). MT1-MMP and MMP-2 have been shown to be localized in the tumor-stroma interface in metastatic lesions (Hofmann *et al.* 2000c). MMPs can also be used as a prognostic marker for the development of melanoma (Vaisanen *et al.* 1996). For example, the secretion of MMP-2 in primary melanoma correlates with hematogeneous metastasis (Vaisanen *et al.* 1998). The high serum levels of soluble MMP-1 and MMP-9 have a clinical value in identifying patients at high risk of the progression of melanoma (Nikkola *et al.* 2002; Nikkola *et al.* 2005).

1.2.5.2 Dissemination in the circulation and extravasation

The dissemination of metastasizing melanoma cells occurs through transportation via lymph or blood vessels (Fidler 2003). Intravasating tumor cells enter the vessel lumen through the surrounding basement membrane, forming non-destructive interaction with the endothelial cells (Timar *et al.* 2001). Thereafter, the tumor cells either grow at the penetration site or circulate as individual cells

or tumor emboli (Fidler & Hart 1982). In the circulatory system, melanoma cells have to escape from immunological and non-immunological defense mechanisms (e.g. blood turbulence). To increase the survival, tumor cells build a mechanical defense by forming aggregates with each other (homotypic aggregation) or with platelets or lymphocytes (heterotypic aggregation). Melanoma cells also initiate clot formation with fibrin and platelets that not only enhances the survival of melanoma but also facilitates the arrest of the enlarged emboli in the microcapillaries of the host organ (Timar *et al.* 2001). Despite tumor cell evading mechanisms, the intravascular death of disseminated tumor cells is high and only 0.01% of circulating cells survive to initiate the metastatic colony (Liotta & Stetler-Stevenson 1991; Liotta *et al.* 1991; Fidler 2003).

The direction of the disseminating tumor cell is influenced by organ-derived chemoattractants in the organ selective homing process (Fidler 2003). The "seed and soil theory" proposed first by Paget in the 19th century suggested that the attraction of one type of cancer cell (seed) to certain host organs (soil) is not random, and different organs provide the optimal growth conditions for certain cancers (Paget 1889). Nowadays, it is known that the certain combinations of chemokines and their receptors and adhesion molecules play an important role in organ-specific metastatic cascade, thus determining the final destination of metastasizing tumor cells (Murphy 2001). Melanoma cells have been found to express the chemokine receptors 7 (CCR7) and 10 (CCR10) whereas skin and lymph nodes, which are major metastatic sites of melanoma, express ligands for these receptors (Muller *et al.* 2001). In addition, chemokines such as IL-8, melanoma growth stimulating activity (MGSA), and the monocyte chemotactic protein-1 (MCP-1) have been proposed as the chemokines associated with melanoma metastasis (Payne & Cornelius 2002).

The arrival of tumor cells to the capillary bed of a host organ is followed by cell arrest in the microvasculature. Besides mechanical filtering, tumor cells form a firm adhesion with the endothelium by expressing similar cell adhesion molecules to leukocytes, thus mimicing the leukocyte homing process (Timar *et al.* 2001; Fidler 2003). This is an efficient primary selection mechanism because the endothelium expresses such adhesion molecules that fit to the metastasizing melanoma cell phenotype. After melanoma adhesion to the endothelium, the retraction of adjacent endothelial cells exposes the underlying subendothelial matrix. Using protrusions, pseudopods, melanoma cells penetrate the endothelial cell junction and became intercalated by endothelial cells (Sandig *et al.* 1997). The melanoma transmigration through the endothelium, i.e. diapedesis, involves many multiple steps of adhesive interactions between melanoma and endothelium. Several adhesion molecules, such as VE and N-cadherins, integrin $\alpha_v\beta_3$ and adhesion molecule L1 have been shown to be involved in a highly

restricted manner in the diapedesis (Voura *et al.* 1998a; Voura *et al.* 2001). After extravasation, tumor cells have to accommodate to the new organ-specific environment of the subendothelial matrix. The successful growth of the secondary neoplasm involves the proper response to organ-specific factors. Those tumor cells incapable of adjusting their phenotype to the new tissue parenchyma will die; however, cells with a potential to proliferate and express growth factor receptors for those present in the host organ will survive and initiate a new colony formation (Timar *et al.* 2001; Fidler 2003).

1.2.5.3 Adhesion molecules involved in metastasis

Several adhesion molecule families are involved in melanoma metastasis. Adhesion molecules mediate the melanoma attachment to extracellular matrix components, thus enhancing melanoma migration in ECM as well as in skin capillaries during intravasation and extravasation (Johnson 1999; McGary *et al.* 2002). Adhesion molecules are also required in the positioning of MMPs on the cell surface. For example, the expression of activated MMP-2 and MT1-MMP has been shown to correlate with the expression of $\alpha_v\beta_3$-integrin in the invasive or metastatic melanomas (Hofmann *et al.* 2000a; Hofmann *et al.* 2000b). Adhesion molecules cooperate with receptor tyrosine kinases to activate proliferation and survival pathways: Signaling through integrin $\alpha_v\beta_3$ occurs via the MAPK proliferation pathway, whereas Mel-CAM affects through the antiapoptotic survival AKT/protein kinase B-mediated pathway (Perlis & Herlyn 2004). Li *et al.* have shown that the hepatocyte growth/scatter factor (HGF/SF), which is known as a potential melanocyte and melanoma mitogen, downregulates E-cadherin expression in melanocytic cells through its receptor c-met and the subsequent signaling pathway, MAPK (Li *et al.* 2001b).

1.2.5.3.1 Cadherins

Cadherins form a family of cell surface glycoproteins (Takeichi 1991). They mediate the calcium-dependent cell recognition and adhesion of neighboring cells and serve as a component in the gap junctions (Takeichi 1991; Li & Herlyn 2000). Cadherins are involved in many physiological conditions, such as embryogenesis, morphogenesis and in the cell motility. In normal human skin, E (epithelial)-cadherin is expressed on the surface of keratinocytes, melanocytes and LCs (Li & Herlyn 2000), whereas the VE (vascular endothelial)-cadherin is expressed by endothelial cells lining the vessel wall (Hendrix *et al.* 2001). N(neural)-cadherin is normally expressed by dermal fibroblasts and endothelial cells but not by keratinocytes (Li & Herlyn 2000). P(placental)-cadherin is expressed only by basal keratinocytes (Li & Herlyn 2000). E-cadherin forms the functional and structural contact between melanocytes and keratinocytes, mediating keratinocyte

control over melanocyte proliferation and differentiation (Tang *et al.* 1994). The importance of this interaction has been shown *in vitro*: melanocytes in cell cultures change their phenotype in a similar manner to melanoma cells *in situ* by downregulating their E-cadherin expression followed by a concomitant increase in the expression of the adhesion molecules Mel-CAM and $\alpha_v\beta_3$ on the melanocyte surface (Herlyn *et al.* 2000). However, when melanocytes are co-cultured with the epidermal keratinocytes, the expression of adhesion molecules return to normal status, thus indicating the importance of E-cadherin and keratinocyte-mediated regulation (Herlyn *et al.* 2000; Hsu *et al.* 2000b).

During the progression of melanoma, a decline in E-cadherin expression has been shown to correlate with diminished keratinocyte-mediated control and with the increased proliferation of melanoma cells (Hsu *et al.* 1996; Danen *et al.* 1996). The loss of E-cadherin on the cell surface also results in the inability of melanoma cells to adhere to each other through homotypic interactions within the primary tumor. This leads to the loosening of the primary tumor mass, which is accompanied with the enhanced invasive potential of melanoma cells as observed in RGP melanomas (Li & Herlyn 2000).

The loss of E-cadherin is followed by the increased expression of N-cadherin on the melanocyte surface. This shift in the cadherin profile has been observed both *in vitro* (Tang *et al.* 1994; Hsu *et al.* 1996) and *in vivo* (Hsu *et al.* 1996; Sanders *et al.* 1999) and it is a well-known marker of the invasive phenotype. N-cadherin promotes and mediates the heterotypic adhesion of melanoma cells to fibroblasts and enables cellular communication through gap junctions (Hsu *et al.* 2000a; Li *et al.* 2001a). This enhances the melanoma migration and assists their invasion from the epidermis to the dermis. N-cadherin also increases the melanoma communication with vascular endothelial cells (Meier *et al.* 1998; Li & Herlyn 2000). This improves the ability of the melanoma to intravasate as well as extravasate (Sandig *et al.* 1997; Voura *et al.* 1998b). The N-cadherin expression also appears to mediate the melanocyte survival through the activation of the PKB/AKT pathway leading to the subsequent downregulation of pro-apoptotic proteins (Li *et al.* 2001a).

1.2.5.3.2 Integrins

In addition to cadherins, integrins also play an essential role in the progression of melanoma. Integrins are transmembrane heterodimers formed by different types of non-covalently linked α and β chains (Hynes 1992). The expression of several integrins has been shown to be involved in the progression of melanoma (Hart *et al.* 1991). Integrins $\alpha_4\beta_1$ and $\alpha_v\beta_3$ have been shown to correlate with poor clinical outcome related to the invasive ($\alpha_v\beta_3$) and metastatic ($\alpha_4\beta_1$) properties of melanoma cells (Albelda *et al.* 1990; Schadendorf *et al.* 1993; Danen *et al.* 1995; Van Belle *et al.* 1999).

The β_3 subunit expression has been found to be a specific marker for the progression of melanoma from to RGP to VGP (Natali *et al.* 1995; Natali *et al.* 1997; Hsu *et al.* 1998; Van Belle *et al.* 1999). Integrin $\alpha_v\beta_3$ binds to vitronectin and other ECM components (Montgomery *et al.* 1996). It is also involved in the formation of firm adhesion to endothelial cells that express the ligand of the $\alpha_v\beta_3$, the adhesion molecule L1 (Voura *et al.* 2001). Activation of $\alpha_v\beta_3$ has been shown to prevent melanoma apoptosis (Montgomery *et al.* 1994). $\alpha_v\beta_3$ also appears to be required for angiogenesis (Brooks *et al.* 1994) and for the activation of MMPs (Brooks *et al.* 1996). Through the expression of $\alpha_4\beta_1$, melanoma cells may mimic the leukocyte homing process during transendothelial migration by interacting with VCAM-1 and selecting receptors in the activated endothelium (Okahara *et al.* 1994).

1.2.5.3.3 Immunoglobulin superfamily

Cell adhesion molecules that belong to the immunoglobulin superfamily have also been connected to melanoma metastasis (Shih *et al.* 1994; Johnson *et al.* 1996). Mel-CAM (also known as MUC18, CD146 and L-Endo) is a cell surface glycoprotein that has been identified as an adhesion receptor in benign nevi and also in melanomas, where its upregulation correlates with tumor thickness and metastatic potential (Luca *et al.* 1993; Shih *et al.* 1994; Xie *et al.* 1997; Jean *et al.* 1998). Mel-CAM plays a role in the homotypic binding between melanoma cells (Johnson *et al.* 1997), possibly enhancing cluster formation and capillary arrest in the circulation (McGary *et al.* 2002). Mel-CAM also mediates the adhesion between melanoma cells and endothelium (Shih *et al.* 1997). This may promote both the intravasation as well as the extravasation of melanoma cells (McGary *et al.* 2002). Furthermore, Mel-CAM has been shown to increase MMP-2 expression from melanoma cells (Xie *et al.* 1997), thus enhancing metastatic potential via ECM degradation.

The upregulated expression of intercellular adhesion molecule (ICAM-1) has been observed in malignant melanocytic lesions (Natali *et al.* 1990). It correlates with the increased vertical thickness of primary melanoma and with poor prognosis (Schadendorf *et al.* 1993; Schadendorf *et al.* 1995; Natali *et al.* 1997). The serum of melanoma patients has been demonstrated to contain higher levels of soluble ICAM-1, correlating with disease progression (Hirai *et al.* 1997). However, the exact role of ICAM-1 in the melanoma development is unknown, but it might enhance aggregate formation with leukocytes in the circulation, thus mediating tumor cell survival (Johnson 1999). The activated leukocyte cell adhesion molecule (ALCAM) is also expressed by melanoma cells and its expression correlates with enhanced metastatic potential (Degen *et al.* 1998). It mediates, like ICAM-1, melanoma binding with leukocytes, thus helping them survive in the circulation.

1.2.6 Ultraviolet radiation and melanoma

The exact role of UV in the etiology of malignant melanoma is unclear when compared to NMSC. Unlike NMSC, melanoma occurs in young adults. Whereas SCC development is linked to total lifetime exposure, the development of malignant melanoma is associated with intense, intermittent exposure especially during childhood (Longstreth *et al.* 1998;Autier & Dore 1998;Whiteman *et al.* 2001). The location of the occurrence of melanoma provides further evidence for the intermittent type of exposure: melanomas occur often in areas protected from the sun, such as trunk in men and in the legs in women. The risk of melanoma is often higher for indoor workers that it is for outdoor workers. This can be associated with the intense intermitted type of exposure in leisure time. Autier *et al.* showed that recreational exposure, sunbathing and the number of holiday weeks spent annually in sunny resorts formed a significant risk factor in developing melanoma (Autier *et al.* 1994b). Severe sunburn is a critical factor in the development of melanoma, especially if experienced in childhood; this is also supported by epidemiological studies (Whiteman & Green 1994; Holly *et al.* 1995). XP patients that face an extremely high risk of developing melanoma state the role of UVR as an etiological reason for malignant melanoma.

1.2.6.1 UVR- induced molecular changes involved in melanomagenesis

1.2.6.1.1 UVR-mediated enhanced proliferation and migration

The progression of melanoma occurs through a series of steps, some of which might involve UVR-induced effects (Table 7). UVR induces DNA damage through the formation of single strand breaks (Wenczl *et al.* 1998; Marrot *et al.* 1999) and thymine dimers (Young *et al.* 1998b) that may lead to mutagenesis if they remain unrepaired. UVR also acts indirectly by stimulating the synthesis of soluble mediators by epidermal cells that mediate the proliferation and survival of melanocytes (Imokawa *et al.* 1992; Kadekaro *et al.* 2003; Rouzaud *et al.* 2005; Kadekaro *et al.* 2005). UVR-induced keratinocyte-derived endothelin (ET-1) and α-MSH have been shown to upregulate the expression of MC1R on the melanocyte surface (Scott *et al.* 2002), which in turn leads to enhanced melanocyte proliferation via the cAMP mediated pathway (Im *et al.* 1998). ET-1 and α-MSH have been shown to be mitogenic (Tada *et al.* 2002), "rescuing" melanocytes from UV-induced G1 arrest (Tada *et al.* 1998). They also act as pro-survival agents activating the Akt/PKB antiapoptotic pathway (Kadekaro *et al.* 2003; Kadekaro *et al.* 2005).

UVA and UVB are known to induce cytokines, such as IL-6 and IL-8 (Kirnbauer *et al.* 1991; Singh *et al.* 1995; Morita *et al.* 1997a; Krutmann 1998) that both mediate the autonomous growth of melanoma and serve as markers

Table 7. UVR effects that might enhance the melanomagenesis.

Examined	UV region	Location	Effect	Reference
α-MSH	UVR	Keratinocytes	*MC1R* gene ↑	Scott et al 2002
ET-1	UVR	Keratinocytes	*MC1R* gene ↑	Scott et al 2002
IL-6	UVA, UVB	Epidermoid carcinoma cell line, keratinocytes	↑	Morita 1997a, Kirnbauer et al 1991
IL-8	UVA, UVB	Epidermoid carcinoma cell line, melanocytes	↑	Morita 1997a, Singh et al 2000
$\alpha 3\beta 1$- and $\alpha 6\beta 1$-integrins	UVR	Suprabasal epidermis in nevi	↑	Tronnier et al 1997
E-cadherin	UVB	Keratinocytes, melanocytes, melanoma cells	↓	Seline et al 1996, Jamal and Schneider 2002
P-cadherin	UVB	Melanocytes, melanoma cells	↓	Seline et al 1996
p73, Nupp	UVA	Melanocytes	↑	Zhang and Rosdahl 2003
Id1, p27	UVB	Melanocytes	↑	Zhang and Rosdahl 2003

for melanoma metastasis. UVB-induced keratinocyte-derived ET-1 has been shown to downregulate E-cadherin expression in melanocytes and melanoma cells (Jamal & Schneider 2002). UVR is known to downregulate E and P-cadherin expression directly in both melanocytes and melanoma cells (Seline *et al.* 1996). UVR also upregulates the expression of integrins in the suprabasal layer of the epidermis in the nevus (Tronnier *et al.* 1997). These UV effects on adhesion molecule expression might promote melanoma invasion and metastasis.

Both UVA and UVB radiation upregulate and activate the expression of such proteins in melanocytes that are involved in the progression and metastasis of melanoma. UVA has been shown to enhance the expression of proteins that are associated with cellular growth, vascular invasion and metastasis in the melanoma cell lines (Zhang & Rosdahl 2003). UVB, in turn, increases the expression of proteins involved in cellular proliferation (Zhang & Rosdahl 2003). This suggests that both UVA and UVB might contribute in the melanomagenesis, possibly via separate pathways leading to uncontrolled proliferation and enhanced migration.

Recently published studies postulate an interesting relationship between an overexpression of growth factors and UVB. By combining bFGF, stem cell factor and ET-1 with UVB, invasive melanoma lesions were shown to develop in newborn skin grafts whereas in adult skin, only melanoma in situ lesions were observed (Berking *et al.* 2001; Berking *et al.* 2004). Young skin seems to be very susceptible to the transforming effects of exogenous growth factors when combined with UVR. Noonan *et al.* found that a single high dose of combined UVB and UVA wavelengths was sufficient to initiate melanoma in neonatal (HGF/SF) transgenic mice, but not in adult mice, even after chronic exposure to UVR (Noonan *et al.* 2000; Noonan *et al.* 2001). This result further supports the notion that UVR exposure and sunburn experienced in childhood appear to be a significant risk factor for developing melanoma.

1.2.6.1.2 Melanin and UVA in melanomagenesis

A fair-skinned Caucasian population seems to be more susceptible to developing melanoma than people with darker skin are. This might be due to the lack of photoprotection, but the other possibility could be the different type of melanin, i.e. pheomelanin, synthesized in Caucasian skin (Diffey *et al.* 1995). Certain MC1R gene variants have been associated with red hair, fair skin, freckling as well as with sensitivity to the sun in Northern European and Australian populations (Kadekaro *et al.* 2003; Rouzaud *et al.* 2005). The observation that melanocytes with high pheomelanin vs. eumelanin content are more sensitive to UVR-induced cytotoxicity suggests that pheomelanin might have an important role in skin carcinogenesis (Kadekaro *et al.* 2003; Rouzaud *et al.* 2005). This is further supported by the notion that pheomelanin content has been observed to be higher in the dysplastic nevi when compared to the normal nevus (Jimbow *et al.* 1991).

Pheomelanin is believed to act as a photosensitizing agent that reacts with UVA. UVA has been demonstrated to induce significantly more single strand breaks (Wenczl *et al.* 1998) and DNA damage, like 8-OHdG (Kvam & Tyrrell 1999; Hill & Hill 2000), in heavily pigmented cells when compared to lightly pigmented cells. Moreover, Hill *et al.* demonstrated that although heavily pigmented melanocytes harbored more DNA damage, they were at the same time resistant to UVA-induced cell death (Hill & Hill 2000). However, unpigmented melanocytes have been shown to be much more susceptible to UVA-induced membrane permeability and lipid peroxidation than strongly pigmented cells (Kvam & Dahle 2003). The pheomelanin precursor, 5-S-cysteinyldopa, has been shown to sensitize DNA and to significantly protect melanocytes from membrane damage induced by UVA (Kvam & Dahle 2004; Kvam & Dahle 2005). These results suggest that UVA may be a potential mutagen for cells containing

pheomelanin, causing single strand breaks and oxidative damage. However, pheomelanin may protect cells from membrane damage and cell death. This may enable the survival of severely DNA-damaged cells that possibly further lead to the mutagenesis.

1.2.6.2 Which wavelength is the most effective in melanomagenesis?

Although UVR is the main etiological risk factor for the development of melanoma, the precise wavelengths involved in melanomagenesis are unclear. Epidemiological studies cannot solely identify wavelength dependency and therefore, this information has to be obtained from animal studies. Two animal models, a marsupial and a hybrid fish, have demonstrated the possible role of UVA in melanoma initiation.

The South-American opossum, *Monodelphis domestica*, has been used widely in photodermatological studies because it can repair UV-induced pyrimidine dimers by a visible light-activated photolyase enzyme (Jhappan *et al.* 2003). It is thus far the only non-transgenic animal model in which melanoma has been shown to be inducible by UV radiation alone (Ley 2002). In addition, UVA is capable of inducing the melanoma precursors in this opossum strain after prolonged exposure to broad-spectrum UVA (Ley 1997). However, these precursor lesions do not develop to fully potent malignant melanoma. The limitation of this model is that the genetics of this animal are poorly defined. Moreover, the UV-induced melanocytic precursors are dermal-derived, not epidermal as in humans (Jhappan *et al.* 2003).

The only action spectrum for the development of melanoma has been obtained from studies with hybrid fish, *Xiphophorus*. Setlow *et al.* showed that UVA radiation was capable of inducing melanoma in the hybrid fish after a single, non-erythemogic exposure to monochromatic UVR (Setlow *et al.* 1993). The authors showed that 365 nm would be the most effective wavelength to cause melanoma in the fish. They concluded that if this data is extrapolated to humans, solar UVA causes over 90% of MM incidences (Setlow & Woodhead 1994; Setlow 1999). One problem with this model is its evolutionary distance from mammals. Moreover, tumors do not develop from typical melanocytes and they do not resemble human melanomas (Jhappan *et al.* 2003).

The controversial result has also been published. De Fabo *et al.* showed using the (HGF/SF) transgenic mouse model that only UVB wavelengths were responsible for the induction of MM, whereas UVA was found ineffective, even at high doses (150 kJ/m^2) (De Fabo *et al.* 2004).

1.2.6.3 Sunscreen use as a risk factor

The use of sunscreens has been advocated as an important way of preventing skin cancer. Sunscreens have been shown to protect against photoaging (Harrison *et al.* 1991) and to prevent solar keratosis (Thompson *et al.* 1993; Naylor *et al.* 1995). Sunscreen use is known to prevent DNA damage in humans (Bykov *et al.* 1998; Berne *et al.* 1998; Young *et al.* 2000; Liardet *et al.* 2001) and to abolish the frequency of p53 mutations and NMSC formation in mouse models (Ananthaswamy *et al.* 1997;Ananthaswamy *et al.* 1998;Ananthaswamy *et al.* 1999). These results suggest that sunscreens may protect well against UV(B)-related events, which may lead to non-melanoma skin cancer. Concerning MM, the effect of sunscreens is less clear. One study demonstrated that sunscreen with UVA and UVB filters did not prevent melanoma outgrowth in a mouse transplantation model after a minimal effective dose of UVB, although it completely inhibited erythema and reduced the formation of sunburn cells (Wolf *et al.* 1994). This postulates the notion that the protection of skin from erythema formation does not necessarily imply the prevention of other effects of UVR, such as immunosuppression.

Sunscreens have failed to offer the proper protection against immunosuppression (Fourtanier *et al.* 2000; Nghiem *et al.* 2001; Moyal & Fourtanier 2002; Moyal & Fourtanier 2003). The immunoprotection factor (IPF) of sunscreen, determined using CHS assay after a single dose of SSR, was demonstrated to be about 50% less that the sun protection factor (SPF) (Kelly *et al.* 2003). Poon *et al.* obtained similar results by showing that the IPF does not correlate with the SPF but with the UVA protective capability of the sunscreens (Poon *et al.* 2003). The SPF label in sunscreens illustrates mainly its UVB protection capability because the SPF rating system is based on the prevention of erythema. However, this does not adequately assess UVA protection since UVA is less erythemogenic than immunogenic. It has been suggested that sunscreens, instead of using only SPF, should be rated according to their immune protective capability (IPF) in order to provide a better indication of their protection against UVA radiation (Poon *et al.* 2003). Moreover, sunscreen with high SPF has been shown to fail to protect properly against the production of UVA-induced free radicals during sunbathing (Haywood *et al.* 2003). The authors have shown that the "free-radical factor" based on their results was only 2 whereas the SPF, when measured using erythema as an end-point, was >20. Taken together, the results presented above suggest that SPF is insufficient in predicting the ability of sunscreen to protect against UVA-induced effects.

Several epidemiological studies have been conducted to clarify whether sunscreen is a risk factor for developing melanoma (Wang *et al.* 2001). Some studies have shown a positive correlation between the use of sunscreen and the incidence of melanoma (Autier *et al.* 1994a; Westerdahl *et al.* 1995; Wolf *et al.*

1998), whereas other studies have found no association with the use of sunscreen (Huncharek & Kupelnick 2002).

1.2.6.4 Sun bed use as a risk factor

During a cosmetic indoor tanning session, people submit themselves to a large quantity of UVA exposure. Artificial tanning in solaria has been linked to the development of melanoma (Westerdahl *et al.* 1994; Autier *et al.* 1994a; Westerdahl *et al.* 2000), but epidemiological studies do not provide unequivocal data about the association (Swerdlow & Weinstock 1998; Wang *et al.* 2001; Young 2004). The methodological limitations of retrospective case-control studies make it difficult to make definitive conclusions: (i) the assessment of exposure is based on questionnaires concerning personal sun bed use and recall bias may lead to overestimates, especially among those participants that developed skin cancer; ii) exposure in a solarium is difficult to distinguish from exposure from natural sources; iii) bias is caused by the lack of information on the spectral output of the lamps used in tanning devices (Wang *et al.* 2001). A recent Scandinavian prospective cohort study shows the strongest evidence thus far for a causal relationship between sun bed use and MM (Veierod *et al.* 2003). A total of 106,379 women in Sweden and Norway were monitored for an average of 8.1 years. There was an increased risk of MM with an OR of 2.58 (95% CI, 1.48-4.50) among females in the 20-29 year age group who used a sun bed more than once a month (Veierod *et al.* 2003). This result supported the previous notion that adolescence and early adulthood appear to be the most sensitive age periods for UVR exposure, either from natural or artificial sources. Melanoma is also associated with a high rate of mortality. Diffey has estimated that the current use of tanning devices in the UK results in ~6% of deaths per year (Diffey 2003). Taken together, the epidemiological data suggest a possible positive relationship between the use of solaria and the increased risk of melanoma, but the data are not conclusive and the additional studies are needed.

2. Aim of the present study

A number of studies have demonstrated that UVA radiation is capable of damaging DNA, causing mutations, promoting carcinogenesis and it participates in the pathogenesis of squamous cell cancer and possibly in cutaneous melanoma. However, the effect of UVA on melanoma metastasis has not been studied.

UVA radiation can modulate various cellular processes, some of which might affect tumor metastasis and enhance the metastatic potential of melanoma cells. Considering the depth of UVA penetration and its effects on the expression of adhesion molecules, cytokines and immunosuppression the working hypothesis in this study was that UVA might alter the adhesive properties of tumor cells present in blood circulation in skin capillaries and cause them to become more adhesive to endothelium and subsequently to form more metastases (Figure 4.)

Using C57BL/6-derived mouse melanoma cell lines B16-F1 and B16-F10 and the syngeneic endothelial MS1 cell line, *in vitro* experiments were executed to study the effect of UVA on melanoma cell adhesiveness. These results are described and discussed in Publication II. In Publications I and III, the main emphasis was on the induction of the metastasis *in vivo* C57BL/6 mouse model. This was used to determine the physiological relevance of UVA as an enhancer of the metastatic potential of melanoma cells and to study the mechanisms behind the phenomenon. Finally, studies were carried out on the effects of UVA on gene expression in the B16-F1 mouse melanoma cell line; those results are discussed in Publication IV.

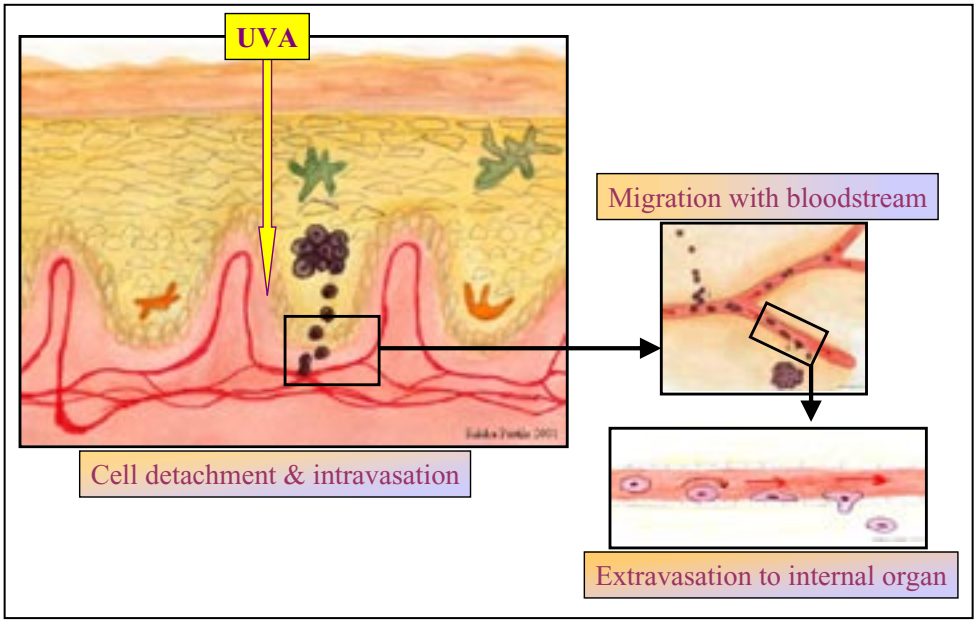

Figure 4. Considering the depth of UVA penetration, the working hypothesis in this study was that UVA might alter the adhesive properties of tumor cells present in skin capillaries and cause them to become more adhesive to endothelium and subsequently, to form more metastases.

3. Materials and methods

The experimental methods used in the present study are listed in Table 8. Detailed information and descriptions are found in the enclosed original publications and the references therein. Brief descriptions of the methods are presented below.

Table 8. Methods used in the present study

Methods	Publication
Cell culture	I, II, III, VI
UV dosimetry *in vitro*	II, III, IV
UV dosimetry *in vivo*	I, III
Cell viability	II
Adhesion assay between melanoma and endothelium	II
Aggregation assay between melanoma cells	II
Analysis of cadherins	II
Animals and *i.v.* injections	I, III
CHS assay	III
cDNA array	IV
Western blot analysis of cyclin G	IV
Cell cycle analysis	IV
The UVA transmittance through mice skin	unpublished

3.1 UVA radiation source and dosimetry

A facial tanner lamp, Philips HB 171/A was used as the radiation source. UVB radiation was filtered with a 5-mm thick glass filter. The irradiances were measured at the same distance from the lamp as in the actual *in vitro* and *in vivo* UV-exposures. The spectral irradiances were measured with a temperature stabilized Optronic and Bentham DM150 double-monochromator spectroradiometer at 0.5 nm intervals from 250 nm to 400 nm. The spectroradiometer was calibrated against a 1000 W halogen standard lamp traceable to the National Institute of Standards and Technology. The spectroradiometer measurement uncertainty was estimated at ± 8%.

The attenuation of the irradiance caused by the UVB glass filter in the *in vivo* experiment or by the UVB filter, dish cover and the culture medium in the *in vitro* experiments was taken into account when measuring the irradiance. The UV irradiance that reached the mice or the melanoma cells was 3.5-4.2 mW/cm^2. The UV spectrum used throughout this study was 310-400 nm, from which the UVA portion was 99.99% and UV-B was 0.01%. When the other error sources

during the exposure, e.g. the radiation distribution, distance, the exposure timing, lamp drift during individual exposure and the whole experiment, are taken into account, the uncertainty of the exposure dose was estimated at ± 12% for *in vitro* experiments and ±14% for the *in vivo* experiments.

3.2 Cell culture

These studies utilized C57BL/6 mice-derived melanoma cell lines B16-F1 (low metastatic potential) and B16-F10 (high metastatic potential) as well as the C57BL/6 mice-derived MS-1 endothelial cell line. The melanoma cell lines were grown in an RPMI-1640 cell culture medium and the MS-1 endothelial cell line in Dulbecco's MEM cell culture medium. The cell culture media were supplemented with 10% heat-inactivated FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml), and L-glutamine (4 mM). All cell culture supplies were purchased from Gibco BRL, Paisley, UK.

3.3 *In vitro* experimental set-up

3.3.1 *In vitro* irradiations

The melanoma cells were irradiated in plastic Petri dishes through a 5-mm glass filter, the dish cover and culture medium. The B16-F1 and B16-F10 melanoma cells were exposed to a single dose of UVA at 2, 4, 8, and 12 J/cm^2. In some experiments, cells were irradiated with four UVA doses of 2 J/cm^2 with 1-hour intervals between each exposure. All irradiations were performed at room temperature in a dark room on a black support in order to avoid the effects of reflected radiation. The temperature of the cell culture medium did not exceed 37ºC during irradiation. The control cells were sham-treated by keeping them at room temperature in a dark room.

3.3.2 Cell viability and cell morphology assessment

The melanoma cells, both those adhering to the bottom of the culture dishes as well as these floating in the culture medium, were harvested with versene solution 1, 4, and 24 hours after the end of UVA irradiation. Thereafter, the melanoma cells were washed once with PBS and suspended in PBS. Cell viability was assessed using the trypan blue exclusion method. Cell morphology and apoptosis were examined using cytospin cell smears stained with the standard May-Grünwald-Giemsa (MGG) method.

3.3.3 Melanoma-endothelium binding assay

MS-1 endothelial cells were grown in flat-bottomed 96-well plates that were coated overnight at 4ºC with bovine collagen. Confluent endothelial monolayers formed within 1 day after seeding and they were used in the tumor cell attachment assay. B16-F1 and B16-F10 melanoma cells used in the binding assay were metabolically labeled with ^{3}H-thymidine for 48 hours. The ^{3}H-thymidine labeled the semi-confluent monolayers of B16-F1 and B16-F10 melanoma cells were exposed to a single dose of UVA at 2, 4, 8, and 12 J/cm^2. The melanoma cells were harvested with versene at 1, 4 or 24 hours after the end of irradiation. The adhesion assay between UVA-exposed melanoma cells and endothelial monolayers was performed using the method of Pauli and Lee (Pauli & Lee 1988). Microtiter plates were then incubated for 30 minutes at 37ºC to allow binding between both cell types. At the end of incubation, the unattached melanoma cells were removed by gentle centrifugation. The cells that remained in the microwells (endothelial monolayers with bound ^{3}H-thymidine-labeled melanoma cells) were solubilized overnight at 37ºC with NaOH. The solubilized cell suspension was mixed with 1 ml of scintillation fluid and the ^{3}H content was measured in an LKB 1210 Ultrobeta scintillation counter. Each adhesion experiment was made in 8-12 separate microwells (replicates).

3.3.4 Melanoma-melanoma aggregation assay

To examine the homotypic aggregation of the B16-F1 and B16-F10 melanoma cells, the semi-confluent monolayers of the B16-F1 and B16-F10 cells were exposed to a single UVA dose at 8 J/cm^2 or to fractionated dose of 4 x 2 J/cm^2. Cells were harvested using versene at 1, 4, and 24-h after the end of irradiation, washed twice with culture medium and re-suspended in culture medium at a concentration of 1x10^6 cells/ml. Aliquots of 500 µl/well of a single-cell-suspension were placed in 24-well plate and incubated for 60 minutes at 37ºC on a rotary shaker slowly rotating at 78 rpm. Thereafter, the cells were gently aspirated from the wells and number of single cells and cell aggregates were counted in microscope using haemocytometer.

3.3.5 Flow cytometry analysis of cadherin expression

For the flow cytometry analysis of cadherins expression, the semi-confluent monolayers of the B16-F1 and B16-F10 cells were exposed to a single UVA dose at 8 J/cm^2. The expression of N and E-cadherin was determined at 1, 4 or 24-h after irradiation using polyclonal goat anti-mouse antibodies. The melanoma cells were harvested with versene and incubated with the appropriate anti-cadherin

antibody solution for 1h. Thereafter, the cells were washed and incubated for 30 min on ice with fluorescein-conjugated donkey anti-goat IgG second antibody. Following this, the cells were washed once with cold PBS and fixed with formalin. The cadherin expression was analyzed by flowcytometer.

3.4 *In vivo* experimental set up

3.4.1 Animals

Female C57BL/6 (C57BL/6JOlaHSd) mice, with Specific Pathogen Free (SPF) status according to Felasa Health Monitoring Guidelines, were purchased from Harlan, The Netherlands, and housed in Viikki Laboratory Animal Center, University of Helsinki, Finland. The ethical evaluations of the experiments were reviewed and approved by the Institutional Animal Use and Care Committee of the University of Helsinki and the State Provincial Veterinarian Office of Southern Finland. The care, welfare and use of the animals were in accordance with national, institutional and European guidelines. The mice were at 8–10 weeks of age at the beginning of the experiments. The mice were housed and arranged for the experiments to groups of five.

3.4.2 *In vivo* UVA irradiation of mice

Hair on the abdomens of the mice was shaven off to allow UVA irradiation of the skin. A suspension of 50,000 of B16-F1 or B16-F10 cells in 0.2 ml of saline was *i.v.* injected into the tail vein of anesthetized C57BL/6 mice (Figure 5).

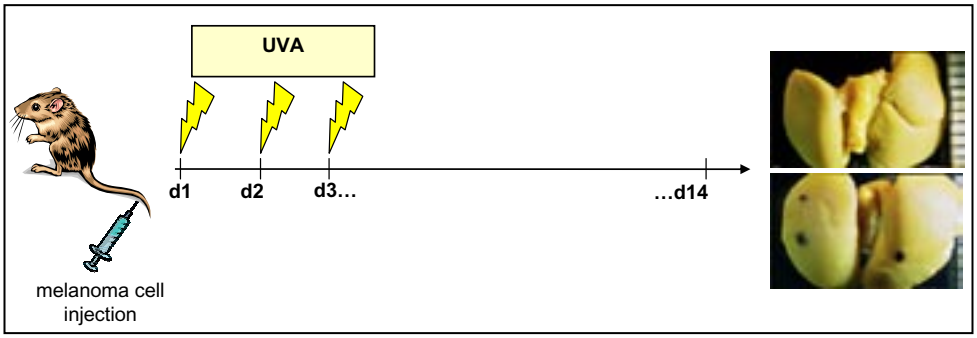

Figure 5. C57B/6 mice were injected with 50.000 non-exposed B16 melanoma cells *i.v.* into the tail vein, after which the mice were UVA exposed with single dose of 8J/cm^2 or 3x8J/cm^2. Their lungs were collected and examined 14 days after the melanoma cell injection

Immediately following the B16-F1 melanoma cell injection, the abdominal side of the mice was exposed to a single UVA dose at 8 J/cm^2. Some of the animals were exposed to two more UVA doses (8 J/cm^2) on two consecutive post-injection days. The non-exposed, anesthetized control mice groups were injected with B16-F1 or B16-F10 cell lines. The mice were terminated 14 days after the melanoma injection and their lungs were removed and fixed in Bouin's solution, after which the tumor colonization in their lungs was evaluated.

3.4.3 *In vitro* UVA irradiation of B16-F1 cells before *i.v.* injection

The B16-F1 melanoma cells were exposed to a single UVA dose of 8 J/cm^2 and the irradiation was performed at room temperature in a dark room on a black support in order to avoid the effects of reflected radiation. The non-irradiated control B16-F1 and B16-F10 cells were sham-treated by keeping them at room temperature in a dark room for the irradiation time. After UVA exposure or sham treatment, a suspension of 50,000 of B16-F1 or B16-F10 cells in 0.2 ml of saline was *i.v.* injected into the tail vein of anesthetized C57BL/6 mice (Figure 6). The mice were terminated 14 days after injection and their lungs were removed and fixed in Bouin's solution, after which the tumor colonization in their lungs was evaluated.

3.4.4 Evaluation of the metastases

The quantitative and qualitative evaluation of the lung metastases was performed under a dissecting microscope. The metastases visible on the lung surface were counted and scored according to their size, color, growth pattern

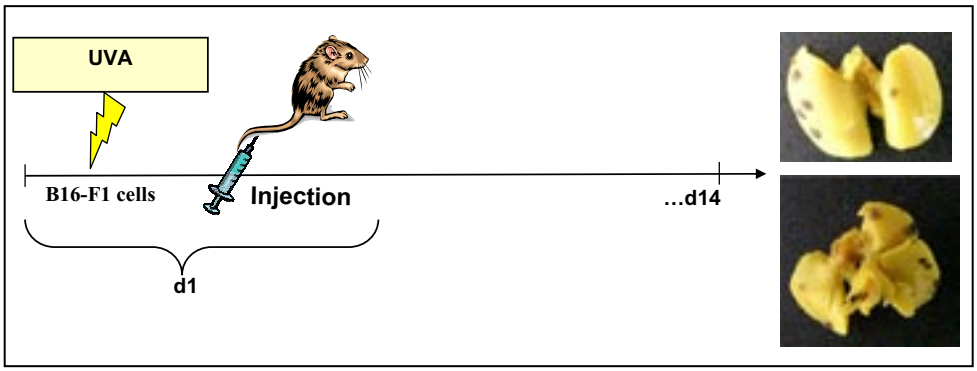

Figure 6. B16-F1 cells were irradiated *in vitro* with an UVA dose of 8 J/cm^2, after which 50.000 UVA-exposed melanoma cells were *i.v.* injected into the tail vein of C57BL/6 mice. Their lungs were collected and examined 14 days after the melanoma cell injection.

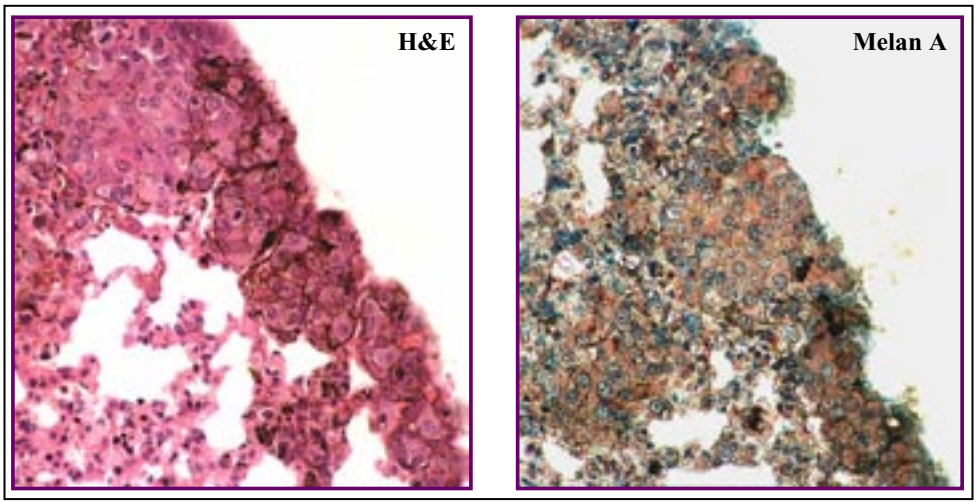

Figure 7. The cross sections of lungs were stained with H&E to determine lung tissue morphology and the appearance of metastases. H&E staining revealed that the vast majority of metastases were located close to the lung surface and only very rarely in the lung parenchyma. The majority of metastases were filled with melanin droplets (seen in black). The cross sections were immunostained with antibody against Melan-A to confirm that the tumor metastases were indeed of melanoma origin.

and uniformity. After quantitative and qualitative evaluation, the lungs were embedded in paraffin, cut into 5µ-sections and stained for histological analysis.

3.4.5 Immunohistochemistry

After quantitative and qualitative evaluation, the lungs were embedded in paraffin, cut into 5µ-sections and stained with hematoxyline-eosine in a standard fashion in order to evaluate tissue morphology and to examine for the presence of invisible micrometastases in tissue parenchyma under a dissecting microscope (Figure 7.) To confirm the melanoma origin of the metastases, Melan-A (Chen *et al.* 1996), a marker antigen of melanoma cells was detected using indirect immunohistochemistry as well as the expression levels of cadherin N and E in order to determine the cadherin profile of the metastases (Figure 8.). Paraffin sections were immunoassayed with goat polyclonal primary antibodies against Melan-A, E-cadherin and N-cadherin using horseradish peroxidase-conjugated secondary and third antibodies and an AEC-chromogen staining kit.

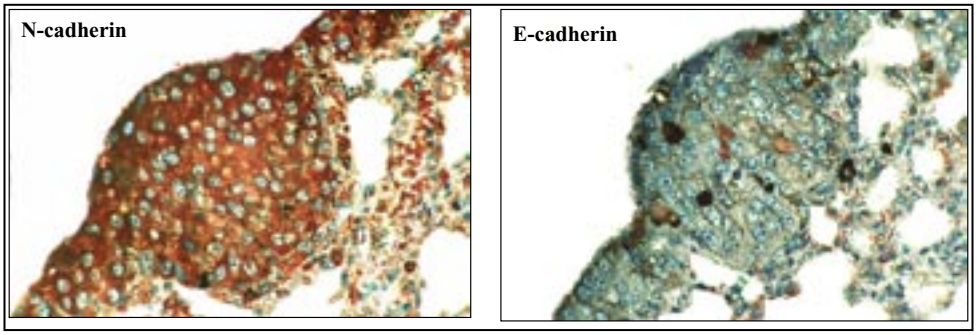

Figure 8. The cross sections of lungs were immunostained with antibodies against E-cadherin and N-cadherin. The cadherin expression profile showed that the lung metastases were strongly N-cadherin positive whereas E-cadherin staining remained weaker.

3.4.6 Contact hypersensitivity (CHS) assay

UVA-induced systemic immunosuppression was studied using contact hypersensitivity assay according to the same irradiation protocol and doses of UVA (8 J/cm^2 and 3x8 J/cm^2) as in the previous *in vivo* experiments. The mice that were irradiated on the shaved abdomen to one UVA dose of 8 J/cm^2 belonged to groups A1 and C1, and the mice that were irradiated to three UVA doses of 8 J/cm^2 applied in consecutive days (3x8 J/cm^2) belonged to groups A2 and C2. Groups C1 and C2 formed the irritant controls for groups A1 and A2. The ears of the UVA-exposed mice were covered with a "cap" made from black plastic and insulation tape in order to avoid UV-mediated alterations in CHS response when challenging ears 10 days later (Reeve 2002). The non-exposed, anesthetized, positive control groups for CHS formation were designated B1 and B2 and the irritant control groups for these mice were designed D1 and D2. Three days after the last UVA dose, 2% oxazolone was applied under light anesthesia as a sensitizer (100 µl, corresponding 2 mg of oxazolone) to the shaved back of groups A1, A2, B1 and B2. The challenging dose of the sensitizer (10 µl, corresponding 20 µg of oxazolone) was applied under light anesthesia to both ear pinna 7 days after sensitization for all groups. The thickness of the ears was measured before application and 24h later by using the springmicrometer. The figure for ear swelling for individual mice was calculated from both ears by subtracting the original thickness from the challenged ear thickness and taking the average from these two values. The UVA induced suppression was calculated according to the formula below, where A, B, C, and D were the average swelling figures for the respective animal groups 24 hours after challenge (Laihia & Jansen 1994):
1- [(A-C)/(B-D)] x 100%

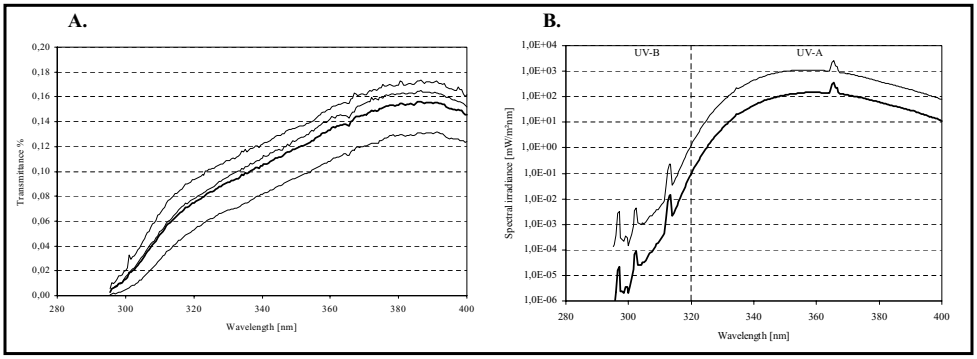

Figure 9. A) The spectral measurements of three mice skins were performed within 30 minutes after skin removal. The relative transmittance varied in the samples from 5–15% in the UVA region. The average transmittance from the three measurements is indicated by a thick line. **B)** The spectral transmittance of the in vivo study was determined from the average value of the relative transmittance. The spectral irradiance of the Philips face solaria is indicated with a thin line and the transmitted spectral irradiance is indicated with a thick line.

3.4.7 The relative transmittance of UVA through mice skin

Skin transmittance was measured using the Bentham DM 150 spectroradiometer (Bentham Instruments Ltd., England). A Philips HP 3136 sun lamp was used as a light source. Although the irradiance of the Philips HP 3136 sun lamp is higher when compared with the Philips HB 171/A face solaria used in the animal experiments, the relative transmission through mice skin remains the same.

The abdominal hair of the mice was shaven, the mice were sacrificed and their skin was removed. The abdominal location and the size of removed skin were identical to that used in the *in vivo* mice experiments. The transmittance was measured within 30 minutes after skin removal. The lamp spectrum was first measured without the mouse skin, after which spectral measurements were performed on three mice skins. The relative transmittance was calculated by dividing the spectrum of mice skin by the spectrum without the skin (Figure 9A). Finally, the spectral irradiance transmitted through the mouse skin during *in vivo* exposures was determined by multiplying (weighting) the spectrum of relative spectral transmission with the spectrum of Philips HB 171/A face solaria (Figure 9B). This spectrum depicts the true spectral transmittance during the animal experiments.

3.5 Gene expression analysis

3.5.1 Differentially expressed genes

Large-scale screening was performed using Atlas™ complementary (cDNA) mouse cancer 1.2 array (Clonetech Laboratories, Palo Alto, CA). The B16-F1 melanoma cells were exposed to a single UVA dose of 8 J/cm^2 and the irradiation was performed at room temperature in a dark room on a black support in order to avoid the effects of reflected radiation. The non-irradiated control B16-F1 cells were sham-treated by keeping them at room temperature in a dark room for the irradiation time.

RNA was isolated 4 hours after the end of UVA exposure. The cells were collected by brief trypsinization and washed twice with ice cold PBS. The total RNA was isolated from melanoma cells using Nucleospin® RNA II kits (Clonetech Laboratories, Palo Alto, CA) and the RNA concentrations were determined spectrophotometrically. The poly A+ RNA enrichment of 50-µg total RNA and ^{32}P-labeled cDNA probe synthesis made by reverse transcription were performed according to the Atlas™ Pure Total RNA Labeling System (Clonetech Laboratories, Palo Alto, CA). Precisely the same amounts of ^{32}P-labeled cDNA from control and UVA exposed melanoma cells were used as a probe in Atlas™ Mouse Cancer 1.2 cDNA expression arrays containing 1176 tumor related genes immobilized on a nylon membrane. Hybridization and washing procedures were performed according to the recommendations of the manufacturer. The x-ray film was exposed at -70ºC simultaneously to the membrane hybridized with a control probe and to the membrane hybridized with a UVA exposed probe.

The hybridization signals on the autoradiograms were scanned using a GS-710 Calibrating Imaging Densitometer and the intensity of the gene spots was analyzed with AtlasImage 2.0 Software. The analysis of the membranes was performed according to the manufacturer's instructions. The ratio of two corresponding gene spots between control and UVA exposed spots was calculated by dividing the intensity of the UVA exposed gene by the intensity of the non-exposed control gene. To select the genes with altered expression level, the significance of upregulation was set at ratio ≥ 1.7 and downregulation at ratio ≤ 0.6. Subsequently, the existence of the spots on the film was verified visually. The student t-test was performed on selected genes in order to calculate statistical significance.

3.5.2 Western blot analysis

The B16-F1 melanoma cells were exposed to the single UVA dose of 8 J/cm^2 at room temperature. The non-irradiated control B16-F1 cells were sham-treated by keeping them at room temperature for the irradiation time. The B16-F1 melanoma cells were harvested by versene immediately after the end of UVA exposure and thereafter at 1-6, 8 and 10 h time-points. The melanoma cells were washed with PBS and lysed with 2.5% sodium dodecylsulfate supplemented with 1% proteinase inhibitor cocktail. The protein concentration was measured according to Lowry (Lowry *et al.* 1951). Samples containing 20 µg protein per lane were resolved using 7.5% SDS-PAGE gel and blotted on PVDF membranes. The membranes were blocked in room temperature for one hour in a 2% blocking solution, followed by overnight incubation first in an anti-cyclin G antibody solution at +4ºC. Thereafter, the membranes were washed and incubated for 1 h in a secondary antibody solution of horseradishperoxidase-conjucated anti-mouse immunoglobulin G at room temperature. The membranes were washed with PBS and the chemiluminiscence signal was detected by exposing an x-ray film to the membrane. Autoradiograms were scanned by GS-710 Calibrating Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA) and the intensity of protein bands was analyzed by Phoretix analysis software (1D v2003.01) from three different experiments. The student t-test was performed to calculate the statistical significance of the change.

3.5.3 Cell cycle analysis

The cell cycle was studied by examining the DNA content of the B16-F1 cells by using the propidium iodide staining method (Leszczynski *et al.* 1995). The B16-F1 melanoma cells were exposed to the single UVA dose of 8 J/cm^2 at room temperature. The non-irradiated control B16-F1 cells were sham-treated by keeping them at room temperature for the irradiation time. B16-F1 melanoma cells were collected either immediately after the end of UVA exposure, or at the same time-points thereafter than Western blot samples, by brief trypsinization. Cells were washed twice with cold PBS, and fixed in methanol. After fixation, the melanoma cells were washed twice with cold PBS followed by incubation in an RNAse solution in PBS (100 units/ml) for 30 minutes at 37ºC. The melanoma cells were incubated in propidium iodide solution in PBS (10 µg/ml) overnight at +4ºC. The fluorescence was measured using FACScan flow cytometry (Becton Dickinson, USA) and analyzed with the ModFitLT V3.1 (PMac) cell cycle analysis application (Becton Dickinson, USA). The student t-test was performed to calculate the statistical significance of the change.

4. Results

The main results of this study are presented and discussed in Publications I–IV. Here, a summary of the results is presented. The numbers (I–IV) located in the titles refers to the publication where detailed descriptions are found. Additional data from the transmittance of mice skin is included.

4.1 UVA effects on melanoma adhesiveness *in vitro* (II)

To determine whether UVA affects tumor cell adhesiveness to endothelium, the binding assays were executed between non-irradiated endothelial monolayer and UVA exposed melanoma cells. The single doses of UVA at 2 J/cm^2 and 4 J/cm^2 caused small, statistically non-significant, changes in cell adhesion at tested time-points in both cell lines. A UVA dose of 8 J/cm^2 affected adhesiveness 24 hours after irradiation. At that time, the adhesiveness of the B16-F1 cells increased by 88% ($P << 0.001$) whereas the adhesiveness of the B16-F10 cells increased only by 28% ($P< 0.05$) The highest used dose of UVA (12 J/cm^2) caused 25% ($P< 0.05$) increase in B16-F1 adhesiveness already 4 hours after exposure. At this time point, there were no detectable changes in the adhesiveness of the B16-F10 cells. Later, 24 hours after the irradiation, the adhesiveness of both cell lines increased by 32% (B16-F1, $P< 0.005$) and 55% (B16-F10, $P<< 0.001$). The highest used UVA dose (12 J/cm^2) appeared to be less effective than the lower dose (8 J/cm^2).

To study whether UVA exposure induces melanoma cell apoptosis, which could affect the adhesiveness of melanoma cells and thus altering the outcome of the adhesion assay, the morphology of the B16-F1 and B16-F10 melanoma cells exposed to UVA was examined. Cell viability and apoptosis were examined at 1, 4 and 24 h after UVA exposure at doses of 2, 4, 8 and 12 J/cm^2. Neither cell viability nor apoptosis were affected by UVA doses up to 8 J/cm^2. However, the highest used dose (12 J/cm^2) caused up to 20.1% of cell death among the highly metastatic B16-F10, but not among the low-metastatic B16-F1 cells.

UVA dose of 8 J/cm^2 split into four fractions of 2 J/cm^2 with three one-hour intervals between exposures increased the adhesiveness of the low-metastatic B16-F1 cells by 149% ($P<< 0.001$) already one hour after the end of the last of 2 J/cm^2 exposures. Thereafter, the adhesiveness of the B16-F1 declined and 24 hours after the exposure the increase in adhesiveness was only 64.1% ($P< 0.01$). In the highly metastatic B16-F10 cell line, repetitive exposure protocol increased adhesiveness by 132% ($P << 0.001$), but the effect was observed only 24 hours after the end of the last exposure. The UVA effect on melanoma-melanoma

adhesion (aggregation) was examined using a UVA dose of 8 J/cm^2, which was found to be the most efficient by inducting the melanoma adhesion to endothelium. The UVA caused almost 60% ($P< 0.05$) decline in B16-F1 and B16-F10 aggregation, which was observed 1 hour after exposure. This effect was no longer statistically relevant at the 4-h time-point, although the tendency to declined aggregation remained. Exposing of cells to 8 J/cm^2, but delivered as 4 doses of 2 J/cm^2 that were separated by 1-h intervals, had a weaker effect and caused decline in the B16-F1 and B16-F10 aggregation only by ca. 35% ($P< 0.05$ and P= n.s., respectively). The tendency of decline in cell aggregation caused by the fractionated-dose-exposure was also observed 4 hours after exposure, but the results were not statistically relevant.

To examine the effect of UVA exposure on the expression of cadherins, two separate pilot experiments were performed to determine the expression of N and E-cadherin on the surface. The expression level of N-cadherin was increased by UVA in the B16-F1 cells at 1-h and 4-h time-points in both experiments. The E-cadherin expression was downregulated in the B16-F1 cells at the 1-h time-point in both experiments. In the highly metastatic B16-F10 cells, the UVA caused no significant changes in E-cadherin expression, but the N-cadherin expression slightly increased 24 hours after UVA exposure.

Table 9. Metastases formation in mice lungs after *in vivo* irradiation of mice

	Mice	Tumors	Score
F1	10	**2**	15
F10	10	**25**	274
F1 + UVA	10	**27**	258
F1 + 3xUVA	10	**27**	170

	Mice	Tumors	Score
F1	10	**27**	259
F10	10	**70**	511
F1 + UVA	10	**117**	978
F1 + 3xUVA	10	**72**	519

F1 / F10 = i.v. 50,000 cells/animal; UVA = 8J/cm^2
UVA only or NaCl only did not induce spontaneous metastases

4.2 UVA effects on the metastatic potential melanoma *in vivo* (I, III)

The UVA effects on melanoma metastasis were studied using C57BL/6 mice and syngenic C57BL/6-derived B16-F1 and B16-F10 melanoma cell lines. The outcome of the two animal experiments is summarized in Table 9. The control mice injected with saline or exposed to UVA, but not injected with melanoma cells, developed no spontaneous metastases, as expected. The injection of low-metastatic potential B16-F1 without UVA-irradiation led to the development of a smaller number of metastases when compared to the positive control animals, which were injected with high-metastatic B16-F10 cells. Animals injected with low-metastatic B16-F1 cells and irradiated with a single dose of UVA (8 J/cm^2) developed 12-fold and 4.3-fold more pulmonary metastases in comparison with the non-exposed control mice (Table 9). The exposure of mice injected with B16-F1 to three consecutive doses of UVA (8 J/cm^2 each) caused no additional increase in melanoma metastasis. Interestingly, the score obtained for the metastases from animals exposed to three doses of UVA was lower than the score obtained for the animals exposed to a single dose of UVA.

Histological evaluation of haematoxylin-eosin stained tissue sections as well as immunohistochemical analysis of the expression of the Melan-A/MART-1 antigen confirmed that the metastatic nodules appearing in the lungs were of melanoma origin (Figure 7). The expression level of cadherin E and N-cadherin was determined to show that lung metastases were strongly N-cadherin positive, whereas E-cadherin staining remained weaker (Figure 8).

The transmittance of the UVA wavelengths in mice skin was measured in order to determine whether UVA reaches the dermis and thus, presumably the circulating melanoma cells in the capillary network. According to the measurements, the transmission varied from 5% to 15% in the UVA wavelengths used in this study (Figure 9).

To study the direct effect of UVA on the pulmonary colonization capacity of melanoma, B16-F1 cells were exposed *in vitro*, whereas the control cells were sham-treated. Animals injected with control B16-F1 cells again developed fewer metastases when compared to the animals injected with the positive control B16-F10 cells (Table 10). The *in vitro* UVA exposure applied directly on B16-F1 melanoma prior to the injection into mice increased the metastatic capacity of melanoma cells 1.5-fold.

In the positive control group for CHS induction, the mean ear swelling responses were 31.2 x 10^{-2} mm (Table 11). When the C57BL/6 mice were irradiated with 8 J/cm^2 on the abdomen, the mean ear swelling responses were 26.9 x 10^{-2} mm, representing a 13.8% decline in ear swelling when compared to

Table 10. Metastases formation in mice lungs after *in vitro* irradiation of B16-F1 melanoma cells.

	Mice	Tumors	Score
F1	10	**22**	157
F10	10	**82**	515
F1 + UVA	10	**34**	202
F1 + 3xUVA	-	**-**	-

Table 11. Suppression of oxazolone induced CHS in C57BL/6 mice (10 mice per group).

Group	*UVA 8 J/cm²*	*Sens*	Ear swelling ± SD 10^{-2} mm
A	+	+	39.1 ± 4.4
B	-	+	42.9 ± 5.3
C	+	-	12.2 ± 3.3
D	-	-	11.7 ± 4.0

Group	*UVA 3x8J/cm²*	*Sens*	Ear swelling ± SD 10^{-2} mm
A	+	+	38.1 ± 5.6
B	-	+	40.2 ± 6.0
C	+	-	9.7 ± 3.5
D	-	-	11.9 ± 2.9

Mean ear swelling (A-C) 39.1 - 12.2 = 26.9
Mean ear swelling (B-D) 42.9 - 11.7 = 31.2

Mean ear swelling (A-C) 38.1- 9.7 = 28.4
Mean ear swelling (B-D) 40.2-11.9 = 28.3

A: UVA-irradiated abdomen, sensitized back and challenged ear
B: Sensitized back, challenged ear
C,D: Irritant controls

the non-UVA-exposed group (Table 11). Irradiation of mice with three consecutive UVA doses did not induce decline in CHS induction as shown in Table 11.

4.3 Transcriptional analysis of UVA affected genes (IV)

Gene expression analysis was performed in order to examine UVA-induced changes on the transcriptional level in B16-F1 mouse melanoma 4 hours after UVA irradiation. The cDNA study revealed that a physiologically relevant UVA dose induced nine differentially expressed genes in the melanoma cells exposed to UVA when compared to the unexposed control cells (Table 12). Seven genes

Table 12. Differentially expressed genes after UVA dose of 8 J/cm^2

Gene family	No. of arrays[a]	Control[b] ± SD	UVA exposed[c] ± SD	t-test	Ratio[d]
Stress induced					
Heat shock 86-kDa protein (HSP86; HSP90α)	3 ↑	0,32 ± 0,23	1,04 ± 0,44	0,43	3,23
Heat shock cognate 71-kDa (HSC70; HSP73)	2 ↑ 1 ↓	0,15 ± 0,12	0,27 ± 0,18	0,39	1,81
Alpha crystallin B-subunit	3 ↑	0,07 ± 0,03	0,21± 0,07	0,05	3,26
Oxidative Stress					
Oxidative stress-induced protein	2 ↑ 1 ↓	0,16 ± 0,14	0,31± 0,15	0,27	1,92
glutathione S-transferase mu2	3 ↑ 1 ↓	0,07 ± 0,04	0,14 ± 0,09	0,23	2,09
Cell cycle control					
cyclin G	5 ↑	0,13 ± 0,04	0,65 ± 0,36	0,03	4,85
Angiogenesis					
Vascular endothelial growth factor (VEGF)	4 ↑	0,07 ± 0,03	0,22 ± 0,08	0,03	2,94
Skeleton & Motility proteins					
G-actin cytoplasmic	3 ↓	1,02 ± 0,37	0,47 ± 0,18	0,10	0,47
non-muscle cofilin 1	3 ↓	0,40 ± 0,10	0,17 ± 0,01	0,05	0,41

[a] The number of arrays (out of five) in which differences in the gene expression were observed.; The arrows indicate the upregulation or downregulation of the gene. In the remaining arrays no difference was observed.

[b] The average of the normalized gene intensity in control cells ± standard deviation;

[c] The average of the normalized gene intensity in UVA exposed cells ± standard deviation.

[d] Ratio of UVA exposed genes versus control genes.

out of nine were upregulated, involving stress response (HSC70, HSP90α/HSP86, α-B-crystallin), oxidative stress (GST mu2, Oxidative stress induced protein), angiogenesis (VEGF), and cell cycle regulation (cyclin G). However, two genes out of nine were downregulated, involving cell motility (G-actin, non-muscle cofilin).

Cyclin G was examined further, since it was the most UVA-affected gene. The protein expression of cyclin G in B16-F1 melanoma cells was examined immediately after the end of exposure and at different time-points thereafter. In spite of the statistically significant almost 5-fold upregulation in the gene expression, the cyclin G protein expression was only moderately affected by

UVA. There was a 1.36-fold increase in the cyclin G expression at 6h time point; however, this was statistically non-significant.

The cyclin G functions in the nucleus as the cell cycle regulator. Thus, the effect of UVA the cell cycle arrest in G2/M restriction point in B16-F1 cell line was determined at the time-points where the protein analysis was performed. The UVA exposure induced cell cycle arrest, beginning at the 4-h time-point. The observed G2/M arrest has increased in a time- dependent manner, peaking at 8-h time-point ($p<0.05$), after which it somewhat declined.

5. Discussion

5.1 The UVA effect in melanoma adhesiveness *in vitro*

This study demonstrated that UVA induced alterations in the adhesiveness of mouse melanoma cell lines B16-F1 and B16-F10 and the syngeneic endothelial MS1 cell line. This *in vitro* study shows for the first time that melanoma cells became less "sticky" among themselves but become more adhesive to the endothelial monolayer after UVA irradiation. A single dose of UVA was shown to induce an increase in melanoma-endothelium adhesion and a decline in melanoma-melanoma adhesion. Small, repetitive doses of UVA had a stronger effect on melanoma-endothelium adhesion than a single-bolus dose does. This suggests that at least *in vitro* repetitive exposure to small UVA doses might be more efficient in inducing cell adhesiveness when compared with a single-bolus dose of the same total amount. The multiple dose irradiation protocol had a weaker effect on melanoma-melanoma aggregation, especially at latter time-points.

Interestingly, our UVA-derived *in vitro* results appear to have a direct effect because the changes in the adhesiveness and in the adhesion molecule expression occurred in the absence of any keratinocyte-derived soluble factors. This *in vitro* data suggests that UVA exposure might help melanoma to form metastases by easing the detachment of melanoma cells from the primary tumor (a weakening of melanoma-melanoma adhesion) and by enhancing their extravasation in target organs (an increase in melanoma-endothelial adhesion). Therefore, these results have confirmed the original hypothesis and they agree with the *in vivo* data obtained.

Then *in vitro* evidence presented in this study is in agreement with previous studies that have shown enhanced cell adhesion after UVA irradiation. UVA has been shown to alter the adhesive properties of cells by increasing the adhesion of endothelial cells to peripheral blood derived granulocytes, lymphocytes and monocytes (Heckmann *et al.* 1997). UVA is known to stimulate fibroblast adhesion to collagen (Tupet *et al.* 1999) and melanocyte adhesion to fibronectin (Neitmann *et al.* 1999) through the expression of integrins. The cumulative effect of UVA radiation on melanoma-endothelial adhesiveness presented in this study is also consistent with studies where the cumulative effect of repetitive exposure to UVA and UVB radiation were observed on skin (Parrish *et al.* 1981; Lavker *et al.* 1995a; Chouinard *et al.* 2001; An *et al.* 2001).

The adhesiveness at the highest UVA dose in this study might be affected by cell death, since agents inducing cell apoptosis might also induce these cells

to be more adhesive to healthy cells, thus altering the outcome of the adhesion assay. Apoptotic cells begin to express phospahatidyl-serine residues, which mediate the attachment and the phagocytosis of dying cells to the neighboring cells. Apoptotic death was not observed in either B16 cell line in any time-point. Only the highest used dose (12 J/cm^2) caused up to 20.1% of cell death among the highly metastatic B16-F10, but not among the low-metastatic B16-F1, cells. This suggests that although the adhesiveness data obtained with the highest UVA dose (12 J/cm^2) might be compromised by the cell apoptosis-associated adhesiveness, the adhesiveness data obtained at lower doses (2-8 J/cm^2) are not.

The initial step of melanoma metastasis is the detachment of cells from the primary tumor and invasion to the surrounding tissue. The strength of melanoma-melanoma interaction in the primary tumor mass is mainly regulated by E-cadherin, and its downregulation has been shown to play an important role in loosening the primary tumor (Hsu *et al.* 1996; Johnson 1999; Li & Herlyn 2000). The experiments performed in this study suggest that UVA exposure might upregulate the expression of N-cadherin and simultaneously downregulate the E-cadherin expression. This change might be an indicator of the acquisition of pro-metastatic phenotype by the UVA exposed melanoma cells. Furthermore, our pilot observations concerning the UVA-induced decline in the E-cadherin expression one hour after the exposure support the observed decline in the aggregation of melanoma cells that also occurred at the 1-h time-point. Such effect, if also occurring *in vivo*, could enhance the detachment of single cells from the solid tumor mass and their subsequent invasion to the host stroma and thus, enhance the metastatic potential of melanoma.

UVA radiation might also alter the adhesive properties of tumor cells by affecting the expression of cell surface adhesion molecules via increased PKC expression (Leszczynski *et al.* 1995; Leszczynski *et al.* 1996). Moreover, PKC activation and translocation to the plasma membrane has been shown to be involved in melanoma metastasis (Gopalakrishna & Barsky 1988; Rusciano 2000). The high level of PKC expression together with a low E-cadherin expression has been shown to contribute to the high migratory activity of colon carcinoma cells (Masur *et al.* 2001) as well as to low homotypic cell aggregation potential (Batlle *et al.* 1998). Thus, one can assume that also UVA-mediated downregulation of E-cadherin might act in concert in UVA-induced PKC activation and thus decrease the homotypic-binding between tumor cells and simultaneously enhance their metastatic potential. Whether this hypothesis also holds true in B16-F1 and B16-F10 cell lines should be determined in further studies that investigate molecular mechanisms and intracellular signal transduction pathways.

5.2 UVA effect on melanoma metastasis in the mouse model

The *in vivo* studies have shown a novel finding; the irradiation of mice with single dose of UVA that have low-metastatic melanoma cells in their blood circulation increases the formation of pulmonary melanoma metastases. In both animal studies, the metastases formation of B16-F1 cells after UVA exposure was comparable with the level of metastases formation by the highly metastatic B16-F10 cell line. These results suggest that UVA exposure has caused an induction of high-metastatic potential in the normally low-metastatic potential melanoma cells.

The UVA dose of 8 J/cm^2, which was used throughout the *in vivo* study, was selected based on the result of the *in vitro* adhesion assay between irradiated melanoma cells and non-irradiated endothelium: the dose of 8 J/cm^2 was demonstrated to be the most effective in affecting cell adhesiveness without causing cell death. Moreover, it roughly corresponds to the UVA dose received approximately within 1 hour on a sunny summer day in Finland, thus being physiologically relevant.

Since the consecutive UVA exposures increased dramatically the adhesion of UVA-exposed low-metastatic B16-F1 cells to endothelium in the *in vitro* adhesion assay, the multiple dose effect was also tested in the *in vivo* mouse model by exposing the mice to the additional UVA doses of 8 J/cm^2 at 24 and 48 hours after the melanoma cell injection. The repetitive exposure pattern mimics human behavior when tanning in a solarium. The exposure of the animals on a three consecutive days had an unexpected effect in that the number of metastases in their lungs did not increase when compared to the animals that received a single dose. This postulates the possibility that consecutive exposures might cause some kind of suppressive effect on the generation of metastases.

The two animal studies had a different baseline for the formation of metastases. All the treatments, the animal handling procedures and melanoma cells lines were kept the same in both studies. Thus, the differences might be caused by differences between the animal batches or by the changes in B16 cell lines. Nevertheless, the phenomenon remained the same in both experiments, showing the capability of UVA to enhance the metastatic potential of the low-metastatic mouse melanoma B16-F1 cell line. Moreover, the injection of low-metastatic potential B16-F1 without UVA irradiation led to the development of a smaller number of metastases when compared to the positive control animals injected with high-metastatic potential B16-F10 cells in both experimental set-ups. These results are in agreement with the previously established low and high metastatic potential for B16-F1 and B16-F10 melanoma cell lines (Fidler 1973; Fidler 1975), thus confirming the validity of the experimental set-ups.

Cell adhesion molecules play an important role in the regulation of metastatic cascade. Changes in the expression of adhesion molecules are shown to facilitate the metastatic spread of tumor cells (Hart et al 1991).The immunohistological analysis of the cadherin expression profile of melanoma metastases in lungs revealed a strong N-cadherin positive staining pattern and weak E-cadherin expression. This result is in agreement with our *in vitro* result that showed a shift in the cadherin expression profile from E to N-cadherin. The loss of E-cadherin expression has been found to correlate with the progression and invasive phonotype of carcinoma cells *in vivo* (Perl *et al.* 1998). This may result in disturbed cell-cell interactions and enhance tumor formation and invasion (Hsu *et al.* 1996; Meier *et al.* 1998; Li & Herlyn 2000).

Some 20–50% of UVA radiation delivered to the skin penetrate into the dermis in Caucasian skin, and dermal microvascular endothelial cells have been shown to be a direct target of the UVA (Heckmann *et al.* 1997). The thickness of mouse epidermis is approximately 10 µm and for dermis 250 µm (Hansen *et al.* 1984), thus being significantly thinner when compared to human skin (1–4 mm). UVA most likely also reaches the dermal compartment in mice skin. According to our measurements, approximately 90% of the UVA wavelengths used in this study were absorbed in the skin and ~10% was transmitted through it. Thus, in our set-up the UVA radiation was likely to penetrate into the dermis layer of mouse skin and had the potential to irradiate the melanoma cells in the blood circulation.

It is not, however, possible to say whether UVA truly reaches the metastazing melanoma cells in mice skin capillaries. In order to study how much of the 8 J/cm2 dose directly affects the metastatic potential of B16-F1 melanoma cells, the low-metastatic potential B16-F1 cells were *in vitro* irradiated with a single dose of UVA prior to injection. This treatment increased the metastatic capacity of the melanoma cells only 1.5-fold, which means that mechanism(s) other than the direct UVA-induced adhesiveness of melanoma cells may play a role in the UVA-induced increase of metastasis. UVA may have caused the generation of soluble factors by the skin cells, which might enhance the pro-metastatic effect of UVA. UVA has been shown to cause a release of some keratinocyte and fibroblast-derived soluble factors, e.g. cytokines (Corsini *et al.* 1997; Kondo & Jimbow 1998; Kondo 1999), eicosanoids (Hanson & DeLeo 1989) and proteolytic enzymes, such as MMPs (Wlaschek *et al.* 1994; Petersen *et al.* 1995).

UVA-induced immunosuppression occurred either locally (Bestak & Halliday 1996; Halliday *et al.* 1998; Damian *et al.* 1999) or systemically (Byrne *et al.* 2002) and might also be responsible for increased melanoma metastasis by impairing the rejection of invasive tumor cells in the UV-exposed host. This study measured systemic immunosuppression by performing the widely

used CHS assay. It showed that a single UVA dose at 8 J/cm^2 caused a 13.8% increase in systemic immunosuppression when compared to the non-exposed control animals. Byrne *et al.* have also showed that UVA irradiation suppressed the systemic CHS reaction by using the same C57BL/6 mice strain as we had used (Byrne *et al.* 2002). UVA-induced immunosuppression of systemic CHS, however, was abrogated when three consecutive UVA doses were applied. This suggests the possibility that the consecutive exposures, i.e. the cumulative UVA dose, might diminish the immunosuppressive effect caused by a single UVA dose. A high dose of UVA has been shown to protect mice from UVB-induced immunosuppression, possibly via UVA-induced antioxidant activity (Reeve & Tyrrell 1999; Reeve & Domanski 2002; Allanson & Reeve 2004; Allanson & Reeve 2005). Moreover, in contrast to the primary UVA-induced immunosuppression of C57BL/6 mice, the further UVA irradiation of mice enhanced secondary immune responses (Byrne *et al.* 2002). Halliday has suggested that low doses of UVA may initiate the ROS production that mediates immunosuppression, whereas higher UVA doses stimulate the production of protective antioxidant enzymes such as HO-1, which reverses UVB-induced immunosuppression through the antioxidant effect (Halliday 2005). The results presented in this study agree with previously published studies and suggest that a single UVA dose could be immunosuppressive, but further doses can abolish this immunosuppressive effect. This result can also explain the observation that the UVA exposures delivered at the three consecutive days has weaker pro-metastatic effect than the single dose does.

As earlier results gathered mostly from animal studies have shown, UVA may have a role in the development of melanoma. The additional data from epidemiological studies suggest that the use of a sunscreen and artificial tanning may increase the risk of the onset of malignant melanoma due to, at least partially, the UVA burden gained during tanning sessions (Wang *et al.* 2001). The results presented in this thesis offer new evidence that UVA exposure increases the metastatic potential of the melanoma cells present in the blood circulation in a mouse model. The enhancement in the metastatic potential of melanoma is possibly caused in part by UVA-induced systemic immunosuppression and by the increased adhesiveness of UVA-irradiated melanoma cells. These results suggest that if occurring also in humans, exposure to UVA radiation, either solar or solaria-derived, might have the potential to cause an increase of melanoma metastasis in people who might be unaware of having early, yet undiagnosed, melanoma lesions. Whether similar UVA-induced pro-metastatic effects occur in people sunbathing or using solaria remains to be determined. In addition, it remains to be determined whether other tumors respond to UVA exposure in a similar manner. Considering the recent findings of UVA-induced DNA

mutations (Agar *et al.* 2004), this study further supplements and supports the notion that exposure to UVA radiation might be a more potential health hazard than previously suggested.

5.3 UVA effect on gene expression in melanoma cells

The cDNA study revealed that a physiologically relevant UVA dose might have more health implications than had previously been expected. From the analysis of 1176 cancer related genes, there were only 4 genes (cyclin G, VEGF, α-crystallin and non-muscle cofilin) with statistically relevant changes ($p \leq 0.05$) after UVA treatment. This suggests that the observed genes might be candidates involved in UVA-induced cellular stress. The small overall influence of UVA on the 1176 cancer genes that were studied also suggests that melanoma cells, which are already transformed and have malignant phenotype, might be resistant to the UVA. The four-hour time-point was selected in order to give melanoma cells enough time to respond to UVA radiation; however, not too much time was given in order to avoid the indirect effects that take place over a longer period. The 4-h time-point was also used in the *in vitro* set-up, where alterations in the adhesive properties of the melanoma cells were observed 4 h after irradiation. The main problem with this experimental set-up was the uneven expression profile of differentially expressed genes as in many genes, both up and downregulation was detected in the different repeats. In addition, poor reproducibility was seen in the lack of a detectable spot in every repeat (there were a total of 5 repeats).

With respect to the statistically relevant altered genes, the changes in the gene expression of cyclin G and VEGF genes might be related to the metastatic process. Cyclin G is one of the cell cycle regulators, localized in the nucleus and it is a transcriptional target for the p53 tumor suppressor protein (Okamoto & Beach 1994), thus contributing to G2/M arrest in response to DNA damage (Shimizu *et al.* 1998; Jensen *et al.* 1998). In our study, the expression of the cyclin G gene was significantly upregulated with a 4.85-fold increase after UVA exposure in all five repeats.

Angiogenesis is believed to be an early event in tumorigenesis and may facilitate tumor progression and metastasis. This study clearly demonstrated the upregulation in VEGF gene expression from UVA. The increase in the gene expression level was 2.9-fold, and it was repeated in 4 out of 5 experiments. UVA has been shown to mediate the induction of VEGF in the primary human keratinocytes (Mildner *et al.* 1999) and epidermal fibroblasts (Trompezinski *et al.* 2000). Our findings agree with these results and show that UVA had an effect on VEGF expression in melanoma cells.

The data obtained from a cDNA array is usually validated by some other method, such as the reverse transcriptase-polymerase chain reaction (RT-PCR). However, the most important issue concerning cell physiology is whether the upregulated mRNA is translated to the protein. Since the cyclin G gene was mostly affected by UVA, the protein expression was studied further. Western blot analysis revealed that the expression of cyclin G was affected by UVA radiation only moderately, as compared to the gene expression change. Therefore, the effect of UVA on cell cycle arrest, which is regulated by cyclin G analysis, was determined. Cell cycle analysis showed that UVA causes cell cycle arrest in G2/M check point beginning at the 4-h time-point and peaking 8 hours after the end of irradiation. Interestingly, at the same 4-h time point the 4.85-fold mRNA expression of cyclin G occurs. This observation postulates the notion that UVA-induced cyclin G may induce, among other G2 checkpoint regulators, such as the cyclin B-cdk2-complex and p16 protein (Gabrielli *et al.* 1999;Pavey *et al.* 1999), cell cycle arrest. In addition to cell cycle arrest, the p53-regulated cyclin G gene has been shown to promote cell growth in human colon carcinoma cells and fibroblasts (Smith *et al.* 1997) instead of arresting the cell cycle. Therefore, future studies should be devoted to determining the effect of UVA-induced cyclin G upregulation on the proliferation of melanoma cells.

Our results suggest that UVA radiation-induced changes in the expression of several genes. Some of these changes, as shown an example of cyclin G, might affect cell physiology. The significance of these gene expression results should be further confirmed by examining the protein expression levels both *in vitro* in cell lines and *in vivo* in UVA exposed animals. The experimental approach using cDNA arrays could be further utilized to discover the genes responding to UVA in physiological studies aimed at detecting the health risks associated with UVA exposure. Moreover, the UVA effects on melanoma metastasis in humans cannot be studied using the same experimental procedures used in this study with mice. Therefore, large-scale screening methods, such as DNA arrays, provide a powerful tool to determine the new metastasis markers for humans.

6. Conclusions

An understanding of the physiological consequences of UVR exposure is of crucial importance in the prevention of melanoma. Despite highly developed diagnostic methods and effective clinical treatment and therapy, the metastatic potential of melanoma remains the main reason for mortality fro melanoma. To understand the melanoma metastasis process itself, we need to clarify which parameters, such as environmental factors, might be involved in the transformation of a primary melanoma phenotype into a metastatic phenotype.

The effect of UVA on melanoma tumor metastasis has not been studied. This study presents the first evidence suggesting that UVA exposure might be a physiologically relevant factor in regulating melanoma metastasis. The evidence supports the hypothesis that UVA increases the metastatic potential in mouse melanoma. This increase is likely due to the direct effect on the melanoma cell expression of adhesion molecules and to an indirect effect by causing systemic immunosuppression. Some other systemic effect(s), still unidentified, might also be involved.

The observations derived from the mouse model could be extended to humans. For a large number of patients, melanoma metastasis has taken already place by the time of their diagnosis. Thus, there is the possibility that people who are unaware of already having a primary melanoma tumor and even hematogenously metastasizing melanoma might expose themselves to UVR. Our observations can be further extended into a hypothesis that suggests UVA exposure might enhance the metastatic potential and extravasation of other, non-melanoma cancer cells, which might be present in the blood circulation of people exposed to UVA. If UVA-derived immunosuppression is the key factor involved in increasing the metastatic potential of melanoma, it most likely affects other cancer cells in a similar manner. Future research should determine whether solar or solaria-derived radiation, containing the residual UVB component, has the same effect on melanoma metastasis because UVB/SSR is known to be more immunosuppressive than UVA alone. Furthermore, what was not included in this thesis study, it would be of great importance to determine whether UVA or SSR induces the generation and/or secretion of well-known pro-metastatic chemokines, such as IL-8, from skin cells and from the melanoma tumor itself. Such studies would offer new insights into the possible role of environmental UV exposure on the spread of cancer metastasis.

Despite the scientific evidence indicating the health risks resulting from exposure to UVR and UVA, the majority of people still actively seek a tan. There appears to be a lack of understanding about a UVR-induced tan being neither

healthy nor protective and that it is a visible warning sign of UVR-induced cellular damage. The possibility of the pro-metastatic effects of exposure to UVA might not be of very high relevance for daily exposure. However, the effects of UVA might gain physiological relevance after periods of extensive sunbathing or tanning in a solarium. In the light of the results presented in this thesis, avoidance of solaria use could be well justified.

While effective UVB sunscreens are available, they are not as efficient in the UVA range. The lack of the adequate UVA filters combined with prolonged exposure might lead to an increased dose of UVA. Therefore, there is an urgent need to develop better protective UVA blockers. However, independently of the UVA/UVB blocking quality of the available sunscreens, the focus of UV protection should be aimed at increasing public awareness about the harmful effects of both UVB and UVA radiation. To be effective, these campaigns need to be based on scientific evidence that explains the detrimental health effects of UV exposure, such as the evidence provided in this thesis, and to encourage people to avoid unhealthy habits and behavior in getting a tan.

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